Complex kinase requirements for EPEC pedestal formation

To the editor:

We are writing in response to the letter of Phillips *et al.*[1], which addresses the identity of the cellular tyrosine kinase(s) required for the formation of actin-filled membrane protrusions (pedestals) induced by enteropathogenic *Escherichia coli* (EPEC). Previous reports have established that the EPEC virulence factor Tir is translocated into the host plasma membrane and binds to the EPEC outer membrane protein intimin[2], thereby allowing tight adhesion of the bacterium to the host cell. Tyrosine phosphorylation of Tir on Y474 is required for recruitment of Nck, N-WASP and the Arp2/3 complex, as well as for actin polymerization, which constructs and braces the pedestal[3,4]. An outstanding question has been the identity of the host tyrosine kinase(s) responsible for phosphorylating Tir. Phillips *et al.* provide evidence that the Src family kinase c-Fyn is both necessary and sufficient for Tir phosphorylation and actin pedestal formation induced by EPEC. Importantly, Phillips *et al.* form actin pedestals by using a dual bacterial system in which the haemagglutinin (HA)-tagged Tir was introduced into the host cell by an EPEC strain lacking wild-type Tir and intimin ("priming"); a K12 strain expressing intimin in the outer membrane was then used to cluster Tir and induce pedestal formation ("challenge").

Our published work[5] indicates that the situation after infection with a wild-type strain of EPEC (WT-EPEC, strain 2348/69) using a standard infection protocol is more complex. In particular, we demonstrate that cells lacking the three kinases Src, Fyn and Yes (the same cells used by Phillips *et al.*), are still capable of forming actin pedestals when infected with WT-EPEC, suggesting that Fyn is not necessary for pedestal formation[5]. Using WT-EPEC, we also show that several tyrosine kinases, including Abl and Arg, localize and are persistently activated beneath attached EPEC, and that Abl and Arg are each sufficient for pedestal formation, but neither alone is necessary. Indeed our results suggest that several functionally redundant kinases can suffice, perhaps allowing the bacterium to increase its host range. We cannot rule out that Fyn, similar to Abl and Arg, functions in a redundant fashion; however, our data indicate that phosphorylation and dephosphorylation of Tir are dynamic and require sustained local kinase activity; furthermore, Fyn is not evident beneath attached EPEC in our experiments, nor beneath K12-intimin strains in the experiments of Phillips *et al.* Thus, the dual bacteria system used by Phillips *et al.* has different requirements for host effector molecules than wild-type EPEC strains. The apparent incongruities between data obtained with the dual bacteria approach of Phillips *et al.* and that obtained with WT-EPEC raise important questions relevant to treating disease caused by pathogenic *E. coli*. Kinases phosphorylating Tir represent targets for drug development, and our data suggest that effective drugs will probably have to target multiple kinases.

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Hayward *et al.* reply:

Our recent Letter[1] and the report of Swimm *et al.*[2] provide intriguing new insights into actin pedestal formation by EPEC. Both groups investigated how the receptor (Tir) of this important human pathogen is tyrosine phosphorylated: we defined a specific requirement for the Src-family kinase (SFK) c-Fyn using "priming and challenge" and *in vitro* assays to uncouple Tir delivery and receptor clustering, whereas Swimm *et al.* investigated the potential pharmaceutical application of pyrido[2,3-d]pyrimidine inhibitors using only a single EPEC wild-type strain (EPEC-WT). Their data implicated several redundant kinases, including SFKs, c-Abl and c-Arg; Swimm *et al.* comment that their particular EPEC-WT forms pedestals on *src*$^{-/-}$ *yes*$^{-/-}$ *fyn*$^{-/-}$ (SYF) cells, which seems to be in contrast to our findings.

How might these data be explained? The potential for variation in kinase specificity is not unexpected to us. Different EPEC-WTs were used, (JPN15(ΔpMar2) for priming[1] and 2348/69 for infection[2]), and it is widely accepted that 'WT' pathogens encode a diverse multifactorial virulence factor repertoire that often exhibits functional redundancy[3]; this is certainly true of EPEC. Correspondingly, sporadic pedestal formation on SYF cells seems to be restricted to a subset of EPEC-WTs (R.D.H., N.P. and V.K., unpublished observations). We cleanly dissected a c-Fyn-dependent Tir signalling mechanism, but do not declare it exclusive. As EPEC Tir Y474F point mutants still form some actin pedestals[1], and the closely related EHEC Tir functions independently of tyrosine phosphorylation[4], an assortment of compensatory pathways probably exist, contributing to the spectrum of disease symptoms. Thus, neither priming and challenge (as we stated), nor infecting cultured cells with one EPEC-WT necessarily mimics 'bacterial pathogenicity' *in vivo*, clearly a much more complex issue.

Are the findings really incongruous? Tir is a c-Fyn substrate *in vitro*[1], but whether c-Abl/c-Arg function directly remains unknown. Unlike c-Fyn, c-Abl/c-Arg are stable pedestal components[2]. It is now critical

to address whether recruited c-Abl/c-Arg actively contribute to pedestal formation, or are merely passive 'bystanders'. Indeed, active c-Abl/c-Arg interact with Nck, actin and CrkII, which are all concentrated in EPEC pedestals[5]. As pyrido[2,3-d]pyrimidine inhibitors block both c-Abl/c-Arg and SFKs[2], and c-Abl/c-Arg are activated by SFK-dependent tyrosine phosphorylation[6], it is not inconceivable that c-Fyn functions upstream of c-Abl/c-Arg as in PDGF-receptor signalling[7]. Further studies are now essential to address how c-Abl/c-Arg are activated, which activities determine pedestal association and whether c-Abl-CrkII-dependent actin polymerization is involved in pedestal persistence. Provocatively, recent reports reveal that c-Src induces c-Abl-CrkII signalling during *Shigella* entry[8,9]. Thus, to assume that the mechanism is 'black and white' would be naive. Nevertheless, careful stepwise biochemical analysis and reconstitution of contributory pathways continues to be central to dissecting complex biological processes.

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