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Non-classical export of epimorphin and its adhesion to αv-integrin in regulation of epithelial morphogenesis

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Summary

Epimorphin (also known as syntaxin 2) acts as an epithelial morphogen when secreted by stromal cells of the mammary gland, lung, liver, colon, pancreas and other tissues, but the same molecule functions within the cell to mediate membrane fusion. How this molecule, which lacks a signal sequence and contains a transmembrane domain at the C-terminus, translocates across the plasma membrane and is secreted to become a morphogen, and how it initiates morphogenic events is not clear. Here, we show that epimorphin is secreted through a non-classical mechanism, similar to that previously described for secretion of the leaderless protein FGF1, and we identify the key molecular elements responsible for translocation and secretion from the cell. We also show that secreted epimorphin binds to αv-integrin-containing receptors on target epithelial cells, leading to activation of specific downstream signaling pathways and induction of epithelial morphogenesis. These findings provide key insight into how epimorphin functions as an epithelial morphogen.

Supplementary material available online at
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Key words: Epimorphin, Integrin, Morphogenesis, Membrane translocation, t-SNARE

Introduction

Morphogenesis, the orchestration of tissue remodeling with cell proliferation, differentiation and apoptosis, is controlled by the concerted action of diffusible morphogens and elements of the insoluble extracellular matrix (ECM) (Gumbiner, 1992; Lubarsky and Krasnow, 2003; Sakai et al., 2003). Whereas many morphogens are delivered to the extracellular space through the ER/Golgi secretory pathway, there is a growing class of morphogens that becomes localized to the extracellular space through non-classical secretory pathways (Prudovsky et al., 2003). The morphogen epimorphin (also known as syntaxin 2) lacks a canonical signal peptide for processing through the ER/Golgi, yet is found in the extracellular space (Hirai et al., 1998; Hirai et al., 1992). Although a number of different mechanisms have been proposed for non-classical secretion (Nickel, 2003; Wickner and Schekman, 2005), some common patterns are emerging. In the best characterized example, fibroblast growth factor 1 (FGF1) is transported along cytoskeletal networks to the cytoplasmic surface of the plasma membrane, where it adopts a molten-globule conformation in a multimeric protein-release complex that becomes translocated across the membrane via phosphatidyl-dependent flipping (Prudovsky et al., 2003; Ptitsyn, 1995); the FGF1 protein-release complex includes annexin II, S100A13 and synaptotagmin 1, the latter component binds to members of the syntaxin protein family (Bai and Chapman, 2004).

Epimorphin is distinct from many other proteins that undergo non-classical secretion in that it has a well-defined intracellular function. It is a member of the t-SNARE protein family that mediates vesicle targeting and fusion (Bennett et al., 1993; Pelham, 1993; Sieber et al., 2006). Epimorphin (syntaxin 2) is highly similar to syntaxin 1A, which consists of a three-helical N-terminal bundle (3-Hlx) connected by a flexible linker to a C-terminal sequence containing the SNARE and transmembrane (TM) domains (Chen and Scheller, 2001). Deletion analyses of epimorphin have revealed that the morphogenic and vesicle-fusion functions are separable: the the C-terminal SNARE and TM domains are essential for syntaxin-mediated vesicular fusion (Chen and Scheller, 2001; Giraudo et al., 2006) but they are dispensable for epimorphin-mediated morphogenic activity (Hirai et al., 2001). Despite these differences and the many studies in a diverse range of tissues in which extracellularly presented epimorphin has been shown to regulate developmental processes (Bascom et al., 2005; Fritsch et al., 2002; Hirai et al., 1998; Hirai et al., 2001; Hirai et al., 1992; Lehnert et al., 2001; Oka and Hirai, 1996; Oka et al., 2006; Qin et al., 2005; Radisky et al., 2003; Takebe et al., 2003; Tulachan et al., 2006; Yoshino et al., 2006), the

extracellular action of epimorphin as a morphogen has remained controversial because the specific molecular mechanism by which epimorphin becomes secreted from the cell has not been defined and the extracellular receptor that mediates the morphogenic action of epimorphin has not been identified.

In this study, we determine the motif responsible for the membrane translocation of epimorphin, and define the processing site and the critical amino acid residue for its subsequent secretion. We find that the key elements for epimorphin secretion are similar to those defined for non-classical export of FGF1 and interleukin 1 beta (IL1B). We also identify the cell surface receptor responsible for controlling morphogenesis induced by secreted epimorphin. These results not only elucidate how epimorphin can act as an extracellular morphogen, but also shed light on a new morphoregulatory mechanism, in which a protein with a defined intracellular function can become externalized and processed as a morphogen for signaling to target cells.

Results

Identification of a domain for extracellular presentation and secretion

Epimorphin localizes to the cytoplasmic surface of the plasma membrane (Quinones et al., 1999) and shows substantial sequence homology with other members of the syntaxin protein family (Bennett et al., 1993). Epimorphin can also exist extracellularly as a soluble morphogen (Hirai et al., 2001). To determine whether other syntaxins could also be secreted, we developed a system to compare the extracellular presentation of epimorphin with that of highly homologous syntaxin 1A. For these studies, we used primary mammary fibroblasts, because these are the principal source of epimorphin in the mammary gland (Fig. 1A) (Hirai et al., 1998). We previously found a soluble 30 kDa form of epimorphin in the lactating mammary gland and in supernatants from epimorphin-expressing cultured cells (Hirai et al., 2001). Here, cells were transiently transfected with plasmids containing expression constructs for mouse epimorphin (TE) or mouse syntaxin 1A (TS) both tagged with the T7 peptide epitope. Although the full-length 34 kDa transgene products were detected in cell lysates, a 30 kDa soluble form was found only in supernatants of TE-transfected cells (Fig. 1B). These results suggested that epimorphin secretion is determined by specific sequence(s) that differ between epimorphin and syntaxin 1A.

We defined the motif responsible for extracellular presentation and secretion of epimorphin by using TE and TS in combination with constructs that express a mutant epimorphin lacking the SNARE domain (TEΔS) and chimeric molecules in which the 3-Hlx domains of epimorphin and syntaxin 1A were fused to the C-terminal SNARE/TM domains of the other (TES, TSE; Fig. 2A). All five protein products (TE, TS, TEΔS, TES, TSE) were found in the lysates of transfected cells, but only TE, TS, TES, and TSE reacted with membrane-impermeant biotinylation reagents (Fig. 2B); these results demonstrated that the presence of the SNARE/TM domain from either epimorphin or syntaxin 1A was sufficient to direct extracellular presentation. However, only constructs containing the SNARE and TM domains of epimorphin were secreted from the cell surface into the extracellular medium (Fig. 2C), demonstrating that solubilization of epimorphin is defined by distinct signaling elements not present in syntaxin 1A. Whereas the amount of protein secreted varied between transfection experiments, we typically observed 5-20% secretion of the total TE and TSE. These results were also obtained in other cultured fibroblast cell lines, including mouse embryonic NIH 3T3, 10T1/2 and mammary g6 cells (K.T. and Y.H., unpublished data). The size of the secreted form and the maintenance of its N-terminal epitope tag suggested that release from the cell surface is accomplished through proteolytic cleavage within the SNARE domain. Mass spectrometric analysis of the tryptic fragments of secreted 30 kDa epimorphin identified only sequences contained within the N-terminal domain, with the most C-terminal peptide ending

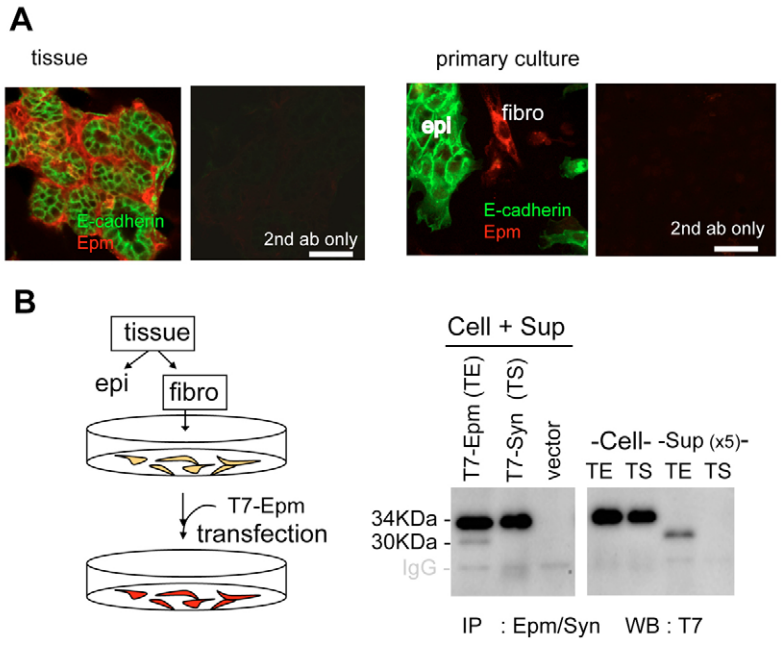

Fig. 1. Extracellular secretion of epimorphin in mammary stromal cells. (A) Epimorphin is produced in the E-cadherin-negative compartment in the mammary gland. Cryosections (left panel) and primary cells (right panel) of glands from mid-pregnant (day 13) mice (ICR, Shimizu Laboratory Supplies) were stained with goat anti-E-cadherin antibodies (E-cadherin; green) and affinity-purified rat anti-epimorphin antibodies (Epm; red). Non-immunized rat serum and secondary antibodies did not label cells. Bars, 100 μm. (B) Secretion of the 30 kDa soluble epimorphin but not of highly similar syntaxin 1A. Epimorphin-producing mammary cell populations were transfected with expression plasmid for T7-tagged epimorphin or syntaxin 1A (TE and TS, respectively), and proteins were immunoprecipitated from the whole-cell culture (cell + sup), cell (cell) or supernatant (sup) with pan-syntaxin antibodies followed by western blot analysis using anti-T7 mAb. Loaded amount of supernatant was five times higher than that of cell lysate.

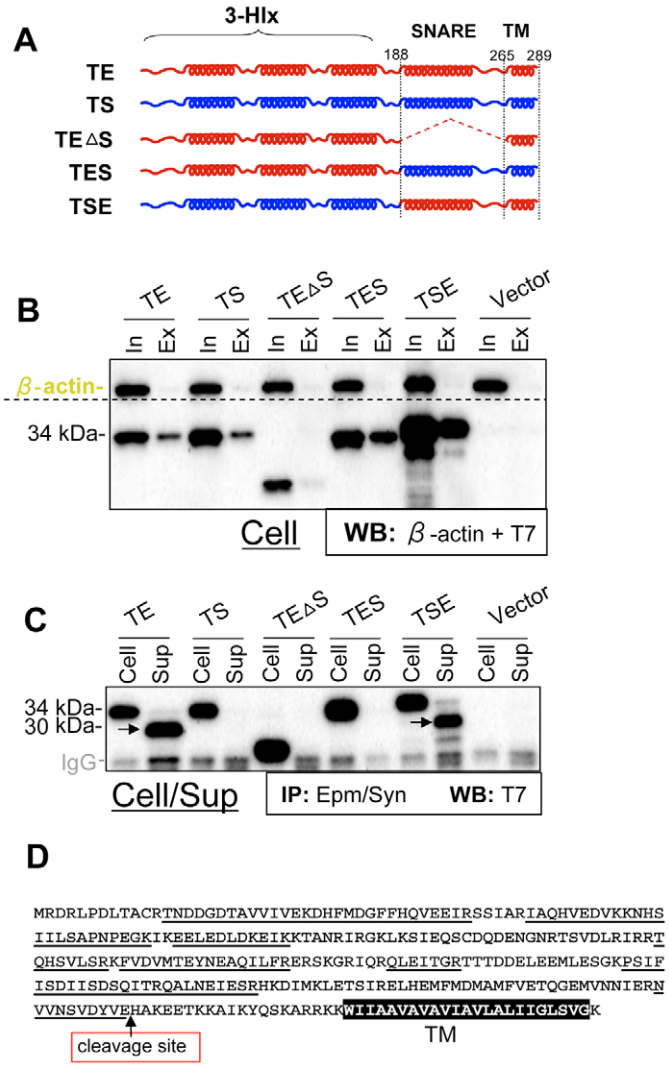

Fig. 2. Extracellular secretion of epimorphin is dependent on the SNARE/TM domain. (A) Schematic of chimeric and mutant constructs. 3-Hlx, triple-helical N-terminal domain; SNARE, SNARE domain; TM, transmembrane domain. Epimorphin (Epm) is shown in red, syntaxin 1A is shown in blue. (B) Cell surface presentation of epimorphin (TE), syntaxin 1A (TS) and the domain-swapped molecules (TES, TSE), but not Epm lacking the SNARE/TM domain (TEΔS) or the cytoplasmic β-actin protein. PT67 cells transiently transfected with indicated constructs were labeled with membrane-impermeant biotinylation reagents. Cell lysates were incubated with streptavidin-agarose beads and bound (Ex) or unbound (In) fractions were probed with antibodies against β-actin and the T7 tag. (C) Transfection with TE or TSE causes secretion of a soluble form of the molecules into the medium (sup), whereas transfection with TS, TES, or TEΔS does not. Loaded amount of supernatant was five times higher than that of cell lysate. (D) Proteomic analysis of secreted 30 kDa epimorphin. Supernatant of TE transfectant was collected, the 30 kDa soluble epimorphin was retrieved with anti-T7-coated beads and separated by SDS-PAGE. Protein amount from cells of one culture dish (10-cm diameter) was visible with Coomassie Brilliant Blue staining. The protein band was isolated and tryptic fragments were analyzed by mass spectrometry (MS-MS). Identified sequences are underlined. The most C-terminal peptide identified ends with glutamic acid (E), indicating the cleavage site.

with glutamic acid at position 245 (E245). This result suggested that E245 is the C-terminus of secreted epimorphin (Fig. 2D). Consistent with this result, antibodies against the C-terminal part of epimorphin SNARE that react with cellular 34 kDa epimorphin failed to recognize the secreted 30 kDa form (see supplementary material Fig. S1).

Determination of the critical amino acid residue involved in epimorphin release

The sequences of epimorphin and syntaxin 1A are highly conserved, especially within the SNARE domain; however, only epimorphin is cleaved and secreted. Comparison of the amino acid residues adjacent to the cleavage site of different syntaxin molecules revealed a histidine residue at position 246 (H246) in epimorphin from mouse, human, quail and sheep. However, in mouse and human syntaxin 1A, as well as mouse syntaxin 3 and syntaxin 4, this position is occupied by an arginine (R246) (Fig. 3A). Moreover, also conserved in epimorphin from all four species examined is a KEE sequence at positions 248-250 that is not present in syntaxin 1A, syntaxin 3 and syntaxin 4 (these syntaxin isoforms contain a VSD sequence instead; Fig. 3A). The release of epimorphin from the cell surface is dramatically reduced upon a H246R mutation, whereas mutation of KEE to VSD did not reduce release into the supernatant (Fig. 3B). Conversely, after a R246H mutation in syntaxin 1A, the resultant protein was released as a 30 kDa soluble form, demonstrating the importance of H246 (Fig. 3B). However, it is noteworthy that mutation of the syntaxin 1A sequence VSD to KEE resulted in the limited release of this molecule into the supernatant (Fig. 3B), suggesting that this sequence may play a secondary role.

Cell-stress-mediated release of epimorphin

While the production of the soluble 30 kDa form of epimorphin was always observed in transiently transfected cells, we found that stable transfectants expressing the full-length TE transgene (34 kDa) showed very little basal extracellular secretion (Fig. 4A). Since cell stress and Ca^{2+}-signaling mechanisms have been implicated in the non-classical secretion of FGF1 and interleukin 1 alpha (IL1A) (Prudovsky et al., 2003), we reasoned that stress associated with the transfection procedure may have played a role in the secretion of epimorphin from transient transfectants. We found that exposure of stable transfectants to subtoxic levels of cycloheximide, actinomycin D or camptothecin resulted in the cell surface projection of epimorphin as well as the appearance of a 30 kDa form in the culture medium, whereas the solvent alone had no effect (Fig. 4A). The Ca^{2+} ionophore A23187 and other reagents that trigger Ca^{2+} influx (Schanne et al., 1979) also showed similar effects (Fig. 4A; Y.H., unpublished). The calpain inhibitor calpeptin, which inhibits later stages of apoptosis and partially prevents cycloheximide-induced cell death (Squier et al., 1999), partially blocked cycloheximide-induced secretion of epimorphin (Fig. 4B). We found that epimorphin secretion was not a trivial consequence of cell death, because exposure to EDTA at subtoxic concentrations and transient heat stress (42°C for 1.5 hours) resulted in dramatic cell death but not the secretion epimorphin (Fig. 4B). Previous studies have shown that cell-stress-induced non-classical secretion of FGF1 is

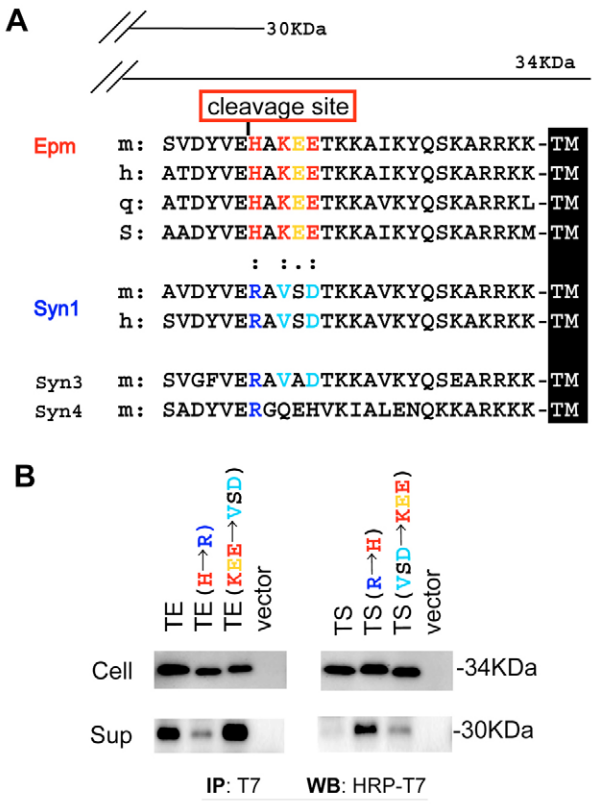

Fig. 3. Identification of critical amino acids for epimorphin secretion. (A) Comparison of the sequences close to the cleavage site of epimorphin (Epm) and conserved syntaxin family members. Syntaxin 1A, syn1; syntaxin 3 (NM001025307), Syn3; and syntaxin 4 (NP033320), Syn4. Histidine H246 (red) is conserved in epimorphin from mouse (m, D10475), human (h, D14582), quail (q, AB076670) and sheep (s, E32546); other syntaxins have arginine R246 (blue). Adjacent to H246, the KEE sequence (red-yellow-red) is also conserved in epimorphin, whereas the VXD sequence (blue-black-blue) occupies this position in syntaxin 1A and syntaxin 3. (B) H246 is crucial for processing and secretion of epimorphin. The H246R mutation (CAT→AGG) in the T7-epimorphin sequence dramatically suppressed the secretion of 30 kDa epimorphin. The R246H (AGG→CAT) mutation in T7-syntaxin-1A resulted in the secretion of a 30 kDa form.

accomplished through assembly of a release complex containing annexin II, an extravesicular 40 kDa form of the SNARE-binding Ca^{2+} sensor syntaptotagmin and S100A13. The first two molecules directly bind to phosphatidylserine at the inner leaflet of the plasma membrane (Bai and Chapman, 2004; Prudovsky et al., 2002). Immunoprecipitation experiments revealed that cellular 34 kDa epimorphin associates with annexin II and synaptotagmin 1 (65 kDa full-length form and the extravesicular 40 kDa domain) regardless of cycloheximide treatment. However, a protein complex containing the 30 kDa form of epimorphin, annexin II and a 40 kDa form of synaptotagmin 1 is secreted in response to cycloheximide treatment accompanied by phosphatidylserine-flipping (Fig. 4C). The cell-stress-dependent externalization of epimorphin, and not intracellular β-actin, was confirmed by immunocytochemistry (Fig. 4D).

Secreted epimorphin binds to target cells and activates focal adhesion kinase

To define how secreted epimorphin elicits a morphological response, we next investigated the association of secreted epimorphin with target epithelial cells. We performed quantitative cell adhesion assays using the non-tumorgenic murine mammary epithelial SCp2 cell line that does not endogenously express epimorphin (Hirai et al., 1998; Hirai et al., 2001). We tested the association between SCp2 cells and the secreted protein complex containing 30 kDa epimorphin, 40 kDa synaptotagmin and annexin II. The cells bound and spread onto the intact protein complex (Fig. 5A); this effect was blocked by antibodies against epimorphin but not by antibodies against synaptotagmin or annexin II, suggesting that a specific association with epimorphin is responsible for cell adhesion (Fig. 5A). As this adhesion and spreading onto epimorphin was reminiscent of interactions with ECM proteins, we measured phosphorylation of focal adhesion kinase (FAK), a direct downstream signaling molecule of integrin ECM receptors (Kumar, 1998). Binding to epimorphin activated FAK, which was blocked by anti-epimorphin antibodies, suggesting that cells bind epimorphin via integrin receptors (Fig. 5A). When purified recombinant epimorphin, corresponding to the secreted form of epimorphin (r-Epm30), was added to the medium it immediately bound the cell surface and induced FAK phosphorylation (Fig. 5B). A similar cell-surface-binding effect has been observed with recombinant epimorphin lacking the entire SNARE domain (r-Epm) that has been demonstrated to be functionally substitutive for full-length epimorphin (Hirai et al., 2001). No cellular binding was detected with the recombinant form of the SNARE domain (r-SNARE). We also detected the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), downstream effectors of FAK but not of epidermal growth factor receptor (EGFR) (Fig. 5B, right). These observations indicate that secreted epimorphin binds to integrins on the cell surface and activates FAK and ERK.

Identification of αv-integrin complex as epimorphin receptor

We next tried to identify the integrin subtype that associates with epimorphin to mediate intracellular signaling using the cell adhesion assay with primary human mammary epithelial cells (HMECs). HMECs clearly bound to r-Epm, but not r-SNARE, and this interaction was completely disrupted by addition of anti-epimorphin antibodies. Candidate-based screening revealed that function-blocking monoclonal antibodies against αv-integrin and β1-integrin selectively inhibit epimorphin association, whereas a function-stimulating antibody against β1-integrin substantially enhanced this association, suggesting that αv- and β1-integrins mediate cell-epimorphin interactions (Fig. 6A). Antibodies against EGFR that block epimorphin function on intestinal cells did not influence cell-epimorphin interaction in this adhesion assay (Y.H., unpublished). As shown using SCp2 cells, association of HMECs with epimorphin induced FAK phosphorylation, and this effect was blocked by antibodies against αv- or β1-integrin (Fig. 6B). We found that epimorphin-immobilized beads selectively pulled down 120-kDa to 140-kDa proteins from cell-surface components in HMECs, which contained β1- and αv-integrin subunits (Fig. 6C), suggesting that epimorphin either directly or indirectly interacts with the integrins. We also

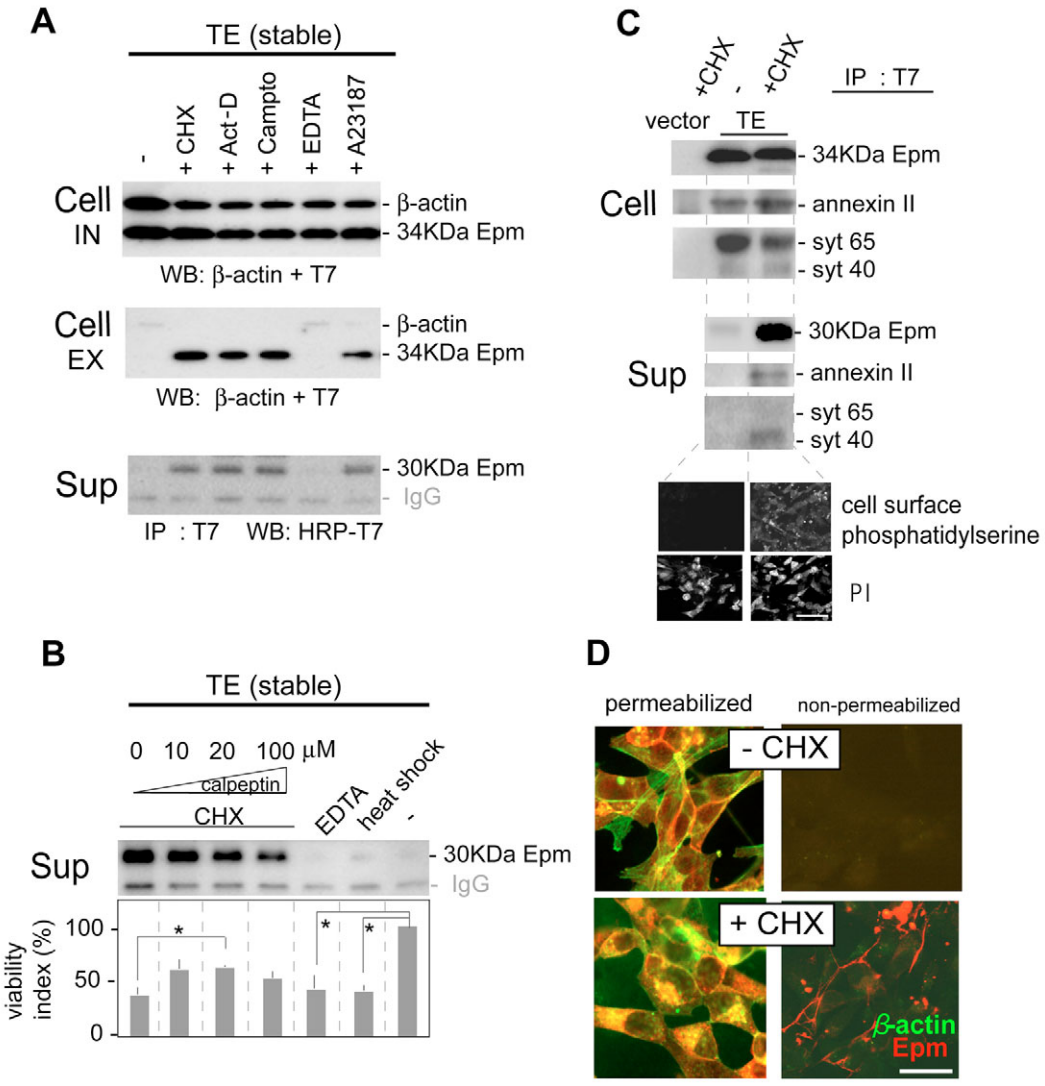

Fig. 4. Induction of epimorphin secretion in stable transfectants. (A) Appearance of a 34 kDa full-length epimorphin at cell surface (Ex) and a 30 kDa form in the supernatant (Sup) of PT67 cells stably expressing the TE transgene following treatment with subtoxic levels of cycloheximide (CHX), actinomycin D (Acti-D), camptothecin (Camp), or the Ca^{2+} ionophore A23187. Treatment with DMSO (–) or the Ca^{2+}-chelator EDTA did not lead to membrane translocation. (B) Epimorphin secretion is not a consequence of cell death. The calpain inhibitor Calpeptin decreased epimorphin secretion by partially inhibiting cycloheximide-dependent cell death. However, neither cell stresses (0.5 mM EDTA) nor temporal heat shock (42°C for 1.5 hours at day 2) resulted in a detectable increase in epimorphin secretion. Cell viability at day 3 was quantified with alamarBlue™ assay with non-treated cells being treated as 100% viable. Data are the mean ± s.d., n=4, *P<0.05. (C) Epimorphin coprecipitates with annexin II and synaptotagmin1 (full-length 65 kDa synaptotagmin and the 40 kDa extravesicular domain) from lysate of the transfectants, regardless of cycloheximide treatment (upper panel). Epimorphin is secreted to medium in response to cycloheximide-treatment in a secretory complex with annexin II and the extravesicular domain of synaptotagmin, both of which directly bind to phosphatidylserine (middle panel). Cycloheximide induces phosphatidylserine externalization as judged by binding of FITC-labeled annexinV (Biovision) to the non-permeabilized cells (lower panel). Cell nuclei were visualized with propidium iodide (PI) after permeabilization. Bar, 25 μm. (D) Anti-epimorphin antibodies (red) bind to the surface of non-permeabilized PT67 cells expressing TE transgene following treatment with CHX, whereas binding of anti-β-actin antibodies (green) requires cell permeabilization. Bar, 10 μm.

found that antibodies against the cytoplasmic domains of αv–and β1-integrins selectively co-precipitate r-EPM from the lysates of SCp2 cells cultured on r-Epm (see supplementary material Fig. S2). Thus, αv- and β1-integrins, most likely the αvβ1 subset, are involved in cell-epimorphin interaction for both of these normal mammary epithelial cells. Experiments assessing other cell types confirmed that αv-integrins are always involved in binding to epimorphin, but that the β-integrin subtype varied. We tested epimorphin association using human skin carcinoma (HSC-5), lung carcinoma (A549) and human breast adenocarcinoma (MCF7) cell lines, and human umbilical vein endothelial cells (HUVECs) and found that antibodies against αv-integrins blocked epimorphin binding of all these cell types, whereas antibodies against β1-integrin specifically blocked binding of HSC-5 cells and HUVECs and those against αvβ5-integrin blocked binding of

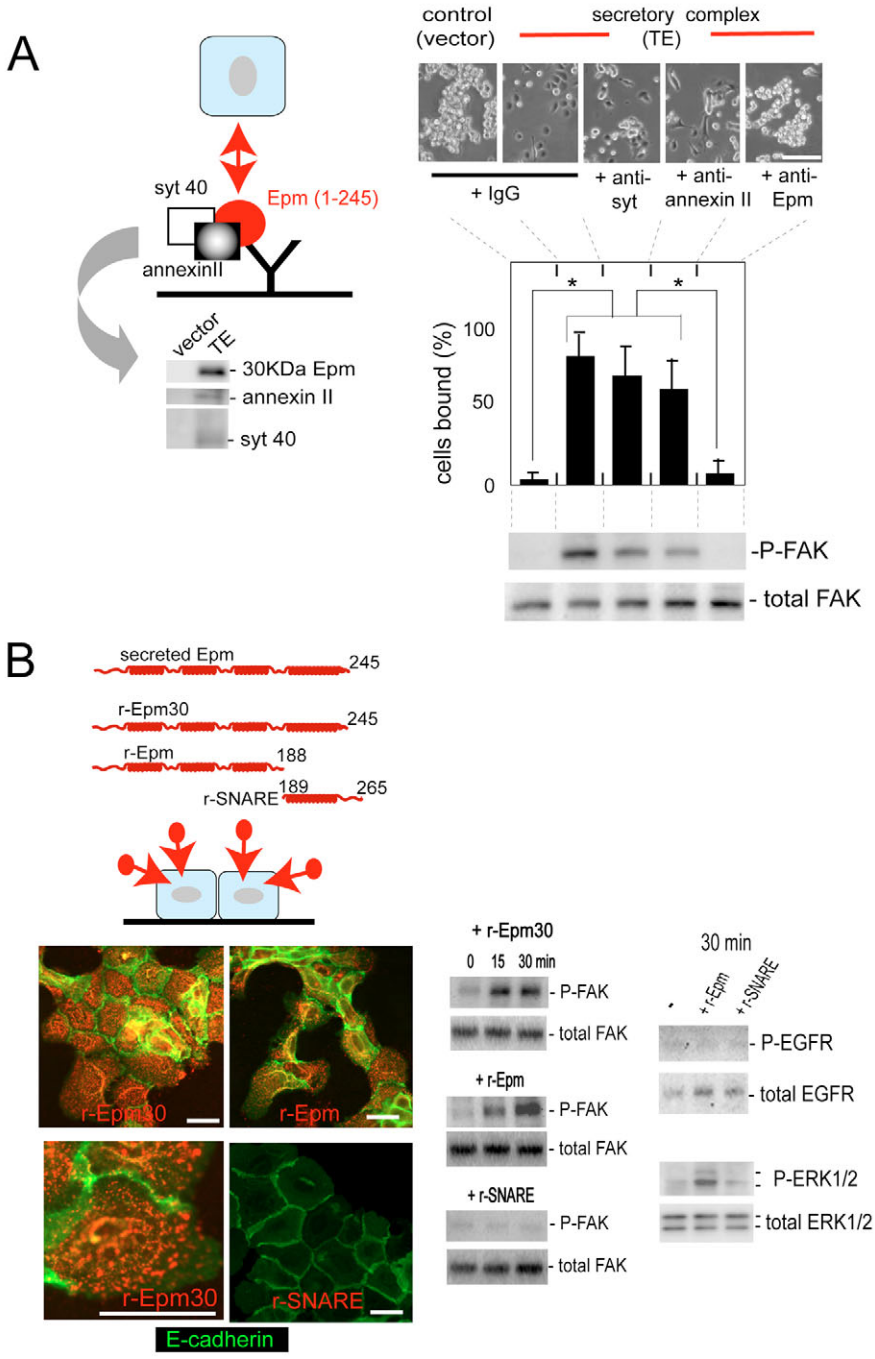

Fig. 5. Cells adhere to secreted epimorphin and activate focal adhesion kinase. (A) SCp2 cells adhere to intact secretory protein complex containing 30 kDa soluble epimorphin, 40 kDa synaptotagmin and annexin II, accompanied by FAK activation. This activity is selectively blocked by anti-epimorphin antibodies. The immobilization procedure was carried out under physiological conditions using the T7-tag as the target site for the immobilization.Western blotting of the immobilized materials confirmed successful immobilization of 30 kDa epimorphin, annexin II and 40 kDa synaptotagmin after incubation with supernatant from TE transfectants but not from the control cells (Vector). The amount of captured epimorphin on the substrate was about 100 ng/cm^2, as determined by a luminescent image analyzer Las1000plus using purified recombinant epimorphin as reference. The number of cells bound to each well in 4 hours was counted and the relative number to collagen-coated wells was calculated. Data are the mean ± s.d., n=4, *P<0.05. P-FAK, FAK phosphorylated at Y397. Bar, 50 μm. (B) Recombinant forms of secreted epimorphin r-Epm30 (aa 1-245) and r-Epm (aa 1-188), but not recombinant epimorphin SNARE domain r-SNARE (aa 189-265), bind to SCp2 cells with FAK/ERK activation. Images show cells starved for 24 hours, incubated with the indicated recombinant protein for 30 minutes; bound protein (red) and E-cadherin (green). Bars, 20 μm. Blots on the right show phosphorylation of FAK in cells treated with a recombinant protein for 15 and 30 minutes (first column of blots). phosphorylation of ERK but not EGF receptor was detected in cells treated with r-Epm for 30 minutes (second column of blots).

MCF7 and A549 cells (Fig. 6D). The importance of αv-integrin in cell-epimorphin interaction was further confirmed using human mammary epithelial cells (HMECs), in which this α subunit was transiently silenced with mission^{TM} shRNA knockdown constructs (Fig. 6E). Although αvβ1- and αvβ5-integrins are known to function as receptors for fibronectin, epimorphin-integrin complexes did not co-precipitate with this ECM component, indicating that the interaction between epimorphin and integrin may not be mediated by ECM molecules (Fig. 6C). To test whether epimorphin interacts directly with αv-integrin, we incubated epimorphin-immobilized beads with purified αvβ5-integrin, which is the only commercially available integrin with an epimorphin-binding potential. Purified αvβ5- and not α1β1-integrin bound to epimorphin-coated beads; this binding was not attenuated by RGD-containing peptide, indicating that epimorphin directly associates with αv-integrin and that possible impurities containing the canonical integrin-binding RGD motif are not involved in this protein-protein interaction (Fig. 6F).

Interaction between epimorphin and αv-integrin is crucial for 3D morphogenesis

We next evaluated whether association of epimorphin with αv-integrin was responsible for regulation of epithelial morphogenesis. Our previous investigations showed that mouse mammary epithelial cell lines cultured in collagen I gels

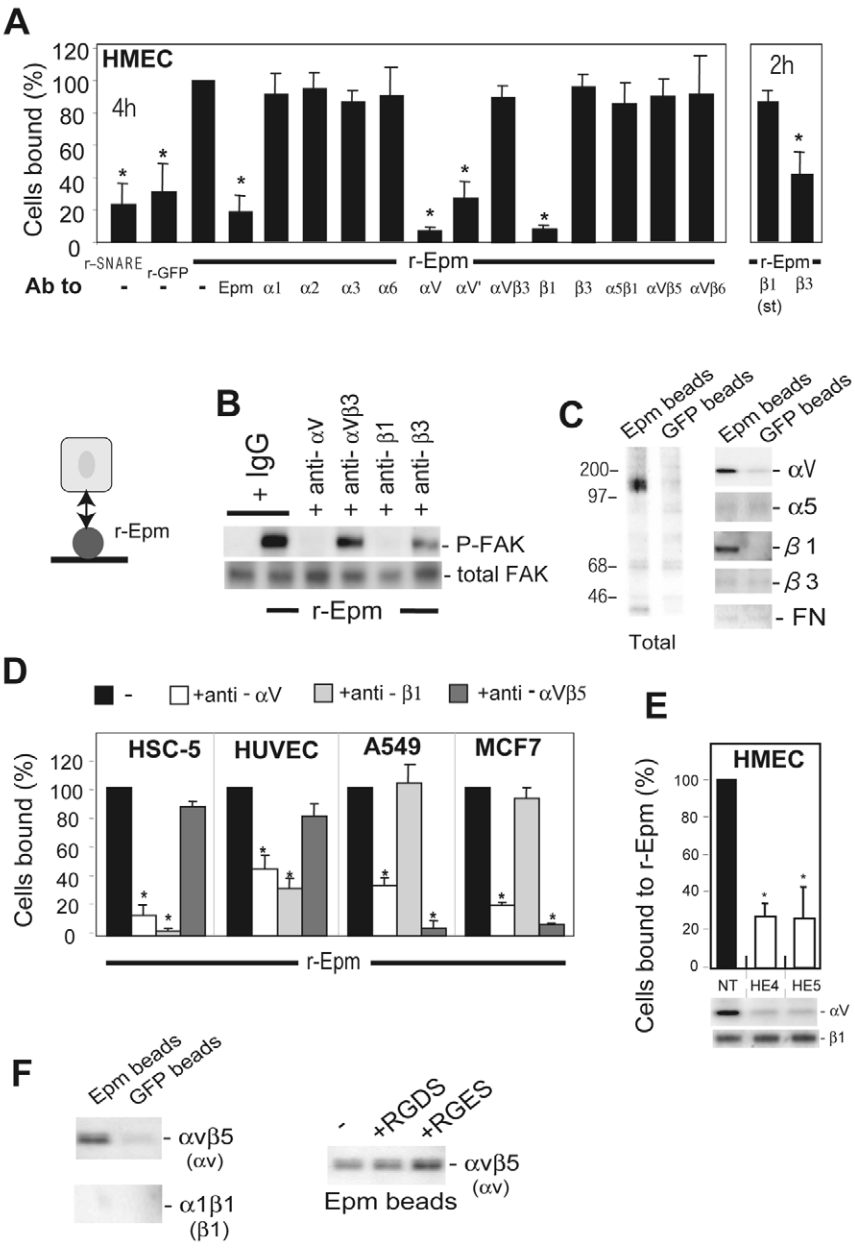

Fig. 6. Cell adhesion to epimorphin is mediated by αv-integrins. (A) Quantification of HMEC adhesion to recombinant epimorphin in the presence of anti-integrin antibodies. Adhesion to r-Epm is inhibited by antibodies that block αv- and β1-integrins (left). In the presence of function-stimulating antibody for β1-integrin (st) most of the cells bound to epimorphin within 2 hours (right). αv; clone P3G8, αv'; clone AV1. Data are the mean ± s.d., n=6, *P<0.05 vs r-Epm only. (B) Epimorphin adhesion-induced FAK phosphorylation is inhibited by antibodies against αv- and β1-integrins. (C) Immobilized epimorphin specifically binds to αv- and β1-integrins from HMECs lysates. Epimorphin beads selectively pull down 120-kDa to 140-kDa proteins (non-reduced condition) from HMEC-surface components labeled with membrane-impermeant biotinylation reagents. HRP-labeled streptavidin was used to visualize the proteins (left blot). Immunoblot of the proteins bound to r-Epm beads under the reduced condition. The cell surface proteins that bound to Epm beads appeared to include αv- and β1-integrins. FN, fibronectin (right blot). (D) Function-blocking antibodies against αv- and β1-integrins block adhesion of HSC-5 and HUVECs, whereas inhibition of αv- and αvβ5 integrins blocks adhesion of A549 and MCF7 cells. Data are the mean ± s.d., n=6, *P<0.05 vs control. (E) The transient silencing of αv-integrin results in a dramatic attenuation of epimorphin association of HMECs. The cells infected with lentivirus without (NT) or with the knockdown construct HE4 or HE5 were assessed for the expression of αv- and β1-integrins and cellular adhesion to r-Epm at day 3. Data are the mean ± s.d., n=4, *P<0.05 vs NT. (F) Epimorphin beads pull down purified αvβ5-integrin but not α1β1-integrin, and this integrin-epimorphin association is not affected by addition of RGDS or RGES peptide. αv- or α1β1-integrin bound to the beads was detected with antibodies against αv-integrin or β1-integrin, respectively.

undergo distinct types of morphogenic processes depending on the orientation of the presented epimorphin: exposure to epimorphin only on the outer surface of cell clusters (polar presentation) stimulated branching morphogenesis, whereas exposure to all the cells throughout the cell cluster (apolar presentation) stimulated luminal morphogenesis (Hirai et al., 1998). To evaluate the role of αv-integrins in epimorphin-mediated morphogenesis, we developed a streamlined version of the assay, which involved exposure of cell clusters to adenovirus carrying constructs of epimorphin fused to the signal peptide of interleukin 2 (IL2) (Av-SE). The IL-2 signal peptide has been shown to efficiently direct the entire population of exogenously expressed epimorphin to the cell surface via the normal secretory pathway (Fig. 7A) (Hirai et al., 1998; Oka et al., 2006). Intriguingly, this method of secretion results in a glycosylated 37 kDa form of cell surface epimorphin that appears not to be released into the medium, providing a tool to more precisely control the mode of epimorphin presentation (see supplementary material Fig. S3 and Discussion). In 3D collagen gels, HMEC clusters with polar presentation of epimorphin underwent branching morphogenesis, whereas those with apolar presentation generated cysts with large central lumina (Fig. 7A). We found that addition of antibodies against αv-integrin or epimorphin substantially inhibited both branching and luminal morphogenesis, suggesting that the interaction between epimorphin and αv-integrin is required for its activity as a morphogen (Fig. 7B). Taken together, these results suggest a model for extracellular epimorphin function, in which epimorphin is secreted by non-classical pathways through specific interactions with the SNARE/TM domain, and mediates morphogenesis by binding to αv-integrins (Fig. 8).

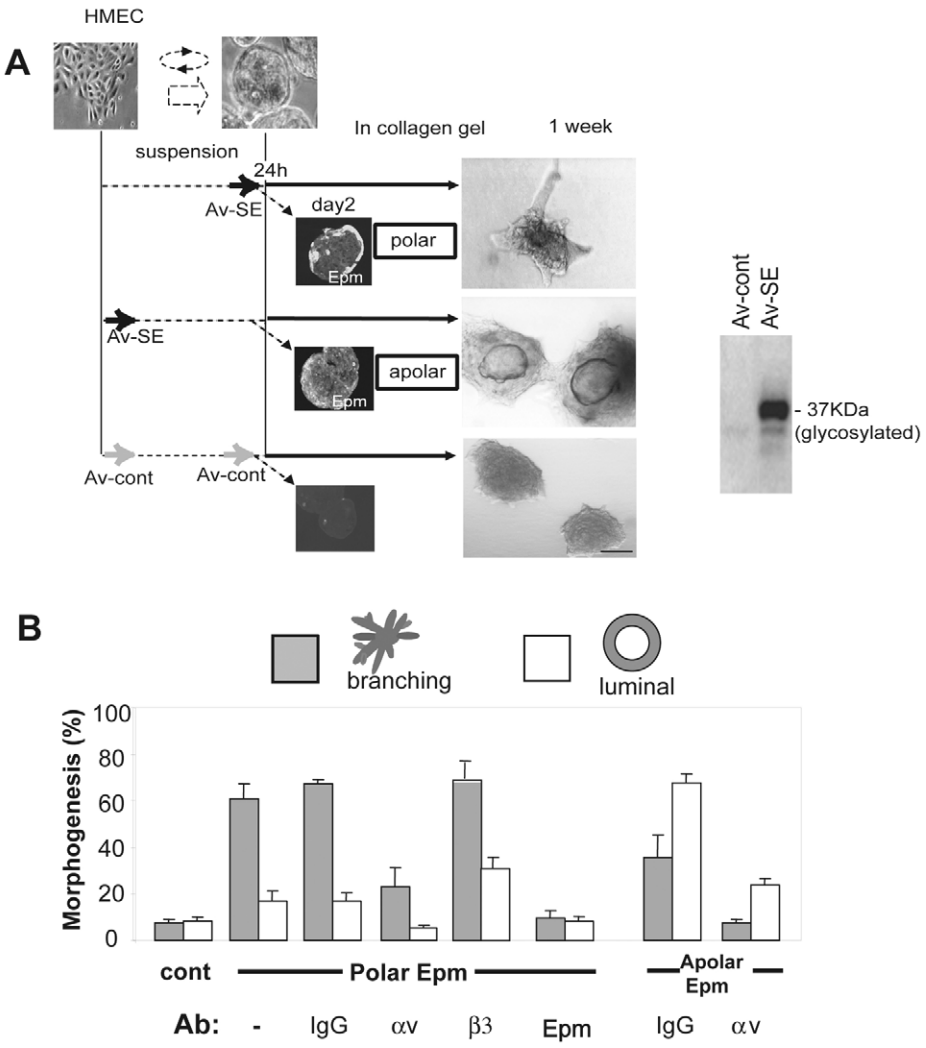

Fig. 7. Inhibition of αv-integrins blocks epimorphin-mediated epithelial morphogenesis. (A) Extracellular epimorphin stimulates luminal or branching morphogenesis in HMECs, depending upon mode of presentation. In suspension culture HMECs form clearly rounded spherical aggregates after 24 hours (see top two micrographs). Adenovirus carrying signal peptide-tagged epimorphin (Av-SE) was added to the suspension culture for the final two hours (cells form well-packed aggregate and Av-SE infects only outer cells in the aggregate) for polar presentation, or first 2 hours (when cells remain single and Av-SE infects all cells in the aggregate) for apolar presentation; embedding of the resultant cell aggregates in 3D collagen gels produced branching or luminal morphogenesis, respectively. Bar, 50 μm. Blot on the right shows HMECs treated with Av-SE producing 37 kDa glycosylated epimorphin. (B) Antibodies against αv-integrin block both branching and luminal morphogenesis. Data are the mean ± s.d., n=3. The phenotypic appearance (branching or luminal) was evaluated as described previously (Hirai et al., 1998).

Discussion

Epimorphin lacks a signal peptide and predominantly exists on the cytoplasmic surface of the plasma membrane where it functions in vesicle docking and fusion (Hirai, 2001). Here, we show that the leaderless protein epimorphin can also become translocated to the extracellular space following cell stress or Ca^{2+} influx. Ca^{2+} has been extensively investigated as a crucial mediator of morphogenic processes (Webb and Miller, 2003), and it is an intriguing possibility that cellular Ca^{2+} influx during development affects morphogenic processes through stimulation of non-classical secretion of morphogens such as epimorphin. Cell-stress-induced or Ca^{2+}-influx-induced protein secretion has been demonstrated for FGF1, IL1A and IL1B, canonical examples of proteins secreted by non-classical mechanisms (Prudovsky et al., 2003; Rubartelli et al., 1990). Membrane translocation of these proteins is accomplished through the action of a multimeric protein-release-complex that contains the extravesicular domain of the syntaxin-binding protein synaptotagmin (syt p40), phosphatidylserine-bound annexin II and S100A13 (Prudovsky et al., 2003; Tarantini et al., 1998). We found clear association of cellular and secreted epimorphin with synaptotagmin and annexin II (Fig. 4C), suggesting that the mechanism of epimorphin secretion is related to that of FGF1, IL1A and IL1B. Fig. S3, in the supplementary material, compares elements for secretion of epimorphin to that FGF1 and IL1 (Jackson et al., 1992; Nickel, 2003; Prudovsky et al., 2003; Rubartelli et al., 1990). Considering that annexin II and synaptotagmin are known to directly bind Ca^{2+} and phosphatidylserine (Bai and Chapman, 2004; Montaville et al., 2002), and that phosphatidylserine inducible flipps from the inner to the outer surface of the cell membrane at cell stress, Ca^{2+} influx or other physiological stimuli (Bevers et al., 1999; Smrz et al., 2007), it is likely that epimorphin becomes translocated through a phosphatidylserine-flipping-dependent process. It is also noteworthy that the phosphatidylserine externalization can be detected during embryogenesis (van den Eijnde et al., 2001), suggesting a mechanism for embryonic extracellular presentation of epimorphin. Although heat shock triggers the secretion of FGF1 and IL1, a process in which S100A13 protein plays an important role, this was not the case for epimorphin. However, heat shock stimulates redistribution of FGF1 and IL1 from a diffuse cytosolic pattern to one where both proteins are located near the inner surface of the plasma membrane, in a process that is S100A13-dependent (Prudovsky et al., 2002). However, this process may not be

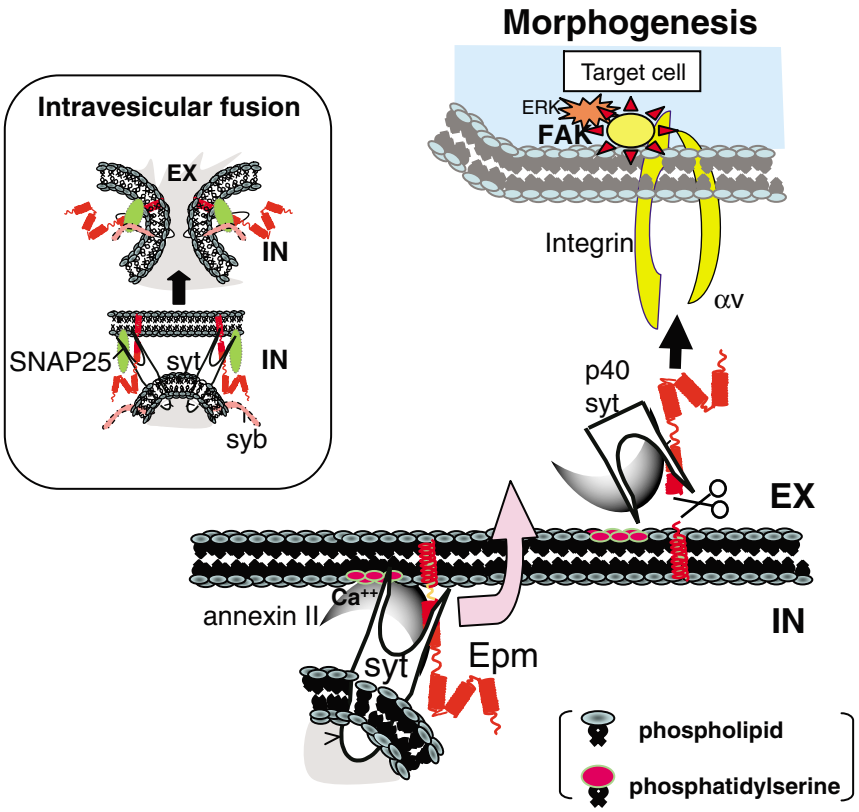

Fig. 8. Cell surface presentation and secretion of epimorphin and its extracellular action for epithelial morphogenesis. Intracellular epimorphin becomes complexed with synaptotagmin (syt) and annexin II, both of which can directly bind to phosphatidylserine at the inner leaflet of the plasma membrane. In response to stress and/or Ca^{2+} influx, this release complex translocates across the membrane with externalization of phosphatidylserine. The extracellularly presented epimorphin is then cleaved at the SNARE domain and the secreted form of epimorphin is captured by αv-integrin on the target epithelia leading to FAK activation and initiation of morphogenesis. The role of cytoplasmic epimorphin as a t-SNARE protein in SNARE-mediated membrane fusion (inset) . EX, extracellular space. IN, intracellular space. Syb, synaptobrevin, Epm, epimorphin.

involved in the extracellular localization of epimorphin because epimorphin has a transmembrane domain and is already found at the inner surface of the plasma membrane.

Our identification of synaptotagmin 1 in the epimorphin secretory complex is consistent with our finding that the synaptotagmin-binding SNARE domain is a crucial domain for epimorphin secretion. Synaptotagmin 1 has been indicated as a neuronal-specific protein, however, NIH 3T3 cells are known to express this molecule (Tarantini et al., 1998) and we confirmed its expression in our NIH 3T3-cell-derived PT67 packaging cell line by RT-PCR (Y.H., unpublished). It is also noteworthy that we observed extracellular presentation of a subpopulation of syntaxin 1A in transiently transfected cells (Fig. 2B, Fig. 3B). Whereas most studies on syntaxin 1A have focused on its intracellular functions, several reports suggested that it can adopt an extracellular localization as well (Brimhall et al., 1999; Smirnova et al., 1993a; Smirnova et al., 1993b).

Altough epimorphin and syntaxin 1A both can be localized to the cell surface, only epimorphin is secreted from the cell (Fig. 2C, Fig. 3B). We identified H246 as the crucial residue for the processing of epimorphin and determined E245 as the C-terminus of secreted epimorphin (Fig. 2D, Fig. 3A,B). Epimorphin from mouse, human, quail and sheep all contain a H246, and a R246H mutation in syntaxin 1A generates a mutant molecule that releases from the cell (Fig. 3B). As R246 is conservered in other syntaxins, and a H246R mutation in epimorphin prevents its release from the cell (Fig. 3B), it is likely that this residue is the key determinant to render epimorphin a soluble morphogen, distinct from other syntaxin isoforms. Generation of soluble epimorphin by cleavage at its membrane-proximal domain might be necessary for its function as a morphogen, because the basement membrane prevents direct contact between epimorphin-producing stromal cells and target epithelial cells (Gurdon and Bourillot, 2001; Hirai et al., 1992). It is possible, however, that syntaxins that are not released as soluble molecules still play a role in juxtacrine cell signaling processes. For example, extracellular syntaxin 1A could function at pre- and postsynaptic junctions or neuron-glia interfaces where no physical barrier exists between the syntaxin-1A-producing cells and the target cells. Surprisingly, we were unable to detect epimorphin secretion when the molecule was directed to the cell surface after fusing it to the IL-2 signal peptide (see supplementary material Fig. S4). It might be that glycosylation within the ER-Golgi pathway prevents proteolytic processing of epimorphin; another possibility is that epimorphin processing takes place in association with the synaptotagmin–annexin-II release complex.

We defined αv-integrin as the core component of the epimorphin receptor using cell adhesion assays, pulldown assays, immunoprecipitation analysis and the 3D morphogenesis assay, whereas recent work also implicated involvement of EGF receptors in the cell-epimorphin interaction in intestinal epithelia (Iizuka et al., 2007). αv-Integrin and its downstream effectors FAK and ERK (which also acts as an effector of EGFR family members) are known to play important roles in the developmental processes of many organ systems (Bader et al., 1998; Bayless et al., 2000; Eliceiri and Cheresh, 2000). In addition to key roles in morphogenesis, αv-integrins have anti-apoptotic functions in both normal and pathogenic developmental processes (Bao and Stromblad, 2004; Kumar, 1998). Iizuka et al. recently reported that

extracellular supplementation of epimorphin prolongs the lifetime of nutrient-deprived intestinal cells and also attenuates apoptosis induced by cytotoxic reagents (Iizuka et al., 2007). Consistent with this, we found that exposure to soluble extracellular epimorphin was sufficient to confer apoptosis resistance in HMECs that were maintained in unsupplemented medium, even after 4 weeks (see supplementary material Fig. S5), suggesting that epimorphin–αv-integrin–FAK signaling is involved in cell survival pathways and that epimorphin secreted from the damaged cells can, in turn, protect epimorphin-receiving cells from environmental insult. A general pathway by which stressed and apoptotic cells can release epimorphin may provide insight into why dramatic morphogenesis is often accompanied by apoptotic cell death, and how tissue repairing processes can be stimulated in injured tissues. Our identification that alterations in Ca^{2+} levels can stimulate extracellular secretion of epimorphin suggests a new link between Ca^{2+}-dependent signaling and morphogenesis (Webb and Miller, 2003). Investigation of the role of Ca^{2+} signaling in the dramatic morphological and functional differentiations triggered by epimorphin is now underway.

Finally, our study clearly provides evidences that a subpopulation of an intracellular vesicular docking mediator can translocate across the membrane, get processed to be secreted and exert new extracellular function as epimorphin. Elucidation of the functional link between these intracellular and extracellular functions, and the regulation mechanism for the spatiotemporal export of epimorphin may identify a new type of signaling pathway for sophisticated tissue morphogenesis.

Materials and Methods

Cell culture

Murine mammary epithelial (SCp2), human breast adenocarcinoma (MCF7), lung carcinoma (A549) cells, human umbilical vein endothelial cells (HUVECs) and the packaging cell line PT67 (Clontech) were maintained in DMEM/F12 medium supplemented with 10% FCS (DH10). Human skin carcinoma (HSC-5) cells were maintained in Iscov-modified DMEM medium supplemented with 10% FCS. Primary human mammary epithelial cells (HMECs) and HUVECs (Cambrex) were expanded according to the manufacturer's protocols and frozen in aliquots, each of which was used for only one experiment. 3D culture was carried out by embedding cell aggregates into collagen gels as described previously (Hirai et al., 1998). For adenoviral infection of cell aggregates, adenovirus containing expression constructs for signal peptide-fused epimorphin (5×10^8 pfu per ml) was added to medium before (first 2 hours then changed to virus-free medium) or after (last 2 hours) aggregate formation, to generate apolar or polar presentation of epimorphin, respectively. For stress induction with drugs, cells were treated with 100 μM cycloheximide, 200 nM actinomycin D, 300 nM camptothecin (BioVision), or 0.5 mM EDTA for 3 days, concentrations at which about 50% of the cells died within a week. The Ca^{2+} ionophore A23187 (Calbiochem) was used at a concentration of 2 μM. These chemicals were prepared as 1000× stock solution in DMSO (for cycloheximide, actinomycin D, camptothecin and A23187) or PBS (for EDTA). To prevent apoptotic cell death induced by cycloheximide, inhibitors for calpain (calpeptin; Calbiochem) were added at 10-100 μM 2 hours prior to cycloheximide treatment. Heat shock was performed by keeping the epimorphin-producing cells at 42°C for 1.5 hours at day 2. Cellular and secreted epimorphin in the supernatant was assessed 3 days after these treatments. For some culture, alamarBlue™ (BioSciences) diluted with medium (1:10) was added to the cells and viability was assessed after 4 hours following the manufacturer's protocol. Non-treated healthy cells were regarded as 100% viable.

Expression constructs

To prepare expression plasmids for epimorphin and the derivatives tagged with T7 peptide, cDNA of mouse epimorphin (D10475), mouse syntaxin 1A (BC047133) or their chimeric molecules fused to the T7 peptide sequence at the N-terminus were generated using PCR and introduced into expression vectors containing either CMV or SRα promoters; both promoters produced similar results. The vectors were p-QCXIN (Clontech), p-TARGET (Promega) and SRα 296 (Takebe et al., 1988). To introduce amino acid mutations in expression plasmids for epimorphin and syntaxin 1A, standard site-directed mutagenesis with Pfu polymerase and restriction enzyme *Dpn*I (Stratagene) was carried out following the manufacturer's protocol. H246 (AGG) at aa position 246 or the following KEE aa sequence (AAGGAAGAG) in epimorphin were mutated to R(CAT) or VSD(GTGTCAGAC), respectively, and the corresponding R(CAT) or VSD(GTGTCAGAC) in syntaxin 1A was mutated to H(AGG) or KEE(AAGGAAGAG), respectively. For the transient expression of the transgene products, subconfluent cells were transfected with the expression plasmids using lipofectamine or lipofectamine 2000 (Invitrogen) and analyzed 3 days after transfection. To generate stable cells expressing T7-tagged epimorphin, PT67 cells were transfected with the T7-epimorphin cDNA cloned in an expression plasmid p-TARGET or p-QCXIN and selected in the presence of 400 μg/ml of G418. Three independent clones TE-3, TE-4 and TE_5 displayed similar properties in terms of epimorphin expression, although we used the TE-5 clone for the results presented here. Adenovirus for extracellular epimorphin was generated using AdenoX expression system (Clontech), according to manufacturer's protocols, using an expression construct in which the IL2 signal peptide was fused with the cDNA-encoding ORF of mouse epimorphin (Hirai et al., 2001). The lentivirus for silencing αv-integrin subunit were prepared using Mission RNAi constructs HE4 and HE5 (Sigma) and the Invitrogen ViraPower Lentiviral Expression System (Invitrogen), according to the manufacturer's protocols. The hairpin insert of the constructs are: CCGGCTCTGTTGTATATCCTTCATTCTCGAGAATGAAGGATATACAACAGAGTTTTT (HE4) and CCGGGACTGAGCTAATCTTGAGAATCTCGAGATTCTCAAGATTAGCTCAGTCTTTTT (HE5).

Cell adhesion assays

Adhesion assays were carried out essentially as previously described (Hirai et al., 1998). To prepare surfaces displaying intact secretory complex containing 30 kDa epimorphin, 48-well plates (non-cell culture; Iwaki) were coated with monoclonal antibody against the T7-tag (Novagen) t 2 μg/cm^2, followed by a 3-hour incubation with supernatant from TE5 cells treated with cycloheximide for 3 days. As the control, supernatant from PT67 cells that had been transfected with the empty vector was used. Other proteins used to coat dishes were recombinant epimorphin lacking the SNARE and TM domains (r-Epm, aa 1-188) (Hirai et al., 2001), recombinant epimorphin with the SNARE domain only (without the TM domain) (r-SNARE, aa 189-265) (Hirai, 1994), green fluorescent protein (GFP) generated in bacteria (r-GFP) and collagen type I (Koken). Wells were coated with protein at 2 μg/cm^2, dried under sterile conditions and washed three times with PBS. Cells (1×10^4) suspended in DMEM/HamF12 medium containing 20 mg/ml BSA were then seeded onto each well. In some wells, affinity-purified rabbit antibodies against epimorphin or control rabbit IgG were added to the medium at the final concentration of 100 μg/ml. Cell adhesion was assessed after 4 hours. To prepare cell lysates for detection of phosphorylated FAK, adhered and unbound cells collected by centrifugation were mixed and dissolved in 1× lysis buffer (Tris-buffered saline containing 1% Triton X-100, 1% NP-40 and protease inhibitor cocktail) (Nacalai Tesque, Inc.). To assess the activity of soluble epimorphin to bind to and activate the target cells, 20 μg/ml recombinant epimorphin corresponding to the secreted form (r-Epm30, aa 1-245), r-Epm or r-SNARE was added to cells that has been maintained in serum-free medium for 24 hours. Cells were collected for western blotting after 15 and 30 minutes, or washed and fixed for immunocytochemistry with ice-cold methanol after a 30-minute incubation.

Antibodies

For immunocytochemistry, affinity-purified rabbit and rat polyclonal antibodies against epimorphin (Hirai et al., 1998), goat polyclonal antibodies against E-cadherin (R&D systems), mouse monoclonal antibody against the T7 tag (Novagen) and β-actin (Sigma) were used. Antibodies used for cell-binding assay were affinity-purified anti-epimorphin polyclonal antibodies, rabbit polyclonal antibodies against annexin II (Santa Cruz) Chicken polyclonal antibodies against synaptptagmin-1 (Abcam, ab8037-100) and function-blocking and function-stimulating monoclonal antibodies against integrins purchased from Chemicon. These antibodies were added to medium at a concentration of 25 μg/ml. Monoclonal antibodies against integrins were against α1-integrin (FB12), α2-integrin (PIE6), α3-integrin (ASC-1), α6-integrin (GoH3), αv-integrin (P3G8 and AV1), β1-integrin (6S6 and B3B11), β3-integrin (25E11), α5β1-integrin (JBS5), αVβ5-integrin (P1F6), αVβ6-integrin (10D5) and αVβ3-integrin (LM609). To test the inhibitory effect on 3D morphogenesis these antibodies were added to medium at a concentration of 200 μg/ml. Antibodies used for immunoprecipitation were affinity-purified rabbit anti-epimorphin polyclonal antibodies that cross-react with syntaxin 1A (Hirai et al., 1998; Takebe et al., 2003), rabbit antiserum against the cytoplasmic domains of αv-integrin, α5-integrin, β1-integrin (Chemicon) and β3-integrin (Santa Cruz) and mouse monoclonal antibodies against the T7 peptide (Novagen). Primary antibodies used for western blotting were mouse monoclonal antibodies against the T7 peptide (Novagen), β-actin (Sigma), phosphotyrosine (Takara) and cellular fibronectin (Sigma), rabbit polyclonal antibodies against FAK (Santa Cruz), FAK phosphorylated at Y97 (Sigma and Biosource), ERK1/2, and ERK1/2 phosphorylated at Y187/Y204 and T185/202, EGFR, Annexin II (Santa Cruz), cytoplasmic domain of αv-integrin, α5-integrin, β1-integrin (Chemicon) and β3-

integrin (Santa Cruz), goat polyclonal antibodiesagainst phosphorylated EGFR at Y1173 (Santa Cruz) and chicken polyclonal antibodies against synaptotagmin 1 (Abcam, ab8037-100). As for anti-β-actin and the T7 peptide, HRP-labeled forms were also used for some western blots without secondary HRP-labeled antibodies.

Immunoprecipitation

To prepare lysates of adherent cells, supernatant was collected out of one well of a six-well plate, in which sub-confluent cells had been cultured in 1 ml medium and the cells had been treated with 1 ml of 1× lysis buffer for 30 minutes at room temperature. After removal of insoluble precipitates from supernatant and cell lysate by centrifugation at 18,500 ***g*** for 30 minutes, specific antibodies were added to them and the immunocomplex was retrieved with ProteinG-sepharose beads (GE Healthcare). The bound materials were dissolved in 50 μl (for supernatant sample) or 250 μl (for lysate sample) of SDS sample buffer, so that the amount of supernatant sample for laoding was five times that of the cell lysates sample. To prepare whole-culture lysates (cells plus supernatant), same volumes of 2× lysis buffer and supernatant were added directly to the culture. To collect epimorphin-bound materials, beads immobilized with r-Epm were prepared. A 1:1 mixture of Affigel 10 and Affigel 15 (Bio-Rad) was incubated with 1 mg/ml of r-Epm or r-GFP in PBS. After 4 hours the beads were treated with 100 mM ethanolamine and washed several times with 1× lysis Buffer. Thus prepared r-Epm-beads were incubated with protein samples in 1× lysis buffer containing the lysis-cations $CaCl_2$, $MgCl_2$ and $MnCl_2$ at a final concentration of 1 mM for 12 hours, washed with lysis-cations three times and bound proteins were analyzed by western blotting. In some tubes, the synthetic peptide RGDS or RGES (Gibco BRL) was added at a concentration of 20 μg/ml. The protein sample includes 1 μg/ml of purified αvβ5-integrin or α1β1-integrin (Chemicon).

Biotin labeling

For biotinylation of r-Epm and r-GFP, 1 mg/ml of the proteins was mixed with 1 mg/ml of Sulfo-NHS-biotin (Pierce) in PBS and incubated for 2 hours followed by extensive dialysis against PBS. To obtain lysate containing labeled cell surface proteins, living cells were treated with membrane impermeant Sulfo-NHS-biotin (100 μg/ml) in Hepes-buffered saline for 30 minutes, washed several times with serum-free medium and dissolved in lysis buffer. After removal of insoluble materials by centrifugation, the lysate was applied to streptavidin-agarose beads (Life Technologies) or r-Epm-beads to collect total cell surface proteins or the epimorphin-binding components, respectively.

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