Cortactin, an oncoprotein targeted by pathogens during infection

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1. Introduction

Cell motility and locomotion are important for many cellular functions. The cell skeleton or cytoskeleton remodels itself during multiple cellular tasks. For example, the actin cortical cytoskeleton undergoes significant changes during endocytosis, cell migration, adhesion and bacterial invasion [1].

The cytoskeleton is made up of actin, intermediate and tubulin filaments. There is crosstalk among these constituents, allowing then to behave as a complex network. We are just beginning to understand some of the aspects of this network and to flesh out the broad strokes of how it is regulated. We are confronted by great biological complexity, so we must try to simplify it in order to understand it. Thus, it would contribute to our knowledge of the cytoskeleton as a whole if we focus on some details of a particular protein that helps to orchestrate some of these changes. This review focuses on cortactin, a protein that has emerged as an important convergence node in the regulation of the cytoskeleton during numerous biological tasks. More than to give an exhaustive review, our intention is to give a general vision of the best known aspects of this protein, while highlighting with a personal view some more controversial aspects that may require further studies. We recommend some recent reviews of cortactin that complement the present one [2,3].

2. From the first studies of cortactin to the present

Cortactin was originally described as a protein located at the cell cortex and as a substrate of the Src kinase [4]. At nearly the same time, it was identified as the product of the CTTN gene (formerly EMS1), located in a chromosomal region, 11q13, frequently amplified in different human carcinomas [5]. Today, cortactin is considered an oncoprotein and a *bona fide* invadopodia marker. Invadopodia are actin-rich protrusions of the cell membrane that penetrate the extracellular matrix and degrade it, mainly through the accumulation and action of metalloproteinases [6,7]. At the same time, cortactin is a preferential target for bacterial and viral pathogens that subvert the cytoskeleton to their own benefit. For this reason, it has been called 'the Achilles' heel of the actin cytoskeleton' [8].

3. Cortactin is an important node in the regulation of the actin cellular network

Cortactin is a modular protein that participates in many signals that converge on the alteration of the actin cytoskeleton. Actin polymerization occurs when the globular monomeric form (G-actin) ensembles into a filamentous form (F-actin). The initial formation of a dimeric or a trimeric nucleus is unstable, and it is promoted and controlled by proteins that facilitate the process. One such protein is the Arp2/3 complex, which comprises two subunits, called actin-related proteins 2 and 3, as well as five other subunits. This complex is able to add a 'branch' to the side of a preexisting filament, giving rise to branched filaments with a characteristic 70-degree angle [1].

Cortactin has an N-terminal acidic motif (NTA) that directly binds and activates the Arp2/3 complex, thereby behaving as a nucleation-promoting factor (NPF). The NTA domain is followed by a six and a half repeats of amino acids that bind to F-actin, binding that is required for cortactin activity [3]. However cortactin is a weak activator of the Arp2/3 complex *in vitro*, which raises the question of whether wild-type (WT) cortactin is active or whether it requires some post-translational modification to be fully active. Indeed, although cortactin has a predicted molecular weight of approximately 65 kDa, it migrates as an 80/85 kDa doublet in SDS-PAGE. To add to the complexity, cortactin is expressed as several isoforms that differ in the number of repeats, which seems to be related to the location of these isoforms to cell-cell contacts in epithelial cells [9]. In particular, a detailed analysis of cortactin expression is needed for immunological cells, since in most situations it is assumed that a paralog protein HS1 plays the role of cortactin. We favor the hypothesis that the pattern of expression of cortactin is similar to that of N-WASP/WASP (see next paragraph). Clearly, we do not understand some basic aspects of cortactin expression and post-translational modification.

There is another way in which cortactin can promote actin nucleation: its SH3 terminal domain binds directly and activates the neural Wiskott-Aldrich syndrome protein (N-WASP) [10, 11]. The first described activators of the Arp2/3 complex were the WASP family of proteins. The representative member of the family, WASP, which is expressed exclusively in the immune system, is mutated in the immune deficiency called Wiskott-Aldrich syndrome (WAS). N-

WASP is more ubiquitous than WASP and is coexpressed with this protein in some cell types, such as macrophages and dendritic cells. The mechanism by which these proteins promote actin polymerization (NPFs class I) differs from that of cortactin: they bind G-actin and activate the Arp2/3 complex through a verprolin cofilin acidic (VCA) domain [10].

4. Cellular location of cortactin

Following various stimuli such as growth factor stimulation, binding and integrin engagement, cortactin translocates from a perinuclear/Golgi location to areas of cytoskeleton remodeling, such as lamellipodia at the leading edge [3]. The molecular mechanism of this translocation is still unknown. More recently, cortactin has been detected in the nucleus, where it has been proposed to participate in the separation of the centrosomes in preparation for mitosis [12]. Again the details of this translocation remain to be elucidated.

5. Post-translational modifications of cortactin

5.1 Cortactin phosphorylation

As previously mentioned, cortactin is traditionally known as a substrate of Src kinase. Until the past decade, that was the only known cortactin post-translational modification of cortactin. In the murine protein the major phosphorylation sites are tyrosines 421, 466 and 482. It is assumed that Src phosphorylation of cortactin stimulates cortactin activity. More recent data, however, paint a more complicated picture of the effect of Src-mediated phosphorylation of native cortactin [3].

In addition to Src kinase, one study has indicated that extracellular response-activated kinase (Erk) phosphorylates cortactin on serines 405 and 418 [13]. Long forgotten, this report came to light again in view of the action of cortactin on N-WASP activity, which we discuss later in this review.

In a comprehensive and meritorious effort, the Cell Migration Consortium mapped cortactin phosphorylation sites [14] describing 17 new ones. This gives us a more realistic perspective on the complexity of the regulation of this protein. Most of these sites are serines and threonines.

At the present it is known that cortactin is phosphorylated by various kinases, including Fer, Abelson and related kinases (Abl/Arg), p21-activated kinases (Paks) [15], and protein kinase D (PKD) [3]. In contrast, protein-tyrosine phosphatase 1B (PTP1B) is the sole phosphatase known to act on cortactin [16,17]. The physiological importance of cortactin dephosphorylation has probably been misrepresented due to the scarce data on the subject.

5.2 Cortactin acetylation

Recently, regulation of cortactin by reversible lysine acetylation has been described [18]. It can be acetylated by the acetyltransferase PCAF and p300, and deacetylated by histone deacetylase 6 (HDAC6) and SIRT1. According to this study, 11 lysines can be acetylated. When acetylated, the molecule loses two otherwise positively charged patches of lysines, most of which are located in the area of the repeats. Consequently, the ability of cortactin to bind F-actin diminishes. The authors of that study proposed that acetylation of cortactin inactivates the protein. However, it is very important to address the consequences of this acetylation on cortactin activity using *in vitro* actin polymerization assays and an acetylation mimic cortactin mutant. In this way, some important conclusions might be drawn about the activity of the protein and the effect of the acetylation on cortactin structure.

This regulation has already been implicated in quality control (QC) autophagy, a mechanism that, under physiological conditions, is important for removal of protein aggregates and turnover of organelles, such as mitochondria. HDAC6 recruits and deacetylates cortactin, which promotes the F-actin remodelling required for the fusion of autophagosome and lysosome and subsequent substrate degradation [19].

6. Cortactin as a cytoskeletal switch

Apart from its intrinsic activity, cortactin can activate the WASP/N-WASP family of nucleators, which establishes a functional connection between the two main families of proteins capable of activating the Arp2/3 complex.

It was shown that cortactin binds and activates N-WASP through its SH3 terminal domain. However, full-length cortactin was less potent than the isolated SH3 domain in activating N-WASP, which suggests that cortactin, at least *in vitro*, is in a closed conformation that masks the SH3 domain. The interaction between cortactin and N-WASP was studied using *in vitro* actin polymerization assays involving both phosphomimetic mutants and non-phosphorylatable mutants of cortactin, as well as recombinant cortactin phosphorylated *in vitro*. Erk-mediated serine phosphorylation of cortactin opens the molecule, and allows the cortactin SH3 to interact with N-WASP. This interaction is terminated by Src-mediated tyrosine phosphorylation of cortactin. These findings led to the proposal of an 'on/off switching' mechanism called the 'S-Y Swicth' model, in which phosphorylation of cortactin by Erk and Src kinases controls the

ability of cortactin to activate N-WASP. Thus, cortactin binds and activates N-WASP only when phosphorylated at serines 405 and 418. The phosphorylation of cortactin by Src at tyrosines 421, 466 and 482 terminates this interaction (Fig. 1) [11].

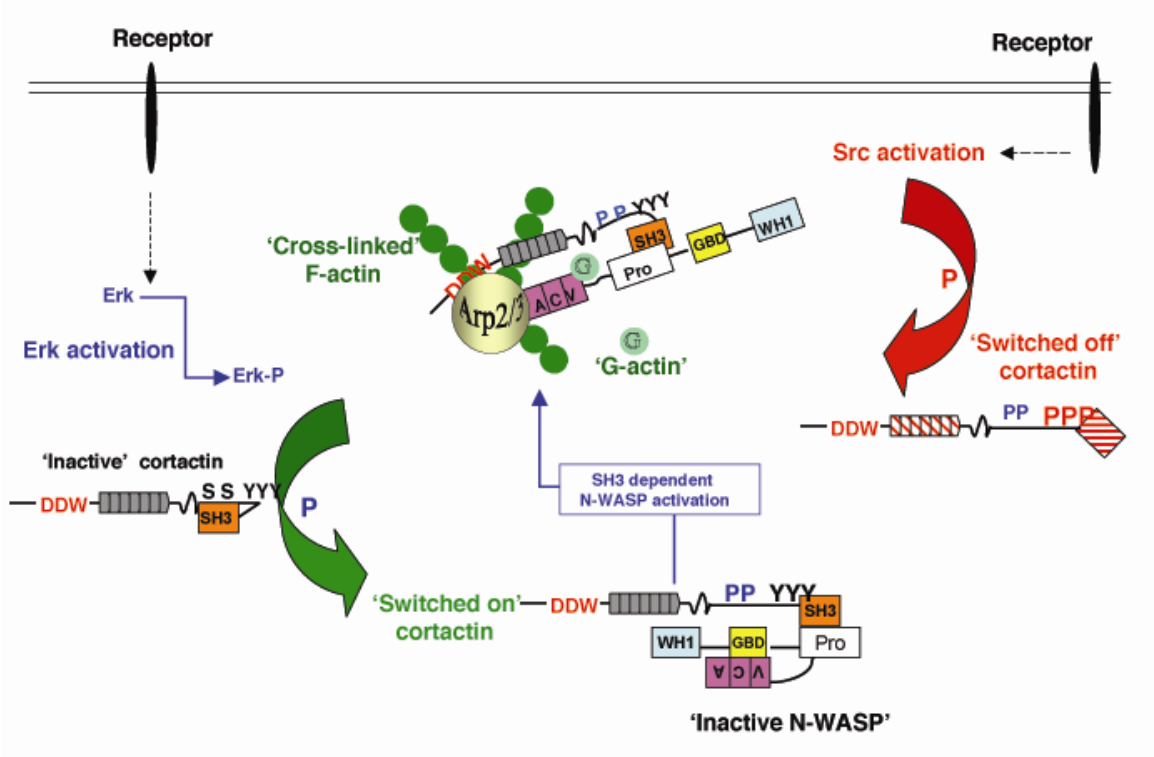

Figure 1. Coupled cortactin-N-WASP activation according to the 'S-Y Switch' model proposed by Martinez-Quiles *et al* (2004).

The most easily testable prediction of the 'S-Y Switch' model is the understanding that cortactin can be regulated by a conformational change. The structure of cortactin has been resolved by circular dicroism, chemical crosslinking and X-ray scattering. These results showed that cortactin adopts a closed globular conformation through interaction between the cortactin SH3 domain and the repeats region [20]. These results are in agreement with our proposed 'S-Y Switch' model and contradict a previous description of cortactin as a thin elongated monomer [21]. It would be very important to use structural techniques to asses how Src-mediated phosphorylation of cortactin affects its structure and activity. As mentioned above, it seems that the *in vitro* effect of this phosphorylation is different from that of Erk phosphorylation, which seems to open the molecule and liberate the SH3 domain.

Immediately after Erk- and Src-mediated phosphorylation was shown to regulate cortactin, these findings were tested in different *in vivo* settings because of their repercussions on the fields of cellular motility and molecular microbiology [8, 22]. In a clear relation to the 'S-Y Switch' model, studies examined the effects of different serine and tyrosine phospho-mutations in cortactin on lamellipodial protrusion, actin assembly within cells, and focal adhesion dynamics. Cortactin mutants mimicking serine phosphorylation appeared to affect predominantly actin polymerization, whereas mutation of tyrosine residues altered turnover of focal adhesions [23].

The 'S-Y Switch' model was also tested in different settings [24, 25], and phosphorylation of cortactin not only by Erk and Src but also by Pak was shown to be important for invadopodia formation, where 'a fine balance between different phosphorylation events induces subtle changes in structure to calibrate cortactin function during invadopodia formation' [24].

Finally, the importance of studying the different states of cortactin phosphorylation during bacterial invasion has been highlighted [8]. Under the new prism of the 'S-Y Switch' model, our group decided to assess the contribution of cortactin to actin polymerization during pedestal formation by enteropathogenic *Escherichia coli* (EPEC). We chose EPEC as a model system in order to analyze signaling to the actin cytoskeleton across the plasma membrane in response to external stimuli.

7. Cortactin and bacterial pathogens

7.1 Role of cortactin in pedestal formation by EPEC and EHEC

Numerous pathogens have evolved mechanisms to subvert for their own benefit the cellular regulatory complexes that control actin polymerization. Among such bacteria, enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are non-invasive pathogens. EPEC are responsible for severe diarrhoea and EHEC can cause bloody diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome. Both food-borne pathogens are an important cause of infant mortality in developing countries. These bacteria colonize the intestinal epithelium through the formation of attaching and effacing (AE) lesions, which are characterized by a localized loss of microvilli, close adherence of bacteria to the host cell membrane and the generation of filamentous (F)-actin-rich structures beneath these bacteria called pedestals [26,27].

To generate these actin pedestals both pathogens translocate their bacterial effectors into the mammalian cell using a type III secretion system (TIIISS). Translocated intimin receptor (Tir) is a translocated receptor that inserts into the plasma membrane with a hairpin loop topology. The extracellular domain binds to the bacterial outer membrane protein intimin inducing clustering of Tir. Tyrosine 474 in the C-terminal cytoplasmic domain is phosphorylated. This phosphotyrosine recruits the SH2 domain-containing mammalian adapter proteins Nck1 and Nck2 (hereafter referred to collectively as Nck). Nck recruits and is thought to activate N-WASP, which in turns binds and activates the Arp2/3 complex to promote actin polymerization [28].

For many years this has been the only pathway considered both necessary and sufficient for actin polymerization. When our research group as new comers to the field, examine the literature on this pathway, several questions occur to us. When does the phosphorylation of Tir occur? After the insertion in the membrane or is it a necessary step for insertion? Does it occur before or after clustering? If we extrapolated from signaling in immune receptors, we would expect the clustering to precede the phosphorylation. A more general question is, why, ultimately, do these bacteria form pedestals? This is an intriguing question because bacteria also adhere without them. Are pedestals made for the sole purpose of strengthening bacterial adhesion? If so, why are then many other proteins recruited to pedestals, including cortactin [29,30]? What is the reason for recruiting two activators of the Arp2/3 complex, when one (N-WASP) is sufficient [31]?

It is known that cortactin localizes to actin pedestals, and that overexpression of truncated forms of the protein blocks pedestal formation [31]. This indicates that cortactin is an important player whose role should be reconsidered in view of its newly described capacity to directly activate both the Arp2/3 complex [21] and N-WASP [11]. Greater importance for cortactin is also suggested by the recently proposed 'S-Y Switch' model of regulation [11]. Indeed, using a very similar approach two research groups [32, 33] examined the effect of serine and tyrosine phospho-mimic and non-phosphorylatable mutants of cortactin on pedestal formation. Both studies concluded that tyrosine phosphorylation inhibits pedestal formation. However, the Erk phospho-mimic mutant had no effect on pedestal formation, in contrast to the inhibitory effect of the corresponding non-phosphorylatable mutant [33]. Therefore it seems that serine phosphorylation of cortactin is needed for pedestal formation, whereas tyrosine phosphorylation inhibits it.

A very important finding reported independently by both groups is that EPEC infection induces tyrosine phosphorylation of cortactin [32, 33]; this phosphorylation is abolished in N-WASP-deficient cells [33] but not in Nck-deficient cells (our own unpublished results). Therefore cortactin tyrosine-phosphorylation depends on the presence of N-WASP, more than on the presence of the pedestal itself, because pedestals do not form in the absence of either N-WASP or Nck. On the contrary, serine phosphorylation of cortactin does not require N-WASP. Moreover, the N-terminal domain of cortactin directly binds the N-terminal part of Tir *in vitro* [33, 32], in a tyrosine and serine phosphorylation independent manner [32, 33]. More importantly, the interaction between cortactin and Tir_{EPEC} promotes Arp2/3 complex-mediated actin polymerization by activating cortactin [33].

We propose a model of cortactin action on pedestals induced by EPEC (Fig. 2) [33]. Taking into account that cortactin binds and activates N-WASP though its SH3 domain, and that this activation by cortactin is regulated by tyrosine and serine phosphorylation of cortactin, we proposed that cortactin binds Tir through the N-terminus and N-WASP through the SH3 domain. In this situation, cortactin phosphorylated on serine would bind both Tir and N-WASP, whereas cortactin phosphorylated on tyrosine would bind only Tir. In other words, cortactin may act on the Tir-Nck-N-WASP pathway by controlling a possible cycling activity of N-WASP that may underlie pedestal motility (Fig. 2) [33].

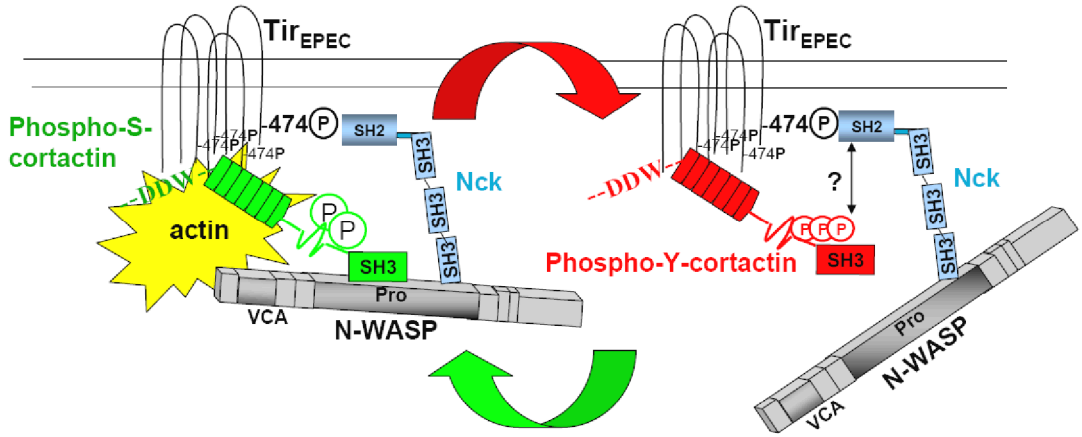

Figure 2. Proposed model of the coordinated actions of cortactin and N-WASP during EPEC infection. Modified from Cell Commun Signal. 2009 May 6; 7:11.

Current research on pedestal signaling to trigger actin polymerization shows an increasingly complicated picture: no longer is there a reassuringly linear Tir-Nck-N-WASP pathway. EspF is another translocated effector of both EPEC and EHEC that seems to participate in pedestal formation. EspF contains several C-terminal repeats, each of which possesses a segment for N-WASP binding and a proline-rich sequence. The latter is recognized by sorting nexin 9 (SNX9), a protein that contains an SH3 domain and a highly conserved Bin-Amphysin-Rvs (BAR) domain that binds and deforms membranes inwardly. The role of EspF-SNX9 interaction is intuitively difficult to understand because pedestals are structures that protrude [34]. The implication of cortactin in this recently described and apparently secondary pathway has not been addressed. Therefore it would be very interesting to test whether cortactin contributes to actin assembly through EspF.

Despite the high homology between the Tir proteins from EPEC and EHEC strains, the latter lack tyrosine 474 which has 'forced' the development of a distinct mechanism to promote actin synthesis in EHEC. In this pathotype, Tir_{EHEC} does not require Nck for actin assembly. Two groups reported that the effector encoded within prophage U, EspFu [35], also known as Tir cytoskeleton coupling protein, Tccp [36], is able to recruit and to activate N-WASP. EspFu is recruited to sites of bacterial attachment, and this recruitment depends on the NPY_{458} (Asn-Pro-Tyr_{458}) motif located in the C-terminal domain of Tir_{EHEC}. It is unclear how the motif recruits EspFu, since EspFu does not bind directly to it.

Interestingly, this motif is homologous to tyrosine 454 on Tir_{EPEC}, which is responsible for a minor Nck-independent pathway whose physiological importance is uncertain. This raises the possibility that both EPEC and EHEC use a common Nck-independent pathway for actin polymerization. How can it be that what is an inefficient mechanism in EPEC has turned into the major mechanism in EHEC? The expression of EspFu by EHEC has converted this otherwise secondary pathway into the principal one. The picture gets even more complicated when we consider that EHEC express both EspFu and EspF effectors which share 25% sequence identity and 35% similarity.

As another example of bacterial molecular mimicry, EspFu is able to activate N-WASP by disrupting the intramolecular interaction that maintains N-WASP in a closed conformation [37]. Although WIP is known to keep N-WASP in a closed conformation [38], the role of WIP in modulating the effect of EspFu on N-WASP has not been addressed.

7.1.1 Cortactin as the 'missing link' in EHEC

Although N-WASP and EspFu complex with Tir neither protein binds Tir directly. An important area of investigation in EHEC signaling to actin is the search for the 'missing link' that bridges Tir and N-WASP. Cortactin has been suggested as an appropriate candidate [39]. Cortactin binds both Tir_{EHEC}, and EspFu simultaneously: the C-terminal SH3 domain of cortactin is responsible for binding EspFu [39]. Subsequently, in *in vitro* organ cultures (IVOC) of human terminal ileal tissue, it was shown that cortactin is recruited to the site of EHEC adhesion independently of EspFu and N-WASP. This suggests that cortactin plays a more important role during infection of mucosal surfaces *in vivo* than has been observed using *in vitro* cultured cells [40].

Interestingly, at early stages of infection, EHEC induces the tyrosine phosphorylation of cortactin, after which it is rapidly dephosphorylated [40]. The authors proposed a model where at early stages of infection cortactin binds Tir and EspFu. Soon thereafter, cortactin is tyrosine-dephosphorylated and released from EspFu. Finally this liberated cortactin helps to activate N-WASP, promoting actin polymerization through the Arp2/3 complex [40] (Fig. 3).

Various other proposed 'missing links' has been proposed, such as the 53KDa insulin receptor substrate (IRSp53) and insulin receptor tyrosine kinase substrate (IRTKS), which are homologous to each other [41-43]. Both contain inverse-BAR (I-BAR) domains that deform PI(4,5)P(2)-rich membranes outwardly, mainly through electrostatic interactions [44]. We should also take into account that Tir_{EPEC} phosphorylated on tyrosine 454 forms complexes with an active phosphatidylinositol 3-kinase (PI3K), probably to stimulate the production of PI(3,4,5)P(3) beneath EPEC attachment sites [45]. If we extrapolate this mechanism to the tyrosine 458 of EHEC, we can speculate that IRSp53 is implicated in the deformation of the membrane, which in turn is coupled to actin remodeling through a process involving the WAVE family of proteins [46]. Although researchers that advocate for these missing links tend to underestimate the contribution of cortactin, we think that both IRSp53/IRTKs and cortactin might participate in the process, and that contradictory views arise from the incompleteness of the data at presently known. To be sure, we could propose many different models to support various views of the signaling process and of the identity of the missing link in EHEC. However, we believe that when doing research on signal transduction it is important to keep an open mind [47] and to interpreter incomplete findings cautiously. Dogmas are sustained until knowledge abrogates them, and one example is the finding that a recently described category of EPEC forms intracellular pedestals [48].

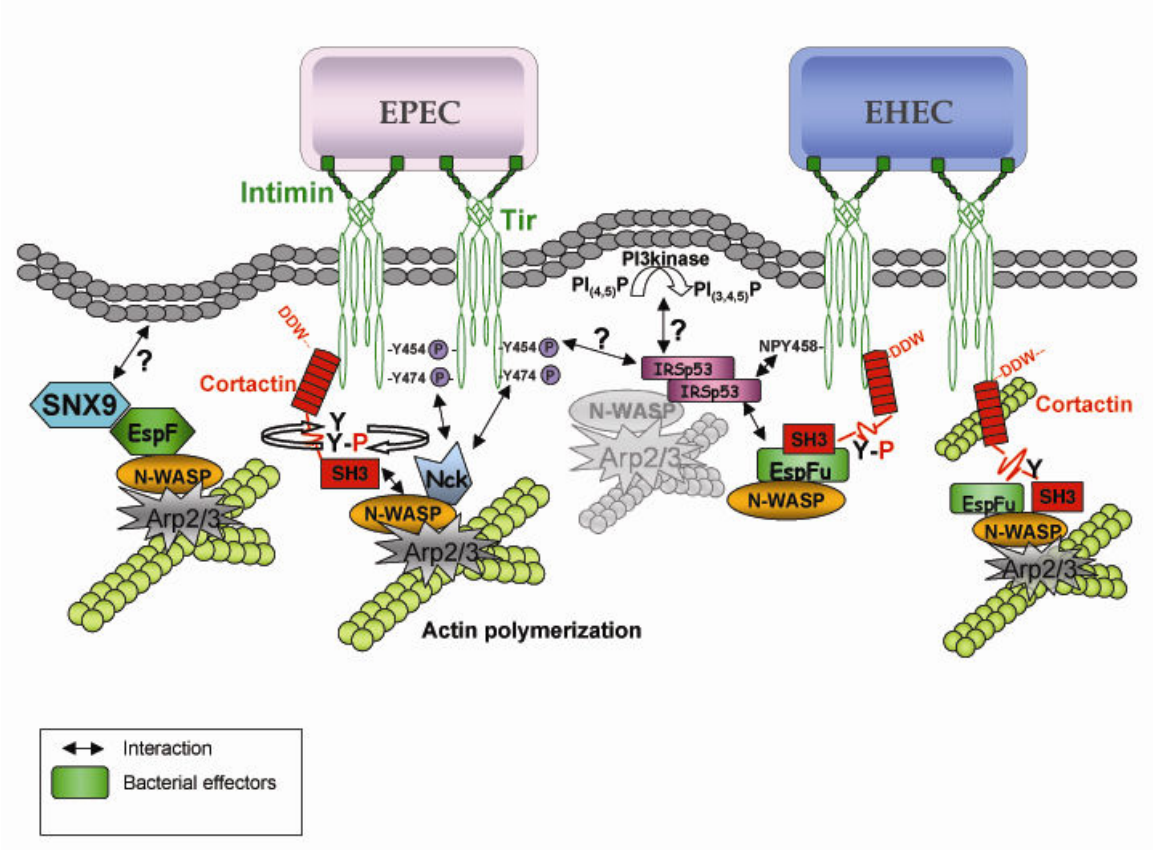

Figure 3. Signaling during pedestal formation by EPEC and EHEC

7.2 Cortactin phosphorylation during the invasion of other pathogens

During *Chlamydia trachomatis* infection, pathways involving Abl kinase or platelet-derived growth factor receptor (PDGFR) are activated, leading to tyrosine phosphorylation of cortactin. Tyrosine phosphorylation of cortactin likely participates in the remodelling of actin cytoeskeleton in order to internalize bacteria. Phospho-cortactin is also recruited to the site of bacterial entry [49]. There are many other examples where bacteria control cortactin phosphorylation as part of a complex mechanism of entry, such as *Shigella* [8]. Many other pathogens will be shown to alter not only cortactin tyrosine phosphorylation but many other post-translational modifications.

7.2.1 Cortactin as a bacterial target for acetylation

To block the immune response, the acetyltranferase YopJ, an effector of *Yersinia* species, acetylates the critical serine or threonine of the activation loop of a MAP kinase, impeding its subsequent activation by phosphorylation [50].

It would be very interesting to test whether *Yersinia* also inhibits cortactin by acetylating it. In addition to deacetylating cortactin, HDAC6 deacetylates alpha-tubulin and this is required for invasion by uropathogenic

Escherichia coli (UPEC) [51]. Future studies will probably establish many situations in which acetilation/deacetilation is subverted by pathogens [52].

7.2.2. Cortactin and the innate immune response to *Candida*

Immature dendritic cells (DC) express C-type lectins that recognize fungi. A newly described type of protrusion, named fungipod, has been implicated in the recognition of *Candida parapsilosis*. Fungipods are dynamic structures that contain clathrin, actin and cortactin. They may promote yeast phagocytosis by DCs [53].

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