Regulation of EGF-stimulated activation of the PI-3K/AKT pathway by exocyst-mediated exocytosis

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The phosphoinositide-3 kinase (PI-3K)/AKT cell survival pathway is an important pathway activated by EGF signaling. Here we show, that in addition to previously described critical components of this pathway, i.e., the docking protein Gab1, the PI-3K/AKT pathway in epithelial cells is regulated by the exocyst complex, which is a vesicle tether that is essential for exocytosis. Using live-cell imaging, we demonstrate that PI(3,4,5)P3 levels fluctuate at the membrane on a minutes time scale and that these fluctuations are associated with local PI(3,4,5)P3 increases at sites where recycling vesicles undergo exocytic fusion. Supporting a role for exocytosis in PI(3,4,5)P3 generation, acute promotion of exocytosis by optogenetically driving exocyst-mediated vesicle tethering up-regulates PI(3,4,5)P3 production and AKT activation. Conversely, acute inhibition of exocytosis by using Endosidin2, a small-molecule inhibitor of the exocyst subunit Exo70 (also designated EXOC7), or inhibition of exocyst function by siRNA-mediated knockdown of the exocyst subunit Sec15 (EXOC6), impairs PI(3,4,5)P3 production and AKT activation induced by EGF stimulation of epithelial cells. Moreover, prolonged inhibition of EGF signaling by EGF receptor tyrosine kinase inhibitors results in spontaneous reactivation of AKT without a concomitant relief of EGF inhibition. However, this reactivation can be negated by acutely inhibiting the exocyst. These experiments demonstrate that exocyst-mediated exocytosis—by regulating PI(3,4,5)P3 levels at the plasma membrane—suberves activation of the PI-3K/AKT pathway by EGF in epithelial cells.

Receptor tyrosine kinases (RTKs) are an important class of signal-transducing receptors that reside on the plasma membrane (1). By binding to extracellular ligands such as growth factors, RTKs initiate intracellular signaling cascades that regulate essential cellular processes, such as proliferation, cell survival, differentiation, migration, and metabolism (2). Like most RTKs, the epidermal growth factor receptor (EGFR) undergoes ligand-induced dimerization, which activates the receptor through autophosphorylation of tyrosine residues within its cytoplasmic domain (3). The resulting phosphorytrosines serve as docking sites to recruit cellular signaling molecules that activate multiple downstream signaling pathways including the phosphoinositide-3 kinase (PI-3K)/AKT and mitogen-activated protein kinase (MAPK) pathways (4). Aberrant activation of these signaling pathways, through genetic alterations of RTKs or their downstream effectors, can lead to cancer and other diseases (5).

The PI-3K/AKT pathway promotes cell survival, proliferation, growth, and metabolism through regulation of many downstream targets (6). PI-3 kinase is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110), whose enzymatic activity is promoted when the regulatory domain, through two SH2 domains, binds to phosphorytrosines in RTKs or associated docking proteins (7). For EGF stimulation, PI-3 kinase activation primarily occurs through the docking protein Gab1 (Grb2-associated binder-1), which contains three canonical p85 binding motifs that are phosphorylated by EGFR (8). Activated PI-3 kinase then catalyzes phosphorylation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to produce phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], which in turn can recruit AKT to the plasma membrane through its pleckstrin homology (PH) domain. At the membrane, AKT is then phosphorylated by other kinases to become activated (6).

It is well established that cell signaling by EGFR and other RTKs can be down-regulated by receptor endocytosis (2, 9). Whether EGF signaling is regulated by the converse membrane trafficking process, exocytosis, is undetermined. Exocytosis relies on vesicle tethers, which are protein complexes that bridge transport vesicles to their target membranes to facilitate intracellular membrane fusion (10). The exocytosis is a type of multivesicle unit tether (11) that is essential for exocytosis of vesicles which recycle between the pericentriolar recycling endosome and the plasma membrane (12). It consists of eight subunits: Sec3 (EXOC1), Sec5 (EXOC2), Sec6 (EXOC3), Sec8 (EXOC4), Sec10

Significance

Epidermal growth factor receptor (EGFR) signaling regulates many critical cellular processes, such as cell proliferation. The role of membrane trafficking in EGF signaling has been studied for over four decades, with a major focus on EGFR endocytosis after its activation. However, it has long been known that EGF promotes exocytosis of vesicles emanating from the endosomal recycling compartment. These vesicles rely on a tethering factor, the exocyst complex, to attach to the plasma membrane prior to exocytosis. Here, we show that exocyst-mediated exocytosis locally generates PI(3,4,5)P3 and that this process underlies EGF-stimulated activation of the phosphoinositide-3 kinase (PI-3K)/AKT cell survival pathway in epithelial cells. We propose a positive feedback mechanism in which exocytosis regulates a key signaling pathway downstream of EGF.

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(EXOC5), Sec15 (EXOC6), Exo70 (EXOC7), and Exo84 (EXOC8). Three of these subunits have key known interactions with membranes. Exo70 and Sec3 (13, 14) both mediate vesicle tethering by interacting with PI(4,5)P2 on the plasma membrane, while Sec15 mediates attachment of the exocyst complex to the vesicle by binding the small GTPases Rab8 or Rab11 on the vesicle membrane (15). After a recycling vesicle is transported to the plasma membrane, the exocyst mediates an active tethering mechanism that promotes full vesicle fusion, leading to the complete merger of the vesicle with the membrane (16). Exocyst-mediated exocytosis is important for several cellular processes such as cell polarization (17), cell survival (18), migration (19–22), and glucose transport (23).

In this paper, we demonstrate an unexpected relationship between RTK signaling and exocytosis. Based on our recent finding that optogenetically controlled exocyst-mediated exocytosis induces membrane expansion in cells (16), we investigated whether exocytosis up-regulates PI(3,4,5)P3, as this phosphoinositide is known to play a key role in membrane remodeling (24). Here, we used a combination of approaches to test this hypothesis—including live-cell imaging, gain- and loss-of-function assays of the exocyst, and biochemic cell signaling assays. We show that exocyst-mediated exocytosis up-regulates PI(3,4,5)P3 and that this process underlies the activation of the PI3K/AKT pathway downstream of EGF stimulation in epithelial cells.

**Results**

**Exocytosis Coincides Spatiotemporally with an Increase in PI(3,4,5)P3 Levels.** Previously, we showed that vesicle tethering by the exocyst can be controlled by using a light-inducible heterodimerization system (16). This was achieved by first impairing membrane binding of the Exo70 exocyst subunit—by mutating two conserved lysines (K632A, K635A) near the C-terminal end that are critical for interactions with PI(4,5)P2 at the plasma membrane—and then attaching the photoreceptor cryptochrome 2 (CRY2) to the C-terminus of Exo70. This allows membrane binding by the mutant Exo70 to be rescued by light-inducible heterodimerization of Exo70(K632A, K635A)-CRY2 with CIB1, the binding partner of CRY2, on the plasma membrane. One of the unexpected findings using this exocyst optogenetics system was that acute promotion of exocytosis causes the plasma membrane to expand, through formation and elongation of filopodia. Importantly, this membrane expansion was spatiotemporally associated with vesicle fusion events at the base of the expanding region. However, because the number of fusion events could not feasibly account for the extent of the membrane expansion, we reasoned that the membrane expansion could not merely reflect the delivery of membrane (i.e., lipids and proteins). Rather, we wondered whether exocytosis promoted membrane expansion by increasing the levels of PI(3,4,5)P2 on the plasma membrane since this phosphoinositide is a key signaling molecule in membrane remodeling (24).

To test the hypothesis that exocytosis regulates PI(3,4,5)P3 levels, we explored PI(3,4,5)P3 dynamics at the membrane in live cells. When we transiently expressed the PI(3,4,5)P3 biosensor PHAKT–GFP (25) at low levels in HeLa cells and imaged it using total internal reflection fluorescence (TIRF) microscopy, we found that the fluorescence of the biosensor at the membrane fluctuated on a minutes time scale (Fig. 1A, Left). Such fluctuations were not seen with CIB1–GFP-CAAX, a protein that is localized on the plasma membrane via a lipid anchor (Fig. 1A, Right), thus ruling out the possibility that PHAKT–GFP fluctuations are due to undulations of the plasma membrane, i.e., changes in the proximity of the plasma membrane to the glass coverslip.

To test whether PHAKT–GFP fluctuations coincide with exocytosis, we performed two-color imaging experiments with the pH-sensitive exocytosis reporter transferrin receptor-pHluorin (TRRC-pH; ref. 26), which emits in the green channel (~509 nm), and the PI(3,4,5)P2 biosensor with its GFP replaced by TagRFP-T, a fluorescent protein that emits in the red channel (~584 nm; ref. 27). Fig. 1B shows an example of such a two-color movie. Here, an initial rise in PHAKT–TagRFP-T fluctuation (dashed circle) coincided with a burst of exocytic fusion events in that area. Notably, the duration of the PHAKT–TagRFP-T fluctuation lasted for several minutes, while the elevation in the fusion rate quickly subsided after momentarily increasing by ~fourfold. This
difference in duration between $\text{PH}_{\text{AKT}}$-TagRFP-T fluctuation and fusion rate increase was recapitulated at the level of a single vesicle: When a brighter “stag” version of TagRFP-T (28) was attached to the PI(3,4,5)P3, biosensor (PH$_{\text{AKT}}$-stagRFP), and fusion events of multiple vesicles ($n = 31$) were time-aligned and averaged, the PI(3,4,5)P$_3$ signal appeared to increase as a ~2-micron wide cloud of fluorescence that persists for over ~1 min after an exocytic event (Fig. 1C). Evidently, an increase in PH$_{\text{AKT}}$-stagRFP fluorescence can be spatially confined at the site of vesicle fusion, long after a vesicle has fused. These results suggest that vesicle fusion locally generates PI(3,4,5)P$_3$ and that multiple vesicles can sustain a longer lasting PI(3,4,5)P$_3$ fluctuation by undergoing exocytosis around the same time and place.

**Acute Promotion of Exocytosis Up-Regulates PI(3,4,5)P$_3$.** We wished to test whether acutely promoting exocytosis can cause an increase in PI(3,4,5)P$_3$ levels. To this end, we transiently coexpressed Exo70(K632A, K635A)-CRY2-mCherry and plasma-membrane targeted CIB1-CAAX to control exocytosis optogenetically (Fig. 2A) and PH$_{\text{AKT}}$-GFP to visualize PI(3,4,5)P$_3$. Illumination of cells using 100-ms pulses of 488-nm light (to activate CRY2 and image PI(3,4,5)P$_3$ simultaneously) caused a steady rise in PH$_{\text{AKT}}$-GFP fluorescence that plateaued after ~5 min (Fig. 2 B–D). Consistent with the fluorescence increase reflecting upregulation of PI(3,4,5)P$_3$, a much smaller increase was seen when we imaged a version of PH$_{\text{AKT}}$-GFP carrying a mutation (R25C) that has greatly diminished affinity for PI(3,4,5)P$_3$ (29). To assess the relative magnitude of PI(3,4,5)P$_3$ generation by exocyst optogenetics, we next imaged PI(3,4,5)P$_3$ during EGF stimulation (10 ng/mL) under the same imaging conditions. Interestingly, while EGF stimulation caused PH$_{\text{AKT}}$–GFP fluorescence to rise ~twofold higher compared to exocyst optogenetics, the fluorescence peaked earlier (~2 min) and returned to baseline, unlike with exocyst optogenetics where it remained elevated. Again, a much smaller increase in PH$_{\text{AKT}}$–GFP fluorescence was seen with the R25C mutant of the PH$_{\text{AKT}}$–GFP biosensor in EGF-stimulated cells compared to the wildtype sensor.

If exocyst optogenetics generates PI(3,4,5)P$_3$, as the above imaging experiments suggest, then it might promote cell signaling activated by PI(3,4,5)P$_3$, such as the AKT pathway. To test this, we performed whole-dish optogenetic experiments to check for AKT activation using biochemical techniques. HeLa cells were transiently transfected with Exo70(K632A, K635A)-CRY2-mCherry and CIB1-CAAX and then optically stimulated en masse with a light-emitting diode (LED) array for 5 min at 37°C. After stimulation, cell lysates were subjected to SDS/PAGE analysis followed by immunoblotting with anti-phospho-AKT antibodies. Fig. 2E shows that exocyst optogenetics could indeed induce AKT phosphorylation, although to a lesser extent than EGF stimulation (100 ng/mL, 5 min at 37°C). While this difference is consistent with the greater upregulation of PI(3,4,5)P$_3$ by EGF in the imaging experiments described in Fig. 2 B–D, it should be noted that AKT phosphorylation in the whole-dish optogenetics experiment was likely suboptimal given that only a fraction of cells are transfected with all components of the optogenetic system. Notwithstanding this point, the above results demonstrate that acute promotion of exocytosis not only up-regulates PI(3,4,5)P$_3$ but also activates AKT.

**Acute Inhibition of Exocytosis Down-Regulates PI(3,4,5)P$_3$.** We reasoned that if acute promotion of exocytosis up-regulates PI(3,4,5)P$_3$, then the converse experiment, acute inhibition of exocytosis, might have the opposite effect. To explore this possibility, we performed a series of experiments with Endosidin2 (Es2), a small-molecule inhibitor of the exocyst (Fig. 3A; ref. 30). Mechanistically, Es2 binds to Exo70, which inhibits not only Exo70 binding to the plasma membrane but also transferrin recycling in HeLa cells (30). We confirmed the inhibitory effect of Es2 on exocytosis by performing TIRF microscopy of exocytosis, again by imaging TIR-c-pH in HeLa cells, and measuring the fusion rate before and after a 30-min treatment with increasing concentrations of the drug. We found that Es2 inhibited exocytosis in a dose-dependent manner, with an IC$_{50}$ of 25.8 ± 7.3 μM ($n = 5$; Fig. 3B).
was necessary to image PI(3,4,5)P₃ for tens of minutes to allow that showed robust TfRc-pH fusion activity. Second, because it “active” vis-à-vis exocytosis (our observations), we selected cells designing these experiments. First, since not all cells are typically and after drug treatment. The fusion rate (events/μM (n = 5 cells), 80 μM (n = 3 cells), 60 μM (n = 5 cells), 80 μM (n = 4 cells), or 120 μM (n = 4 cells) Es2 for 30 min at 37°C, and finally reimagined for 5 min, to record exocytosis before and after drug treatment. The fusion rate (events/μM/min) was calculated for each period and the normalized rate change (post/pre) for each experiment was plotted against Es2 concentration. Data points were fitted (red) using a dose–response curve fitting function (Origin). (C) The effect of Es2 on basal PI(3,4,5)P₃ levels. (Upper) TIRF micrographs of PHAKT–TagRFP-T in HeLa cells before treatment with either DMSO or Es2 (80 μM). (Scale bar: 20 μm.) (Middle) Kymographs of regions outlined by the yellow boxes in the upper panels. White dashed line indicates start of DMSO or Es2 treatment. Fluorescence is presented using a fire LUT (ImageJ). (Lower) Time-dependent changes in PHAKT–TagRFP-T fluorescence during EGF stimulation in cells pretreated with DMSO (n = 11 cells), Es2 (n = 13 cells), and wortmannin (100 nM; n = 14 cells). Data are shown as mean ± SEM. Note that the fluorescence decrease was less than 30% (dashed line in the lower plot P = 0.082). It should be noted that the fluorescence decrease does not exceed 30% (dashed line), even for wortmannin. (D) The effect of Es2 pretreatment (30 min at 37°C) on EGF stimulation of PI(3,4,5)P₃ production. (Upper) TIRF micrographs of PHAKT–TagRFP-T in HeLa cells before treatment with either DMSO or Es2 (80 μM). (Scale bar: 20 μm.) (Middle) Kymographs of regions outlined by the yellow boxes in the upper panels. White dashed line indicates start of EGF (10 ng/mL) stimulation. Fluorescence is presented using a fire LUT (ImageJ). (Lower) Time-dependent changes in PHAKT–TagRFP-T fluorescence during EGF stimulation in cells pretreated with DMSO (n = 11 cells), Es2 (n = 13 cells), and wortmannin (100 nM; n = 14 cells). Data are shown as mean ± SEM. Note that traces are normalized to prestimulation intensity (dashed line).

consistent with the reported micromolar affinity of Es2 for Exo70 (30). It should be noted that maximal inhibition of exocytosis was only 49.2 ± 7.3% at the highest concentration tested. However, this is consistent with the ~50% colocalization of TfRc-pH-containing vesicles with the exocyst (31).

Next, we used Es2 to test the effect of inhibiting exocytosis on basal PI(3,4,5)P₃ levels. There were several considerations in designing these experiments. First, since not all cells are typically “active” vis-à-vis exocytosis (our observations), we selected cells that showed robust TfRc-pH fusion activity. Second, because it was necessary to image PI(3,4,5)P₃ for tens of minutes to allow drug diffusion into cells, we used a slower acquisition rate (0.2 Hz compared to 2 Hz for imaging exocytosis) to avoid photobleaching of PHAKT–TagRFP-T. Finally, after recording a sufficiently long (~8.3 min) baseline signal, we added either Es2 (80 μM), the PI-3 kinase inhibitor wortmannin (100 nM), as a positive control, or the drug vehicle DMSO as a negative control. The results presented in Fig. 3C show that Es2 caused a time-dependent decrease in PHAKT–TagRFP-T fluorescence that was not seen with DMSO (13.0 ± 1.6% vs. −0.4 ± 5.8%, P = 0.042), but somewhat smaller than that seen with wortmannin (13.0 ± 1.6% vs. 26.2 ± 5.9%, P = 0.082). It should be noted that the fluorescence decrease was less than 30% (dashed line in the lower plot of Fig. 3C) even with wortmannin because the penetration depth of the evanescent field is greater than the thickness of the membrane (~100 nm vs. ~10 nm), which allows for much of the fluorescence to be derived from PHAKT–TagRFP-T molecules in the cytosol, not just at the membrane.

The above experiments demonstrate that exocytosis contributes to steady-state levels of PI(3,4,5)P₃. To test whether exocytosis is involved in the upregulation of PI(3,4,5)P₃ by RTK signaling, we performed EGF stimulation experiments with cells that were starved
up-regulates PI(3,4,5)P3 (Fig. 2); (iii) acute inhibition of exocyst-mediated exocytosis using Es2 down-regulates steady-state (Fig. 3C) and EGF-stimulated PI(3,4,5)P3 levels (Fig. 3D). As such, we tested whether Es2 inhibits AKT activation by EGF stimulation. HeLa cells were starved in serum-free media and then treated with increasing concentrations of Es2 for 30 min at 37°C before stimulation with EGF (100 ng/mL) for 5 min. Cell lysates were then subjected to immunoblotting with anti-pAKT antibodies, as well as anti-pMAPK (pERK1/2) antibodies, to monitor AKT and MAPK (ERK1/2) stimulation, respectively. Surprisingly, we found that Es2 caused very weak inhibition of AKT phosphorylation (Fig. 4D). One possible explanation for this result is that PI(3,4,5)P3 upregulation by endogenous exocytosis is not tightly coupled to AKT stimulation in HeLa cells. Because it has been reported that shRNA-mediated knockdown of the exocyst subunit Sec8 or Sec10 significantly reduces phospho-AKT levels in murine mammary gland NMuMG cells (18), we wondered whether the

Acute Inhibition of Exocytosis Inhibits AKT Stimulation in Epithelial Cells. The data presented so far suggest that (i) exocytosis of individual vesicles generates PI(3,4,5)P3 (Fig. 1C); (ii) acute optogenetic promotion of exocyst-mediated exocytosis up-regulates PI(3,4,5)P3 (Fig. 2B–D) and AKT phosphorylation (Fig. 2E); and (iii) acute inhibition of exocyst-mediated exocytosis using Es2 down-regulates steady-state (Fig. 3C) and EGF-stimulated PI(3,4,5)P3 levels (Fig. 3D). As such, we tested whether Es2 inhibits AKT activation by EGF stimulation. HeLa cells were starved in serum-free media and then treated with increasing concentrations of Es2 for 30 min at 37°C before stimulation with EGF (100 ng/mL) for 5 min. Cell lysates were then subjected to immunoblotting with anti-pAKT antibodies, as well as anti-pMAPK (pERK1/2) antibodies, to monitor AKT and MAPK (ERK1/2) stimulation, respectively. Surprisingly, we found that Es2 caused very weak inhibition of AKT phosphorylation (Fig. 4D). One possible explanation for this result is that PI(3,4,5)P3 upregulation by endogenous exocytosis is not tightly coupled to AKT stimulation in HeLa cells. Because it has been reported that shRNA-mediated knockdown of the exocyst subunit Sec8 or Sec10 significantly reduces phospho-AKT levels in murine mammary gland NMuMG cells (18), we wondered whether the

Fig. 4. The effects of Es2 on EGF signaling in different cell lines. (A) HeLa cells were cultured for 1 d, starved for 1 d, treated with increasing concentrations of Es2 (0–320 μM) for 30 min at 37°C, and then stimulated with EGF (100 ng/mL) for 5 min. Cell lysates were subjected to SDS/PAGE and analyzed for activation of AKT and MAPK (ERK1/2) by immunoblotting with antibodies for pAKT (Ser473) and pMAPK (Thr202, Tyr204), respectively. (B) Upper) MCF7 cells were cultured for 7 d, starved for 2 d, treated with increasing concentrations of Es2 (0–320 μM) for 30 min at 37°C, and then stimulated with EGF (100 ng/mL) for 5 min. AKT and MAPK activation was analyzed as above. (Lower) Analysis of the dose-dependent inhibition of AKT phosphorylation by Es2 (n = 3). Data are shown as mean ± SEM. Datapoints were fitted (red) using a dose–response curve fitting function (Origin). (C) A431 cells were cultured for 3 d, starved for 6 h, treated with increasing concentrations of gefitinib (0–3 μM) for 30 min at 37°C, and then stimulated with EGF (10 ng/mL) for 5 min. AKT and MAPK activation was analyzed as above. (D) MCF7 cells were cultured for 7 d, starved for 2 d, treated with Es2 (240 μM) for 30 min at 37°C, and then stimulated with EGF (100 ng/mL) for 5 min. EGFR was immunoprecipitated from cell extracts by using anti-EGFR antibodies, and EGFR stimulation and expression were analyzed by immunoblotting the precipitates with anti-pEGFR and anti-EGFR antibodies, respectively. AKT and MAPK activation was analyzed as above. (E and F) DLD1 cells and HepG2 cells were cultured for 4 or 5 d, respectively, starved for two different durations (6 and 24 h), treated with Es2 (320 μM) for 30 min at 37°C, and then stimulated with EGF (100 ng/mL) for 5 min. AKT and MAPK activation was analyzed as above.
upregulation of P1(3,4,5)P3 by exocyst-mediated exocytosis is coupled to AKT stimulation specifically in epithelial cells.

To test this notion, we turned to MCF7 cells, which are a human mammary epithelial cell line. MCF7 cells were cultured for 7 d (first 3 d with 0.1 mg/mL insulin) to near confluence (~80%), starved in serum-free media (0.2% BSA) for 2 d, and then treated with increasing concentrations of Es2 for 30 min at 37°C before stimulation with EGF (100 ng/mL) for 5 min. Cell lysates were then subjected to immunoblotting with anti-pAKT antibodies and anti-pMAPK antibodies to monitor AKT and MAPK stimulation, respectively. The results presented in Fig. 4B show that Es2 completely inhibited AKT phosphorylation in a dose-dependent manner with an IC50 of 60.7 ± 6.1 μM (n = 4), demonstrating that EGF-induced AKT stimulation is dependent on exocyst-mediated exocytosis.

In MCF7 cells, Es2 also inhibited phosphorylation of MAPK, although higher concentrations of the drug were needed to inhibit MAPK stimulation than AKT stimulation (Fig. 4B). A dependence of MAPK stimulation on PI-3 kinase has been reported in MCF7 cells (32), which we confirmed with wortmannin (SI Appendix, Fig. S2). Interestingly, we found that the differential sensitivity of AKT and MAPK stimulation to Es2 was similar to that of wortmannin (SI Appendix, Fig. S2) and the EGFR tyrosine kinase inhibitor gefitinib (Fig. 4C), since the latter two drugs also inhibited MAPK stimulation at higher concentrations than AKT stimulation. These results suggest that if exocyst-mediated exocytosis regulates EGFR signaling, then it might do so by regulating a signaling node close to the activation of EGFR itself. Thus, a simple but possibly trivial explanation for the ability of Es2 to inhibit AKT and MAPK stimulation is that Es2 inhibits EGFR activation. To test this possibility, we examined the phosphorylation of EGFR after Es2 treatment. We repeated the previous experiment, but this time, after EGF stimulation, we immunoprecipitated EGFR from cell extracts by using anti-EGFR antibodies and monitored EGFR stimulation and expression by immunoblotting the precipitates with anti-pEGFR and anti-EGFR antibodies, respectively. Immunoprecipitation of EGFR was necessary since MCF7 cells express very low levels of the receptor, which precludes its detection by immunoblotting of cell lysates. As shown in Fig. 4D, Es2 (240 μM) strongly inhibited AKT phosphorylation without inhibiting EGFR phosphorylation or affecting EGFR levels. These results demonstrate that Es2 inhibits AKT and to a lesser degree MAPK stimulation downstream of EGFR activation.

Because MCF7 cells harbor an activating PIK3CA mutation (33), we tested whether inhibition of exocyst-mediated exocytosis by Es2 affects AKT stimulation in two other epithelial cell lines with different genetic backgrounds: human colorectal DLD1 cells, which carry an oncogenic mutation in KRAS, and human hepatoma HepG2 cells, which are nonmutagenic. DLD1 and HepG2 cells were cultured for 4 d to confluence and 3 to 80% confluence, respectively, then starved in serum-free media (for 6 or 24 h), and treated with Es2 (320 μM) for 30 min before stimulation with EGF (100 ng/mL) for 5 min. Cell lysates were then subjected to immunoblotting with anti-pAKT antibodies to monitor AKT stimulation. Fig. 4E and F show that Es2 strongly inhibited AKT phosphorylation in both cell lines, suggesting that exocyst-mediated exocytosis is coupled to EGF-induced AKT stimulation in multiple different types of epithelial cells.

Inhibition of Exocyst by Sec15 Knockdown Inhibits AKT Stimulation. In the next set of experiments, we studied a fourth epithelial cell line, human epidermoid A431 cells. These cells have high levels of EGFR expression, making it convenient to monitor the stimulation and expression of EGFR by immunoblotting cell lysates. We first tested whether A431 cells are sensitive to Es2 regarding EGF-induced AKT stimulation. A431 cells were cultured for 3 d until ~80% confluent, starved in serum-free media (for 6 or 24 h), and treated with Es2 (320 μM) for 30 min before stimulation with EGF (10 ng/mL) for 5 min. Cell lysates were then subjected to immunoblotting with anti-pAKT antibodies and anti-pMAPK antibodies to monitor AKT and MAPK stimulation, respectively. As with the other epithelial cell lines, Es2 strongly inhibited both AKT and MAPK stimulation (Fig. 5A), without affecting EGFR activation (SI Appendix, Fig. S1). Interestingly, however, in A431 cells (Fig. 5A)—but less so in DLD1 (Fig. 4E) or HepG2 cells (Fig. 4F)—both basal and EGF-stimulated AKT but not MAPK phosphorylation was greater after 24 h starvation than with 6 h starvation. This suggests that suppressing cell signaling in A431 cells sensitized AKT but not MAPK stimulation.

To corroborate the results obtained with acute drug (Es2) inhibition of exocyst-mediated exocytosis with an orthogonal approach, we turned to an exocyst gene knockdown (KD) approach. To this end, we transfected A431 cells with siRNA targeting either the exocyst subunit Sec15—the subunit which mediates attachment of the exocyst complex to vesicles via binding to Rab11—or with scrambled siRNA as a negative control. Sec15 KD phenocopies inhibition of exocyst function by Rab11 KD (12). We used siRNA with modifications from the manufacturer (see Materials and Methods) that reduced off-target toxicity compared to unmodified siRNA (our observations), and we used these siRNAs at low concentrations (250–500 pM) to further minimize cell death. After transfecting the cells with siRNA, we cultured them for 3 d, replaced the media on the day of the experiment, and after ~9 h stimulated the cells with EGF (10 ng/mL) for 5 min. The results demonstrate that 55.5 ± 3.5% (n = 3, P = 0.0029) Sec15 KD caused a 60.2 ± 5.7% (n = 3, P = 0.0215) inhibition of EGF-induced AKT phosphorylation without significantly affecting total EGF levels or EGFR phosphorylation (Fig. 5B and C). Thus, inhibition of the exocyst by Sec15 KD inhibited AKT stimulation but not EGFR activation, similar to the effect of Es2 on cell signaling by EGFR in MCF7 cells (Fig. 4D) and A431 cells (SI Appendix, Fig. S1). We surmise that the lack of MAPK inhibition by Sec15 KD may be due to insufficient inhibition of exocytosis by the partial KD of Sec15 since higher concentrations of Es2 are needed to inhibit MAPK than AKT stimulation (Fig. 4B).

Es2 Inhibits a Novel form of Drug Resistance in A431 Cells. As described above, prolonged serum starvation sensitizes AKT but not MAPK stimulation in A431 cells (Fig. 5A). One explanation for this is that suppressing cell signaling causes a compensatory adaptation that up-regulates AKT activation. To explore this idea, we examined the effect of EGFR inhibitors on AKT stimulation over time. A431 cells were cultured for 3 d, starved for 6 h, and then treated with increasing concentrations of the EGFR TKI gefitinib to inhibit EGF signaling for either 1 or 18 h. Immediately after each TKI treatment period, the cells were stimulated with EGF (10 ng/mL) for 5 min. The results presented in Fig. 5D show that AKT phosphorylation was strongly inhibited in a dose-dependent manner after 1 h of TKI treatment. As in an earlier experiment using a short treatment period (30 min; Fig. 4C), AKT and MAPK inhibition displayed different dose responses to gefitinib, with MAPK inhibition requiring higher drug concentrations. However, after the 18 h treatment period, AKT phosphorylation levels rose again at submaximal gefitinib concentrations (~2 µM), shifting the dose response of AKT inhibition to the right so that it looked similar to the dose response of MAPK inhibition. Importantly, this change occurred without a commensurate change in the dose

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response of EGFR inhibition to gefitinib. Thus, A431 cells rapidly developed resistance to gefitinib with respect to AKT stimulation.

Finally, we wondered whether the reemergence of AKT stimulation during prolonged EGFR inhibition depended on exocyst-mediated exocytosis. This could be tested by treating cells first with an EGFR TKI to inhibit EGFR and then with Es2 to inhibit exocytosis. Initial experiments indicated possible drug–drug interactions between gefitinib and Es2, which necessitated sequential but separate drug treatments. As such, we turned to a different EGFR TKI, afatinib. Unlike gefitinib, this TKI irreversibly inhibits EGFR, allowing us to add Es2 separately after TKI treatment without potentially reversing EGFR inhibition. As in the previous experiment, A431 cells were cultured for 3 d and starved for 6 h. The cells were then treated with afatinib at two different concentrations (75 and 90 nM) to ensure sufficient but submaximal AKT inhibition for either 2 or 18 h. Immediately after each TKI treatment period, the cells were treated with Es2 at three ascending concentrations (160, 200, and 240 μM) and then finally stimulated with EGF (10 ng/mL) for 5 min. The results presented in Fig. 5E show that AKT phosphorylation by EGF was inhibited down to prestimulation levels during the first 2 h of afatinib treatment but reappeared after 18 h even though EGFR phosphorylation remained low. Importantly, subsequently treating cells with Es2 after 18 h strongly inhibited the reappearance of phosphorylated AKT in a dose-dependent manner. Thus, these results demonstrate that reactivation of AKT during prolonged EGFR inhibition with TKIs can be negated by inhibiting exocyst-mediated exocytosis.

**Discussion**

The PI-3/AKT pathway is one of the main cellular signaling pathways that are activated by RTKs and other cellular cues to mediate a critical antiapoptotic signal, i.e., cell survival. While several
RTKs, e.g., PDGFR and ErbB3, activate PI3-kinase through formation of direct contacts between the regulatory p85 subunit of PI3-kinase with specific tyrosine phosphorylation sites on activated RTK, other RTKs, e.g., EGFR and INSR, utilize closely associated tyrosine phosphorylated docking proteins such as Gab1 and IRS1, respectively, for p85 recruitment and PI3-kinase activation. EGFR utilizes an additional indirect mechanism for PI3-kinase recruitment and activation through EGF-induced heterodimerization with ErbB3 resulting in tyrosine phosphorylation of several specific docking sites for PI3-kinase in the cytoplasmic domain of ErbB3. It is noteworthy that both Gab1 and AKT utilize their PH domains for translocation to the cell membrane by specific binding to PI(3,4,5)P3 molecules; membrane translocation of Gab1 and AKT is an essential step in a highly regulated EGF-induced positive feedback mechanism of PI3K/AKT activation (34). In this report, we present several lines of evidence that exocyst-mediated exocytosis regulates AKT stimulation downstream of PI3K signaling primarily in epithelial cells. First, our live-cell imaging demonstrates that when an individual recycling vesicle undergoes exocytosis, the local level of PI(3,4,5)P3 increases for ~1 min. And, when multiple vesicles undergo exocytosis around the same time, a longer lasting PI(3,4,5)P3 fluctuation occurs at the membrane. We then demonstrate that exocytosis and PI(3,4,5)P3 levels are causally related by controlling the exocyst through acute gain- and loss-of-function methods. We show that acutely promoting exocytosis through exocyst optogenetics up-regulates PI(3,4,5)P3 and AKT phosphorylation to an extent comparable to that of EGF stimulation. Conversely, we show that acutely inhibiting exocyst-mediated exocytosis using Es2, a small-molecule inhibitor of Exo70, down-regulates PI(3,4,5)P3 levels to an extent that is comparable to that by the PI3-kinase inhibitor wortmannin. Collectively, these imaging data not only support our biochemical experiments showing that exocyst-mediated exocytosis regulates AKT stimulation, but they also shed light on potential mechanisms underlying this regulation by revealing the spatiotemporal dynamics of PI(3,4,5)P3 generation associated with exocytosis.

Regarding the biochemical experiments, an intriguing finding is that Es2 only weakly inhibits AKT phosphorylation in HeLa cells even though it inhibits both basal and EGF-stimulated PI(3,4,5)P3 levels in the same cells. Based on (i) the observed spatial confinement of PI(3,4,5)P3 generation by single exocytic events and (ii) a reported dependence of basal PI(3,4,5)P3 levels on the exocyst in a mammary epithelial cell line (NMuMG; ref. 18), we speculate that PI(3,4,5)P3 generation by exocytosis might be functionally coupled to AKT activation in epithelial cells but not in HeLa cells. This notion is supported by experiments showing that Es2 can strongly inhibit AKT stimulation in four different epithelial cell lines. The epithelial cell lines tested not only originate from different tissues but also have different genetic backgrounds: HepG2 cells (liver) are nontumorigenic, whereas MCF7 (mammary) and DLD1 cells (colon) carry oncogenic mutations in PIK3CA and KRAS, respectively, and A431 cells (epidermis) overexpress EGFR. It remains to be determined whether nonepithelial cells, like HeLa cells, are generally less sensitive to Es2 with respect to AKT stimulation. The lack of coupling between exocyst-mediated exocytosis and AKT stimulation in some cells may reflect a spatial aspect of signaling, as activation and signaling of RTKs do not occur uniformly on the cell membrane (35–37).

An important aspect of our signaling experiments is that Es2 also inhibits MAPK stimulation, but at ~threefold higher concentrations. This is analogous to the differential sensitivity of AKT and MAPK stimulation to the EGFR TKI gefitinib. Thus, Es2 behaves like a low-affinity EGFR TKI. However, because Es2 does not inhibit EGFR autophosphorylation in MCF7 or A431 cells, it likely targets a signaling node that is downstream of EGFR activation but close to this initial signaling event. This idea is supported by the unexpected finding that AKT is reactivated during prolonged treatment with an EGFR TKI in A431 cells, and that this reactivation, which does not involve the relief of EGFR inhibition, can be negated by treating cells with Es2. Thus, it appears that exocyst-mediated exocytosis wholly subserves AKT stimulation by EGFR, in agreement with the ability of exocyst optogenetics to induce AKT stimulation. To our knowledge, this rapidly acquired resistance of AKT stimulation to EGFR TKIs has not been described before. Additional studies will be required to elucidate the upregulation of exocyst-mediated exocytosis in response to suppression of EGFR signaling, but it may involve the MAPK pathway as the reactivation of AKT stimulation does not occur when MAPK is completely inhibited (Fig. 5D).

Importantly, the main results with Es2 are corroborated using an independent approach to inhibit exocyst-mediated exocytosis—by genetically downregulating the exocyst subunit Sec15 (12). Like Es2, Sec15 KD significantly inhibits EGF-induced AKT stimulation without inhibiting EGFR activation or affecting EGFR levels. Notably, EGF-induced MAPK stimulation was not inhibited by Sec15 KD. However, given the differential sensitivity of AKT and MAPK stimulation to Es2 (with MAPK inhibition requiring higher Es2 concentrations), the differential sensitivity of AKT and MAPK stimulation to Sec15 KD may reflect the incomplete (~56%) knockdown of Sec15 in these experiments. It should be noted that greater levels of Sec15 KD are associated with experimentally prohibitive levels of cell death.

We propose the following model of the regulation of EGF-induced AKT1 signaling by exocyst-mediated exocytosis (SI Appendix, Fig. S3). In the first step, EGFR signaling promotes exocytosis of recycling vesicles. It has been known that EGF can stimulate exocytosis of recycling vesicles for over two decades (38, 39), but the mechanism underlying this process is still unclear. However, it may involve regulation of the exocyst, either by direct phosphorylation of Exo70 by MAPK to stabilize the exocyst complex (40), or through an interaction between the Rho family GTPase TC10 and Exo70 to promote binding of Exo70 to the plasma membrane (23, 41). In a previous study (16), we showed that a vesicle needs to be tethered at the membrane by enough exocyst complexes for it to undergo full vesicle fusion; otherwise, the vesicle undergoes kiss-and-run fusion, which is a reversible mode of exocytosis that does not deliver vesicular cargo effectively. Thus, if EGFR signaling promotes exocytosis by regulating vesicle tethering, it could do so by affecting the number of exocyst complexes that engage with the plasma membrane.

In the second step of the model, vesicles undergo exocytosis which in turn promotes PI(3,4,5)P3 synthesis at the membrane. While the mechanism underlying exocyst-induced PI(3,4,5)P3 production is presently not clear, we propose that essential components or regulatory elements are delivered to specific sites at the membrane to generate PI(3,4,5)P3. This hypothesis is based on the observation that the cloud of PI(3,4,5)P3 that forms after a single exocytic event is spatially confined to the fusion site for a relatively long time (~1 min). Such local and persistent confinement of PI(3,4,5)P3 production suggests the potential presence of scaffolding proteins near the fusion site that couples the exocytic delivery of the unknown factors to PI(3,4,5)P3 synthesis. An attractive scaffolding candidate is the ubiquitously expressed IQ-motif-containing GTPase-activating protein 1 (IQGAP1), a multidomain protein that can associate with EGFR, the exocyst, and components of several signaling pathways, including the

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Pl-3K and MAPK pathways (42). Moreover, IQGAP1 has been shown to assemble all the phosphoinositide kinases—PI(4)KIIβ, PIKKφ (a.k.a. PI(4)P5K), and PI(4,5)P3K—that are necessary to sequentially convert phosphatidylinositol to PI(3,4,5)P3 (43). In this regard, it is interesting that recycling vesicles carry PI(4)P (44) and that the exocyst complex interacts with PIKKφi2 (22), as well as EGFR (45). A scenario thus emerges whereby exocyst-mediated exocytosis could feed into the local synthesis of PI(4,5)P2, at the membrane to sustain PI(3,4,5)P3 levels. We note that AKT activation by EGF may also involve clathrin scaffolds, but not endocytosis per se (46), which would be in agreement with EGFR signaling occurring primarily at the plasma membrane (47, 48). However, spatial regulation of PI(3,4,5)P3 may be context dependent, as AKT activation on endosomes has been reported (49).

In summary, we describe a novel role of the exocyst in EGFR signaling. We conclude that AKT activation by EGF in epithelial cells depends on the delivery of factors to the plasma membrane by exocyst-mediated exocytosis that promote the local generation of PI(3,4,5)P3. The scheme presented in SI Appendix, Fig. S3, depicts a model for how exocyst-mediated PI(3,4,5)P3 production will enhance both Gab1 and AKT recruitment to the cell membrane to facilitate a robust positive feedback mechanism for EGF stimulation of the PI-3K/AKT cascade in epithelial cells. Since exocytosis itself is promoted by EGF, AKT stimulation in epithelial cells thus involves the reciprocal regulation of EGFR signaling and exocytosis of recycling vesicles.

Materials and Methods

Plasmids and Reagents. To generate PHAKT–TagRFP-T plasmids, DNA sequences for wildtype and R25C mutant of PHAKT from PH-Akt-GFP plasmid ($51465, Addgene) and PH-Akt(R25C)-GFP plasmid ($51466, Addgene) were amplified by PCR and cloned into pmTagRFP-T-N1 (50) using XhoI and BamHI sites. PHAKT–stagRFP was generated by site-directed mutagenesis of TagRFP-T sequences for wildtype and R25C mutant of PH AKT from PH-Akt-GFP plasmid.

Cell Culture and Transfection. HeLa cells. For live-cell imaging experiments, HeLa cells (CCL-2, ATCC) were maintained in T-75 flasks (Falcon) at 37°C and 5% CO2 in DMEM (Gibco) supplemented with 4.5 g/L glucose, 1 mM sodium pyruvate, 1× nonessential amino acids (Gibco), 10% (vol/vol) FBS (Sigma), and 100 μM penicillin-streptomycin mix (Gibco). Cells were split every 3−4 d, before they reached confluence. To transiently transfect HeLa cells for live-cell imaging experiments, a Nepa21 Type II electroporator (Nepa Gene) was used according to the manufacturer’s instructions. Briefly, 106 cells were resuspended in ~100 μL Opti-MEM (Gibco), 2 μg PHAKT–stagRFP, and 5 μg TfRc-pH. The cells were split at a 1:2 ratio every 2 d, using 1× TrypLE Express Enzyme (Gibco) to dissociate cells. For biochemical experiments, 1,875 × 104 cells were plated onto 35-mm treated polystyrene dishes (Corning), cultured for the first 3 d in complete media, cultured for the next 4 d in media without insulin, and then finally starved for 2 d in serum-free media containing 0.2% BSA (Fraction V, low endotoxin grade; Genentech Bio). For signaling experiments involving Es2, HeLa cells were maintained in T-75 flasks (Corning) at 37°C and 5% CO2, in EMEM (ATCC) supplemented with 0.01 mg/mL human recombinant insulin (Gibco) and 10% (vol/vol) γ-irradiated FBS (Corning). Cells were split at a 1:3 ratio every 4 d, using 1× TrypLE Express Enzyme (Gibco) to dissociate cells. For biochemical experiments, 2.5 × 106 cells were plated onto 35-mm treated polystyrene dishes (Corning), cultured for 3 d to 80% confluence, and then starved for either 6 or 24 h in serum-free media containing 0.2% BSA (Fraction V, low endotoxin grade; Genentech Bio).

For Sec15 KD, cells were reverse transfected with siRNAs using RNAiMAX (ThermoFisher Scientific), according to the manufacturer’s instructions. Briefly, 250–500 pM Sec15A and Sec15B siRNA or negative control (NC) siRNA (1−2 pmol each) were mixed with 6 μL RNAiMAX reagent in 250 μL Opti-MEM (Gibco), incubated for ~10 min in a microcentrifuge tube, and then transferred to 35-mm treated polystyrene dishes (Corning). Then, 2.5 × 105 cells in 4 mL of media were plated onto the dishes containing the siRNA lipid complexes and allowed to grow for 2 d before replacing the media. The following day, the media was replaced again ~9 h before the cells were used in signaling experiments.

LDLD1 cells. DLD1 cells (CCL-221, ATCC) were maintained in T-75 flasks (Corning) at 37°C and 5% CO2 in RPMI-1640 medium (ATCC) supplemented with 10% (vol/vol) γ-irradiated FBS (Corning). Cells were split at a 1:4 ratio every 2 d, using 1× TrypLE Express Enzyme (Gibco) to dissociate cells. For biochemical experiments, 5 × 105 cells were plated onto 35-mm treated polystyrene dishes (Corning), cultured for 4 d to 100% confluence, and then starved for either 6 or 24 h in serum-free media containing 0.2% BSA (Fraction V, low endotoxin grade; Genentech Bio).

HepG2 cells. HepG2 cells (HB-8065, ATCC) were maintained in T-75 flasks (Corning) at 37°C and 5% CO2 in EMEM (ATCC) supplemented with 10% (vol/vol) γ-irradiated FBS (Corning). Cells were split at a 1:4 ratio every 2 d, using 1× TrypLE Express Enzyme (Gibco) to dissociate cells. For biochemical experiments, 1.25 × 106 cells were plated onto 35-mm treated polystyrene dishes (Corning), cultured for 3 d to 80% confluence, and then starved for either 6 or 24 h in serum-free media containing 0.2% BSA (Fraction V, low endotoxin grade; Genentech Bio).

TIRF Microscopy. Live-cell imaging was done as previously described (16) using a custom TIRF microscope equipped with a 488- and 568-nm solid state lasers (Melles Griot), an EMCCD camera, and a 60 × 1.49 NA TIRF objective (Olympus). All experiments were done at 37°C (using a custom incubator chamber) in phenol red-free DMEM with 25 mM HEPES, pH 7.4, with or without 10% FBS. Image frames were acquired in time-lapse recordings at 2 or 0.2 Hz with 150-ms exposures. Pixel size was 160 nm. For PI(3,4,5)P3 imaging with PHAKT–stagRFP, cells were imaged at 37°C and 5% CO2 in a cage incubator (Okolab) housing a Nikon Eclipse Ti2 microscope (Nikon) equipped with a motorized Ti-LA-H1TIRF module with 15 mW LU-N4 488- and 561-nm lasers, using a CFI Plan Apochromat Lambda 100 ×/1.45 Oil TIRF objective and a Prime95 cMOS camera (110 nm pixel size; Teledyne Photometrics). Images were acquired using a 100-ms exposure time at 1 Hz.

Immunoprecipitation and Immunoblotting. For immunoprecipitation, cells were lysed using lysis buffer (50 mM HEPS, pH 7.5, 150 mM NaCl, 1 mM
EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 25 mM NaF, 1 mM MgCl₂, 1 mM sodium orthovanadate (Na₃VO₄), and complete protease inhibitor cocktail (Roche)) and lysates were clarified by centrifugation at 13,000 g. Protein A/G Agarose beads (Thermo Fisher Scientific) and anti-EGFR antibody EGFR (100:1 dilution, D38B1; Cell Signaling Technology, #4267) were added directly to the cell lysate. After overnight incubation of antibodies, beads, and lysate at 4°C with shaking, immune complexes were collected and washed three times with 1 mL washing buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5% glycerol) at 4°C. 2× loading buffer was added, and samples were boiled at 95°C for 5 min, spun to pellet the beads, and subjected to SDS-PAGE. Western blot analysis was performed using antibodies specific to Sec15 (155266; Kerafast, ED2003), EGFR (D38B1; Cell Signaling Technology, #4267), pEGFR (53A5, Tyr1173; Cell Signaling Technology, #4407), AKT (Cell Signaling Technology, #9727), pAKT (D9E, Ser473; Cell Signaling Technology, #4060), pMAPK (Thr202/Tyr204; Cell Signaling Technology, #43603), and γ-tubulin (GIU-88; Sigma, #6557). Immunoreactive bands were detected with horseradish peroxidase secondary antibodies through chemiluminescence.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix.

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