Cell-surface attachment of pedestal-forming enteropathogenic *E. coli* induces a clustering of raft components and a recruitment of annexin 2

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Summary

Annexin 2 is a Ca^{2+}-regulated membrane- and F-actin-binding protein implicated in the stabilization or regulation of membrane/cytoskeleton contacts, or both, at the plasma membrane and at early endosomal membranes. To analyze the dynamic nature of such action we investigated whether annexin 2 could be found at sites of localized actin rearrangements occurring at the plasma membrane of HeLa cells infected with noninvading enteropathogenic *Escherichia coli* (EPEC). We show that adherent EPEC microcolonies, which are known to induce the formation of actin-rich pedestals beneath them, specifically recruit annexin 2 to the sites of their attachment. Mutant EPEC (EPEC*tir*), which lack a functional receptor for intimate attachment (Tir, translocated intimin receptor) and which fail to produce full pedestal formation, are still capable of recruiting annexin 2 to the bacterial contact sites. Accumulation of annexin 2 at sites of EPEC or EPEC*tir* attachment is accompanied by a recruitment of the annexin 2 protein ligand S100A10. EPEC and EPEC*tir* attachment also induces a concentration of cholesterol and glycosyl phosphatidylinositol-anchored proteins at sites of bacterial contact. This indicates that membrane components present in rafts or raft-like microdomains are clustered upon EPEC adherence and that annexin 2 is recruited to the cytoplasmic membrane surface of such clusters, possibly stabilizing raft patches and their linkage to the actin cytoskeleton beneath adhering EPEC.

Key words: Actin organization, Calcium, Membrane microdomain, Raft

Introduction

Interactions between cellular membranes and the underlying cytoskeleton play a pivotal role in determining cell shape, morphogenesis and motility. They can be brought about by a large number of bivalent or multivalent proteins acting as linkers between the actin-based cortical cytoskeleton and membrane lipids or membrane-embedded proteins. In addition to providing the general membrane scaffold, the cortical cytoskeleton also defines structures or domains within the plasma membrane where signals are received or processed, or both. To fulfil such roles the membrane/cytoskeleton interactions have to be dynamic in nature and have to be regulated both in spatial and temporal terms, e.g. following modulation of the intracellular free Ca^{2+} concentration. Among the proteins implicated in connecting intracellular Ca^{2+} signalling to the regulation of membrane-cytoskeleton contacts and the organization of membrane domains are members of the annexin family.

Annexins comprise a multigene family of bimodular Ca^{2+}-binding proteins that interact with acidic phospholipids preferentially found at the cytosolic face of cellular membranes (Gerke and Moss, 1997). This interaction is mediated by the annexin core domain, a Ca^{2+}- and lipid-binding module consisting of four or eight homology segments, the annexin repeats. The second principal module in each annexin protein is the N-terminal domain, which is highly variable in sequence and length and specifies individual annexin properties. N-terminal domains of annexins can harbour binding sites for cellular protein ligands and also play a role in specifying the intracellular target membrane of a given annexin, i.e. the membrane to which an annexin is located at resting or elevated Ca^{2+} levels. Target membranes for annexins include the plasma membrane as well as membranes of the endosomal and the secretory apparatus (Creutz, 1992; Gerke and Moss, 1997; Gruenberg and Emans, 1993; Raynal and Pollard, 1994).

In addition to binding to cellular membranes, several annexins also interact in a Ca^{2+}-regulated manner with elements of the actin cytoskeleton, in particular F-actin and spectrin. The actin-binding sites mapped within annexins so far are also found in the protein core (Filipenko and Waisman, 2001; Jones et al., 1992) and it is not yet known whether phospholipid and F-actin binding are mutually exclusive. However, at least in the case of annexins 2 and 6, membrane and actin-cytoskeleton binding could occur simultaneously, thus allowing for a bridging function. Annexin 6 is the only member of the family comprising eight annexin repeats, which are folded into two separate core modules, each having the potential of binding to a membrane surface or the cytoskeleton

(Avila-Sakar et al., 2000; Benz et al., 1996). Annexin 2, however, is a four-repeat annexin but it can form a heterotetrameric complex with its specific ligand, the S100A10 protein. In this complex, a central S100A10 dimer links two annexin 2 chains in a highly symmetrical manner, thereby providing a physical connection between two membrane- and F-actin-binding cores (Gerke and Weber, 1985; Lewit-Bentley et al., 2000; Rety et al., 1999).

Both annexin 2 and 6 are found associated with membranes of the endosomal system and the plasma membrane (Gerke and Moss, 1997). This association appears to occur preferentially at sites of membrane microdomains rich in cholesterol, glycosphingolipids and glycosyl phosphatidylinositol (GPI)-anchored proteins (Babiychuk and Draeger, 2000; Harder and Gerke, 1994; Harder et al., 1997; Oliferenko et al., 1999). Such microdomains, also known as rafts, represent lateral lipid assemblies serving as signalling platforms in a number of cellular processes including T-cell receptor and Ras signalling (Simons and Toomre, 2000). Although the characteristic lipid components of rafts are elements of the exoplasmic leaflet, less is known about lipids enriched in the inner leaflet of such microdomains and their interaction with the cortical cytoskeleton. Annexin 2 appears to be one of the few structural proteins located specifically at this raft-cytoskeleton interface, at least in polarized mammary epithelial and smooth muscle cells (Babiychuk and Draeger, 2000; Oliferenko et al., 1999).

Signalling through the plasma membrane to the underlying actin cytoskeleton also occurs when certain microorganisms attach to the surface of their host cells. Such events are particularly evident in the case of enteropathogenic *Escherichia coli* (EPEC) – diarrhoea-causing pathogens that form microcolonies on intestinal epithelial cells of the infected host. Once attached, the bacteria use a specialized secretion system to deliver into the host cell a number of *E. coli*-secreted proteins (Esps), which include a receptor for the EPEC outer membrane protein intimin. The translocated intimin receptor (Tir) not only mediates intimate bacterial adhesion but also recruits several components that regulate the actin cytoskeleton including α-actinin, ezrin, Wiskott-Aldrich syndrome protein (WASP) and the actin-related protein 2 and 3 (Arp 2/3) complex. This recruitment ultimally leads to the localized loss of microvilli and the formation of actin-rich pedestals beneath the attached bacteria (Frischknecht and Way, 2001; Goosney et al., 2000).

To analyze whether cholesterol-rich membrane domains and cytoplasmically associated annexins could participate in the attachment of EPECs, we localized different annexins as well as green fluoresecent protein (GFP)-tagged GPI and plasma membrane cholesterol in EPEC-infected HeLa cells. We show that annexin 2 concentrates at sites of attached EPEC clusters and that the membrane beneath such clusters is rich in cholesterol and GPI-anchored GFP. This indicates that raft components and annexin 2 attached to the inner leaflet of rafts are involved in initiating rearrangements of the actin cytoskeleton that occur upon EPEC contact.

Materials and Methods

Expression constructs

Enhanced YFP, a bright yellow variant of GFP, was attached to the amino-terminus of human S100A10 (p11) by cloning full-length S100A10 cDNA into the appropriately linearized pEYFP-C1 vector (Clontech, Heidelberg, Germany). The annexin 2-GFP mutant has been described previously (Rescher et al., 2000). A fusion construct containing the GPI-anchoring sequence of the folate receptor attached to GFP (GPI-GFP) was kindly provided by Satyajit Mayor (National Centre for Biological Sciences, Bangalore, India). Following cloning, plasmids were amplified in *E. coli* and purified using the Jetstar 2.0 Plasmid Kit (Genomed, Bad Oyenhausen, Germany).

Cell culture and transfections

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, glutamine and antibiotics in a 7% CO_2 incubator at 37°C. For transient transfections, cells were grown on coverslips in 35 mm dishes and then transfected with Effectene (Qiagen, Hilden, Germany) using 0.4 μg DNA per well. Cells were used for infection with EPEC 24 hours after transfection.

Bacterial culture and infection

EPEC strains used in this study were EPEC 2348/69 (wild-type), EPEC 2348/69 SE896 (EPEC*tir*, a mutant expressing a C-terminally truncated Tir protein not inserted into the host cell membrane or cytoplasm) (Elliott et al., 1999) and EPEC 2348/69 CVD452 (a mutant defective in type III-dependent secretion) (Jarvis et al., 1995). All EPEC strains were grown in Standard I-medium (Difco, Augsburg, Germany) at 37°C in overnight cultures with shaking at 150 rpm. HeLa cells grown on cover slips were infected with an overnight EPEC culture (approximately 100 bacteria per HeLa cell) in DMEM, 2% fetal calf serum, 1 mM glutamine, 10 mM HEPES and 1% methyl-α-D-mannose for 3 hours at 37°C in a 10% CO_2 incubator. Subsequently, cells were washed intensively with PBS.

Fluorescence microscopy

To visualize endogenous annexin 2, EPEC-infected HeLa cells were permeabilized for 5 minutes on ice with 0.2% Triton-X-100 in PBS containing Ca^{2+} and Mg^{2+} (Dulbecco's PBS/Ca^{2+}/Mg^{2+}; BioConcept, Umkirch, Germany) and then fixed in 4% paraformaldehyde (PFA) in the same buffer for 10 minutes at room temperature (RT). Following fixation and washing, cells were treated with 50 mM NH_4Cl in PBS to quench free aldehydes and then incubated with undiluted hybridoma supernatant containing the monoclonal anti-annexin 2 antibody HH7 (Thiel et al., 1992) for 45 minutes at RT. Following washing with PBS, secondary Cy2-labelled goat anti-mouse antibodies (Dianova, Heidelberg, Germany), diluted in PBS containing 2% BSA, were applied in the same way.

To analyze cells transfected with the different GFP or YFP fusion constructs, fixation was carried out with 4% PFA in PBS for 10 minutes. For cholesterol labelling, cells were incubated in PBS containing 2% BSA and 50 μg/ml filipin III (Sigma). Filamentous actin was stained with rhodamine-conjugated phalloidin, which was added either during incubation with the secondary antibody or with filipin. Coverslips were mounted in mowiol with 4% *n*-propyl-gallate as antifade agent and the cells were inspected using a DM RXA fluorescence microscope (Leica, Wetzlar, Germany). Confocal images were obtained using a TCS NT confocal laser scanning microscope (Leica, Wetzlar, Germany).

Results

Annexin 2 is recruited to adhering EPEC microcolonies in a Ca^{2+}-dependent manner

We have shown recently that annexin 2 is present in actin tails propelling macropinosomes formed in rat basic leukaemia cells under mildly hyperosmotic conditions. The protein was

specifically enriched at the membrane-actin interface and this association appeared to depend on the presence of the native pinosome membrane, given that annexin 2 was not targeted to actin tails of rocketing *Listeria* (C. J. Merrifield et al., unpublished). Prompted by this observation, we wanted to elucidate whether annexin 2 could be recruited to specific sites at the plasma membrane where a dynamic rearrangement of the actin cytoskeleton occurred. We chose to induce such sites by using enteropathogenic *E. coli*, which trigger without invading the host cell actin accumulation into pedestals underlying the plasma membrane at sites of bacterial contact (Frischknecht and Way, 2001; Goosney et al., 2000).

HeLa cells infected with EPEC for 3 hours showed the characteristic reorganization of the actin cytoskeleton beneath microcolonies of adhering bacteria. Visualization of endogenous annexin 2 with the monoclonal antibody HH7 revealed that the protein accumulates at the sites of actin polymerization (Fig. 1A-C). By contrast, annexins 1 and 3, which had not been located to membrane/cytoskeleton contacts before, failed to be enriched at sites where EPEC attach, thus indicating that the recruitment of annexin 2 is specific (not shown). When inspected more closely, it appears that the annexin 2 staining is not uniform throughout the actin-rich pedestals but that it surrounds pedestals formed underneath individual bacteria in the juxtamembrane region (insets in Fig. 1).

Annexin 2 is a member of the family characterized by three so-called type II Ca^{2+}-binding sites, which are located in annexin repeats 2, 3 and 4, respectively. Inactivation of such sites by single point mutations leads to a mutant derivative (CM annexin 2) which fails to incorporate into the cortical cytoskeleton. This indicates that Ca^{2+} binding is a prerequisite for cortical targeting of annexin 2 (Thiel et al., 1992). Here, we expressed the same mutant as a GFP fusion protein in HeLa cells and recorded its intracellular distribution following EPEC infection. As compared to endogenous annexin 2 and an ectopically expressed annexin 2-GFP chimera (see below), CM annexin 2-GFP showed a somewhat reduced recruitment to sites of EPEC attachment (not shown). Although this indicates that Ca^{2+} binding is required for targeting of annexin 2 to sites of EPEC microcolonies, a residual recruitment of CM annexin 2 is still evident. Most likely the latter is due to the fact that, by binding to the S100A10 dimer, CM annexin 2 can form a complex with endogenous annexin 2, which is present at sites of EPEC attachment (see also below).

To analyze the annexin 2 distribution in living cells, we had previously employed an annexin 2-GFP fusion protein. When expressed in HeLa cells, this annexin 2-GFP chimera is found uniformly distributed along the entire plasma membrane with a diffuse fluorescence signal also being present in the cytoplasm (Rescher et al., 2000). The same GFP chimera was employed in the current study to circumvent the potential problem of causing an artificial mislocalization of annexin 2 when differing Ca^{2+} concentrations were present in the permeabilization medium. Fig. 2 (left panels, A,C,E,G) shows that EPEC attachment induces a recruitment of annexin 2-GFP to the attachment sites that is indistinguishable from that observed for the endogenous protein in cells permeabilized with Triton X-100 in the absence of EDTA. This indicates that the recruitment observed occurs at Ca^{2+} levels met in the infected cells and is not a phenomenon induced only following Triton X-100 permeabilization in the presence of Ca^{2+}.

Intracellular annexin 2 exists in part as a monomer, but mainly in its heterotetrameric complex with the S100A10 protein, which is bound to the unique N-terminal domain of annexin 2. To test whether S100A10 is also directed to the EPEC attachment sites, we used for the infection HeLa cells transfected with a chimeric YFP-S100A10 construct. Controls employing colocalization and co-immunoprecipitation had shown previously that the YFP tagged S100A10 is capable of forming a complex with annexin 2-CFP and that complex formation is required for cortical targeting of S100A10 (Zobiack et al., 2001). As shown in Fig. 2 (right panels, B,D,F,H), EPEC attachment also triggers an accumulation of the YFP-S100A10 at sites where the bacteria adhere. Thus, as observed for the general cortical localization of annexin 2 and S100A10, formation of the annexin 2-S100A10 complex appears to be a prerequisite for recruitment to EPEC attachment sites.

Tir is not required for the targeting of annexin 2 to EPEC attachment sites

Following EPEC adherence, several virulence factors that are encoded on a chromosomal pathogenicity island, the locus of enterocyte effacement (LEE), are transferred into the infected host cell by means of a type III secretion system (Jarvis et al., 1995). One of the proteins transferred, Tir, is inserted into the host plasma membrane and acts as a recruitment factor for a number of actin-regulating proteins including WASP and Arp 2/3 (Frischknecht and Way, 2001; Goosney et al., 2000). This recruitment triggers pedestal formation, which has been shown to depend on phosphorylation of Tir at tyrosine 474 (Kenny, 1999). In line with this crucial role of Tir in inducing pedestal formation, a mutant EPEC strain (EPEC*tir*, SE896), which only expresses a C-terminally truncated Tir protein comprising the N-terminal 233 residues (Elliott et al., 1999), is not capable of inducing pedestals upon attachment (S.L. and M.A.S., unpublished).

To determine whether annexin 2 translocation to the sites of EPEC attachment is mediated via Tir, we made use of this mutant strain. HeLa cells expressing annexin 2-GFP were infected with EPEC*tir* (SE896) for 3 hours and then analyzed for GFP and rhodamine-phalloidin fluorescence. Fig. 3 (left panels, A,C,E,G) shows that, although pedestal formation is compromised in *tir* mutant-infected HeLa, some F-actin accumulation continues to occur at sites of bacterial contact. However, although the phalloidin signal is significantly diminished in the case of the *tir* mutant as compared to wild-type EPEC, annexin 2-GFP continues to concentrate at EPEC*tir* attachment sites. It even appears that the extent of the annexin 2-GFP recruitment is more pronounced in the case of *tir* mutant as compared to wild-type bacteria. Next, we carried out infections with an EPEC mutant strain (CVD452) deficient in secretion of all type III-secreted proteins like, for example, the secreted Esps and Tir (Jarvis et al., 1995). Although adhering CVD mutant bacteria continue to be visible on the infected HeLa cells, they fail to induce the recruitment of annexin 2-GFP (Fig. 3, right panels, B,D,F,H). Thus, the translocation of annexin 2 to sites of EPEC attachment occurs

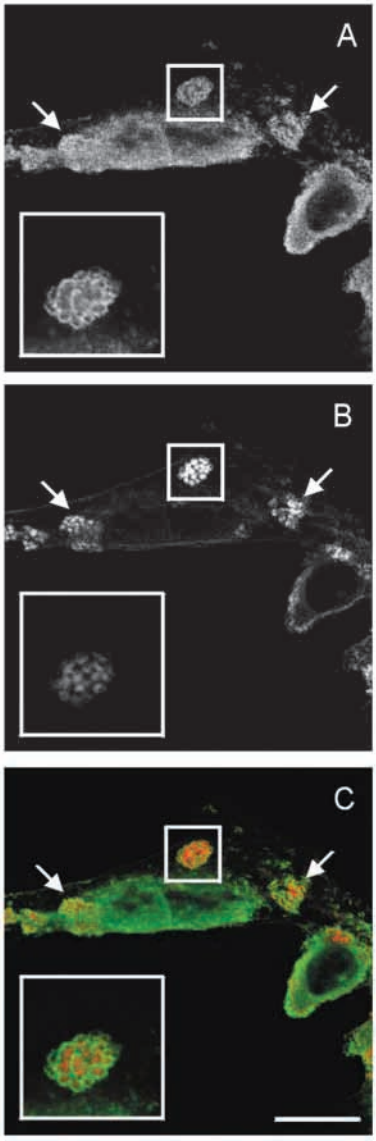

Fig. 1. Annexin 2 is recruited to sites of adhering EPEC. HeLa cells were infected with EPEC for 3 hours, permeabilized and fixed with PFA. The endogenous annexin 2 was visualized using the monoclonal anti-annexin 2 antibody HH7 and F-actin was stained with rhodamine-conjugated phalloidin. Note that EPEC-infected cells show bright annexin 2 (A) and F-actin (B) signals beneath adhering bacteria (arrows) (a merge of the images is given in C). The enlarged insets show a twofold magnification of the boxed region and reveal that annexin 2 seems to be localized in cuplike structures around the recruited actin. Bar, 10 μm.

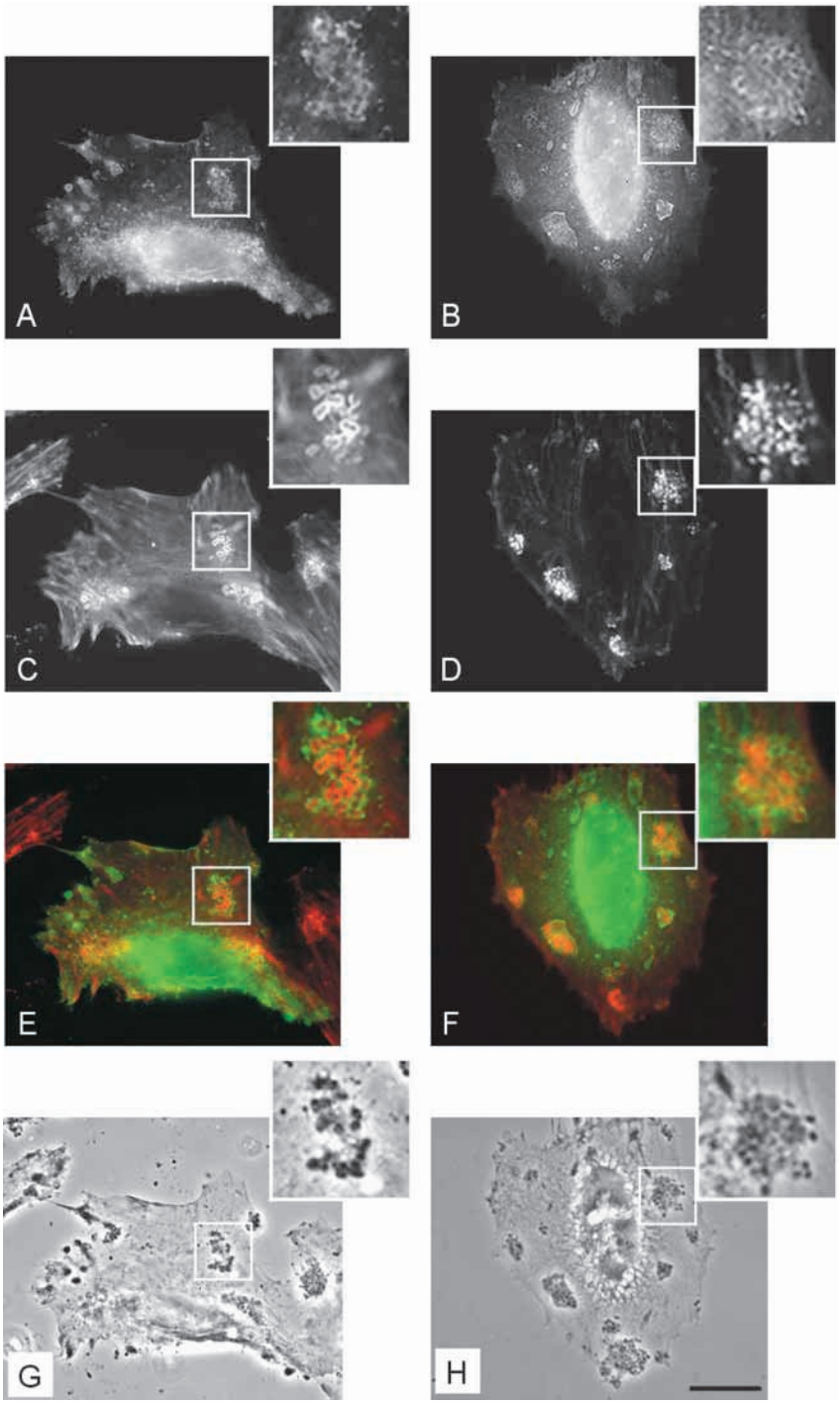

Fig. 2. Accumulation of annexin 2-GFP and YFP-tagged S100A10 at sites of EPEC attachment. HeLa cells ectopically expressing annexin 2-GFP (left panels; A,C,E,G) or YFP-S100A10 (right panels; B,D,F,H) were infected with EPEC and then processed for analysis of the fluorescent protein signals (A,B) and rhodamine-phalloidin (C,D), respectively. Merged images of the fluorescent signals are shown in E and F, respectively, whereas G and H give phase contrast images identifying the adherent bacteria. Localization of annexin 2-GFP (A) around polymerized actin (C) at sites of adhering EPEC (G) is indistinguishable from that of endogenous annexin 2. Likewise, YFP-S100A10 (B) is targeted to actin-positive structures (D) beneath EPEC microcolonies (H). The enlarged insets show a 2.5-fold magnification of the boxed region. Bar, 10 μm.

independently of Tir being inserted into the host cell membrane but requires (a) factor(s) encoded on the LEE pathogenicity island.

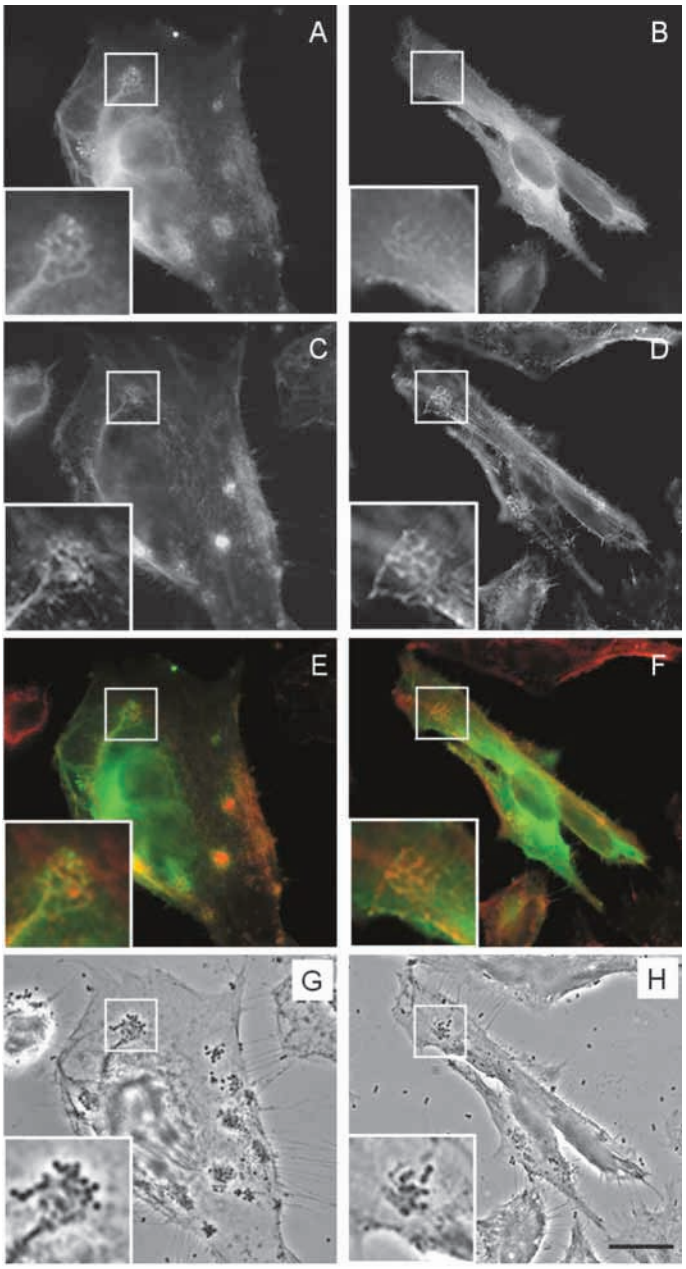

Fig. 3. Recruitment of annexin 2 to sites of attached EPEC does not require Tir but depends on other secreted Esps. HeLa cells expressing annexin 2-GFP were infected with mutant EPEC strains either lacking a functional Tir protein (EPEC*tir* SE896; left panels; A,C,E,G) or defective in type III-dependent secretion (CVD452; right panels; B,D,F,H). The annexin 2-GFP staining (A,B) is compared to that of rhodamine-phalloidin (C,D; merged images in E and F). Phase contrast images revealing the adherent bacteria are given in G and H, respectively. The enlarged insets show a 2.5-fold magnification of the boxed region. Note that annexin 2-GFP clearly accumulates beneath adhering EPEC*tir* (SE896), which do not insert Tir into the host cell membrane (A,C,E,G). Mutant EPEC defective in type III secretion, however, fail to induce an annexin 2 recruitment (B,D,F,H). Bar, 10 μm.

Cholesterol and GPI-anchored proteins accumulated together with annexin 2 at sites of EPEC attachment

Because annexin 2 has been linked to the interface between the actin cytoskeleton and specialized membrane microdomains rich in cholesterol and sphingolipids (rafts), we next visualized the membrane cholesterol distribution in HeLa cells infected with EPEC*tir* (SE896). The bacterial mutant was chosen for this experiment because it induced a more pronounced annexin 2 accumulation and because this occurred independently of pedestal formation, thus likely reflecting an early response to bacterial adherence. Following infection, transfected cells were analyzed for annexin 2-GFP and filipin staining, the latter being chosen as a fluorescent cholesterol marker. Fig. 4A,C,E shows that, in addition to annexin 2-GFP, membrane cholesterol also accumulates beneath the adhering bacteria. A similar clustering of the membrane cholesterol is seen in HeLa cells infected with wild-type EPEC (not shown).

GPI-anchored proteins are a class of proteins with affinity for rafts, and it has been shown that clustering of these proteins can lead to a patching of pre-existing rafts (Friedrichson and Kurzchalia, 1998; Harder et al., 1998; Varma and Mayor, 1998). We next analyzed the distribution of a GPI-anchored protein to further characterize the membrane domains beneath adhering EPEC. Therefore, we made use of a GPI-anchored GFP ectopically expressed in HeLa cells prior to infection with EPEC*tir*. When the distribution of this protein was analyzed and compared to that of membrane cholesterol, an obvious co-clustering at sites of EPEC attachment became evident (Fig. 4B,D,F). This indicates that EPEC attachment induces a clustering of raft components independent of Tir insertion and that this is accompanied by a recruitment of annexin 2 to the cytosolic face of the clustered rafts. Control experiments with uninfected HeLa cells revealed that, prior to EPEC attachment, neither GPI-anchored GFP nor filipin showed a concentration in larger clusters, resembling those observed at sites of EPEC microcolonies (Fig. 4G,H).

Discussion

Several unrelated bacterial pathogens exploit the actin polymerization machinery of the host cell for their infection. The most prominent examples are probably the intracellularly motile *Listeria* and *Shigella* species, which trigger localized actin polymerization at the bacterial surface and thereby induce the formation of actin-rich comet tails, propelling the bacterium through the host cell (Cameron et al., 2000; Frischknecht and Way, 2001). Central to this hijacking of the host's actin polymerization is a bacterial surface protein, ActA in *Listeria* and IcsA in *Shigella*. Both ActA and IcsA induce an activation of Arp2/3, thereby acting similarly to host cell N-WASP protein and activating the Arp2/3 complex, bypassing the tight regulation of the actin polymerization machinery. Recently, actin tail formation has also been observed at endosomal vesicles in live cells following mild hyperosmotic shock (Merrifield et al., 1999) and in vitro (Taunton et al., 2000), and it was proposed that endosomes can use this mechanism for their locomotion. At least in vitro this process appears to require the activation of Arp 2/3, in this case by N-WASP (Taunton et al., 2000), thus resembling the bacterial actin tail formation also in molecular terms. However, comparably little is known about the events linking N-WASP

recruitment to processes occurring at the cell membrane, e.g. the formation and pinching off of endosomal vesicles. One component possibly involved in such cell-membrane-dependent events is annexin 2, which was shown recently to accumulate at the membrane/actin tail interface of rocketing pinosomes (C. J. Merrifield et al., unpublished). Such accumulation was not observed at the interface of the bacterial membrane and the actin tail in the case of motile intracellular *Listeria*, indicating that annexin 2 does not interact directly with the polymerized actin but needs the additional presence of a cellular membrane. This requirement for a cellular membrane prompted us to analyze whether noninvading bacteria that compromise the host's actin cytoskeleton at sites of their membrane adherence could recruit annexin 2, thus displaying fundamental differences to the scenario observed with intracellular *Listeria*. By using pedestal-forming EPEC, we show that this is indeed the case and that the annexin 2 accumulation does not require the action of Tir, a bacterial protein essential for full pedestal formation.

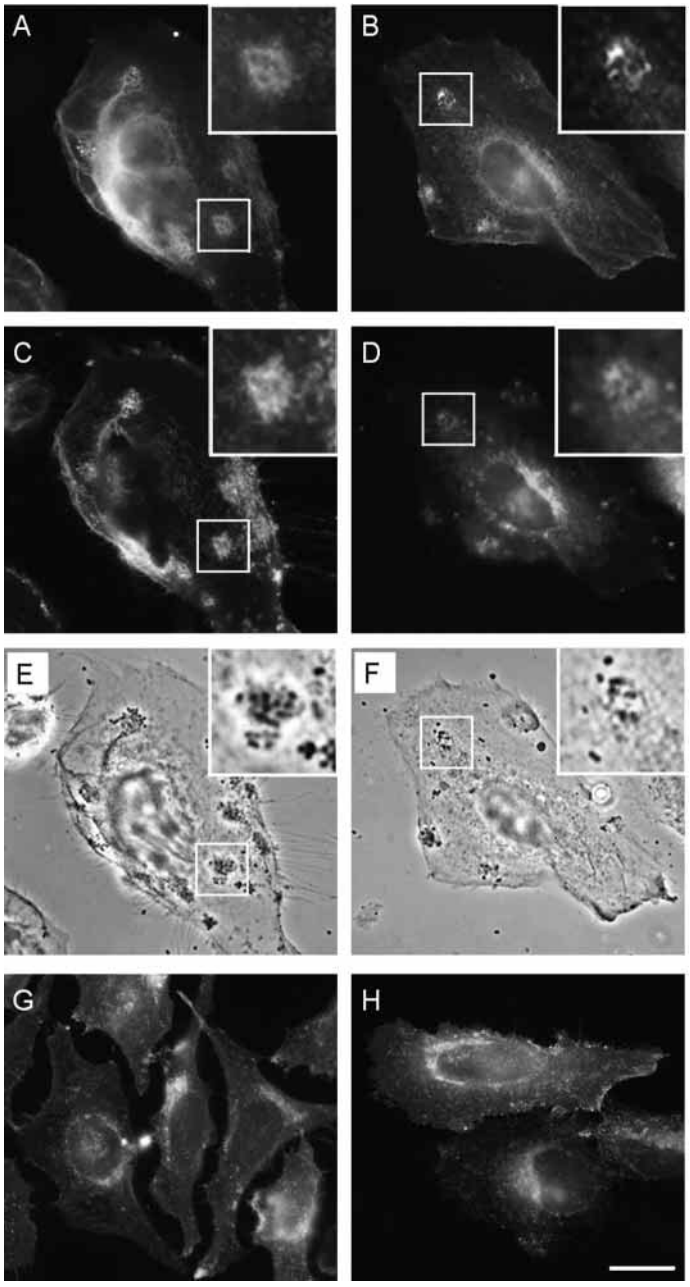

Fig. 4. EPEC attachment induces a clustering of raft components. HeLa cells ectopically expressing annexin 2-GFP (A,C,E) or GPI-GFP (B,D,F) were infected with EPEC*tir* (SE896) and then processed for analysis of the fluorescent protein distribution (A,B) or membrane cholesterol distribution by filipin staining (C,D). Phase contrast images revealing the adherent bacteria are given in E and F, respectively. The enlarged insets show a 2.5-fold magnification of the boxed region. Note that both GPI-anchored GFP, as well as annexin 2-GFP, accumulate together with membrane cholesterol at sites of EPEC attachment. Panels G and H show controls of uninfected HeLa cells stained with filipin (G) or transfected with the GPI-GFP expression construct and processed for fluorescent protein analysis (H). Bar, 10 μm.

Annexin 2 is a Ca^{2+}-regulated phospholipid and F-actin-binding protein (Gerke and Moss, 1997), and the somewhat reduced recruitment of the Ca^{2+}-defective CM annexin 2 mutant to sites of EPEC attachment suggests that this process requires Ca^{2+} binding to annexin 2. Although the role of Ca^{2+} signalling in EPEC-induced actin rearrangement is not clearly established, our data employing a GFP-tagged version of annexin 2 indicate that the Ca^{2+} concentrations met in EPEC-infected HeLa are sufficient to trigger annexin 2 accumulation beneath EPEC microcolonies. Thus, the accumulation could reflect a Ca^{2+}-dependent binding of the protein to membrane components or F-actin enriched at EPEC attachment sites, or both. The annexin 2 ligand S100A10 is also found enriched at sites of bacterial adherence indicative of the presence of the heterotetrameric annexin 2-S100A10 complex. This complex represents an entity capable of binding phospholipids and F-actin simultaneously and thus could serve as a bridging function between the host cell membrane and the rearranged actin cytoskeleton beneath EPEC microcolonies. Such linking function is supported by the observation that annexin 2 appears to be concentrated around the actin-rich pedestals in close proximity to the plasma membrane (Fig. 1).

Which membrane component(s) could specify the recruitment of annexin 2 to the sites of EPEC attachment? Tir, a bacterial protein incorporated into the host cell membrane, can be excluded because a Tir-defective mutant strain is still capable of inducing a strong annexin 2 accumulation. However, other bacterial Esps, in particular those discussed to be inserted into the host membrane (DeVinney et al., 1999; Wachter et al., 1999), could represent anchors for annexin 2, given that they are produced in the EPEC*tir* strain (Elliott et al., 1999). Likewise, it is possible that membrane components of the host are involved. In uninfected HeLa cells annexin 2 assumes, at least at the light microscopical level, a uniform distribution along the plasma membrane and in the cortical cytoskeleton (Rescher et al., 2000; Thiel et al., 1992). Thus, it seems plausible that annexin 2-binding structure(s) are distributed over the entire membrane in noninfected cells and only become concentrated when EPEC or EPEC*tir* adhere. Our analyses identify cholesterol and GPI-anchored proteins as membrane components of the host cell that accumulate at EPEC attachment sites. Although a direct binding of annexin 2 to cholesterol or GPI anchors has not been reported, it is evident from a number of studies that the protein can associate with rafts or raft-like structures rich in cholesterol and GPI-

anchored proteins. In BHK cells, membrane-bound annexin 2 can be released specifically upon cholesterol sequestration, and in mammary epithelial cells, as well as BHK and smooth muscle cells, the protein is significantly enriched in the raft fraction in the presence of Ca^{2+} (Babiychuk and Draeger, 2000; Harder and Gerke, 1994; Oliferenko et al., 1999). Although the raft component acting as the actual binding partner still awaits identification, our findings suggest that EPEC attachment is accompanied by a clustering of rafts, which in turn is responsible for – and could be stabilized by – an enrichment of annexin 2 at such sites.

By employing an EPEC strain defective in Tir we could show that both accumulation of raft components and annexin 2 does not require the incoporation of Tir into the host cell membrane. However, Tir has been shown to be essential for pedestal formation, which depends on N-WASP recruitment and Arp 2/3 activation (Frischknecht and Way, 2001; Goosney et al., 2000). It is therefore tempting to speculate that, during initial Tir-independent adherence, EPEC interact with one or more raft components of the host membrane, thereby inducing raft clustering. Although not shown for EPEC so far, binding of bacterial pathogens to raft components, in particular GPI-anchored proteins, is not without precedent. Among other things, it has been shown that diffusely adhering *E. coli* bind via the fimbrial adhesin F1845 to the GPI-anchored decay accelerating factor (Pfeiffer et al., 1998) and that the pore-forming aerolysin produced by *Aeromonas hydrophila* binds to a GPI-anchored protein receptor (Pfeiffer et al., 1998). EPEC-induced clustering of rafts could represent a signal for actin rearrangement, which is required prior to the insertion of Tir and the actual pedestal formation. In line with this hypothesis, the Tir-defective strain triggers some actin rearrangement at sites of bacterial attachment without being capable of inducing the characteristic pedestals. Similar to what was proposed for Ca^{2+}-regulated membrane segregation in smooth muscle cells (Babiychuk and Draeger, 2000), annexin 2 could participate in this scenario by stabilizing the formation of larger raft patches beneath adhering EPEC. By this means annexin 2 may act at an early stage of bacterial infection and possibly independently of actin pedestal formation. The view that EPEC can utilize two separate ways of inducing actin polymerization, one depending on Tir-dependent N-WASP recruitment and the other on raft clustering accompanied by annexin 2 accumulation, is supported by our finding that infection with wild-type EPEC of a liver cell line (HepG2), which expresses only trace amounts of annexin 2, still induces actin rearrangement at sites of bacterial attachment (data not shown). The action of annexin 2 inferred from the cell culture studies described here could be of physiological relevance during EPEC infections occurring in the intestine. Both annexin 2 and S100A10 are particularly abundant in intestinal epithelial cells where they show an enrichment in the apical membrane cytoskeleton below the level of the microvilli (Gerke and Weber, 1984). This localization could facilitate a rapid action of annexin 2 in reorganizing the apical membrane/cytoskeleton following EPEC contact. Future experiments have to define the precise site of annexin 2 action and have to identify the types of EPEC infections that require annexin 2.

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