Active EGF receptors have limited access to PtdIns(4,5)P_2 in endosomes: implications for phospholipase C and PI 3-kinase signaling

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Summary

Although prolonged cell signaling is attenuated by internalization and downregulation of active receptors, it is now appreciated that many receptors continue to signal in intracellular compartments. Employing enhanced green fluorescent protein fusion probes, we have investigated the hypothesis that multiple signaling pathways are affected by the differential trafficking of membrane substrates such as PtdIns(4,5)P_2. A phosphotyrosine-specific probe, but not a PtdIns(4,5)P_2-specific probe, colocalized with internalized EGF as well as transferrin in EGF-stimulated living cells expressing autophosphorylation-competent EGF receptors. Neither probe colocalized with transferrin in the absence of EGF, demonstrating that the reduced level of accessible PtdIns(4,5)P_2 in endosomes is constitutive. Finally, a PtdIns(3,4,5)P_3-specific probe, which monitors phosphorylation of PtdIns(4,5)P_2 by phosphoinositide 3-kinases, was recruited to the plasma membrane but not to EGF- or transferrin-containing endosomes in response to EGF stimulation. These results suggest that while many internalized receptors continue to engage intracellular enzymes, the phospholipase C and phosphoinositide 3-kinase signaling pathways are abrogated by the constitutive lack of accessible PtdIns(4,5)P_2 in endosomes.

Key words: Phosphatidylinositol (4,5)-bisphosphate, EGF receptor, phospholipase C, Endosome

Introduction

Intracellular signal-transduction pathways are typically initiated by transmembrane receptors that interact with both extracellular ligands and intracellular signaling molecules. Long-term receptor-mediated signaling is attenuated by the clustering of receptor-ligand complexes in clathrin-coated pits, which leads to the downregulation of surface receptors relative to the bulk membrane (Mellman, 1996; Trowbridge et al., 1993). Among signaling receptors, trafficking of the epidermal growth factor (EGF) receptor, a receptor tyrosine kinase that binds to EGF and related polypeptide growth factors, is the best characterized. Ligation of the EGF receptor leads sequentially to receptor dimerization and activation of the intrinsic kinase activity, autophosphorylation on multiple tyrosine residues and recruitment of heterologous signaling proteins (Lund and Wiley, 1994). Activation of downstream signal-transduction pathways is accompanied by enhanced endocytosis of receptor-ligand complexes (Carpenter, 2000). Ligated receptor tyrosine kinases in early endosomes maintain their kinase activity and accessibility to cytosolic signaling proteins, suggesting that intracellular signaling may continue from these sorting organelles (Baass et al., 1995). However, the receptor pools associated with the plasma membrane and endosomes may signal differently because they are physically segregated. Indeed, in certain cell types it has been shown that internalized EGF receptors can differ in their autophosphorylation activity, as well as in their ability to phosphorylate specific signaling proteins in complex with the receptor (Di Guglielmo et al., 1994; Emlet et al., 1997; Vieira et al., 1996).

An additional factor to consider is the compartmentalization of downstream, membrane-associated signaling molecules, such as small GTPases and specific phospholipids, which may also experience trafficking processes at rates that differ from the bulk membrane. To the extent that receptor-mediated membrane localization of enzymes acting on these substrates is important, such partitioning will affect specific signaling fluxes irrespective of the internalized receptor properties. A case in point is the EGF-receptor-mediated hydrolysis of PtdIns(4,5)P_2 through γ isoforms of phospholipase C (PLC). It was recently shown that internalized EGF–EGF-receptor complexes remain autophosphorylated and fully retain their ability to phosphorylate PLCγ1, yet fail to participate in PtdIns(4,5)P_2 hydrolysis, in NR6 fibroblasts overexpressing the wild-type EGF receptor (Haugh et al., 1999). This result was attributed, hypothetically, to a lack of accessible lipid substrate in endosomal membranes. However, other explanations for the abrogated PtdIns(4,5)P_2 hydrolysis in endosomes can be formulated on the basis that PLCγ1 is regulated independently of tyrosine phosphorylation (Rhee and Bae, 1997). This distinction naturally leads to a question of selectivity – are there PtdIns(4,5)P_2 pools in intracellular trafficking compartments available to other receptor-responsive enzymes, such as PI 3-kinases? In addition, the intracellular localization of PtdIns(4,5)P_2 is likely to be a

critical factor in the regulation of membrane trafficking and the cytoskeleton (Toker, 1998).

In this study, minimal protein-binding domains fused to enhanced green fluorescent protein (GFP) were employed as molecular markers to track the intracellular localization of activated EGF receptors, PtdIns(4,5)P_2 and lipid products of the PI 3-kinase pathway in living cells. We show that intracellular receptor trafficking compartments do not present a detectable level of PtdIns(4,5)P_2 in stimulated or unstimulated cells, which would explain the previously identified restriction of PtdIns(4,5)P_2 hydrolysis to the plasma membrane. Consistent with the lack of accessible PtdIns(4,5)P_2, we further report that the PI 3-kinase products PtsIns(3,4,5)P_3 and PtdIns(3,4)P_2 are not detected in compartments containing internalized EGF receptors.

Materials and Methods

Cell culture

Except where noted, all cell culture reagents were purchased from Life Technologies. Swiss-3T3-derived, parental NR6 fibroblasts and stable EGF receptor transfectants of this cell line were kind gifts from Alan Wells (University of Pittsburgh). These cells were grown in MEMα medium with added fetal bovine serum (FBS, 7.5%), sodium pyruvate, L-glutamine, non-essential amino acids and penicillin/streptomycin, with selection of transfectants maintained using 350 μg/ml G418. Serum-free medium was the same as the growth medium, omitting FBS and G418, but with added bovine serum albumin (1 mg/ml). Human A431 epidermoid carcinoma cells (American Type Culture Collection) were grown in DMEM with added FBS (10%), L-glutamine, and penicillin/streptomycin. Serum-free medium was made by omitting FBS and adding bovine serum albumin (1 mg/ml).

cDNA constructs and transfection

Fusions of the tandem SH2 domains of PLCγ1 (Stauffer and Meyer, 1997), the PH domain of PLCδ1 (Stauffer et al., 1998) and the PH domain of Akt (Kontos et al., 1998) to the C-terminus of enhanced GFP were made by cloning these constructs into the pEGFP-C1 vector (Clontech). Cells were seeded onto 25 mm circular glass cover slips, precoated with poly-D-lysine, for 24 hours in growth medium. The cells were transiently transfected using Lipofectamine Plus and OptiMEM I as the medium (Life Technologies); transfection and viability of fibroblasts were optimal after only 30–60 minutes incubation, whereas A431 cells were incubated for three hours. After recovering in growth medium overnight, transfected cells were incubated in serum-free medium for three to four hours prior to imaging.

EGF and transferrin internalization

Cells were allowed to internalize EGF–Texas-Red (EGF-TR; 200 ng/ml) or transferrin–Texas-Red (Tf-TR; 10 μg/ml), both from Molecular Probes, in serum-free medium for 20 minutes at 37°C. Tf-TR-treated cells were either given no additional treatment or coincubated with 100 nM unlabeled EGF (Peprotech).

Confocal fluorescence microscopy

Living cells that had internalized Texas Red conjugates were removed from the incubator and washed with ice-cold extracellular buffer (20 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose and 2 mg/ml bovine serum albumin) prior to imaging. For time-course experiments, the living cells were stimulated with EGF, in extracellular buffer prewarmed to 37°C, on the microscope. Cells were imaged using a Zeiss 410 inverted laser scanning microscope, with GFP (488 nm excitation, 500–530 nm emission) and Texas Red (568 nm excitation, >590 nm emission) signals collected sequentially. Four one second scans were line-averaged for each. Each image presented is representative of at least three experiments performed on separate days, each with observations of 5–20 transfected cells.

Results

The phosphotyrosine probe GFP-SH2 colocalizes with internalized EGF in cells expressing autophosphorylation-competent EGF receptors

A construct consisting of the tandem Src homology 2 (SH2) domains of PLCγ1 fused to GFP (GFP-SH2) was used to assess the ability of internalized EGF receptors to recruit cytosolic enzymes. These domains direct association with autophosphorylated tyrosine residues in the cytoplasmic tail of the EGF receptor, and receptors lacking such autophosphorylation sites fail to stimulate PLC activity (Chen

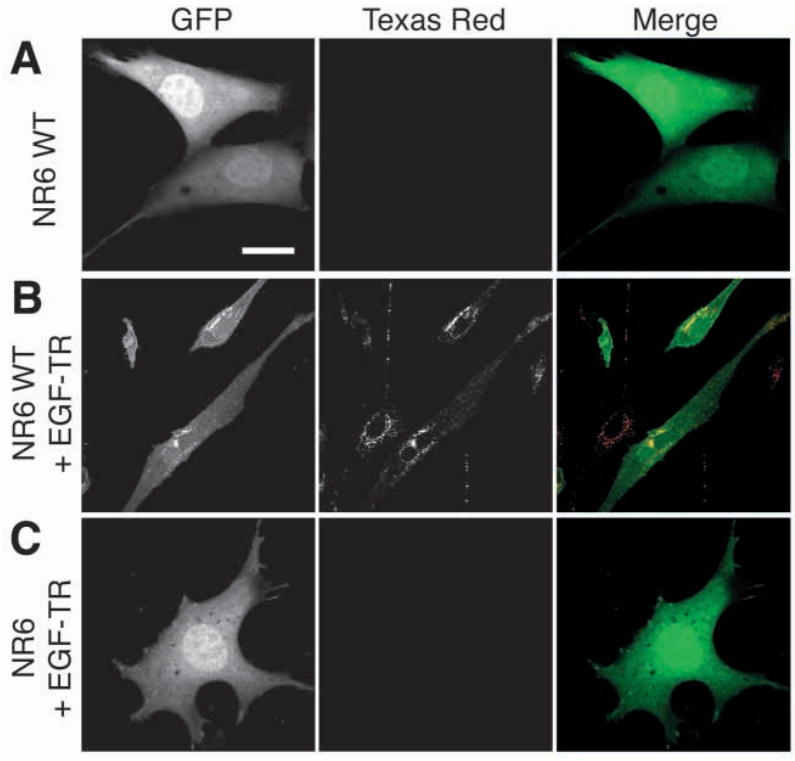

Fig. 1. EGF-receptor-dependent recruitment of SH2 domains to intracellular compartments containing internalized EGF. The tandem SH2 domains of PLCγ1, fused to enhanced GFP (GFP-SH2), were used as a probe to visualize EGF receptor activation. GFP-SH2-transfected NR6 fibroblasts expressing the wild-type EGF receptor (NR6 WT) were either (A) untreated or (B) stimulated with Texas Red-conjugated EGF (EGF-TR) for 20 minutes. The live cells were subsequently visualized by confocal fluorescence microscopy as described in Materials and Methods. (C) GFP-SH2-transfected, EGF-TR-treated parental NR6 cells, which lack EGF receptors, were used as a control. Scale bar, 20 μm.

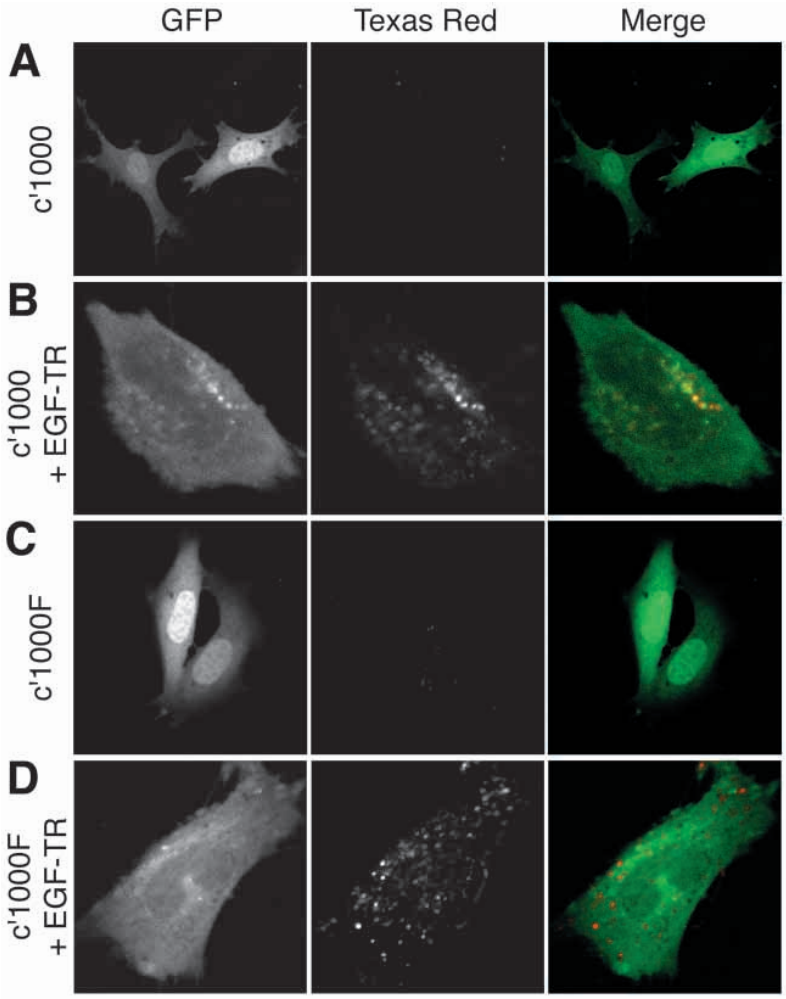

Fig. 2. GFP-SH2 is only recruited by autophosphorylation-competent EGF receptors. GFP-SH2-transfected NR6 fibroblasts expressing either the c'1000 EGF receptor truncation mutant (NR6 c'1000; A,B) or the c'1000 EGF receptor with the mutation Y992F (NR6 c'1000F; C,D) were observed in parallel. The cells were either untreated (A,C) or stimulated with EGF-TR for 20 minutes (B,D). Cells were visualized by confocal fluorescence microscopy.

et al., 1994; Vega et al., 1992). NR6 fibroblasts were used as a model cell line in these studies; parental NR6 cells are devoid of endogenous EGF receptors (Pruss and Herschman, 1977), thus avoiding background signaling in cells ectopically expressing receptor variants (100,000–500,000 per cell). Cells were visualized using confocal fluorescence microscopy. Since the diffraction-limited spatial resolution of this technique is comparable to the size of sorting endosomes (~250-500 nm) (Marsh et al., 1986), staining of endosomes and the plasma membrane are expected to yield similar fluorescence intensities for the same density of bound fluorophore. In contrast, fluorescence associated with small endocytic vesicles (~50 nm) would appear to be diffuse, and so probes bound to such vesicles would be relatively difficult to detect by confocal microscopy.

In unstimulated cells expressing the wild-type EGF receptor (NR6 WT), the transiently transfected GFP-SH2 probe exhibited a homogeneous distribution in the cytosol and was not excluded from the nucleus (Fig. 1A). Following stimulation with Texas-Red-labeled EGF (EGF-TR) for 20 minutes, a dramatic redistribution of GFP-SH2 was observed in these cells. The labeled EGF and GFP-SH2 colocalized in a punctate pattern consistent with early endosomes (Fig. 1B). A series of 1 μm z-sections within a rounded cell confirmed that the spots were on the inside of the cell (results not shown). In contrast, parental NR6 cells lacking the EGF receptor did not exhibit EGF-TR staining or relocalization of GFP-SH2, confirming that detection of EGF internalization and GFP-SH2 recruitment were receptor-specific (Fig. 1C).

To further address the requirements for colocalization of GFP-SH2 with intracellular EGF

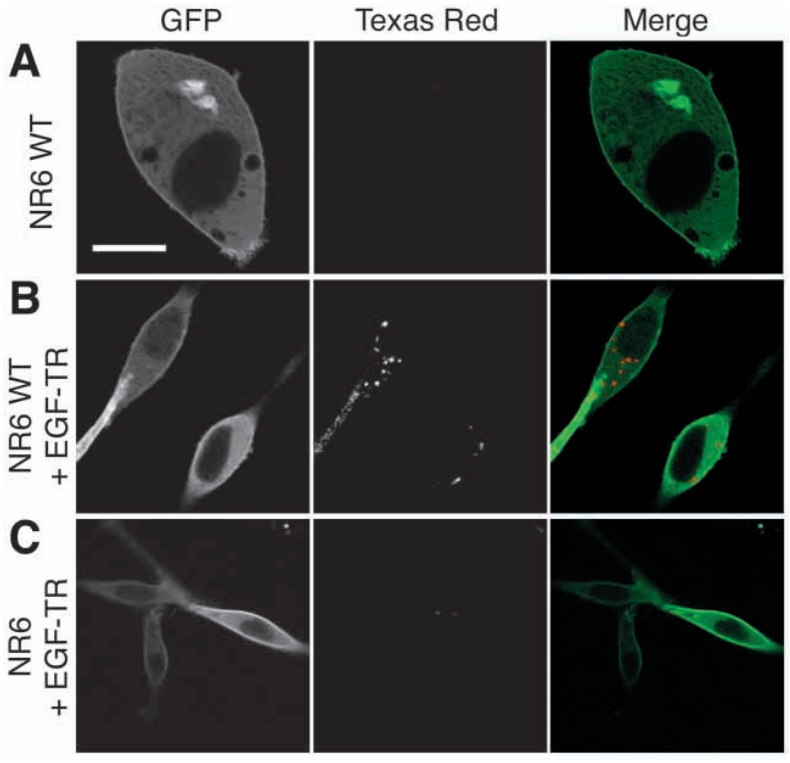

Fig. 3. Intracellular compartments containing internalized EGF lack accessible PtdIns(4,5)P_2. The pleckstrin homology domain of PLCδ1, which is fused to enhanced GFP (GFP-PH), was used as a probe to visualize PtdIns(4,5)P_2. GFP-PH-transfected NR6 WT cells were either (A) untreated or (B) stimulated with EGF-TR for 20 minutes. Cells were visualized by confocal fluorescence microscopy. (C) GFP-PH-transfected, EGF-TR-treated parental NR6 cells were used as a control. Scale bar, 10 μm.

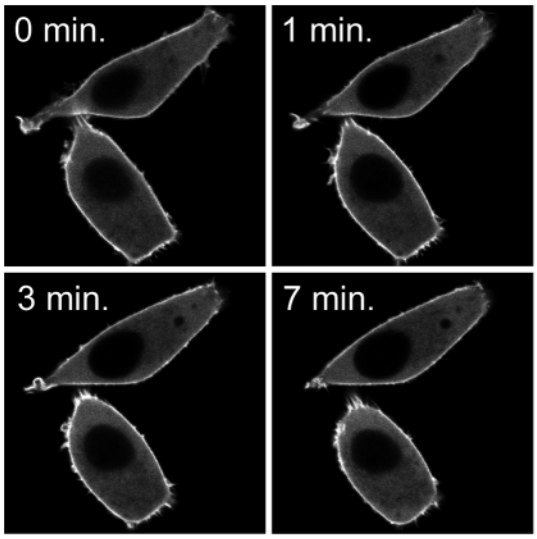

Fig. 4. Living NR6 WT cells transfected with GFP-PH were stimulated with EGF. The distribution of GFP was observed in real time using confocal fluorescence microscopy. Images were acquired every 20 seconds, and panels are shown for the indicated times of stimulation.

receptors, NR6 fibroblasts transfected with EGF receptor mutants were employed. An EGF receptor truncated at residue 1000 (c'1000) retains EGF-stimulated kinase activity, one of its known autophosphorylation sites (Y992) and the ability to elicit PLC activity in NR6 cells expressing this receptor (NR6 c'1000) (Chen et al., 1994). The relocalization of GFP-SH2 in NR6 c'1000 cells stimulated with EGF-TR is consistent with those findings; as in NR6 WT cells, GFP-SH2 is enriched at vesicles containing internalized EGF (Fig. 2A,B). In contrast, cells expressing a truncation variant with the Y992F mutation (NR6 c'1000F) do not support EGF-stimulated PLC activity (Chen et al., 1994), and these receptors failed to recruit GFP-SH2 (Fig. 2C,D). These results confirm that internalized EGF receptors remain autophosphorylated on a site or sites that recruit PLCγ1 to endosomal membranes.

The specific PtdIns(4,5)P_2 probe GFP-PH does not colocalize with internalized EGF

To determine whether cytosol-accessible PtdIns(4,5)P_2 is also a component of EGF-containing intracellular vesicles, a GFP fusion construct of the PLCδ1 pleckstrin homology (PH) domain (GFP-PH) was used. Compared with other lipid-binding PH domains, this probe is uniquely selective for PtdIns(4,5)P_2 in vitro and in vivo (Kavran et al., 1998; Lemmon et al., 1995; Stauffer et al., 1998). In accordance with the presence of appreciable levels of PtdIns(4,5)P_2 in resting cells, GFP-PH-transfected NR6 WT cells exhibited a uniform enrichment in fluorescence at the plasma membrane (Fig. 3A). In addition, some intracellular staining was apparent in many cells. However, GFP-PH did not colocalize with EGF-containing vesicles, and the distribution of the probe after 20 minutes of internalization did not differ qualitatively from that seen in unstimulated cells (Fig. 3B). GFP-PH also failed to colocalize with EGF-TR in either NR6 c'1000 or c'1000F cells (results not shown), and controls with GFP-PH-transfected NR6 parental cells confirmed that detectable internalization of EGF-TR is EGF receptor dependent (Fig. 3C). As the GFP-PH fluorescence at the plasma membrane was typically two-fold higher than the cytosolic background, we conservatively estimate that endosome-associated fluorescence at least five-fold lower than at the plasma membrane would have been detected, indicating that internalized EGF receptors have significantly reduced access to PtdIns(4,5)P_2.

To rule out the possibility that endocytic structures contain measurable levels of PtdIns(4,5)P_2 during shorter periods of

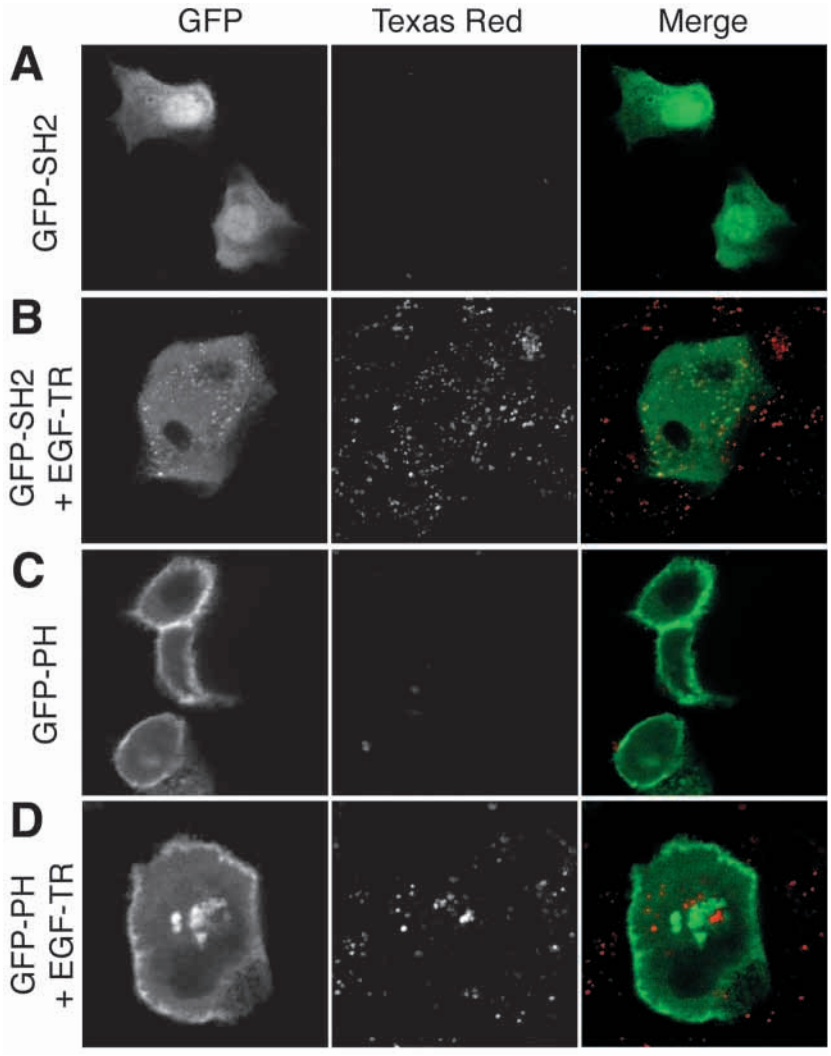

Fig. 5. The segregation of active EGF receptors from accessible PtdIns(4,5)P_2 is not specific to NR6 fibroblasts. A431 cells transfected with GFP-SH2 (A,B) or GFP-PH (C,D) were either untreated (A,C) or stimulated with EGF-TR for 20 minutes (B,D). Cells were visualized by confocal fluorescence microscopy.

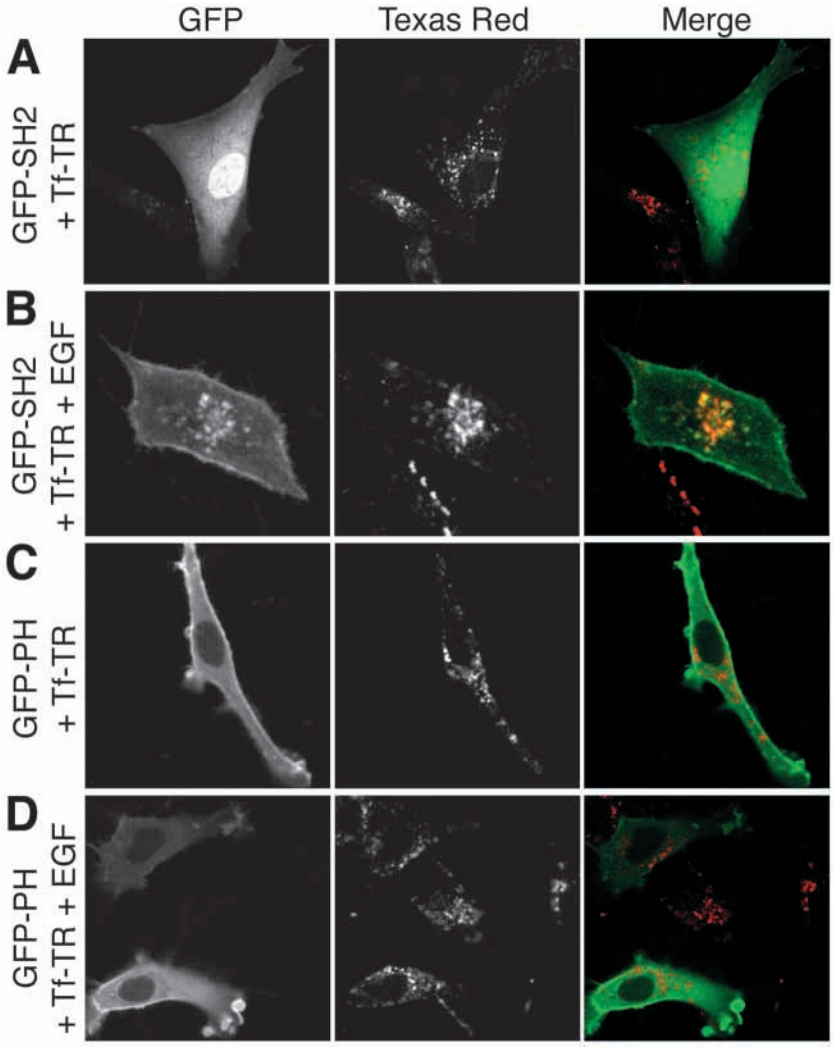

Fig. 6. Accessible PtdIns(4,5)P_2 is not detected in transferrin-containing trafficking compartments. Texas-Red-conjugated transferrin (Tf-TR) was used as a marker for intracellular compartments of the endocytic pathway. NR6 WT cells transfected with either GFP-SH2 (A,B) or GFP-PH (C,D) were treated for 20 minutes with Tf-TR alone (A,C) or with Tf-TR and unlabeled EGF (B,D). Cells were visualized by confocal fluorescence microscopy.

EGF stimulation, GFP-PH-transfected NR6 WT cells were stimulated with EGF and observed in real time (Fig. 4). During the early stages of EGF stimulation, we observed no marked changes in the subcellular distribution of GFP-PH. In contrast, GFP-SH2-transfected cells exhibited a redistribution of fluorescence, consistent with the results shown in Fig. 1, over a similar period. In both cases, EGF caused NR6 WT cells to retract noticeably, as observed by other investigators (Welsh et al., 1991).

Finally, we addressed whether the sequestration of internalized EGF receptors from accessible PtdIns(4,5)P_2 occurs in other cell types. Owing to their high expression of EGF receptors (>2×10^6 per cell), A431 epidermoid carcinoma cells have been used in numerous trafficking and signaling studies; as in NR6 WT cells, GFP-SH2 translocated to internal vesicles containing EGF-TR, whereas GFP-PH failed to colocalize with internalized EGF-TR in A431 cells (Fig. 5).

GFP-SH2, but not GFP-PH, colocalizes with internalized transferrin in EGF-stimulated cells

Though colocalization of the GFP-SH2 probe with autophosphorylated EGF receptors was readily visualized, we sought to confirm that the punctate spots observed were indeed endocytic structures. Thus, cells were treated with Tf-TR, a classic marker for sorting endosomes and recycling tubules. Binding of the transferrin receptor does not elicit tyrosine phosphorylation, and so Tf-TR did not alter the subcellular distribution of GFP-SH2 in NR6 WT cells (Fig. 6A). However, this picture changes dramatically when unlabeled EGF is added along with the Tf-TR marker. Colocalization of GFP-SH2 with Tf-TR-containing structures in EGF-stimulated cells confirms that activated EGF receptors in pre-degradative endocytic compartments were being visualized (Fig. 6B). On the other hand, the GFP-PH probe did not colocalize with Tf-TR in either unstimulated or EGF-treated NR6 WT cells, whereas concentration of GFP-PH at the plasma membrane was apparent (Fig. 6C,D). This strengthens the finding that accessible PtdIns(4,5)P_2 is not detectable in internal membranes of the endocytic pathway. Further, the lack of GFP-PH colocalization with transferrin in the absence of EGF indicates that the exclusion of PtdIns(4,5)P_2 from endosomal membranes is constitutive.

Lipid products of type I PI 3-kinases are not produced at detectable levels in compartments containing internalized EGF

The finding that PLC activities do not have access to PtdIns(4,5)P_2 in intracellular receptor trafficking compartments implies that phosphorylation of PtdIns(4,5)P_2 by type I PI 3-kinases (Vanhaesebroeck and Waterfield, 1999) is also spatially restricted to the plasma membrane. To evaluate this hypothesis, NR6 WT fibroblasts were transiently transfected with a GFP fusion construct of the Akt PH domain (GFP-AH). This probe binds specifically to PtdIns(3,4,5)P_3 and its breakdown product PtdIns(3,4)P_2, and translocation of the probe from the cytosol to the plasma membrane is PI 3-kinase-dependent (Frech et al., 1997; Gray et al., 1999; Kavran et al., 1998; Kontos et al., 1998). The majority of unstimulated, GFP-AH-transfected NR6 WT cells exhibited the typical homogeneous cytosolic and nuclear fluorescence distribution (Fig. 7A), although a fraction of the cells exhibited some prelocalization of GFP-AH at the membrane (not shown). In response to treatment with EGF-TR, all cells showed enrichment of GFP-AH at the plasma membrane but not at EGF-containing vesicles (Fig. 7B). When cells were subsequently incubated with LY294002 (added to 100 μM) for 10 minutes, the membrane-associated probe was redistributed to the cytosol and nucleus (Fig. 7C), confirming the specificity of

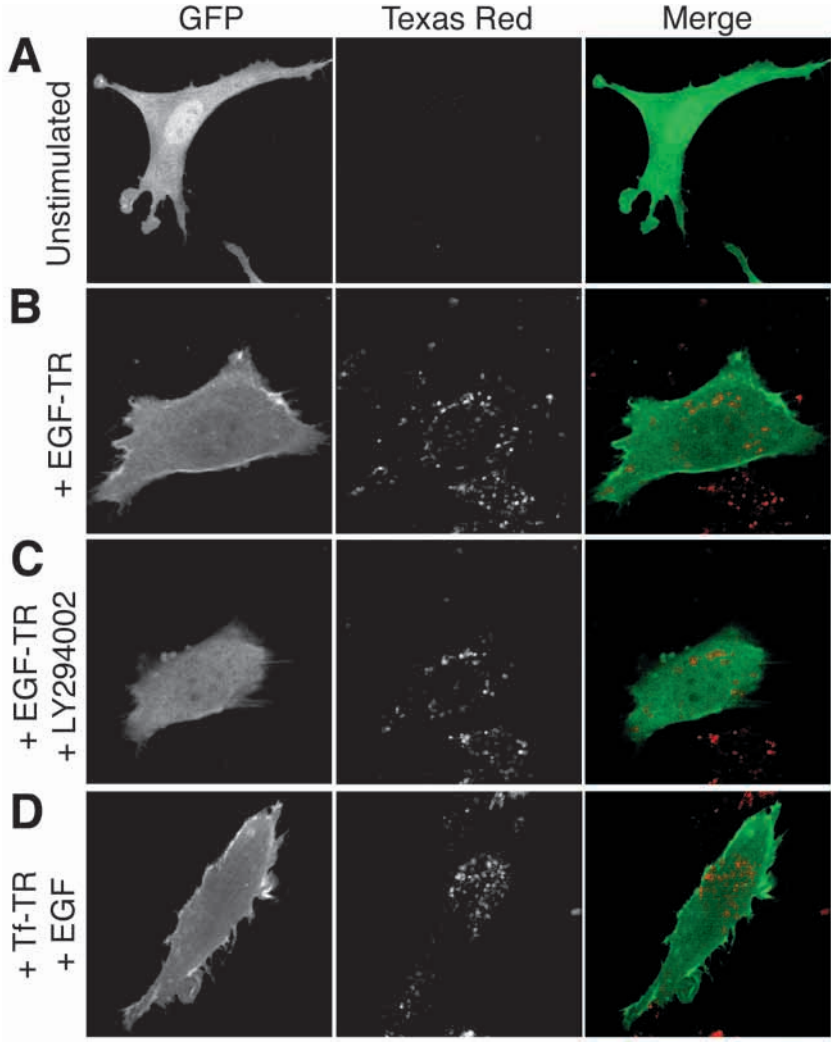

Fig. 7. PI 3-kinase-dependent phosphorylation of PtdIns(4,5)P_2 is not significantly stimulated by internalized EGF receptors. The pleckstrin homology domain of Akt fused to enhanced GFP (GFP-AH) was used to visualize production of PtdIns(3,4,5)P_3. GFP-AH-transfected NR6 WT cells were either (A) untreated or (B) stimulated with EGF-TR for 20 minutes. (C) The cell in B was subsequently treated with LY294002 (100 μM, Calbiochem) for 10 minutes and revisualized. (D) GFP-AH-transfected NR6 WT cells were simultaneously treated with Tf-TR and unlabeled EGF for 20 minutes. Cells were visualized by confocal fluorescence microscopy.

GFP-AH for products of PI 3-kinases. This redistribution upon PI 3-kinase inhibition was also observed in cells that exhibited GFP-AH prelocalization, whereas treatment with 0.2% DMSO alone did not affect the localization of GFP-AH in EGF-stimulated cells (results not shown). Finally, GFP-AH failed to colocalize with Tf-TR-containing vesicles in NR6 WT cells stimulated with unlabeled EGF, while plasma membrane translocation was clearly observed (Fig. 7D). We conclude that EGF receptors do not elicit significant PI 3-kinase-mediated phosphorylation of PtdIns(4,5)P_2 in endosomes. Taken together, our results strongly suggest that the lack of PtdIns(4,5)P_2 in intracellular trafficking compartments affects downstream signaling, because the lipid substrate is not available to either PLC or PI 3-kinase activities coordinated by internalized receptors.

Discussion

It was previously speculated that silencing of the PLCγ signaling pathway upon EGF receptor internalization in NR6 fibroblasts is caused by the absence of cytosol-accessible PtdIns(4,5)P_2 in early endosomes. In support of this hypothesis, we found that SH2 domains, but not the PtdIns(4,5)P_2-specific PLCδ1 PH domain, associate with EGF-containing intracellular vesicles in this cell line, as well as in A431 cells. Using labeled transferrin, we confirmed that these compartments were endocytic structures. These findings are consistent with recent reports that GFP-tagged PLCγ1 and PLCγ2 are recruited to endocytic compartments in EGF-stimulated A431 cells (Matsuda et al., 2001; Wang et al., 2001); these compartments were not noticeably labeled by anti-PtdIns(4,5)P_2 antibodies in fixed cells (Matsuda et al., 2001). Furthermore, we found that the PLCδ1 PH domain fails to colocalize with labeled transferrin in unstimulated fibroblasts, indicating that the lack of endosomal PtdIns(4,5)P_2 was not caused by its consumption in response to EGF stimulation. This suggests that signaling elicited by other internalized receptors would also be affected by the constitutive absence of accessible PtdIns(4,5)P_2 in endosomes and that signaling through enzymes other than PLCγ could be abrogated upon EGF receptor internalization. Indeed, our experiments demonstrated that generation of PtdIns(3,4,5)P_3 through the PI 3-kinase pathway is not significant in endosomes.

We hypothesize that signals regulated at the level of substrate availability control highly localized cell responses. For example, a relevant function of such a signal would be in spatial sensing, with localized modification of the cytoskeleton leading to biased cell migration. Both PLC and type I PI 3-kinase activities play key roles in coordinating random cell motility and chemotaxis, with both pathways acting at least partially through the local modulation of actin dynamics. PLC action results in, among other signals, the mobilization of PtdIns(4,5)P_2-binding actin-modifying proteins, whereas PI 3-kinase products lead to activation of Rho family GTPases responsible for formation of lamellipodia and filopodia (Ridley, 2001; Wells et al., 1998). More direct roles of PtdIns(4,5)P_2 in mediating cell motility include functions in cytoskeletal attachment and actin polymerization (Higgs and Pollard, 2000; Raucher et al., 2000; Rohatgi et al., 2000). PtdIns(4,5)P_2 also plays a significant role in membrane trafficking itself, recruiting AP2 and dynamin, which regulate the assembly of clathrin-coated membrane pits and formation of endocytic vesicles, respectively (Jost et al., 1998; Martin, 2001). In general, the restricted localization of PtdIns(4,5)P_2 could provide a basis for the targeting of regulatory proteins to the plasma membrane, rather than to internal compartments of the cell where receptors remain activated. In line with this

hypothesis, PtdIns(4,5)P_2 has no known function in endosomes. If such a function is revealed, an alternative mechanism for the specific targeting of PtdIns(4,5)P_2- and PtdIns(3,4,5)P_3-binding proteins would need to be offered.

How is PtdIns(4,5)P_2 spatially restricted from early endosomes? The absence of PtdIns(4,5)P_2 in endosomes is consistent with the kinetic-trapping mechanism, in which the rate of consumption of PtdIns(4,5)P_2 at the plasma membrane is much faster than its rate of distribution to internal membranes (Batty et al., 1998). A second, possibly related concept, is the concentration of accessible PtdIns(4,5)P_2 in low density lipid microdomains (Pike and Casey, 1996), which may be excluded from bulk membrane endocytosis. However, depletion of cholesterol from the plasma membrane, which delocalizes PtdIns(4,5)P_2 from these microdomains (Pike and Miller, 1998), failed to induce colocalization of GFP-PH and internalized EGF-TR in our system (results not shown), suggesting that perhaps another mechanism is responsible for the restricted localization of PtdIns(4,5)P_2. Indeed, the fact that PtdIns(4,5)P_2 is intimately and constitutively involved in formation of endocytic vesicles implies the presence of an internalizing PtdIns(4,5)P_2 pool. If PtdIns(4,5)P_2 is concentrated in clathrin-coated pits, we hypothesize that this lipid is depleted prior to the delivery of membrane to sorting endosomes. The enrichment of PtdIns 5-phosphatase activity in coated vesicles suggests that clearance of PtdIns(4,5)P_2 may be important for dissociation of the clathrin coat (De Camilli et al., 1996; Malecz et al., 2000); incidentally, such a clearance mechanism could account for the detectable rate of PtdIns(4,5)P_2 turnover in unstimulated cells (Willars et al., 1998). Thus, tight spatial regulation of PtdIns(4,5)P_2 levels could control both assembly and disassembly of the endocytic machinery and prevent appreciable incorporation of PtdIns(4,5)P_2 into early endosomes, maintaining the ability of the cell to spatially address molecules to the plasma membrane for crucial signaling and regulatory functions.

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