Early Endosome*

Prenylation-dependent Association of Protein-tyrosine Phosphatases PRL-1, -2, and -3 with the Plasma Membrane and the Early Endosome*

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PRL-1, -2, and -3 represent a novel class of protein-tyrosine phosphatase with a C-terminal prenylation motif. Although PRL-1 has been suggested to be associated with the nucleus, the presence of three highly homologous members and the existence of a prenylation motif call for a more detailed examination of their subcellular localization. In the present study, we first demonstrate that mouse PRL-1, -2, and -3 are indeed prenylated. Examination of N-terminal epitope-tagged PRL-1, -2, and -3 expressed in transiently transfected cells suggests that PRL-1, -2, and -3 are present on the plasma membrane and intracellular punctate structures. Stable Chinese hamster ovary cells expressing PRL-1 and -3 in an inducible manner were established. When cells were treated with brefeldin A, PRL-1 and -3 accumulated in a collapsed compact structure around the microtubule-organizing center. Furthermore, PRL-1 and -3 redistributed into swollen vacuole-like structures when cells were treated with wortmannin. These characteristics of PRL-1 and -3 are typical for endosomal proteins. Electron microscope immunogold labeling reveals that PRL-1 and -3 are indeed associated with the plasma membrane and the early endosomal compartment. Expression of PRL-3 is detected in the epithelial cells of the small intestine, where PRL-3 is present in punctate structures in the cytoplasm. When cells are treated with FTI-277, a selective farnesyltransferase inhibitor, PRL-1, -2, and -3 shifted into the nucleus. Furthermore, a mutant form of PRL-2 lacking the C-terminal prenylation signal is associated with the nucleus. These results establish that the primary association of PRL-1, -2, and -3 with the membrane of the cell surface and the early endosome is dependent on their prenylation and that nuclear localization of these proteins may be triggered by a regulatory event that inhibits their prenylation.

Dynamic tyrosine phosphorylation-dephosphorylation is a major regulatory event affecting the functional activities of diverse proteins that participate in many aspects of cellular, physiological, and pathogenic processes. The protein-tyrosine phosphatase (PTP)1 superfamily encompasses a large group of enzymes that undoubtedly play key roles in the regulation of the above events. The PTPs are classified into subgroups depending on their form and substrate specificity, with the receptor-like transmembrane and some intracellular PTPs being specific for phosphophotyrosine, whereas other intracellular PTPs are of dual specificity and dephosphorylate phosphoserine/threonine as well as phosphotyrosine residues (1–3). The cellular roles and substrates of many PTPs remain unknown.

The PRL phosphatases (PRL-1, -2, and -3) are three closely related intracellular enzymes that possess the PTP active site signature sequence CX,R (4–8). All are proteins of about 20 kDa with at least 75% amino acid sequence similarity. They resemble the dual specificity phosphatases of the PTP superfamily in having few of the conserved catalytic domain residues typical of the tyrosine-specific PTPs, although PRL-1 has so far only been demonstrated to dephosphorylate phosphotyrosyl substrates in vitro (4). Furthermore, outside of themselves, their highest homology is to Cdc14p, a dual specificity phosphatase that regulates mitotic exit (9–11), and to PTEN, a dual specificity PTP that functions as a tumor suppressor and has an additional and unique lipid phosphatase activity (12–15). PRL-1 is the founding member of these PRL phosphatases. The PRL-1 gene was originally identified as an immediate early gene whose expression is induced in mitogen-stimulated cells and regenerating liver (16). Overexpression of PRL-1 can lead to cell transformation, suggesting that it may participate in key events that regulate cell growth (4, 6), although the underlying mechanism is currently unknown. A most interesting feature of PRL-1, -2, and -3 is the presence of a consensus C-terminal CAAX sequence for prenylation (17, 18), where C is cysteine, A is an aliphatic amino acid, and X is any amino acid. Three types of prenyltransferase carry out the addition of either farnesyl (C15) or geranylgeranyl (C20) isoprenoids to cellular proteins. Farnesyltransferase (FT) and geranylgeranyl transferase I (GGT I) are heterodimeric α/β enzymes that share a common subunit and mediate prenylation of the CAAX sequence (19, 20), with FT preferring Met, Ser, or Gln in the X position and GGT I preferring Leu in the X position (21). Geranylgeranyltransferase II (GGT II) is a distinct α/β dimer that prenylates XCC, XCXC, or CCXX C-terminal sequences when the substrate protein is bound to a carrier protein called REP (Rab escort protein) (22, 23). The only known substrates of GGT II are the Rab proteins. Protein prenylation is important in targeting proteins to intracellular membranes and in protein-protein interactions (21, 24, 25). This is often critical for protein function, as observed with the dependence of mutant Ras transforming activity on its farnesylation (26, 27). Because of

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; FT, farnesyltransferase; GGT, geranylgeranyl transferase; PCR, polymerase chain reaction; GST, glutathione S-transferase; EM, electron microscope; NLS, nuclear localization signal.
the presence of oncogenic forms of Ras in about 30% of human cancers, FT inhibitors have been investigated as anti-tumor agents and have been shown, for example, to inhibit the anchorage-independent growth and revert the phenotype of Ras transformed cells and to effect tumor regression in Ras oncomice (28–31). Nevertheless, various studies have suggested that FT inhibitors exert these effects on targets other than Ras, with RhoB and other proteins representing possible alternate targets (32, 33). It is thus important to identify such other farnesylated proteins involved in cell growth and transformation and determine their specific cellular actions in relation to their prenylation status.

Previous studies suggest that PRL-1 is localized to the interior of the nucleus (4). Because prenylated proteins are normally associated with cellular membranes and PRL-1 contains the consensus motif for prenylation, we have re-examined the subcellular localization of PRL-1 using a different approach. In addition, because PRL-1, -2, and -3 share high degrees of amino acid sequence homology, antibodies against one may potentially cross-react with others. The subcellular localization of each PRL was thus investigated independently. Our results suggest that PRL-1, -2, and -3 are farnesylated and normally associated with the membrane of the cell surface and the early endosome. We show that this membrane association depends on their prenylation and that unlipidated PRL-1, -2, and -3 are shifted into the nucleus.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO-K1 and other cell lines were obtained from the American Type Culture Collection (Manassas, VA). Synthetic oligonucleotides were from Oligos Etc (Wilsonville, OR). The Pyrococcus furiosus DNA polymerase was a product of Stratagene (La Jolla, CA). The Taq DNA polymerase and Hybrid C-extra nitrocellulose filters were obtained from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat were purchased from Amersham Pharmacia Biotech, and fluorescein isothiocyanate-conjugated-c-Myc antibody (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG was purchased from Amersham Pharmacia Biotech. Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. DNA polymerase and Hybond C-extra nitrocellulose filters were obtained from Amersham Pharmacia Biotech. DNA polymerase was a product of Stratagene (La Jolla, CA). The Taq DNA polymerase and Hybrid C-extra nitrocellulose filters were obtained from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat were purchased from Amersham Pharmacia Biotech, and fluorescein isothiocyanate-conjugated-c-Myc antibody (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG was purchased from Rocko Molecular Biochemicals. Brefeldin A was from Epicentre Technologies (Madison, WI). Wortmannin was purchased from Sigma. The FTI-277 was a gift from Dr. S. M. Sebti (H. Lee Moffitt Cancer Center and Research Institute). [3H]Farnesyl pyrophosphate and [3H]geranylgeranyl pyrophosphate were bought from NEN Life Science Products.

**Expression of Recombinant Proteins in Bacteria**—To construct plasmids expressing wild-type PRLs, the coding regions of PRL-1, -2, and -3 were retrieved by PCR using Pfu polymerase and appropriate forward primers incorporating a BamHI or a SalI restriction site and reverse primers incorporating an EcoRI restriction site. To construct a plasmid expressing a mutant form of PRL-2 lacking the C-terminal four amino acids (amino acids 164–167) of the CAAX box (PRL-2-cd), the PRL-2 cDNA was amplified using the same forward primer as above (with an added SalI site) in conjunction with a reverse primer corresponding to the sequence encoding amino acids 159–163 and with an added EcoRI site. Mutant PRL-2 (C101S), where the essential cysteine residue of the active site of the phosphatase was replaced by serine, was obtained through a first PCR reaction using a forward primer corresponding to the desired nucleotide substitution (5′-TGTTTTGGCAGTGCTAGTTGCGAGATGGGA-3′), and the reverse primer was used to amplify wild-type PRL-2. The completed first PCR reaction was then used as a template in a second PCR reaction employing the forward (with an added BamHI site) and reverse (with an added EcoRI site) primers used to amplify wild-type PRL-2 (above). All PCR fragments were inserted into the BamHI-EcoRI-digested pGEX-KG vector (34) or into the SalI-EcoRI-digested pGEX-3C vector (35) and sequenced to verify the insertions. The plasmids were transformed into the Escherichia coli strain DH5αF′ and GST-PRL fusion proteins produced upon isopropyl-1-thio-

β-D-galactoside induction. Soluble purified PRL proteins were obtained by protease 3C cleavage (35) of affinity-purified GST-PRL-1, -2, and -3 and GST-PRL-2-cd fusion proteins from pGEX-3C-PRL expressing E. coli.

**Preparation of Polyclonal Antibodies**—Rabbits were each injected with 500 μg of GST-PRL-3 emulsified in complete Freund’s adjuvant. Booster injections containing a similar amount of antigen emulsified in incomplete Freund’s adjuvant were administered every 2 weeks. Rabbits were bled 10 days after the third and subsequent booster injections. For affinity purification, serum was diluted twice with phosphate-buffered saline and incubated first with cyanogen bromide-activated Sepharose beads coupled with GST to absorb antibodies against GST. Antibodies against PRL-3 were then affinity purified by incubating with beads conjugated to GST-PRL-3. After extensive washing, specific

![Fig. 1](image_url) In vitro prenylation of the PRLs. Purified recombinant PRL-1, -2, and -3 were incubated with HeLa cell extract and [3H]FPP or [3H]geranylgeranyl pyrophosphate (GGPP) as described under "Experimental Procedures." A and B show the results of experiments conducted with different preparations of the PRL proteins and cell extracts. Prenylation of the PRLs is shown in the top panels, and Coomassie Blue-stained SDS-polyacrylamide gels of the purified PRL proteins are shown in the bottom panels.

![Fig. 2](image_url) Association of PRL-1, -2, and -3 with the plasma membrane and intracellular punctate structures. Constructs encoding N-terminal Myc epitope-tagged PRL-1, -2, and -3 were transiently transfected into CHO cells, and the cells were processed for indirect immunofluorescence microscopy to detect the expressed myc-PRL-1 (A), myc-PRL-2 (B), and myc-PRL-3 (C), respectively. Bar, 10 μm. Arrows indicate the labeling on the plasma membrane.
antibodies were eluted, neutralized, concentrated, and stored at −20 °C in 10% glycerol.

*In Vitro Prenylation*—HeLa cell lysate was used as a prenyltransferase source and was prepared by scraping cells into 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and then passing the cells through a 26-gauge needle 6–8 times. The lysate was clarified by centrifugation in a microcentrifuge at 12,000 rpm for 30 min at 4 °C, and the supernatant was used in the following reactions. A typical prenylation reaction mixture of 40 μl contained 2 μCi of [³H]FPP or [³H]geranylgeranyl pyrophosphate, 1 μg of purified recombinant PRL protein, and 40 μg of HeLa cell lysate. The reaction mixture was incubated at 30 °C for 4 h and resolved by SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed to film at −80 °C for 1 week.

**Epitope Tagging and Transfection**—To construct the pStar-mycPRL vectors, three pairs of oligonucleotides were used in PCR reactions with the respective PRL cDNA as the template to introduce the Myc epitope at the N terminus of PRL-1, -2, and -3. For PRL-1, oligos A (5'-GCGAATTCCATGGAGACGAGCTGATCTCGCTCGCTC-3') and B (5'-GATGCACATGCCCTTG-3') were used. Oligos C (5'-GCGAATTCCATGGAGACGAGCTGATCTCGCTCGCTC-3') and D (5'-GATGCACATGCCCTTG-3') were used for PRL-2. For PRL-3, oligo E (5'-GCGAATTCCATGGAGACGAGCTGATCTCGCTCGCTC-3') and F (5'-GCGAATTCCATGGAGACGAGCTGATCTCGCTC-3') were used. The PCR fragments were cut with EcoRI and BamHI and inserted into the inducible expression vector pStar as described (36). Recombinant pStar with proper inserts were transiently or stably transfected into CHO cells as described (36). Stable transfecants were screened for tetracycline-induced expression after being cultured overnight in the presence of 4 μg/ml doxycycline-HCl, and stable CHO cell lines expressing myc-PRL-1 (clone 9) and myc-PRL-3 (clone 36) were expanded. To construct the pXJ41-mycPRL-neo vectors, the wild-type PRL-1, -2, and -3 and the mutant PRL-2 (C101S) coding sequences were excised from the pGEX-KG-PRL plasmids with BamHI and XhoI and inserted into the pXJ41-myc-neo vector 3' to and in a continuous reading frame with the Myc tag in the vector. The DNA encoding mutant PRL-2 lacking the C-terminal sequence encoding the CAAX box (PRL-2) on a BamHI site and a reverse primer incorporating an added BamHI site and a reverse primer incorporating an added XhoI site and then directly inserted into the pXJ41-myc-neo vector as described for the wild-type PRL sequences. These PRL expression plasmids were used to transfect NIH 3T3 cells. The stable transfecants were selected by neomycin resistance and confirmed to express the desired Myc-tagged PRL protein by immunoblotting cell lysates with anti-Myc antibody.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed as described previously (37, 38). For treatment with brefeldin A or wortmannin, cells were incubated with brefeldin A (10 μg/ml) or wortmannin (500 nM) for 1 h at 37 °C, washed twice in PBS (phosphate-buffered saline with 1 mM MgCl₂ and 1 mM CaCl₂) and then fixed in 3% paraformaldehyde in PBS. For tetracycline-induced expression, CHO cells were grown in the presence of 4 μg/ml of doxycycline-HCl for 24 h. For farnesyltransferase inhibition, stable lines of NIH 3T3 cells were cultured in the presence of 10 μg/ml FTI-277 for 16 h prior to fixing. Fixed cells were then permeabilized and
processed for indirect immunofluorescence microscopy with the respective antibodies.

Electron Microscopy—Expression of Myc-tagged PRL-1 and PRL-3 in stable CHO cells was induced by overnight culture in the presence of 8 μg/ml of tetracycline. Cells were fixed in a mixture of 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Cryosections were processed for immunogold labeling according to the protocol described (39, 40). To mark the early endosomes, cells were incubated with BSA stabilized colloidal gold before fixation (39–41).

Immunoperoxidase Labeling—This was performed using a Vectastain ABC kit from Vector Laboratories (Burlingame, CA) according to the procedure provided by the supplier.

RESULTS

In Vitro Prenylation of Mouse PRL-1, -2, and -3—Purified recombinant PRL proteins were incubated with HeLa cell lysates and labeled farnesyl or geranylgeranyl pyrophosphates. When farnesyl pyrophosphate was used as the isoprenoid donor, all the PRLs were farnesylated (Fig. 1). When geranylgeranyl pyrophosphate was used as the isoprenoid donor, PRL-1 and -2 were geranylgeranylated, but PRL-3 was not (Fig. 1). To ensure that the lack of detected geranylgeranylation of PRL-3 was not merely due to its relatively lesser amount in the reaction (Fig. 1A, bottom panel), new preparations of PRL-1 and -3 were prenylated using freshly prepared cell extract in side-by-side reactions. As shown in Fig. 1B, equal amounts of PRL-1 and PRL-3 were equally well farnesylated. However, in contrast to PRL-1, PRL-3 was still not modified by geranylgeranylation (Fig. 1B, top panel), thus confirming that even when efficiently farnesylated, PRL-3 is not an in vitro substrate of a GGT. The mutant PRL-2 protein lacking the CAAX sequence (PRL-2-cd) was never observed to be prenylated with either isoprenoid donor (data not shown). Thus the PRLs are prenylated in vitro, and PRL-3 exhibits a marked difference in its ability to be modified by C15 and C20 prenyltransferases.

PRL-1, -2, and -3 Are Associated with the Plasma Membrane and Intracellular Punctate Structures in Transiently Transfected Cells—Because PRL-1, -2, and -3 are highly homologous proteins and antibodies against them may potentially cross-react with each other, we took an alternative approach to examine their subcellular localization by expressing N-terminally Myc epitope-tagged versions of these proteins in transiently transfected CHO cells. Constructs for Myc-tagged versions of these proteins were cloned into the pStar plasmid, a tetracycline-inducible expression vector for mammalian cells (36). As shown in Fig. 2, the Myc-tagged PRL-1, -2, and -3 exhibited labeling characteristic of the plasma membrane (see the labeling on the cell edges marked by arrows) as well as intracellular punctate structures scattered throughout the entire cytoplasm but concentrated in the perinuclear region. These punctate structures resemble those of the secretory and/or endocytotic pathway.

Establishment of Stable CHO Cells Inducibly Expressing PRL-1 and -3—To more precisely define the subcellular localization of PRL-1 and -3, we established stably transfected CHO cells that express these proteins in an inducible manner. CHO cells were transfected with the pStar vector expressing epitope-tagged PRL-1 and -3, and stable cell lines expressing myc-PRL-1 and -3 in a tetracycline-inducible manner were established (Fig. 3). As revealed by immunoblot analysis, expression of myc-PRL-1 was undetectable for clone 9 in the absence of tetracycline and was induced by tetracycline treatment (Fig. 3A, lanes 1 and 2). Similar levels of the Cdk inhibitor p27 were detected in clone 9 cells regardless of the presence of tetracycline in the culture medium (Fig. 3B). The expression of myc-PRL-3 in clone 36 cells was leaky because a significant amount of myc-PRL-3 was detected in the absence of tetracycline (Fig. 3C, lane 1). However, the expression of myc-PRL-3 was greatly enhanced by tetracycline (Fig. 3C, lane 2).

Effects of Brefeldin A and Wortmannin on the Subcellular Distribution of PRL-1 and -3—When Clone 9 was examined by indirect immunofluorescence microscopy, myc-PRL-1 was detected on the plasma membrane and associated with microspike-like plasma membrane processes (Fig. 4A, focusing on the surface labeling). When focused on intracellular labeling (Fig. 4B), distinct punctate structures were detected. Brefeldin A treatment resulted in the redistribution of intracellular PRL-1 into a compact structure near the microtubule-organizing center (Fig. 4C), characteristic of proteins associated with the early endosome and the trans-Golgi network (42, 43). Because the PRL-1 labeling does not resemble that of markers of the trans-Golgi network, this result suggests that the intracellular structures positive for myc-PRL-1 may be early endosomes. Consistent with this interpretation, myc-PRL-1 shifted...
to vacuole-like structures when cells were treated with wortmannin (Fig. 4D). Wortmannin is a phosphatidylinositol 3-kinase inhibitor and known to cause redistribution of endosomal proteins to swollen vacuole-like structures (43–45).

Similarly, when clone 36 was examined by indirect immunofluorescence microscopy, labeling of myc-PRL-3 at both the plasma membrane (Fig. 5A, focal plane at cell surface) and intracellular punctate structures (Fig. 5B, focal plane at cell interior) was observed. Myc-PRL-3 is also detected on microspike-like plasma membrane processes (Fig. 5A). Brefeldin A and wortmannin redistributed myc-PRL-3 to compact and vacuole-like structures, respectively (Fig. 5, C and D). These results suggest that, like myc-PRL-1, myc-PRL-3 is also associated with the plasma membrane and the early endosome.

Association of PRL-1, -2, and -3 with the Plasma Membrane and Early Endosome as Revealed by EM Immunogold Labeling—To define the precise subcellular localization of myc-PRL-1 and -3, clone 9 and 36 cells were allowed to internalize BSA conjugated with 6-nm gold particles (BSA-gold) for 6 min so that the early endosome will be marked by the internalized BSA-gold (41). Cells were processed for cryosections and then labeled with antibodies against the Myc epitope followed by protein A-conjugated with 9-nm gold particles (protein A-gold). As shown in Fig. 6A, myc-PRL-1 is clearly associated with the cytoplasmic side of the plasma membrane (arrowheads). Furthermore, myc-PRL-1 detected in both vacuole-like as well as vesicular-tubular network of the early endosome (Fig. 6B, large arrowheads). The BSA-gold (Fig. 6B, small arrowheads) is present in the vacuole-like but not the vesicular-tubular early endosome. These results establish that myc-PRL-1 is associated with the membrane of the cell surface and the early endosome.

Similarly, EM immunogold labeling using cryosections prepared from clone 36 cells whose early endosomes have been filled with BSA-gold revealed that the majority of myc-PRL-3 is associated with both the vacuole-like and vesicular-tubular early endosomal structures (Fig. 6C). Association of myc-PRL-3 with the cytoplasmic side of the plasma membrane and its processes was also observed by EM immunogold labeling (data not shown).

Inhibition of Prenylation Redirects PRL-1, -2, and -3 into the Nucleus—Association of prenylated proteins with cellular membranes is usually dependent on their prenylation. We have investigated whether association of PRL-1, -2, and -3 with the plasma membrane and the early endosome is a prenylation-dependent event. NIH 3T3 cells stably expressing Myc-tagged PRL-1, -2, or -3 also exhibited the endosomal localization of the phosphatases (Fig. 7, A–C). Endosomal localization was not dependent on the phosphatase activity of PRL-2, because a mutant, inactive PRL-2 generated by substitution of the active site essential cysteine residue (Cys101) showed a localization similar to that of the wild-type, catalytically active PRL-2 (Fig. 7D). Treatment of these cell lines with the selective farnesyltransferase inhibitor FTI-277 (46) for 16 h resulted in the altered localization of all the PRLs to the nucleus (Fig. 7, E–G). PRL-1 exhibited labeling throughout the entire nucleus and was enriched in distinct nuclear speckles (Fig. 7E), suggesting that unprenylated PRL-1 is specifically transported into the nucleus. The nuclear labeling of PRL-2 is distinct from that of PRL-1 in that it was excluded from some regions of the nucleus and no discrete regions of enrichment were observed (Fig. 7G). PRL-3 showed a similar nuclear redistribution to that of PRL-2 upon inhibition of prenylation (Fig. 7F). The PRLs are highly homologous, and the significance and molecular basis of their different nuclear distributions are presently unknown.

Mutant PRL-2 Lacking the Prenylation Motif Is Localized to the Nucleus—The farnesyltransferase inhibitor FTI-277 can have wide ranging cellular effects because it will prevent the prenylation of diverse proteins. Although the observed effect of FTI-277 in redistributing PRL-1, -2, and -3 into the nucleus is likely due to the direct inhibition of their prenylation, it remains possible that the observed nuclear accumulation of the PRLs is due to an indirect effect of inhibiting the prenylation of other proteins whose prenylation is important for plasma membrane and endosome association of PRL-1, -2, and -3. To rule out this possibility, we constructed and stably expressed a mutant PRL-2 (PRL-2-cd) lacking a prenylation signal because of deletion of the CAAX motif. The PRL-2-cd is localized in the nucleus (Fig. 7H), thus independently establishing that prenylation of PRL-2 is indeed necessary for its association with the plasma membrane and early endosome.
Expression of PRL-3 in Small Intestine and Its Association with Non-nuclear Punctate Structures—The above studies investigated the subcellular localization of ectopically expressed, epitope-tagged PRLs. We were also able to examine the subcellular localization of endogenous PRL-3 using a purified polyclonal antiserum, which preferentially recognizes PRL-3. Antiserum from rabbits immunized with recombinant GST-PRL-3 was incubated with GST-beads to remove anti-GST antibodies, and then anti-PRL-3 antibodies were affinity purified using bead-coupled with GST-PRL-3 (see “Experimental Procedures”). Antibody specificity was tested and found to display preferential recognition against PRL-3 as compared with PRL-1 and PRL-2 (Fig. 8). Cells stably expressing myc-PRL-1 (A–C), myc-PRL-2 (D–F), or myc-PRL-3 (G–I) were processed for double labeling using both the affinity-purified rabbit antibodies and monoclonal Myc antibody. As shown, the rabbit antibodies detected myc-PRL-3 as efficiently as the Myc antibody (Fig. 8, compare G and H). Although rabbit antibodies recognized myc-PRL-1 (Fig. 8A), the relatively weaker intensity as compared with that obtained with Myc antibody (Fig. 8B) suggests that the rabbit antibodies are not as efficient as the Myc antibody. Under identical conditions, myc-PRL-2 was essentially not recognized by the rabbit antibodies (Fig. 8D), although Myc antibody gave strong labeling (Fig. 8E). These results suggest that the affinity-purified antibodies recognize preferentially PRL-3, although some cross-reactivity with PRL-1 but not PRL-2 was observed.

We first investigated the subcellular localization of endogenous PRL-3 in diverse cell lines (including A431, NRK, HeLa, Vero, MDCK, and 3T3-L1), but specific labeling was not ob-
observed, possibly because of low levels of expression. We therefore investigated the expression of PRL-3 in various mouse tissues by immunoperoxidase labeling. Frozen sections derived from entire 1-week-old mouse were incubated with affinity-purified PRL-3 antibodies followed by biotinylated anti-rabbit IgG, avidin-horseradish peroxidase complex, and the substrate. After careful examination, PRL-3-specific labeling was only observed in some regions of the small intestine. As shown in Fig. 9B, PRL-3 is present in the differentiated epithelial cells of the villus but not the proliferating crypt cells. The selective detection of PRL-3 in the villus but not the crypt or in other mouse tissues also suggests that the labeling is specific. In support of this, the villus-specific labeling was not observed when PRL-3 antibodies were omitted (Fig. 9A). The labeling of PRL-3 in the epithelial cells is primarily confined to the apical side of the cells facing the lumen of the gut.

The expression of PRL-3 in the epithelial cells allowed us to investigate the subcellular localization of PRL-3 by indirect immunofluorescence microscopy. As shown, PRL-3 was detected in punctate structures in the apical cytoplasm of the epithelial cells and PRL-3-positive structures are enriched in the perinuclear region (Fig. 10A). Importantly, PRL-3 was not detected in the nuclei (Fig. 10A) marked by Hoechst staining of the DNA (Fig. 10B). The labeling of PRL-3 is characteristic of the endosome. These results not only suggest that our conclusions derived from studies using the transfected cells are not due to epitope-tagging or overexpression, they also suggest that PRL-3 may participate in differentiation-associated events in the epithelial cells of the small intestine.

**DISCUSSION**

Evidence suggests that PRL-1, -2, and -3 play important roles in regulating cell growth, including the observations that overexpression of these proteins can lead to cellular transformation (4, 6) and the discovery of their amino acid sequence homology with Cdc14p and PTEN (8), two important players in cell growth control. Establishment of their subcellular localization is thus important for studies aiming to reveal their specific roles and mechanism of action. Indirect immunofluorescent and EM immunogold microscopy of cells expressing any one of the Myc-tagged PRLs show that the PRLs are normally associated with the cytoplasmic face of the plasma membrane and with microspike-like and other plasma membrane processes (Figs. 2, 4, A and B, and 5, A and B). Additionally, the PRLs are resident in the early endosome, as demonstrated by their presence in intracellular punctate structures resembling endosomes (Figs. 4A, 5A, and 8, A–C); their characteristic subcellular redistributions in response to brefeldin A and wortmannin (Fig. 4, C and D, and 5, C and D), two compounds known to alter the structures of endosomal compartments and effect the relocalization of early endosomal proteins such as the transferrin receptor and endobrevin (42–45); and their presence in vacuole-like as well as tubular-vesicular structures of the early endosome marked by BSA-gold (Fig. 6, B and C).

Myc-tagged PRLs were used for these studies, because a survey of many cell lines (including A431, NRK, HeLa, Vero, MDCK, and 3T3-L1) with antibodies raised against PRL-2 and PRL-3 did not reveal specific labeling of subcellular structures, possibly because of the low levels of expression of the PRLs in these cells. The anti-PRL-3 antibodies preferentially recognize PRL-3 (Fig. 8). The examination of different mouse tissues indicates that PRL-3 is preferentially expressed in the small intestine (Fig. 9) where the majority of endogenous PRL-3 is localized to perinuclear punctate structures but is undetectable in the nucleus (Fig. 10), consistent with our results obtained using tagged PRL-1, -2, and -3. Also, the presence of PRL-3 in the differentiated epithelial cells of the villus but not in the proliferating crypt cells suggests that PRL-3 may participate in the differentiation-associated events in the epithelial cells of the small intestine.

All the PRLs can be prenylated in vitro, and it is now clear that in vivo prenylation is key to their subcellular localization. The association of PRL-1, -2, and -3 with the plasma membrane and the early endosome is abolished by treatment with FTI-277, a potent and selective FT inhibitor (46). Under this condition, all the PRLs redistribute to the nucleus. Furthermore, a mutant PRL-2 with the prenylation motif deleted is delivered to the nucleus. These results not only establish that the plasma membrane and endosomal localization of PRL-1, -2, and -3 requires their farnesylation but also suggest that in the absence of farnesylation they are specifically targeted to the nucleus. Consistent with specific nuclear import is the presence of a potential nuclear localization signal (NLS) near the C terminus of all the PRLs. This region, possessing the sequence KRR_KYP_KMRLRNK, resembles a typical bipartite NLS with an initial cluster of basic residues separated from a second more C-terminal basic region by a spacer of 10–12 residues (47, 48), although the latter portion of this potential signal in the PRLs is atypical in its alternation of six basic with nonbasic residues. Whether this is a functional mono- or bipartite NLS needs to be investigated. Nevertheless it is attractive to speculate that this NLS is masked by prenylation of the adjacent C terminus of the PRLs, resulting in their observed
plasma membrane and endosomal localization, and the absence of prenylation permits the specific interaction of the PRLs with nuclear import proteins to effect a nuclear redistribution.

The positively charged region near the C terminus of the PRLs may fulfill another function in membrane targeting, similar to that of the polybasic region of Ki-ras4B. Prenylation and CAAX modification is sufficient to target the ras proteins to endomembranes such as the endoplasmic reticulum and Golgi, and a second signal contained with the ras hypervariable domains is required for subsequent transport to the plasma membrane (49, 50). For Ha-ras, N-ras, and Ki-ras4A, the additional signal is palmitoylation sites, but with Ki-ras4B it is a polybasic domain of six consecutive lysine residues. Each type of signal appears to direct different pathways of ras trafficking to the plasma membrane. The polybasic region may enhance membrane avidity of Ki-ras4B through electrostatic interactions with negatively charged membrane phospholipid head groups or with specific plasma membrane proteins (49). It functions only in conjunction with the CAAX motif, because mutation of the prenylation site diverts the majority of Ki-ras4B protein to the nucleus (49), similar to our observations with PRL-2. Whether an unknown third signal (25) or perhaps variations in the polybasic region can differentially determine the final membrane destination of prenylated proteins is unknown but could explain the observed diversity of membrane compartmentalizations of these CAAX proteins.

How do we explain the apparent difference between our current study and the previous one showing nuclear localization of PRL-1 (4)? Among several possibilities, we propose that the majority of newly made PRL-1, -2, and -3 are prenylated and targeted to the plasma membrane and the early endosome with a small fraction being unprenylated and thus targeted to the nucleus. The antibodies used in previous studies may specifically recognize the unprenylated form of PRL-1 if the prenylation signal itself is part of the epitope. In this way, prenylated PRL-1 was not recognized by these antibodies. Further experiments are needed to resolve this issue.

Our findings describe two potential sites of action for PRL-1, -2, and -3 and provide a regulatory mechanism for switching or altering PRL function. This could occur, for example, in response to cell stress. Little is known of the cellular regulation of prenyltransferase activity, but the regulation of isoprenoid biosynthesis may be more important in this respect. The mevalonate pathway leading to isoprenoid synthesis in mammalian cells has recently been reported to be activated by heat shock, UV radiation, or arsenite treatment with a consequent increase in the prenylation and membrane association of Ras (51). In plants, environmental changes such as heat and light enhance isoprenoid synthesis (52). A prenylated calmodulin was found to relocalize from the plasma membrane to the nuclei of leaf epidermal cells upon shifting from light to dark conditions (53). This was proposed to result from reduced isoprenoid levels and lack of calmodulin prenylation, because a similar shift was observed upon treatment with an inhibitor of mevalonate synthesis.

Several nuclear events occur upon treatment of mammalian cells with FT inhibitors, such as a p53-dependent increase in transcription of p21, and the consequent inhibition of kinase activity of Cdk complexed with cyclins E or A, reduced phosphorylation of Rb, and inhibition of DNA replication (54). In cells lacking p21 or with mutant p53, endoreduplication of DNA is observed and polyploid cells undergo apoptosis (54). It will be important to determine whether the PRLs are involved in mediating any of these changes.

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