Role of TI-VAMP and CD82 in EGFR cell-surface dynamics and signaling

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Summary
The v-SNARE TI-VAMP (VAMP7) mediates exocytosis during neuritogenesis, phagocytosis and lysosomal secretion. It localizes to endosomes and lysosomes but also to the trans-Golgi network. Here we show that depletion of TI-VAMP enhances the endocytosis of activated EGFR receptor (EGFR) without affecting constitutive endocytosis of EGFR, or transferrin uptake. This increased EGFR internalization is mainly clathrin dependent. Searching for defects in EGFR regulators, we found that TI-VAMP depletion reduces the cell surface amount of CD82, a tetraspanin known to control EGFR localization in microdomains. We further show that TI-VAMP is required for secretion from the Golgi apparatus to the cell surface, and that TI-VAMP-positive vesicles transport CD82. Quantum dots video-microscopy indicates that depletion of TI-VAMP, or its cargo CD82, restrains EGFR diffusion and the area explored by EGFR at the cell surface. Both depletions also impair MAPK signaling and enhance endocytosis of activated EGFR by increased recruitment of AP-2. These results highlight the role of TI-VAMP in the secretory pathway of a tetraspanin, and support a model in which CD82 allows EGFR entry in microdomains that control its clathrin-dependent endocytosis and signaling.

Key words: CD82, EGFR, Endocytosis, Tetraspanin, TI-VAMP, L1-CAM, AP-2

Introduction
Exocytosis relies on soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) which have been recognized as the central core for intracellular membrane fusion in all eukaryotes (Galli and Hauke, 2004; Jahn and Scheller, 2006; Pfeffer, 2007). Membrane fusion is mediated by the formation of a complex between a so-called vesicular (v)-SNARE (often referred to as vesicle-associated membrane protein; VAMP) with a target (t)-SNARE. Several additional proteins are required for membrane fusion including complexins, synaptotagmins, tethering factors, Rab GTPases, Sec1/Munc18 (SM) proteins, and other regulators, which add layers of specificity to membrane transport and fusion events (Cai et al., 2007; Shen et al., 2007; Sztul and Lupashin, 2006). The v-SNARE VAMP7 (TI-VAMP; tetanus neurotoxin-insensitive VAMP) localizes to late endosomes and lysosomes (~50%) but also to the trans-Golgi network (TGN) (~40%) and tubulo-vesicular membranes (Advani et al., 1999; Coco et al., 1999), in HeLa and PC12 cells. It has been suggested that TI-VAMP allows for heterotypic fusion between endosomes and lysosomes (Advani et al., 1999; Antonin et al., 2000b; Pryor et al., 2004). TI-VAMP is also involved in fusion of vesicles with the plasma membrane, which is necessary for phagocytosis, neurite outgrowth, lysosomal and granule secretion in polarized cells (Alberts et al., 2003; Braun et al., 2004; Logan et al., 2006; Marcat-Palacios et al., 2008; Martinez-Arca et al., 2000; Martinez-Arca et al., 2001; Mollinedo et al., 2006; Oishi et al., 2006; Pocard et al., 2007; Proux-Gillardieux et al., 2007; Sander et al., 2008; Scheuber et al., 2006). Connections between the roles of TI-VAMP in exocytosis and endocytosis have not been investigated.

Here we examined the role of TI-VAMP in the case of the prototypal endocytosis of epidermal growth factor receptor (EGFR). EGFR is crucial for cell division, differentiation and migration (Yarden, 2001). EGFR (or ERBB1) is a member of the EGFR family, which also includes ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). EGFR is the only member of the ErbB family to undergo ligand-induced internalization (Baulida et al., 1996). In the presence of EGFR, internalization rate increases five- to tenfold and is followed by recycling or delivery to lysosomes for degradation (Sorkin and Goh, 2009). From the cell surface, activated EGFR goes through early endosomes and is then either sent back to the cell surface by exocytosis or to multivesicular bodies and late endosomes to finally be degraded by the lysosomal compartment.

We found that TI-VAMP knockdown (KD) leads to an increase of EGF-EGFR clathrin-dependent endocytosis without significant effect on constitutive endocytosis of EGFR, or transferrin uptake. We further demonstrated that TI-VAMP mediates transport from the Golgi complex to the cell surface. Depletion of TI-VAMP induces a decrease in the expression at the cell surface of the metastasis suppressor CD82, which is known to regulate EGFR endocytosis. Moreover, a rescue experiment after TI-VAMP depletion restores the expression of CD82. Both TI-VAMP and CD82 depletion deeply affect cell surface diffusion of activated receptor, MAPK signaling and AP-2 recruitment. We conclude that TI-VAMP-dependent secretion of CD82 allows EGFR to enter
microdomains that control its clathrin-dependent endocytosis and regulate its signaling.

**Results**

**TI-VAMP depletion accelerates endocytosis of activated EGFR**

To determine whether TI-VAMP knockdown (KD) had an effect on the endocytic pathway we evaluated EGFR endocytosis kinetics by surface immunoochemistry and flow cytometry. We silenced the expression of TI-VAMP in HeLa cells by either targeting the coding region (99% of extinction) or the 3'-UTR of TI-VAMP mRNA (97% of extinction, supplementary material Fig. S1). Efficient silencing required 96 hours incubation and was effective for at least 8 days.

Surface EGFR was labeled at 4°C with an antibody which does not interfere with the binding of EGF. In the absence of EGF, the amount of EGFR internalized was not significantly different in mock- and TI-VAMP KD cells (Fig. 1A, left), indicating that constitutive endocytosis of EGFR was unchanged. In the presence of EGF, however, EGFR endocytosis was significantly enhanced in TI-VAMP-depleted cells (Fig. 1A, right). These results indicate that TI-VAMP depletion accelerates endocytosis of activated EGFR within a few minutes.

The increased endocytosis was not due to an effect on expression of EGFR, since no significant difference in either the total level of EGFR, as determined by western blot (supplementary material Fig. S2E) or in the surface level of EGFR, quantified by cytometry (supplementary material Fig. S2F) was observed. Moreover we did not find any effect of TI-VAMP KD on transferrin internalization, indicating that TI-VAMP KD does not have a general stimulatory effect on cell endocytosis (Fig. 1B).

The increased EGFR endocytosis was confirmed by uptake of fluorescent EGF at 18°C (supplementary material Fig. S2A-C and S3), which blocks trafficking towards degradation compartments and impairs recycling towards the plasma membrane (Futter et al., 1996; Sorkin et al., 1991a). The amount of EGF was higher in TI-VAMP-depleted cells with both TI-VAMP siRNAs and not with the silencing of other v-SNAREs (VAMP3, VAMP4 or VAMP8; supplementary material Figs S2C, S4). To further confirm the specificity of the TI-VAMP KD on the EGF-EGFR endocytosis, we carried out a rescue experiment. Efficient complementation was observed in depleted cells transfected with pcDNA3-rat-TI-VAMP (supplementary material Fig. S2D), confirming that the increased endocytosis of the EGF-EGFR complex is specifically induced by the KD of TI-VAMP.

EGFR was shown to enter the cells, either via clathrin-dependent (CME) or -independent endocytosis (CIE; also referred as raft/caveolar pathway). It has been proposed that EGFR internalization could be dependent on the concentration of ligand (Sigismund et al., 2005), 60% by CME and 40% by CIE (Sigismund et al., 2008) at 100 ng/ml EGF. Other groups have shown that EGFR...
could be internalized by clathrin-dependent endocytosis even at high concentrations (Kazazie et al., 2006; Rapportin and Simon, 2009). We wanted to determine whether the increased EGFR endocytosis evoked by TI-VAMP depletion was clathrin-dependent or not. HEK293 cells were processed for endocytosis in either clathrin-KD cells or in cells treated with dansyl-cadaverine (DCV), a clathrin-dependent endocytosis inhibitor (Haigler et al., 1980; Schlegel et al., 1982). DCV prevents the internalization of cell surface receptors by inhibition of clathrin polymerization (Nandi et al., 1981) leading to stabilization of clathrin cage assembly at the plasma membrane (Phonphok and Rosenthal, 1991).

As control, we checked the endocytosis of transferrin (Tf), which is known to be clathrin dependent. DCV treatment or clathrin siRNA strongly inhibited the endocytosis of transferrin down to 40% and 20% of control levels, respectively (Fig. 1B, bottom panel). The block of Tf endocytosis was thus similar to previously published data which indicated a residual background internalization of Tf of around 20-25% even when clathrin was 95% depleted (Huang et al., 2004; Motley et al., 2003).

We found that the increase in EGFR endocytosis evoked by TI-VAMP depletion was reduced 74% by clathrin siRNA (from 50.2% to 12.85%; Fig. 1D, bottom right panel). These results were confirmed by the use of DCV, which strongly, but not fully, decreased the EGF accumulation (Fig. 1C). These data indicate that, even though EGF is able to enter by CME and CIE, most of the increased EGF-EGFR endocytosis due to TI-VAMP depletion is mediated by CME.

**TI-VAMP controls the expression of CD82**

The fact that TI-VAMP KD increased EGFR endocytosis was surprising since membrane fusion per se is not required for endocytosis. We thus hypothesized that the accelerated EGFR endocytosis could be a consequence of modified expression of regulators at the cell surface. We searched among the many proteins that regulate EGFR trafficking for those that regulate EGFR endocytosis at the plasma membrane and may be cargos of TI-VAMP.

Expression of β-catenin and E-cadherin, known to mediate the signaling cascade of EGFR (Hoschuetzky et al., 1994; Pecce and Gutkind, 2000), were unchanged as measured by flow cytometry (Fig. 2A,A'). Other potential regulators of EGFR endocytosis are tetratraspins. Indeed, CD82 (also called 'metastasis suppressor (Fig. 2A,A') and quantification by flow cytometry indicated a strong increase (49%) in CD82-depleted cells (Fig. 2C, compare green and yellow bars). These results suggest that TI-VAMP controls the transport of several cargos including L1-CAM and CD82 (Fig. 2G). This indicated that, as in the case of TI-VAMP, the knockdown of CD82 induced an increased EGF-EGFR endocytosis. Finally, we overexpressed CD82 in TI-VAMP KD cells and showed that the increased endocytosis of EGF due to TI-VAMP depletion was decreased by 22% (our unpublished observation). These results further suggest a strong functional link between TI-VAMP and CD82.

**Role of TI-VAMP in the secretory pathway**

Our previous results strongly suggest that TI-VAMP may control the trafficking of molecules to the cell surface (Alberts et al., 2006; Braun et al., 2004; Chaineau et al., 2008; Martinez-Arca et al., 2001). We thus wondered if TI-VAMP KD could alter transport from the Golgi to the cell surface. Since TI-VAMP is a cargo of the AP-3 adaptor (Danglot and Galli, 2007; Martinez-Arca et al., 2003; Scheuber et al., 2006), we studied exocytosis of the VSV glycoprotein (VSV-G), which was also shown to be transported in an AP-3-dependent manner (Nishimura et al., 2002), in TI-VAMP silenced cells. As a reporter for generic constitutive secretion, we used the tsO45 temperature-sensitive VSV-G mutant fused to GFP (VSV-G–GFP). This mutant was retained in the endoplasmic reticulum (ER) at 39°C, accumulated in the Golgi at 20°C (Fig. 3A,B) and was released into the secretory pathway upon a shift to 32°C (Fig. 3C). The kinetics of appearance of the VSV-G–GFP at the cell surface was measured (red channel), in GFP-positive cells (green) sorted by flow cytometry. In mock-depleted cells, the kinetics of the fluorescence appearing at the surface adopted a growing slope reaching a plateau after 1 hour as previously shown (Miller et al., 1992; Presley et al., 1997). By contrast, in TI-VAMP-depleted cells, VSV-G–GFP transport was dramatically impaired as illustrated for both siRNAs in Fig. 3D. This demonstrates that TI-VAMP is a crucial v-SNARE for the transport from the Golgi complex to the cell surface. We then checked whether TI-VAMP KD had a general effect on the Golgi complex by comparing the subcellular localization of several key markers in mock and TI-VAMP-depleted cells. Immunocytochemistry experiments showed that there was no major defect in the distribution of the AP-1 or AP-3 adaptors, M6PR and TGN46, in TI-VAMP KD (supplementary material Fig. S5). Thus, TI-VAMP is a v-SNARE operating in the post-Golgi secretory pathway, at least for a subset of cargos including VSV-G in HeLa cells. To gain insight into the transport of CD82 at the cell surface, we investigated the colocalization of CD82 and TI-VAMP in living cells. Fast sequential confocal video-microscopy...
of CD82-YFP and TI-VAMP–RFP showed that CD82 colocalized with TI-VAMP at different times (see arrows, Fig. 3E and supplementary material Movie 1). Our data thus suggest that CD82 is transported, at least in part, in TI-VAMP vesicles. This could then explain why TI-VAMP depletion results in decreased CD82 expression at the cell surface.

Role of CD82 in plasma membrane EGFR diffusion
Given that CD82 has been shown to be necessary for EGFR compartmentalization (Odintsova et al., 2003), we hypothesized that the increased endocytosis of EGFR in TI-VAMP and CD82-depleted cells might arise from modified interactions and/or mobility of EGFR at the plasma membrane.
To test this possibility we monitored the surface mobility of endogenous EGFR by rapid time-lapse imaging of individual quantum dots (QDs) (Dahan et al., 2003) coupled to EGFR antibodies. Activated EGFR were highly mobile and could be tracked for several minutes (supplementary material Movie 2). In the absence of EGF, the instantaneous diffusion coefficient of EGFR ($D_{\text{EGFR}}$) was not statistically different between mock- and TI-VAMP-depleted cells (Fig. 4A, left panel). However, in the presence of EGF, $D_{\text{EGFR}}$ was statistically significantly decreased in TI-VAMP- and CD82-depleted cells compared with the mock-depleted cells (Fig. 4A, right panel). In TI-VAMP-depleted cells, activated EGFR was less mobile and often remained in the same area of the cell. The frequency distribution of $D_{\text{EGFR}}$ showed a uniform shift in the distribution between mock- and TI-VAMP- or CD82-depleted cells. We then categorized the diffusion coefficient of tracked EGFR into three classes: slow, medium and fast. Our results showed that in mock-depleted cells and in the absence of EGF, half of the EGFR is characterized by slow diffusion (Fig. 4B, left panel). The addition of the EGF leads to an increase of the medium class to the detriment of the slower fraction. By contrast, EGFR diffusion in TI-VAMP- or CD82-depleted cells was characterized by a persistent slower fraction at the expense of the fast one (Fig. 4B, right panel). This confirmed that the global population of EGFR was indeed slowed down by TI-VAMP and CD82 KD and that these modifications were only seen in the presence of EGF (Fig. 4A). This result is consistent with the increased endocytosis of EGFR evoked by EGF in TI-VAMP- and CD82-depleted cells previously shown (see Fig. 1A and Fig. 2F,G).

Role of TI-VAMP and CD82 in EGFR signaling and degradation

In TI-VAMP and CD82 KD cells, where EGFR endocytosis is increased, one would expect an altered signaling. EGFR signaling is initiated by its dimerization and phosphorylation on several residues. This in turn activates the mitogen-activated protein kinase (MAPK) pathway. We measured MAPK signaling by immunocytochemical quantification of phospho-ERK upon treatment of EGF as previously described (Lajoie et al., 2007). The peak of activation of phospho-ERK was significantly reduced in TI-VAMP- and CD82-depleted cells after 5 minutes and 10 minutes of stimulation (Fig. 5A,B). We further quantified phospho-ERK translocation into the nucleus as a mark of activation, and found a significant sustained translocation after 10 minutes of EGF in TI-VAMP- and CD82-depleted cells (Fig. 5C). These results confirmed that depletion of CD82 at the plasma membrane impaired early signaling of EGFR.

Moreover, we checked whether depletion of TI-VAMP and CD82 could have an effect on EGFR degradation, which could explain the long-lasting effect on signaling. We found that EGF accumulation in TI-VAMP KD cells did not result from a recycling defect (supplementary material Fig. S6A). We further showed that EGF was still able to reach the degradative compartment (supplementary material Fig. S6B-D) and that EGFR degradation was only slightly slowed down in TI-VAMP KD cells (supplementary material Fig. S6C). This indicates that TI-VAMP is dispensable for EGF degradation and recycling and that there may be an alternate pathway to reach the lysosomes. If TI-VAMP mediates the fusion between endosomes and lysosomes as suggested previously (Advani et al., 1999; Pryor et al., 2004), the function of TI-VAMP may be redundant with another v-SNARE (possibly VAMP8/endobrevin) which would explain why EGF is still degraded in our experiment (Antonin et al., 2000a). Interestingly, in CD82 KD cells (where the TI-VAMP is still present and potentially able to mediate fusion between endosome and lysosome) degradation of EGFR was delayed to a more significant degree (supplementary material Fig. S6E). These results suggest that the depletion of CD82 may alter the composition of tetraspanin-enriched microdomains important for EGFR degradation.

TI-VAMP and CD82 regulate endocytic machinery recruitment

We then wondered what mechanism could trigger an accelerated endocytosis of the receptor in TI-VAMP and CD82 KD cells. Increased phosphorylation or ubiquitylation of EGFR could be a cue to stimulate endocytosis. We thus assayed for ubiquitylation and phosphorylation of EGFR on T669, Y1045 and Y1173 by western blot and by flow cytometry. Phosphorylated tyrosine 1045 (Y1045) is a binding site of the ubiquitin ligase Cbl leading to EGFR ubiquitylation, internalization and degradation (Ettenberg et al.,
Y1173 interacts with Shc, and PLCγ, and is part of the autophosphorylation site involved in internalization (Helin and Beguinot, 1991; Sorkin et al., 1991b). Thr669 is phosphorylated by p38 MAP kinase and is involved in regulation of ligand-induced receptor internalization by interacting with downstream specific EGF receptor tyrosine kinase substrate (Countaway et al., 1990; Vergarajauregui et al., 2006; Winograd-Katz and Levitzki, 2006).

We did not detect any significant increase of ubiquitylation (Fig. 5D), or phosphorylation of Y1045 and Y1173 (Fig. 5E,F). We found, however, a 60% increase in phosphorylation of T669 in TI-VAMP-depleted cells (Fig. 5F). This result is particularly interesting because phospho-T669 has been shown to modulate both internalization and signaling (Bagowski et al., 1999; Li et al., 2008). Therefore increased phosphorylation of T669 is in good agreement with our observation of increased endocytosis and impaired signaling.

Lastly, we hypothesized that differential recruitment of endocytic machinery could favor accelerated endocytosis. The results shown in Figs 4 and 5 suggested that EGFR could recruit endocytic machinery more efficiently in TI-VAMP- and CD82-depleted cells. We found a significant increased colocalization of AP-2 with endocytosed fluorescent EGF after 5 minutes of activation in TI-VAMP and CD82 KD cells (Fig. 6A). Indeed, the proportion of AP-2 colocalizing with EGF was increased 2.1-fold after TI-VAMP depletion and 2.3-fold after CD82 depletion. Moreover, we found an increased density of AP-2 clusters in TI-VAMP- and CD82-depleted cells after 5 minutes of EGF stimulation (Fig. 6B), whereas no difference was detected in absence of EGF stimulation (our unpublished observations). These results support the notion that KD of TI-VAMP and CD82 favors endocytosis of activated EGFR by allowing a more efficient recruitment of AP-2.
Discussion

Our aim was to identify the function of TI-VAMP in the EGF pathway. Our main conclusion is that TI-VAMP acts primarily in the secretory pathway and is involved in the transport of the metastasis suppressor CD82 to the cell surface. In presence of EGF, CD82 depletion favors the recruitment of AP-2, which enhances endocytosis and limits EGF diffusion and signaling at the cell surface, thus unraveling a novel mechanism of EGF regulation. The use of HeLa cells allowed us to reach a high level of transfection and silencing, avoiding analysis of phenotype resulting from partially depleted protein expression (Bethani et al., 2009). The use of two different oligonucleotides targeting two different...
regions of the TI-VAMP mRNA and the rescue experiments that we performed, strongly suggest that the increased endocytosis of EGFR is truly specific to TI-VAMP KD. Cells cytometry allowed the quantification of thousands of cells (typically 30,000 cells in each condition), and thus for unbiased statistical analysis.

**Role of TI-VAMP via CD82 secretion in EGF/EGFR dynamics and endocytosis**

In this study, we report the unexpected increased EGF/EGFR endocytosis following depletion of TI-VAMP. The increase of EGF uptake both at 18°C and 37°C and the accelerated endocytosis of EGFR at 37°C in minute range strongly suggest that, in physiological conditions, TI-VAMP has an indirect role in endocytosis. We propose that TI-VAMP is required for the expression of cell surface factors which in turn regulate EGFR at the cell surface. Here we show that knockdown of TI-VAMP leads to decrease of CD82 expression and that depletion of CD82 recapitulates the effect of TI-VAMP KD on EGRF endocytosis. Furthermore, we found that TI-VAMP and CD82 were, at least partially, colocalized in the same vesicles. In addition, we know that TI-VAMP associates with lipid microdomains, which are rich in cholesterol in epithelial cells (Lafont et al., 1999). Biosynthesis of complex gangliosides (Tettamanti, 2004) and tetraspanin-specific interactions take place in the Golgi complex (Hemler, 2003; Levy and Shoham, 2005; Odintsova et al., 2006). TI-VAMP is necessary for the fusion of endosomal and lysosomal vesicles with the plasma membrane (Braun et al., 2004; Proux-Gillardeaux et al., 2007; Rao et al., 2004). Here, we showed that TI-VAMP is required for the transport from the Golgi to the plasma membrane, probably of AP-3-dependent cargos, including VSV-G, further suggesting that TI-VAMP is primarily involved in exocytosis of various types of secretory vesicles. Therefore, we propose that in the absence of TI-VAMP, CD82 and possibly associated proteins and lipids may not be loaded into secretory vesicles but degraded, thus accounting for the decreased expression level of CD82 shown here. This in turn would perturb activated EGFR dynamics, signaling and endocytosis at the cell surface.

**Role of CD82 in EGF/EGFR dynamics and endocytosis**

Tetraspanins regulate cell morphology, motility, invasion and signaling in brain, immune system and tumors (Hemler, 2003; Levy and Shoham, 2005). Tetraspanins associate at the plasma membrane and facilitate lateral positioning of various receptors (Berditchevski and Odintsova, 2007; Charrin et al., 2003a; Hemler, 2005) by establishing the tetraspanin web, a network of so-called tetraspanin-enriched microdomains (TEMs). TEMs constitute dynamic platforms in permanent lateral exchange with the rest of the membrane (Espenel et al., 2008). They are considered as physically
and functionally distinct from lipid rafts (Boucheix et al., 2004; Hemler, 2003) but they depend at least in part on lipids, including gangliosides (GM) and cholesterol (Charri

It has been shown that CD82 increases surface expression of several gangliosides and associates with and regulates cholesterol-rich microdomains (Charri

Moreover, cholesterol has a role in the diffusion of EGFR since its depletion led to almost complete confinement of the receptors (Orr et al., 2005). Thus the loss of CD82 at the plasma membrane may alter the amount of gangliosides and cholesterol and thus decrease EGFR motility. Overexpression of CD82 causes the redistribution of EGFR into membrane rafts and results in attenuation of dimerization (Odintsova et al., 2000; Odintsova et al., 2003). In our case, CD82 depletion may favor the exit of the EGFR from lipid microdomains, and thus dimerization and endocytosis.

Role of TI-VAMP and CD82 in EGFR signaling and motility

Our results furthermore demonstrate that EGFR downstream signaling is regulated by its internalization as was originally proposed by Vieira et al. (Vieira et al., 1996). In the latter case, decreased EGFR phosphorylation and MAPK activity resulted from the expression of defective dynamin and inhibited endocytosis. These effects increased with time of EGF activation up to 20 minutes. Here we found decreased ERK phosphorylation after 5 and 10 minutes of activation but a greater sustained response measured by phospho-ERK translocation into the nucleus (Fig. 5).

Fig. 6. TI-VAMP and CD82 KD favor EGFR endocytosis via increased recruitment of AP-2. (A) Double detection of EGF-A488 (green) and AP-2 (red) immunoreactivity in confocal sections after 5 minutes of stimulation. Colocalization between AP-2 and EGF was significantly increased in TI-VAMP and CD82 KD cells. (Student’s t-test: TI-VAMP siRNA 1: P=0.0001; siRNA 2: P<0.0001; CD82 siRNA: P<0.0001.) (B) AP-2 immunoreactivity viewed by standard epifluorescence microscopy after 2 minutes of EGF stimulation. Density of AP-2 clusters was analyzed on 30 cells per condition. Density of AP-2 was significantly increase in TI-VAMP (Student’s t-test: TI-VAMP siRNA 1: P=0.0087; siRNA 2: P<0.0001) and CD82 (P=0.0073) KD cells.

Fig. 7. TI-VAMP-dependent secretion of CD82 regulates cell surface diffusion and endocytosis of EGFR. TI-VAMP mediates the transport from the Golgi to the cell surface of the marker VSV-G and potentially of the tetraspanin CD82 (top panel). One possibility would be that AP3 is present on this vesicle since it has already been involved in the transport of VSV-G from the Golgi complex to the plasma membrane (Nishimura et al., 2002) and since the targeting of TI-VAMP is dependent on AP3 both in HeLa cells and in neuronal cells (for a review, see Danglot and Galli, 2007). At the cell surface, CD82 assembles into tetraspanin-enriched microdomains constituting the tetraspanin web. EGFR strongly interacts with CD82 and is endocytosed by CME and CIE. In TI-VAMP-depleted cells (bottom panel), the transport of CD82 to the cell surface is inhibited, leading to a defect in the tetraspanin web at the cell surface. When stimulated by EGF, the lack of TI-VAMP and/or CD82 results in a diminished lateral diffusion of EGFR, increased recruitment of EGFR in AP-2-coated pits and by increased endocytosis by CME, thus impairing MAPK signaling.
and depleting Ti-VAMP (supplementary material Fig. S6) delay EGFR degradation may explain the increased EGFR signaling after prolonged activation. At short times of EGF activation, however (<10 minutes), our results suggest that altered motility and endocytosis of activated EGFR impair its full activation. This model is in agreement with the proposal by Lajoie et al. (Lajoie et al., 2007) that EGFR may partition in different subdomains at the cell surface with different signaling capabilities. Therefore, EGFR signaling is regulated by endocytosis in a rather complex manner with distinct effects for short and prolonged EGFR activations.

We further showed that the surface area explored by the activated EGFR (trajectory length and radius) is decreased in Ti-VAMP- and CD82-depleted cells. TEMs are known to be connected to the actin cytoskeleton and CD82 is recognized as a link between lipid raft and actin cytoskeleton in lymphocytes (Delaguillaumie et al., 2004). Furthermore EGFR diffusion and endocytosis are regulated by F-actin dynamics (Lidke et al., 2005; Lidke et al., 2004; Orr et al., 2005). Thus TEMs might participate in the EGFR membrane dynamics at least in part by interacting with the actin cytoskeleton. This hypothesis will require further exploration. In the absence of the TEMs of CD82, EGFR may be incorporated into plasmalemmal domains with a different protein and lipid composition and this may change its biochemical properties. In particular, upon activation by EGF, EGFR showed increased phosphorylation of T669, a residue involved in internalization (Fig. 5F).

It has been proposed that receptors such as TIR have a random diffusion at the surface until they reach stable clathrin-coated pits (Ehrlich et al., 2004). By contrast, clathrin-coated pits have limited motility within the membrane due to the attachment to the membrane actin cytoskeleton (Gaidarov et al., 1999). In Ti-VAMP and CD82 KD cells, EGFR traverses a shorter distance before being internalized by a more efficient clathrin-dependent endocytosis, thus suggesting that EGFR reached clathrin coated pits more efficiently. This could be explained either by the de novo generation of clathrin-coated pits and/or increase of EGFR recruitment into pre-existing CCPs. De novo formation of clathrin-coated pits has already been described after EGFR activation (Benmerah and Lamaze, 2007; Johannessen et al., 2006), and represents 27% of EGFR endocytic events (Rappoport and Simon, 2009), the remaining EGFR clusters being recruited at sites of preformed clathrin lattices (Rappoport and Simon, 2009). In our case, the proportion of AP-2 colocalizing with EGF is increased by 2.1- to 2.3-fold in knockdown compared with mock-depleted cells, and the density of AP-2 clusters is specifically increased after EGFR stimulation. These results may suggest the formation of new clathrin-coated pits. However, since the increase of AP-2 clusters density is quite low (×1.1) in comparison with the increased colocalization of AP-2 with EGFR (×2.3), this suggest that a large proportion of EGFR is also specifically recruited to pre-existing coated pits. In both scenarios, increased concentration of EGFR into clathrin-coated pits would favor its endocytosis, thus limiting activated EGFR diffusion and signaling at the cell surface. This could also explain why we observed an effect only on activated EGFR diffusion and endocytosis, and not on TIR internalization or EGFR constitutive endocytosis. Altogether, our data provide new evidence to conclude that Ti-VAMP mediates the transport of the microdomain-associated tetraspanin CD82 to the cell surface, which in turn controls cell surface diffusion, signaling and endocytosis of activated EGFR (see our model, Fig. 7). The lack of CD82 at the plasma membrane, caused by Ti-VAMP depletion, prevents the receptor exploring a wide surface area and favors the recruitment of endocytic machinery (at least AP-2) and consequently favors its internalization by clathrin-dependent endocytosis and decreases MAPK signaling at the plasma membrane.

Understanding the precise orchestration of events during receptor sequestration, diffusion and endocytosis is a significant challenge. Our data underscore the importance of tetraspanin microdomains in EGFR dynamics and points to the tetraspanin web as essential component of the mechanisms of clathrin-coated pits assembly and endocytosis of EGFR.

Further studies on how tetraspanins regulate the lipid and protein composition of microdomains will be fruitful to understand how these complexes contribute to cell growth, motility and metastasis.

Materials and Methods
Antibodies and reagents
Antibodies and reagents are described in supplementary material Table S1.

Cell culture
HeLa cells were cultured at 37°C, in 5% CO2 in DMEM (Dulbecco’s modified Eagle’s medium) with 10% FCS (fetal calf serum) 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Transfection
siRNA
siRNA were delivered by two waves of transfection with Oligofectamine (Invitrogen, Carlsbad, CA, USA). Cells were incubated for at least 96 hours with 20 nM siRNA, passed and used 96-198 hours after the first transfection when extinction was optimal. This allowed for homogenous silencing (virtually all the cells) compared with the use of plasmid shRNA expressed from a pSuper plasmid (supplementary material Fig. S1). The use of two nucleotides allowed us to avoid interferon response (Sledd et al., 2003) and off-targets effects (Jackson et al., 2003; Scales et al., 2000; Snove and Holen, 2004) which are one of the pitfalls of RNAi experiments. For control cells, luciferase siRNA (mock condition) was used: firstly (Photinus pyralis, GenBank M15077, sense: 5’-CGUACCGGGAUAACUUGCATT-3’; antisense: 5’-UCCAAAGUAUUCGCGUACCTT-3’).

Ti-VAMP siRNAs were chosen to target either the coding region (5’-CTGCAAGACGACAGATGTATA-3’ = siRNA1) or the 3’UTR sequence (5’-TGCCA-TAAAATGAAATATATA-3’ = siRNA 2). Sequences were submitted to BLAST searches against the human genome sequence to ensure that only the desired mRNA was targeted [database resseq_rna using Megablast (optimize for highly similar sequences)]. siRNA duplexes were synthesized by Qiagen (Qiagen SA,Courtabeuf, France).

The siRNA targeting VAMP3 [5’-CAG CAT GTT TCT GAT AAT TAT-3’; HS VAMP3_1 HP siRNA (S01095722)], VAMP4 [5’-TTG CTG CAT AAT GTA AAT AAT-3’; HS VAMP4_2 HP siRNA (S01075962)], VAMP8 [HS VAMP3_3 HP validated siRNA (S01265345)] and CD82 [CGCGTTGCGATCGTTCATCAA; HS CD82_2 FP siRNA (S01386650)] were purchased from Qiagen. Clarin siRNA were previously described [5’-AAG CUG GGA AAA CUC UGC AGA-3’] (Motley et al., 2003).

Plasmid transfection
The best ratio of plasmid transfection and survival on the knocked down cells was obtained with FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA) which allowed transfection of HeLa cells that were not subconfluent. Experiments were carried out at least 96 hours after first incubation with RNAi.

Rescue transfection
Rat brain Ti-VAMP was cloned into pcDNA3.1 by RT-PCR from rat brain mRNA. This rat sequence was not targeted by our human oligonucleotide siRNA 2 which targets the 3’UTR human sequence, a sequence absent in the rat construct.

EGF uptake, transferrin recycling and cell surface labeling
EGF uptake
Cells were starved overnight in DMEM without serum, collected in PBS-EDTA, washed in DMEM, 15 mM HEPES pH 7.5, 20 mM glucose, 1% BSA, incubated in the same medium with EGF (100 ng/ml, for 1 hour at 18°C) or transferred (for 45 minutes at 37°C), and washed extensively at 4°C and fixed immediately.

Acid stripping
Where indicated, cells were incubated for 3 minutes at 4°C in acidic buffer (50 mM glycine, 100 mM NaCl, pH 3.0) to remove surface labeling.

Surface labeling for flow cytometry
For the detection of the EGF, CD62, CD63 and L1-CAM molecules at the plasma membrane, cells were harvested with PBS-EDTA, resuspended at 106 cells/ml and
incubated for 1 hour at 4°C with the monoclonal antibodies in DMEM, 15 mM HEPES pH 7.5, 20 mM glucose, 1% BSA, washed in PBS and fixed.

Intracellular antibody staining for flow cytometry
Cells were fixed in 4% paraformaldehyde (20 minutes at 4°C), quenched in PBS-50 mM NH₄Cl (20 minutes), permeabilized (1% PBS-BSA, 0.05% Triton X-100, for 4 minutes) and incubated with primary antibodies (45 minutes at 4°C, on a wheel). Secondary antibodies used were either coupled to Alexa Fluor 488, phycocerythrin (PE) or cyanin 5 (Cy5).

Flow cytometry analysis
Analyses were performed with a CyAn ADP flow cytometer (DakoCytomation, Dako France SAS, Trappes, France) equipped with two solid-state lasers: a 488 nm one for Alexa Fluor 485 and PE, and a 635 nm one for Cy5. Alexa Fluor 488 fluorescence was collected through a 530/40 nm band-pass, PE fluorescence through a 575/25 nm band-pass and Cy5 fluorescence through a 665/20 nm band-pass. The cell populations analyzed were gated on the basis of forward and side (90° angle) scatter criteria to avoid potential contamination by dead cells or debris. Forward angle light scatter and right angle scatter were used to select cells, the resultant fluorescence histograms were constructed from 10,000 to 20,000 cells, as specified. Experiments were usually done three times independently. Data analysis was performed with Summit v 4.3.01. Median fluorescence intensity and mean fluorescence intensity values were determined for both positive and negative populations of cells.

VSVG-GFP secretion assay
Cells were transfected with VSV-G-GFP tO45, incubated at 39°C overnight, rinsed and incubated in DMEM-HEPES-glucose for 2 hours at 20°C to allow progress of the VSVG-GFP protein to the Golgi. At time t=0, cells were incubated with mouse anti-GFP 8G5F11, recognizing an ectoplastic epitope of VSVG-GFP protein, for 30 minutes at 4°C on a wheel. After incubation at 32°C, cells were harvested and rinsed in ice-cold PBS and then incubated with mAb 8G5F11 for 30 minutes at 4°C. After washing, fixation and permeabilization, cells were then incubated with rabbit anti-GFP antibody for 1 hour at 4°C. Primary antibodies were revealed by goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Cy5. Transfected cells were sorted on the basis of the GFP staining, and the intensity of the fluorescence of surface VSVG-G labeling was analyzed in the Cy5 channel. Immunofluorescence of VSVG-GFP secretion assay on coverslips was done with the same protocol but with adherent HeLa cells grown on 12 mm coverslips.

Immunocytochemistry
Cells were processed for immunofluorescence studies as described previously (Danglot et al., 2003) and mounted in Prolong mounting medium (Invitrogen).

Western blotting
Cells were rinsed in ice-cold phosphate-buffered saline (PBS), and scraped in TSE buffer [50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics), leupeptin, aprotinin and phosphatase inhibitor Cocktail (Sigma K0476, Germany)] after 30 minutes at 4°C. Lysates were then centrifuged at 15,000 g for 20 minutes, and the supernatant was frozen. Samples were boiled for 5 minutes in SDS sample buffer. Protein concentration was determined using the Bio-Rad protein assay. SDS-PAGE was performed using 4–12% bis-Tris NuPAGE (Invitrogen) gradient gels and then processed for western blotting. Primary antibodies were then revealed with either secondary antibodies conjugated to horseradish peroxidase (1:10000 Jackson Laboratories) or with fluorescent antibodies, using tubulin or GC3PDH as standards. Peroxidase was detected either by chemiluminescent detection on Hyperfilm using ECL-PLUS (GE Healthcare, Chalfont St Giles, UK), or with a HRP-CPR (Chemiluminescence, Brattleboro, VT, USA). Quantification of immunoblotting was obtained with a densitometer and scanning images were processed using homemade software (Matlab; The Mathworks, Natick, MA, USA).

Immunoprecipitation
Cells were lysed in TSE buffer and incubated with protein-G-coupled Sepharose beads (GE Healthcare) that had been incubated with antibody to EGFR (Calbiochem Abs-1) for 1 hour at 4°C. The beads were washed once in TSE and twice with immunoprecipitation buffer [TSE buffer supplemented with 20 mM NEM, 100 mM MG-132, 5 mM iodoacetamide, 0.5% BSA and 0.25% sodium deoxycholate, 2 mg/ml Phenylmethylsulfonyl fluoride (Applied Biosciences)] before being added to cell lysates. Immunoprecipitation was performed for 1 hour at 4°C before washing four times with immunoprecipitation buffer. The immunoprecipitate was eluted in 2× sample NUPAGE buffer, incubated at 95°C for 5 minutes and subjected to SDS-NUPAGE and immunoblotting. Immunoblots were revealed with anti-ubiquitine antibody (Santa Cruz).

Standard epifluorescence microscopy
Fluorescence images were acquired on a Leica DM2500 microscope (×63, 1.32 objective) using A488-, Cy3-, Cy5- and DAPI-specific filters sets and a high-resolution camera (Coolspap HQ) driven by the Metamorph Image Analysis System. Image files were merged for colocalization using Adobe Photoshop CS3.

Confocal microscopy
Specimens were observed with a ×63 oil immersion objective, followed by a 1.6 digital zoom magnification. Images were acquired on a Leica SP2 confocal microscope by sequential scanning of the emission lines. Alexa Fluor 488 was detected by using the 488 nm-line of an argon laser for excitation; Cy3 and Alexa Fluor 594 were excited by the 543-nm line of a green neon laser, and Cy5 was excited by the 650-nm line of a helium neon laser. Typically, pictures were scanned twice at high frequency (1000 Hz) to optimize the signal to noise ratio and to avoid delay between the YFP and RFP channels.

Single quantum dot tracking and data analysis
Cells were incubated for 10 minutes at 37°C with biotinylated mAb-3 EGFR primary antibody (1 μg/ml), washed intensively and incubated for 3 minutes at 37°C with 1024 nM quantum dots (QDs; Invitrogen) emitting at 605 nm (0.2 nM). Cells were incubated at 37°C (5% CO2) in an open chamber mounted onto an inverted microscope (Leica DMi6000) equipped with a ×63 objective and an intensified EMCCD camera (Cascade 512, Roper Scientific, Tucson, AZ, USA). QDs were detected using a Hg lamp (excitation filter 525/45F and appropriate emission filter 595/460; Omega Filters, Inc., Cheshire, Brattleboro, VT, USA). QDs fluorescent images were obtained with an integration time of 38 ms with up to 500 consecutive frames acquired with Metamorph (Universal Imaging, MDS Inc., Toronto, Canada). Tracking was performed with homemade software (Matlab; The Mathworks, Natick, MA, USA). Single-QDs were identified by characteristic blinking fluorescent emission and uniform size. Because of these random blinking events, the trajectory of each QD could not necessarily be tracked continuously. The center of the spot fluorescence was determined using a Gaussian fit with a spatial resolution of 10-30 nm. Values of the mean square displacement (MSD) were calculated from the trajectories applying the relation: MSD(n=dt)=<(x(i+n–1)–x(i))2+(y(i+n–1)–y(i))2>, where x and y are the coordinates of an object on frame i, N is the total number of steps in the trajectory, dt is the time interval between two successive frames, and n is the time interval over which displacement is averaged. Diffusion coefficients (D) were calculated by fitting the first five points of the MSD curves versus time (t) with the equation MSD=4Dt+4Gt. Trajectory length and radius were determined from short trajectories (100 frames) during which QDs did not blink.

Statistical analyses
All experiments were done at least three times. Results are expressed as mean ± s.e.m. Comparisons of mean values between mock and RNAi cells were performed using an unpaired Student’s t-test or non parametric Mann-Whitney’s test when distributions were not Gaussian. Statistical significance was accepted at P<0.05. Significant differences are indicated by asterisks (**P<0.01; ***P<0.001). Statistical analyses were performed using Statview 5.0.1 software or Prism 5 (Graphpad software).

Nuclear translocation of Erk
Cells were plated on coverslips, serum starved for 24 hours, and stimulated with EGF. After various times with EGF, cells were fixed (10 minutes with 4% formaldehyde at 20°C, then 2 minutes in 100% methanol at –20°C), washed, blocked in PBS plus 0.1% fish gelatin for 1 hour at 37°C and incubated with mAb anti-phospho-Erk1/2 (Zh202/Tyr204; Sigma-Aldrich, 1:1000 overnight at 4°C). The cells were washed three times and Alexa-Fluor-488-labeled anti-mouse Ig (Invitrogen) added at 1/400 with DAPI for 1 hour at 20°C. Images were acquired using a Leica DM2500 microscope (×63, 1.32 objective) with equivalent acquisition settings. Staining intensity was determined individually for 30-50 cells per condition from two independent experiments. Total phospho-ERK intensity was measured and then normalized to cell surface area. The mean intensity of nuclear phospho-Erk was quantified by creating a mask based on DAPI staining using Metamorph software. The ratio of (nuclear:total) intensity values was used to represent the nuclear translocation after the addition of EGF.

Quantitative analysis of colocalization between AP2 and EGF
Fluorescence images were acquired by sequential scanning using a TCSP5 Leica confocal microscope. Typically 1024×1024 pixels were scanned three times. Quantifications were performed using Metamorph software. Images were subjected to the same user-defined intensity threshold to select clusters. AP2 clusters were classified as colocalizing with EGF if clusters in the thresholded AP2 images
had more than 50% of pixels overlapping with an EGFR puncta. All results are means ± s.e.m., referring to the number of region analyzed (30 regions per condition, i.e. 500-700 AP-2 clusters analyzed in each condition).

**Quantitative analysis of AP-2 cluster density**

Since the z-axis of confocal sections can influence the density of clusters, we analyzed AP2 cluster density by wide-field microscopy on a Leica DMRE microscope with a closed aperture objective (×63, 1.32) to maximize the depth of field. Images were filtered using a Log3D macro in ImageJ software to detect all clusters within a cell (Sage et al., 2005). Number of AP-2 clusters were then analyzed with Metamorph software and reported per cell area (30 cells analyzed per condition).

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**Supplementary material available online at** [http://jcs.biologists.org/cgi/content/full/123/5/723/DC1](http://jcs.biologists.org/cgi/content/full/123/5/723/DC1)

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