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APPL Proteins Link Rab5 to Nuclear Signal Transduction via an Endosomal Compartment

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Summary

Signals generated in response to extracellular stimuli at the plasma membrane are transmitted through cytoplasmic transduction cascades to the nucleus. We report the identification of a pathway directly linking the small GTPase Rab5, a key regulator of endocytosis, to signal transduction and mitogenesis. This pathway operates via APPL1 and APPL2, two Rab5 effectors, which reside on a subpopulation of endosomes. In response to extracellular stimuli such as EGF and oxidative stress, APPL1 translocates from the membranes to the nucleus where it interacts with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1, an established regulator of chromatin structure and gene expression. Both APPL1 and APPL2 are essential for cell proliferation and their function requires Rab5 binding. Our findings identify an endosomal compartment bearing Rab5 and APPL proteins as an intermediate in signaling between the plasma membrane and the nucleus.

Introduction

In response to extracellular stimuli cells activate an intricate network of signaling cascades. In the traditional view, signal transduction is initiated at the plasma membrane and, via a series of protein-protein interactions and kinase cascades, transmitted through the cytoplasm to the nucleus where gene expression is modulated. In this model, endocytosis is considered merely as a mechanism for signal termination by downregulation of receptors activated at the plasma membrane and their degradation in the lysosomes. The idea that endosomes can perform a signaling function received support by studies of NGF action in neurons (Grimes et al., 1996). More recently, an increasing number of proteins have been shown to form structurally and functionally distinct signaling complexes with activated receptors along their intracellular itinerary through various endocytic compartments (Di Fiore and De Camilli, 2001; McPherson et al., 2001; Sorkin and Von Zastrow, 2002; Wunderlich et al., 2001). These findings suggest that trafficking through endosomes may play a more active role in the initiation, propagation, and termination of signals than previously anticipated. To which extent, however, endosomes participate in signal transduction remains to be established also in view of other studies arguing against such a role (Johannessen et al., 2000; Kao et al., 1998; Lu et al., 2002). On the other hand, there is compelling evidence that signaling pathways can modulate the endocytosis machinery, as exemplified by the recently uncovered functional connections between the small GTPase Rab5 and signaling molecules (Barbieri et al., 2000; Lanzetti et al., 2000; Tall et al., 2001). Rab5 is a key regulator of transport from the plasma membrane to the early endosomes. Continuous cycles of GDP/GTP exchange and hydrolysis regulate the kinetics of constitutive endocytosis (Rybin et al., 1996) but this nucleotide cycle can also be modulated by extracellular stimuli. Stimulation by EGF enhances the rate of endocytic membrane flow (Barbieri et al., 2000) by increasing the fraction of active Rab5 through stimulation of the Rab5 guanine nucleotide exchange factor (GEF) RIN1 (Tall et al., 2001) and downregulation of the GTPase-activating protein (GAP) RN-tre (Lanzetti et al., 2000). Beside regulating receptor internalization (Lanzetti et al., 2000), RN-tre is also integrated into the EGF signaling pathway via its interactions with the EGF receptor (EGFR) substrate Eps8 and the adaptor protein Grb2, which links EGFR to mSos, a GEF for Ras (Lanzetti et al., 2000; Martinu et al., 2002).

The molecular principles underlying the structural and functional organization of early endosomes are also intimately linked to the function of signaling molecules. On the early endosomes, Rab5 regulates the membrane recruitment and activity of a wide range of downstream effectors (Bucci et al., 1992; Christoforidis et al., 1999a; Rubino et al., 2000), such as Rabaptin-5α/5β/Rabex-5, EEA1, Rabenosyn-5/hVPS45, and phosphatidylinositol-3 kinases (PI(3)Ks) p110β/p85α and hVPS34/p150, which act cooperatively in vesicle tethering, SNARE priming, and endosome motility along microtubules (Christoforidis et al., 1999b; Lippe et al., 2001; Nielsen et al., 2000; Simonsen et al., 1998). Based on these data, Rab5 has been proposed to organize a domain on the early endo-

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somes which is enriched in phosphatidylinositol 3-phosphate (PI(3)P) and a set of PI(3)P binding effectors (Christoforidis et al., 1999b; Zerial and McBride, 2001). PI(3)P is also required for the endosomal localization of various signaling molecules, such as a component of the TGF-β pathway SARA (Smad anchor for receptor activation) (Hayes et al., 2002; Tsukazaki et al., 1998). Intriguingly, dominant-negative mutant of Rab5 affects TGF-β/activin signal transduction in endothelial cells by an as yet unknown mechanism (Panopoulou et al., 2002).

While these examples suggest a link between Rab5 and intracellular signaling, it remains open whether components of the Rab5 machinery and the endocytic organelles harboring them are required for signal transduction. Furthermore, whether canonical early endosomes are the only organelles involved in signal transduction or whether specialized compartments devoted to signaling exist are open questions. Here, we describe a pathway propagating signals from the cell surface to the nucleus involving two previously uncharacterized Rab5 effectors residing on an endosomal compartment.

Results

Identification of Two Rab5 Effectors

In a search for new Rab5 effectors, mass spectrometry analysis revealed that one of the most abundant proteins (~80 kDa) affinity purified on a GST-Rab5:GTPγS column (Figure 1A; Christoforidis et al., 1999a) corresponded to APPL (*A*daptor protein containing *P*H domain, *P*TB domain and *L*eucine zipper motif; accession number AF169797; Figure 1B), a 709 amino acid protein previously identified in a two-hybrid screen as an interacting partner of the AKT2/PKBβ kinase and putative adaptor tethering inactive AKT2 to PI(3)K p110α (Mitsuuchi et al., 1999). Another two-hybrid screen described APPL (therein referred to as DIP13α) as an interactor of the tumor suppressor DCC (deleted in colorectal cancer) and a mediator of DCC-induced apoptosis (Liu et al., 2002). Further sequencing of the GST-Rab5:GTPγS eluate from HeLa cytosol revealed a protein of 664 amino acids (recently named DIP13β, accession number NM_018171), sharing 54% identity and the same domain organization with APPL (Figure 1B). The two proteins, referred here as APPL1 and APPL2, are encoded by genes on human chromosomes 3 and 12, respectively. A potential nuclear localization signal on APPL2 (^{151}PKKKENE^{157}) but not on APPL1 was detected by PSORT II program (Nakai and Horton, 1999). Furthermore, by SMART analysis (Schultz et al., 1998) we identified a BAR domain (*B*IN1/*A*mphiphysin/*R*VS167; Sakamuro et al., 1996) at the N terminus of APPL1 (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/116/3/445/DC1). Given the relatively high homology between APPL1 and APPL2 in this region (54% identity and 74% similarity), APPL2 is assumed to contain a BAR domain. Interestingly, PSI-Blast searches (Altschul et al., 1997) with the BAR domain of APPL1 or APPL2, as well as structural predictions using 3D-PSSM (Fischer et al., 1999) indicate that the BAR domain is distantly related to arfaptins, which bind ARF and Rac GTPases (Supplemental Figure S1 available on *Cell* website; Tarricone et al., 2001; Van Aelst et al., 1996).

To test whether the interaction with Rab5 is direct and specific, we cloned and in vitro translated both APPL proteins to measure their ability to bind various recombinant GST-tagged Rab proteins. Both APPL1 and APPL2 strongly bound Rab5:GTPγS but neither Rab5:GDP nor Rab4, Rab7, or Rab11 (Figure 1C), indicating that they are specific effectors of Rab5. To confirm that APPL1 and APPL2 colocalize with Rab5:GTP in vivo, we raised antibodies against the C-terminal peptides of both proteins which recognize endogenous levels of the corresponding antigens and do not exhibit any crossreactivity between them (see below). Both endogenous APPL1 (Figure 1E) and APPL2 (data not shown) accumulated on the enlarged endosomes induced by expression of the constitutively active Rab5Q79L mutant (Stenmark et al., 1994). Thus, APPL proteins specifically interact with Rab5:GTP in vitro and localize to membranes harboring this protein in vivo.

APPL1 and APPL2 Localize to Cytoplasmic Membranes

In contrast to other Rab5 effectors exhibiting a typical endosomal staining pattern, we were surprised to observe a more complex intracellular distribution of APPL1 and APPL2. In HeLa (Figure 1F), A431, and BHK (data not shown) cells, APPL1 localized to punctate structures dispersed in the cytoplasm but mostly concentrated underneath the plasma membrane. Similar structures were also labeled for APPL2 (Figure 1G). In addition, both proteins were present in the nucleus. Whereas APPL2 was particularly enriched in the nucleus with respect to the cytoplasmic structures, the intensity of the nuclear staining of APPL1 varied between individual cells.

Given the complexity of the staining pattern, it was essential to exclude antibody artifacts. Five lines of evidence validate the specificity of the staining. First, both anti-APPL1 and -APPL2 antibodies recognize single bands corresponding to the predicted protein size in HeLa cytosol by Western blot (Figure 1D). Second, the immunofluorescence staining was abolished upon preincubation of the antibodies with the respective peptide (data not shown). Third, knocking down both genes by siRNA drastically reduced or abolished both immunofluorescence and Western blot signals (Figure 7A and Supplemental Figure S2 available on *Cell* website). Fourth, myc epitope-tagged APPL1 and APPL2 colocalized with the endogenous proteins in the peripheral structures and on Rab5Q79L enlarged endosomes (Figure 2A and data not shown). Fifth, the presence of APPL1 in a purified nuclear fraction was determined by Western blot analysis (Figure 6A) and this signal was reduced by siRNA treatment (data not shown). Having established the authenticity of the staining pattern, we analyzed the APPL-positive peripheral structures in more detail. Endogenous APPL1 largely colocalized with myc-APPL2 in the same structures (Figure 2A), which were also positive for GFP-Rab5 (Figure 2B). Surprisingly, little overlap (below 11%) between APPL1 and EEA1, a marker of canonical early endosomes (Figure 2C) was observed and, in contrast to EEA1 (Simonsen et al., 1998), immunodepletion of both APPL1 and APPL2 from HeLa cytosol did not inhibit heterotypic and homotypic early endosome fusion (data not shown). Lack of APPL1 colocalization with caveolin1-GFP (Pelkmans et al.,

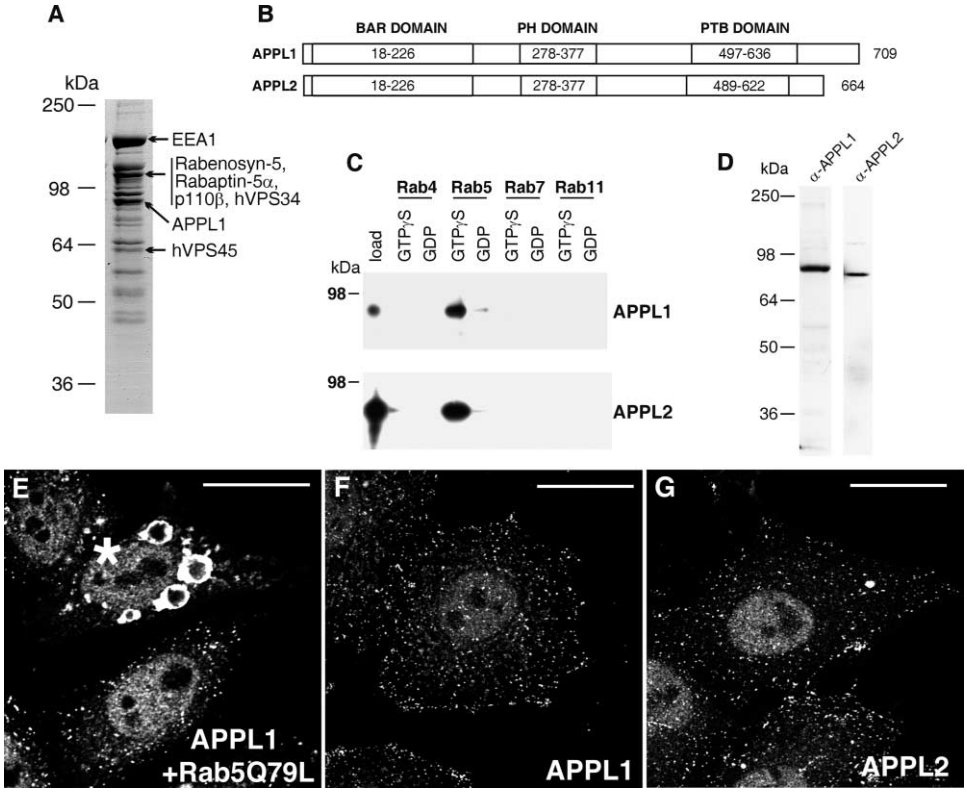

Figure 1. APPL1 and APPL2 Are Rab5 Effectors

(A) Coomassie-stained cytosolic proteins interacting specifically with Rab5:GTPγS.

(B) Domain structure of APPL1 and APPL2 proteins.

(C) APPL1 and APPL2 interact specifically with Rab5:GTPγS. In vitro translated, [^{35}S]methionine labeled APPL proteins were incubated with beads loaded with GST-Rab proteins in the GDP or GTPγS forms; bound proteins were analyzed by SDS-PAGE and autoradiography.

(D) Peptide antibodies to APPL1 and APPL2 recognize single bands in HeLa cytosol by Western blot.

(E) Endogenous APPL1 localizes to Rab5Q79L-enlarged endosomes in vivo. HeLa cells were transfected with Rab5Q79L and stained with antibodies to APPL1. The transfected cell is indicated with an asterisk.

(F and G) Distribution of endogenous APPL1 and APPL2 in HeLa cells, stained with specific antibodies as indicated. Individual confocal sections are shown in (E–G). Scale bar is equal to 20 μm.

2001; Figure 2D) and α-adaptin (data not shown) eliminated the possibility that APPL-positive structures corresponded to caveolae/caveosomes and clathrin-coated vesicles, respectively.

To confirm that the punctate pattern of APPL1 represented membrane structures and not some proteinaceous particles, we performed immunoelectron microscopy on frozen sections. Specific labeling for APPL1 was associated with both tubular and vesicular membrane structures generally in close proximity to the plasma membrane (Figure 3). These data clearly establish that the APPL-labeled structures are membrane bound, consistent with the fact that APPL1 was detectable in preparations of endosomal membrane isolated by floatation on density gradients (data not shown). The lack of colocalization with EEA1 observed by immunofluorescence microscopy raises the possibility that these structures may be different subcompartments of early endosomes or even a distinct type of endosomes, a possibility that requires further investigation.

EGF Is Internalized into APPL Structures and Causes APPL1 Redistribution

We next set out to determine whether the APPL structures are accessible to endocytic cargo internalized either via receptor-mediated (transferrin) or fluid-phase endocytosis (dextran). We observed only a low degree (below 10%) of APPL1 colocalization with internalized transferrin (Figure 4A) and no significant labeling with dextran (data not shown) at any time point, arguing that APPL-positive structures are not pinosomes. Given that the machineries responsible for constitutive (transferrin) and ligand-induced (growth factors) endocytosis can be differentially regulated (Di Fiore and De Camilli, 2001), we tested whether rhodamine-labeled EGF (Rh-EGF) could access APPL structures. Cells were serum-starved overnight and Rh-EGF was internalized for 5, 15, or 30 min in order to progressively label clathrin-coated vesicles, early endosomes, and late endosomes/lysosomes. Unexpectedly, we observed that the APPL1 distribution changed dramatically upon serum starvation and EGF stimulation (Figure 4B). In serum-starved cells, APPL1 was restricted to the punctate structures in cytosol and absent from the nucleus. In sharp contrast, upon treatment of cells with Rh-EGF for 5 min, APPL1 partly translocated from the peripheral structures to the cytoplasm, became particularly enriched on the nuclear envelope and began to appear in the nucleus. Within 15 min of Rh-EGF treatment, APPL1 shifted from the cytoplasm to the nucleus and after 30 min its accumulation in the

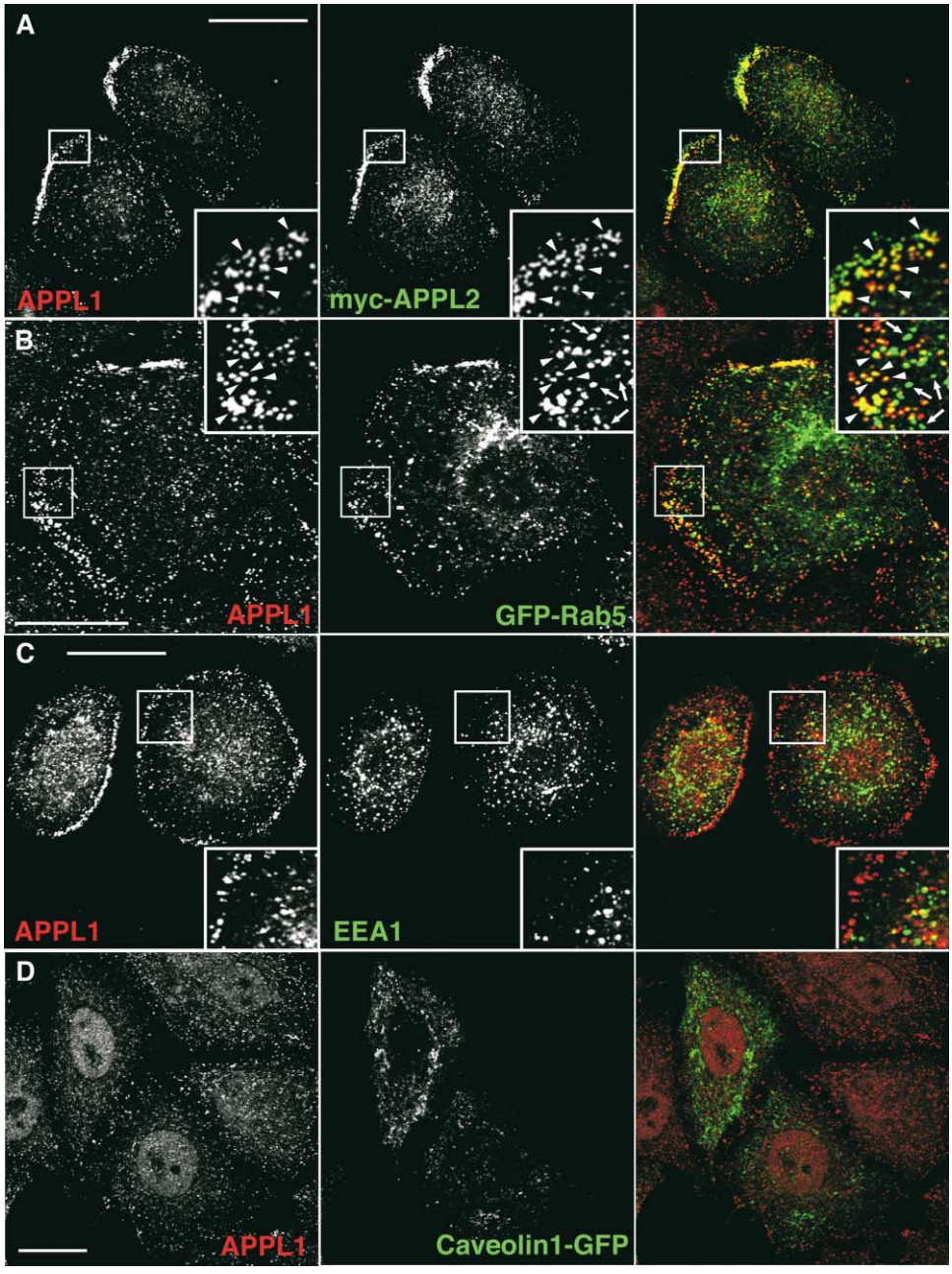

Figure 2. Morphological Characterization of Intracellular Structures Labeled by APPL Proteins

APPL1 and APPL2 colocalize with each other (A) and Rab5 (B) but not EEA1 (C) or caveolin (D) in peripheral punctate structures. HeLa cells were transfected with the indicated plasmids, fixed and stained with antibodies to APPL1, myc, or EEA1. Arrowheads in (A) and (B) indicate the structures shared between APPL1 and myc-APPL2 or Rab5, respectively. Arrows in (B) mark the Rab5-positive structures, which do not contain APPL1. Individual confocal sections are shown. Scale bar is equal to 20 μm.

nucleus subsided and the typical membrane labeling reappeared. The response of APPL1 to EGF indeed correlated with the accessibility of APPL1-positive membranes to this growth factor (Figure 4C). After 5 min of internalization, a fraction of Rh-EGF (between 5%–28%) localized to fine punctate structures harboring APPL1 (the extent of colocalization varied depending on the degree of APPL1 solubilization). At this point, the majority of Rh-EGF (70%–90%) was present in EEA1-positive early endosomes and, presumably, EEA1-negative clathrin-coated vesicles (Rubino et al., 2000). At 15 min, Rh-EGF appeared in EEA1-containing early endosomes (Figure 5A) that expanded in size as shown previously (Barbieri et al., 2000) and colocalization with APPL1 was no longer detectable. These data illustrate two main points. First, the APPL structures are endosomes accessible to cargo such as EGF, but poorly if any, transferrin. Second, APPL1 undergoes regulated cycles of redistribution between endosomes and the nucleus in response to EGF. Unfortunately, the exclusion

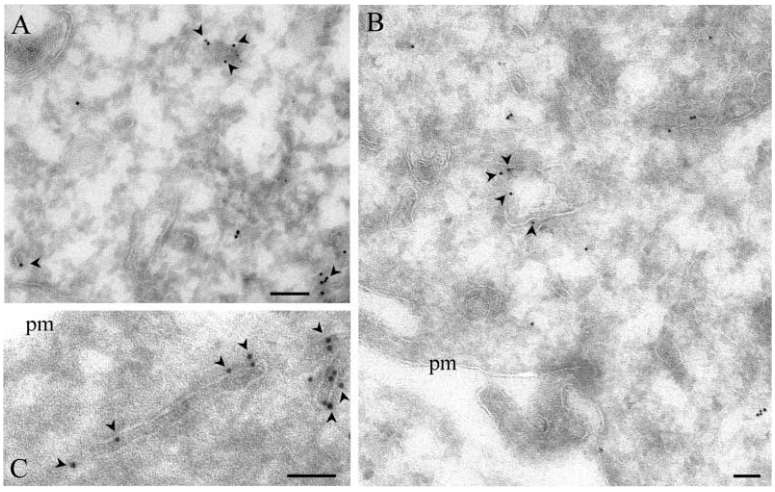

Figure 3. Electron Microscopic Localization of Endogenous APPL1

Serum-starved A431 cells were fixed with paraformaldehyde and processed for frozen sectioning. Sections were labeled with antibodies to APPL1 followed by 10 nm protein A-gold. Specific labeling (arrowheads) is associated with membrane structures that vary in morphology, predominantly located close to the plasma membrane (pm). The figure shows a gallery of representative labeled structures, which include small vesicles (A), vacuolar structures (B) and tubular profiles (C). Scale bar is equal to 100 nm.

of GFP-labeled APPL from the nucleus prevented the possibility to capture this interesting process by video microscopy.

We next tested whether the APPL1 cycle in response to EGF depended on dynamin by performing Rh-EGF uptake in cells expressing a dominant-negative mutant (K44A) of dynamin II (Vieira et al., 1996). Strikingly, although dynamin^{K44A} blocked the transport of EGF into early and late endosomes, as evidenced by the lack of enlarged endosomes labeled with Rh-EGF (Figure 5,

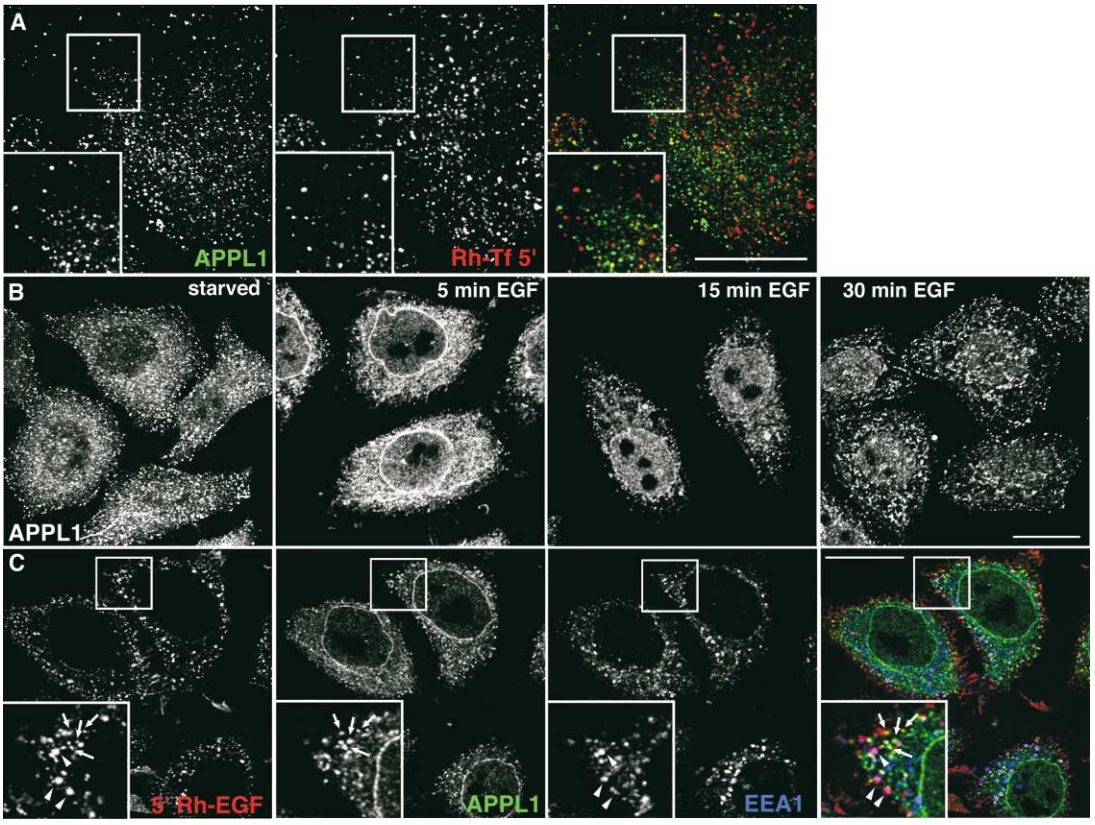

Figure 4. EGF, but Not Transferrin, Is Internalized into APPL Structures and Causes APPL1 Redistribution

(A) HeLa cells were serum-starved for 1 hr and incubated with 30 μg/ml of rhodamine-transferrin (Rh-Tf) for 5 min at 37°C, fixed, and stained with antibodies to APPL1. The degree of colocalization between APPL1 and Rh-Tf was not increased upon longer internalization times (30 min).

(B) HeLa cells were serum-starved overnight and incubated with 1 μg/ml Rh-EGF for 5, 15, or 30 min at 37°C, fixed, and stained with anti-APPL1 antibodies.

(C) HeLa cells were treated with Rh-EGF for 5 min, fixed, and stained with antibodies to APPL1 or EEA1. Arrowheads in (C) indicate the structures labeled by EGF and EEA1, while arrows indicate the EGF- and APPL1-positive membranes. Individual confocal sections are shown. Scale bar is equal to 20 μm.

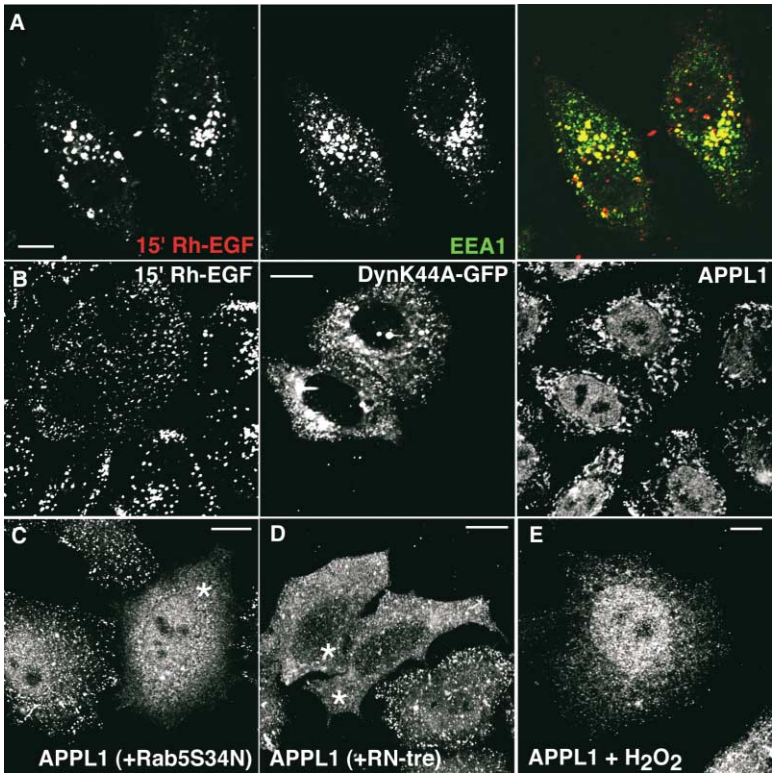

Figure 5. Release of APPL1 from Membranes Is Dependent on Rab5:GTP but Not Dynamin

(A–B) Control HeLa cells (A) or cells transfected with dynamin^{K44A}-GFP (B) were serum-starved overnight, incubated with Rh-EGF for 15 min at 37°C, fixed, and stained with antibodies to EEA1 or APPL1, as indicated.

(C–E) APPL proteins associate with the membrane in a Rab5-dependent manner.

(C and D) HeLa cells were transfected with Rab5S34N or RN-tre, fixed, and stained with antibodies to APPL1. The transfected cells are indicated with an asterisk.

(E) HeLa cells were treated with 1 mM H_2O_2 for 15 min, fixed, and stained with antibodies to APPL1. Scale bar is equal to 10 μm.

compare A with B, 15 min Rh-EGF), a fine punctate labeling of EGF resembling the APPL1 staining and underlying plasma membrane was observed (Figure 5B). Importantly, dynamin^{K44A} did not affect the translocation of APPL1 to the nucleus (Figure 5B). In contrast, blocking EGF endocytosis at 4°C prevented release of APPL1 and its nuclear translocation (data not shown), suggesting that APPL signaling requires EGF internalization and is not triggered by EGF binding to its receptor on the surface or deeply invaginated pits (Vieira et al., 1996). Collectively, EGF internalization into APPL-positive endosomes and EGF-dependent release of APPL1 from them appears to operate dynamin independently.

GTP Hydrolysis by Rab5 Releases APPL1 from Endocytic Structures in Response to Extracellular Stimuli

By which mechanism is APPL1 released from endosomes? GTP hydrolysis on Rab5 could potentially disengage APPL1 from the membranes since APPL binding to Rab5 is GTP-dependent. We tested this possibility in three ways. First, a dramatic redistribution of endogenous APPL1 (Figure 5C) from the membranes to the cytosol was observed in cells expressing Rab5S34N, a mutant preferentially GDP bound. Second, overexpression of the Rab5 GAP RN-tre (Lanzetti et al., 2000) caused a substantial displacement of APPL1 from the peripheral structures, consistent with the reduction of the pool of active Rab5 in these cells (Figure 5D). Third, we took advantage of the fact that under oxidative stress, phosphorylation of RabGDI by p38MAPK results in extraction of Rab5 and its effector EEA1 from the early endosomes (Cavalli et al., 2001). Consistently, upon treatment of HeLa cells with H_2O_2 for 15 min, we observed a progressive loss of APPL1 from the peripheral structures and its accumulation in the nucleus (Figure 5E). These results provide strong evidence that membrane localization of APPL1 depends primarily on the active Rab5 and is sensitive to GTP hydrolysis or reduction in Rab5:GTP levels. Moreover, similar to EGF stimulation, oxidative stress is another signaling pathway that relocates APPL1 to the nucleus.

APPL Proteins Interact with Components of Nucleosome Remodeling and Histone Deacetylase Complex NuRD/MeCP1 and Are Required for Cell Proliferation

We used subcellular fractionation to verify that a pool of APPL1 is present in the nucleus and is dynamically modulated by signaling molecules, in agreement with the immunofluorescence experiments. Purified nuclei devoid of endosomal contaminants, e.g., Rab5 or EEA1, were found to contain APPL1 and this pool was reduced upon serum deprivation (Figure 6A). To gain further insights into the function of APPL1, we undertook a search for interacting partners by coimmunoprecipitation experiments from cytosol and detergent extracts of HeLa cells. Whereas no proteins were coimmunoprecipitated with APPL1 from cytosol (data not shown), a number of proteins were recovered from the detergent extract (Figure 6B). Surprisingly, mass spectrometry sequencing revealed the presence of PID/MTA2, p66, HDAC1, and/or HDAC2 (identified through common peptides) RbAp46, RbAp48, and MBD3, namely 6 out of 10 components of the nucleosome remodeling and histone deacetylase NuRD/MeCP1 complex (Feng and Zhang,

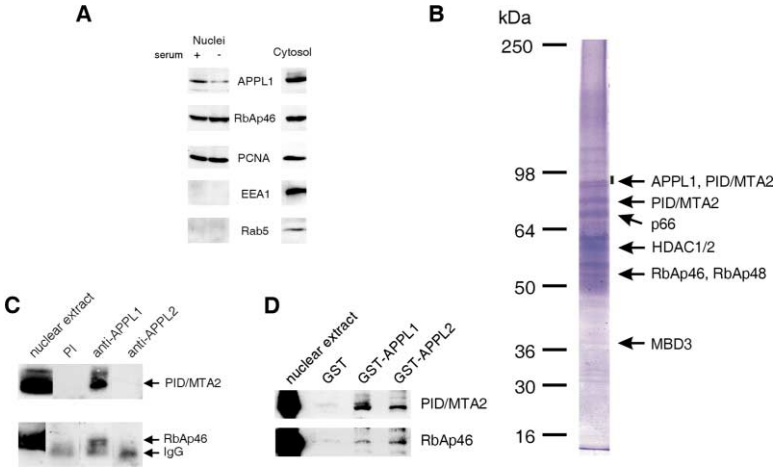

Figure 6. APPL Proteins Interact with the Components of the Nucleosome Remodeling and Histone Deacetylase Complex NuRD/MeCP1

(A) A pool of APPL1 is present in the nucleus and is decreased in serum-starved cells. Nuclei were prepared from HeLa cells grown with or without serum and analyzed for the presence of APPL1 and indicated markers by Western blot (load of 100 μg total protein). Cytosol from serum-grown HeLa cells (100 μg) was loaded as a control.

(B) Coomassie-stained proteins coimmunoprecipitated from HeLa detergent extracts by antibody to APPL1.

(C) Western blot detection of PID/MTA2 and RbAp46 immunoprecipitated from HeLa nuclear extracts by antibodies to APPL1 and APPL2 and a preimmune (PI) serum.

(D) GST pulldown of proteins interacting with APPL1 and APPL2. HeLa nuclear extracts were incubated with the beads loaded with GST alone or fused to APPL1 or APPL2. PID/MTA2 and RbAp46 retained on the columns were detected by Western blot.

2001). Given the reported nuclear localization of the interacting proteins and of APPL1 (Figure 6A), we confirmed the specificity of the coimmunoprecipitation using HeLa nuclear extracts. Western blot analysis (Figure 6C) showed that antibodies to APPL1 but not preimmune serum efficiently and specifically coimmunoprecipitated PID/MTA2 protein and, to a lesser extent, also RbAp46. We could not observe a similar interaction using APPL2 antibodies presumably due to their low efficiency in immunoprecipitation. We furthermore confirmed these interactions by GST pull-down experiments applying nuclear extracts to columns with immobilized GST alone or fused to APPL proteins (Figure 6D). PID/MTA2 and RbAp46 were specifically bound to GST-APPL1 but not GST alone. Interestingly, we also recovered these proteins on the GST-APPL2 column, suggesting that both APPL proteins can interact with the components of NuRD/MeCP1 in the nucleus.

Histone deacetylase activities are required for cell cycle progression and development (Ahringer, 2000; Yoshida et al., 1995). The identification of the NuRD/MeCP1 complex as a binding partner together with the nuclear localization of APPL1 and APPL2 prompted us to investigate their function with respect to cell proliferation. We assayed DNA synthesis under downregulation of endogenous APPL proteins by RNA interference (Elbashir et al., 2001). Forty-eight hours after transfecting the cells with small interfering RNA oligonucleotides specific for APPL1 or APPL2, we observed a pronounced reduction in protein levels of APPL1 and/or APPL2, as evidenced by Western blot (Figure 7A) and immunofluorescence analysis (Supplemental Figure S2 available on *Cell* website). Strikingly, by measuring BrdU incorporation we observed that knockdown of either APPL1 or APPL2 resulted in a 50% reduction in the number of cells entering S phase in comparison with control cells (mock-treated or transfected with unrelated siRNA; Figure 7B). The inhibitory effects on DNA synthesis elicited by knockdown of either APPL1 or APPL2 were not additive (Figure 7B), arguing that the two proteins cannot substitute for each other. No increase in cell death was evident under these conditions, as determined by Trypan blue staining. Flow cytometric analysis of DNA content indicated that knockdown of APPL proteins did not elicit a specific cell cycle arrest (Figure 7C). Collectively, the interaction with the NuRD/MeCP1 complex together with the effects on DNA replication suggest that APPL proteins exhibit essential functions in a signaling pathway leading to cell proliferation.

Binding to Rab5 Is Indispensable for the Functional Cycle of APPL1

We further wished to test whether the interaction between Rab5 and APPL is critical for the regulation of cell proliferation. We first conducted deletion mutagenesis and in vitro binding studies to identify sequences engaged in Rab5 binding on the APPL1 molecule (Figure 7D). Based on the homology of the BAR domain to arfaptins we focused on this region of APPL1 as the potential binding site. Strikingly, the presence of both BAR and PH domains (residues 1–428) was necessary for binding to Rab5:GTP, suggesting that one domain may stabilize the other or both may cooperatively bind Rab5. In contrast to some PH domains that can bind specific membranes due to high affinity for certain phosphoinositides (Dowler et al., 2000), the PH domain of APPL1 was not sufficient for membrane targeting. Remarkably, when overexpressed in vivo as fluorescently tagged proteins, only mutants capable of interacting with Rab5 exhibited membrane localization, underscoring the function of Rab5 as a primary determinant of APPL localization to the endosomes (Figure 7D).

We further investigated the effect of the truncation mutants on DNA synthesis, as measured by BrdU incorporation (Figure 7D). While the overexpressed wild-type or APPL1 mutants capable of Rab5 binding (Δ532−709, Δ429−709) did not affect the rate of DNA synthesis, all mutants unable to interact with Rab5 elicited some inhibitory effects on this process. In particular, the expression of APPL1Δ1-272 lacking the BAR domain and unable to bind Rab5 completely blocked BrdU incorporation. Since the mutant protein accumulated in the cytosol and was excluded from the nucleus, its antiproliferative activity in all likelihood depends on the seques-

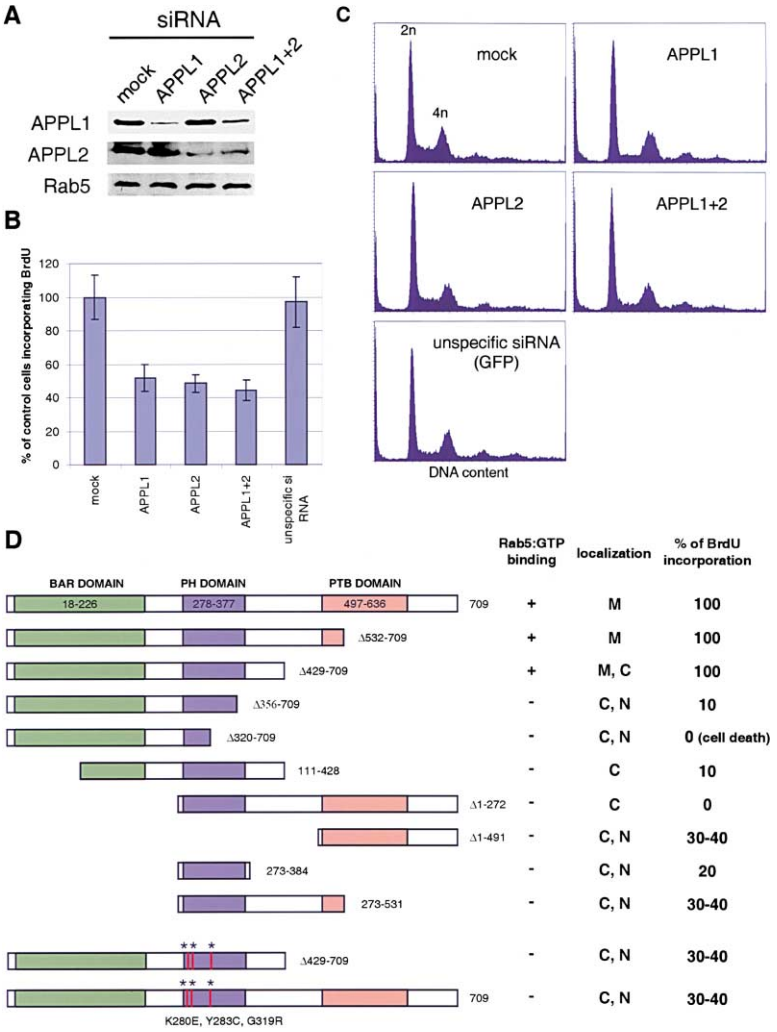

Figure 7. APPL1 and APPL2 Are Required for Cell Proliferation and Rab5 Binding Is Essential for Their Function

(A) Reduced levels of APPL1 and APPL2 48 hr after transfecting HeLa cells with siRNA oligos, as detected by Western blot.

(B) Histogram showing the percentage of cells incorporating BrdU (1 hr pulse) 48 hr after transfection with siRNA oligos. Typically, about 50%–60% of control cells showed BrdU incorporation under these conditions.

(C) DNA content of cells 48 hr after transfection with siRNA oligos measured by flow cytometry.

(D) Schematic representation of APPL1 mutants. Rab5 binding was assayed biochemically as in Figure 1C. All mutants were stably expressed in reticulocyte lysate. Intracellular localization was tested by transfecting YFP fusion constructs in HeLa cells (N, nucleus; C, cytosol; M, cytosolic membranes). Percentage of BrdU incorporation was determined in cells transfected with YFP fusion constructs (BrdU incorporation in cells transfected with YFP alone was set to 100%).

tration of yet unidentified soluble factors acting prior to nuclear import of APPL1. Moreover, expression of APPL1Δ320-705 comprising the BAR domain caused increased cell death, indicating that interference with the activity of APPL may induce a proapoptotic effect.

To avoid a possibility that, in addition to Rab5 binding, other APPL1 functions have been affected by introducing large domain deletions, we performed a random mutagenesis on the residues 1–428 of APPL1 (BAR and PH domains) selecting for point mutations abolishing interaction with Rab5 in vitro and membrane localization in vivo. A mutant fulfilling these criteria carried 3 amino acid changes within the PH domain (K280E, Y283C, G319R). Assuming that the PH domain of APPL1 adopts the same fold as the crystallized RhoGEF Tiam1 (Worthylake et al., 2000), these mutations are unlikely to disrupt the structural core of the domain, being located in loop regions possibly involved in intra- or intermolecular interactions. Similarly to the truncated proteins unable to interact with Rab5, both the APPL1 and APPL1Δ429−709 triple point mutants remained cytosolic, did not localize to Rab5Q79L enlarged endosomes and elicited a dominant-negative inhibition of BrdU incorporation (Figure 7D). Collectively, these data suggest that (1) binding to Rab5 and (2) cytoplasmic interactions following the release from endosomes constitute essential steps of the functional cycle of the APPL1 protein.

Discussion

We report here the discovery of a previously unidentified signal transduction pathway between the plasma membrane and the nucleus. First, this pathway involves two new Rab5 effectors, APPL1 and APPL2, that are localized to an endocytic compartment, which receives low amounts of internalized cargo such as EGF suggesting a specific role in signaling rather than housekeeping endocytosis. Second, following EGF internalization APPL1 is released from the membrane and translocates to the nucleus. Third, this release depends on the GTPase cycle of Rab5, an established regulator of endocytosis. Fourth, both APPL1 and APPL2 proteins interact with components of the nucleosome remodeling and histone deacetylase complex NuRD/MeCP1 and are required for efficient cell proliferation. Fifth, mutagenesis analysis indicates that the interaction of APPL1 with Rab5 is essential for the regulation of cell proliferation. Our data raise important implications concerning the

role of membrane compartmentalization and endocytic transport in signal transduction.

EGF Signaling Via APPL Endosomes

EGF uptake is traditionally a hallmark of clathrin-, dynamin- and Rab5-dependent endocytosis (Lanzetti et al., 2000; Vieira et al., 1996). The existence of an EGF entry route into an APPL-positive compartment indicates that this view is incomplete. Since only a minor pool of EGF is internalized into APPL structures, the physical sequestration of EGF in this subcompartment may fulfill a signaling role rather than ligand-receptor downregulation. Importantly, our data shed more light onto the seminal findings by Schmid and colleagues (Vieira et al., 1996), who reported an enhancement of EGF-dependent proliferation in cells where clathrin-mediated endocytosis was inhibited via the dominant-negative dynamin^{K44A} mutant. A residual EGF uptake (30%) was observed under these conditions. Our data suggest that at least a fraction of this pool is most likely internalized into APPL-positive endosomes. The data of Schmid and colleagues argue further that even if EGF signaling takes place on canonical early endosomes, it is dispensable for the mitogenic response. In contrast, we demonstrate that APPL-dependent signaling pathways are required for efficient cell proliferation, pointing to functional differences between signals emitted from various endosomal compartments. EGF elicits activation of intracellular signaling cascades through a subclass of high affinity receptors constituting only 5%–10% of the total EGFR pool (Defize et al., 1989). Since loss of dynamin function does not impair mitogenic signaling upon EGF stimulation (Vieira et al., 1996), an exciting possibility raised by our observations is that at least a fraction of the subclass of high affinity receptors may escape the dynamin block and signal via the Rab5-APPL-mediated signaling pathway.

Coupling the GTPase Cycle of Rab5 to Signal Transduction

Remarkably, our study indicates that the cell utilizes the simplest mechanism to couple the regulation of receptor trafficking to that of growth factor signaling: the shared GTPase switch of Rab5. We propose a model whereby such regulation is exploited both in time and space. Rab5 is present on distinct intracellular organelles: plasma membrane, clathrin-coated vesicles, and early endosomal compartments, such as EEA1-positive endosomes, where it recruits different sets of effectors (Bucci et al., 1992; Christoforidis et al., 1999b; Miaczynska and Zerial, 2002; Rubino et al., 2000). Upon EGF stimulation, Rab5 is activated at the plasma membrane and on EEA1-positive early endosomes, allowing for efficient EGF internalization and further transport to early endosomes, late endosomes, and lysosomes (Barbieri et al., 2000; Lanzetti et al., 2000). Stimulation of GTP hydrolysis by Rab5 is, in contrast, required for the release of APPL1 from the membrane. Our data imply that this dual regulation of the kinetics of the GTPase cycle exploits the physical separation of APPL from the canonical Rab5-, EEA1-, and PI(3)P-transport machinery. APPL proteins may be localized to a subcompartment of early endosomes, if not to a distinct population of Rab5-positive membranes. Such physical segregation would provide the advantage of independent regulation of the Rab5 GTP/GDP cycle in response to growth factors as compared with a single compartment. The Rab5 GEF RIN1 and the GAP RN-tre are subjected to regulation by EGF (Barbieri et al., 2000; Lanzetti et al., 2000; Tall et al., 2001) and, thus, these or some yet uncharacterized family members could regulate the Rab5 cycle on APPL endosomes. It is conceivable that the kinetics of the Rab5 nucleotide cycle may also determine the residence time of EGF in APPL endosomes. Following GTP hydrolysis, the level of Rab5:GTP must be reequilibrated to restore the localization of APPL proteins to the membrane within 30 min of EGF stimulation (Figure 4B). In addition, reactivation of Rab5 would enable clearance of EGF from the APPL endosomes and its further trafficking along the canonical endocytic route. This possibility is supported by (1) the finding that expression of Rab5Q79L relocates APPL proteins to enlarged endosomes, suggesting a mixing of the two compartments and (2) a certain degree of colocalization between APPL and EEA1 structures.

Our data uncover an essential role of APPL proteins in a pathway leading to cell proliferation. By which mechanisms could APPL proteins exert this function? Two important clues were provided by the observations that APPL proteins undergo nucleocytoplasmic shuttling and interact with the NuRD/MeCP1 complex. As histone deacetylase activities are required for cell cycle progression (Yoshida et al., 1995), APPL binding to NuRD/MeCP1 may serve the purpose of subjecting this function to regulation by extracellular signaling molecules. To our knowledge, there are no data linking the histone deacetylase/chromatin remodeling activities to endocytosis and our findings may indicate the first example of such regulation. Our data delineate a multistep process in which (1) the interaction with Rab5 followed by (2) the release from endosomes triggered by extracellular signals and (3) the import from cytoplasm to the nucleus constitutes crucial steps of the APPL1 cycle. Interestingly, the interaction with Rab5 appears to be part of a control mechanism to couple the release of APPL1 from the endosomes to growth factor signaling and trafficking. Our mutagenesis analysis in fact points at the Rab5-dependent localization being a prerequisite for downstream cytoplasmic interactions that are required for transmitting proliferative signals. This hypothesis is supported by the findings that all mutants unable to interact with Rab5 accumulated in cytosol and/or nucleus and exerted dominant-negative effects on DNA synthesis. These effects are most likely due to interference with the activity of endogenous APPL1 through sequestration of cytoplasmic and/or nuclear factors. Although our data point at the nucleus as a primary site of APPL-NuRD/MeCP1 complex interactions, we cannot exclude that these may also take place in the cytoplasm, as we observed significant cytoplasmic pools of PID/MTA2 and RbAp46 in addition to their nuclear localization (Figure 6A and data not shown), as also reported for PID/MTA2 (Humphrey et al., 2001). However, these components do not colocalize with APPL proteins on Rab5Q79L enlarged endosomes (data not shown), suggesting that Rab5-APPL and APPL-NuRD/MeCP1 binding might be mutually exclusive. Continuous rounds of binding of

APPL1 to Rab5 and dissociation from the membrane may be necessary to ensure the reversibility of interactions with downstream factors but also regulate their activity. For example, it is plausible that posttranslational modifications or conformational changes taking place on the Rab5-positive membranes may regulate the ability of APPL1 to functionally interact with other partners, e.g., the NuRD/MeCP1 complex, making cycling through endosomes an obligatory step in APPL signaling.

Cellular Functions of APPL Proteins

With the discovery of the interaction with Rab5 and the localization to endosomes, some of the earlier data on APPL1 will now have to be reexamined. Originally, APPL1 was shown to interact with the inactive form of the multifunctional antiapoptotic kinase AKT2 (Mitsuuchi et al., 1999). Since inactive AKT kinases are predominantly cytosolic, it is unlikely that AKT2 colocalizes with APPL proteins on endosomes. Another reported interactor of APPL1 is the tumor suppressor DCC, a plasma membrane receptor for an axon-guiding molecule netrin-1 (Keino-Masu et al., 1996; Liu et al., 2002). In the absence of ligand, DCC induces apoptosis via activation of caspase-3 and -9 in a process that requires APPL1 (Forcet et al., 2001; Liu et al., 2002). Neither the intracellular trafficking nor the ligand-dependence of the DCC-APPL1 interaction has been addressed, but an attractive possibility suggested by our work is that DCC could signal via APPL endosomes in neurons. Another exciting implication of our data concerns the possible link between APPL-mediated processes, such as DCC-induced apoptosis, to the action of p53, one of the substrates of NuRD/MeCP1. Activation of p53 induces either growth arrest or apoptosis, depending on the set of its transcriptional targets activated under various conditions (Vousden, 2000). In this context, it appears particularly interesting that deacetylation of p53 mediated by a direct interaction with PID/MTA2 (one of the most abundant proteins in the APPL1 immunoprecipitate) reduces its activity and apoptotic potential (Luo et al., 2000). Notably, the BAR domain of amphiphysinII/BIN1 has been shown to possess proapoptotic activity (Elliott et al., 2000) and we observed increased cell death upon overexpression of the BAR domain of APPL1 (Figure 7D).

We anticipate that the function of APPL proteins is not restricted to the response to a single growth factor (EGF) but that these proteins act at the crossroad of several signaling pathways. The observed release of APPL1 from the endosomes upon oxidative stress confirms this prediction. It is appealing to consider that the fraction of growth factors sorted into APPL- versus canonical early endosomes and the resulting differences in the quality of generated signals may be regulated depending on the cell type or developmental stage, as it is known that the same growth factor can elicit either proliferation or differentiation response in various cells (Yarden, 2001). Interestingly, an inhibition of DNA synthesis upon knocking down APPL proteins seems to be a more general phenomenon, as it was observed also in cell lines derived from colon (DLD-1) and breast (MDA-MB-231) cancer (our unpublished data). The observed APPL-NuRD/MeCP1 interaction suggests that signaling via APPL proteins could be directly linked to chromatin remodeling, a process of crucial importance in development. This view is supported by recent studies demonstrating that the components of *C. elegans* NuRD are required for embryonic viability, patterning, and Ras signaling (Ahringer, 2000; Lu and Horvitz, 1998; Unhavaithaya et al., 2002). APPL proteins do not have homologs in *C. elegans* or *Drosophila* but are present in all vertebrates and could play a signaling role during development, e.g., in axon guidance (Liu et al., 2002).

In summary, the identification of a signaling pathway involving the APPL proteins opens several exciting lines of investigation. The Rab5-dependent regulatory cycle of APPL proteins and their residence on an endosomal (sub)compartment raise possibilities for therapeutic intervention based on antiproliferative agents without affecting the housekeeping functions of the early endosomes. Further dissection of the function of the APPL proteins will likely bring unexpected insights into how ligand-stimulated, Rab5-mediated endocytosis can affect chromatin structure, gene expression, and apoptosis.

Experimental Procedures

Rab5 Affinity Chromatography, APPL Cloning, Antibody Production, and Random Mutagenesis

GST-Rab5 affinity chromatography with cytosol or in vitro translated proteins was performed as described (Christoforidis et al., 1999a, 1999b). APPL1 and APPL2 were cloned from human full-length adult leukocyte cDNA library (Invitrogen Life Technologies) and by RT-PCR from HeLa mRNA, respectively. Peptides SSSQSEESDLGEGG KKRESEA+C and NDQPDDDDGNPNEHRGA+C derived from the sequence of APPL1 and APPL2, respectively, were synthesized and injected into rabbits (Eurogentec, Belgium). Sera were affinity purified using peptides immobilized on Sulfolink beads (Pierce). Random mutagenesis was performed using Diversify PCR Kit (Clontech).

Protein Identification by Mass Spectrometry

Gel separated, Coomassie-stained proteins were excised from the gel slab and in-gel digested with trypsin as described (Shevchenko et al., 1996). Tryptic peptides were sequenced by nanoelectrospray tandem mass spectrometry on hybrid quadrupole time-of-flight mass spectrometers Q-TOF I (Micromass Ltd, Manchester, UK) and QSTAR Pulsar i (MDS Sciex, Concord, Canada) as described (Shevchenko et al., 1997). Database searching was performed by Mascot software (Matrix Science, Ltd., London).

Cell Culture, Transfections, Immunofluorescence, Immunoelectron Microscopy, Endosome Fusion Assay and BrdU Incorporation, and Flow Cytometry Analysis

HeLa and A431 cells were grown and immunofluorescence labeling was performed according to standard procedures. MetaMorph6.1r3 program (Universal Imaging Corp.) was used for quantifying the percentage of overlap between immunofluorescence signals. For transient expression studies, cells were transfected using FuGENE 6 (Roche) and analyzed 20 hr posttransfection. For immunoelectron microscopy, cells were processed for frozen sections as described (Luetterforst et al., 1999). BrdU incorporation was performed using Labeling and Detection Kit (Roche). Endosome fusion assay was performed as described (Horiuchi et al., 1997). Antibodies against PID/MTA2, RbAp46, and PCNA were obtained from Oncogene Research Products, Affinity BioReagents Inc. and BD Transduction Laboratories, respectively. Flow cytometry analysis was performed as described (Liu and Erikson, 2003).

siRNA Preparation and Transfection

Duplex siRNA (APPL1: 5′-CACACCUGACCUCAAAACUTT and 5′-AGUUUUGAGGUCAGGUGUGTT; APPL2: 5′-GUGGUGGAUGAGCU UAAUCTT and 5′-GAUUAAGCUCAUCCACCACTT) were purchased

from Proligo (Paris, France) and transfected using Oligofectamine (Invitrogen).

Preparation of Nuclei, Immunoprecipitation, and GST Pulldown

Nuclei from HeLa cells were prepared as described (Ryseck et al., 1989). For large-scale immunoprecipitation and GST pulldown sHeLa cells grown in suspension (4 l) were pelleted, broken in the lysis buffer (50 mM HEPES [pH 7.4], 150 mM KCl, 2 mM $MgCl_2$) by 10 passages through a cell cracker (EMBL, Heidelberg), and fractionated by centrifugation to obtain nuclei (4000 × g) and cytosol (100,000 × g). To produce total or nuclear detergent extracts, sHeLa cells or nuclei were homogenized in the lysis buffer containing 1% Triton X-100, followed by 3 hr solubilization with rotation at 4°C and centrifugation at 100,000 × g to remove particulate material. For immunoprecipitations, antibodies were crosslinked with dimethyl pimelimidate (Pierce) to protein A agarose, incubated with extracts or cytosol at 4°C overnight and washed extensively with the respective lysis buffers containing 500 mM KCl before elution with 100 mM glycine [pH 2.5] (with 1% Triton X-100 in case of detergent extracts). For GST pulldown, glutathione-Sepharose beads complexed with GST, GST-APPL1, and GST-APPL2 were incubated with nuclear extracts at 4°C overnight, washed with the lysis buffer containing 1% Triton X-100, and eluted with the wash buffer supplemented with 25 mM glutathione.

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