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A bacterial effector targets host DH-PH domain RhoGEFs and antagonizes macrophage phagocytosis

Na Dong, Liping Liu and Feng Shao

Corresponding author: Feng Shao, National Institute of Biological Sciences, Beijing

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1st Editorial Decision 28 October 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three express significant interest in your identification of EspH as a bacterial effector that targets RhoGEF. However, all three also raise significant concerns that would have to be addressed by a substantial revision before we could consider publication of your manuscript. I will not go into all the details here, but most notably, all three referees raise major concerns as to the directness and the specificity of the interaction between EspH and RhoGEFs. Moreover, all also find that further data on how this interaction affects phagocytosis would be important.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor

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The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This interesting study provides evidence that the type III secreted effector, EspH interferes with the actin cytoskeleton dynamics of host cells by binding to the DH-PH domain in RGS-RhoGEFs. Thus, EspH blocks activation of RhoA, thereby inhibiting downstream signaling. The authors then show that EspH plays a role in inhibiting bacterial phagocytosis by cultured mouse macrophages. The study is convincing and well written. The authors should address the following points:

1. Although this paper thoroughly shows that the DH-PH domain of RhoGEFs and EspH interact, it is not clear whether this interaction is direct. One difficulty is that the authors have been unable to purify recombinant EspH. By co-immunoprecipitation assays in transfected mammalian cells, they show that no other bacterial proteins are required for EspH-RhoGEF interaction. The authors could similarly test whether any mammalian factors are required for this interaction by expressing the DH-PH domain of RhoGEF in EHEC (or an E. coli K12 strain expressing EspH) and test for complex formation by co-immunoprecipitation. Interaction in this assay would show that the only mammalian factor necessary for an interaction between EspH and RhoGEF is the DH-PH domain of RhoGEF and support the contention that the interaction is direct.
2. The title implies that the RhoGEF interaction is essential to the antiphagocytic effect. This may well be the case, but in spite of the comprehensive biochemical data showing EspH interaction with RhoGEFs, a clear link between this interaction and inhibition of phagocytosis is lacking. Any evidence for this that the authors could provide would greatly strengthen the manuscript. Possible experiments include co-localization of EspH and RhoGEF in mouse macrophages, or co-immunoprecipitation of EspH and RhoGEF in mouse macrophages, although these experiments may be technically challenging.
3. Figure 3A is lacking error bars, please include. In this and other figures, although it is usually intuitively clear which differences are statistically significant, it may be useful to include asterisks depicting statistical significance.
4. In transfection experiments, described throughout manuscript, the efficiency of transfection might influence the interpretation of results. For example, luciferase assays were performed on entire populations of cells, so that a low transfection efficiency might be predicted to result in a much more modest effect than if all of the cells were successfully transfected. The authors should address this issue in Methods and/or Results.
5. Fig. 3C. A prediction is that the presence of EspH should have no effect on RhoA-GTP when cells are treated with GST-CNF1, and this could be included as a specificity control.
6. Figure 2A and B. The actin staining seems to be very bright in transfected cells. Is this due to bleedthrough of GFP? Please clarify
7. Figure 2C. The transfected cells appear to be somewhat rounded in comparison to untransfected cells. Please clarify.

Minor Points:
8. Figure 1. (A) Presumably, the authors have shown that vector only control (EGFP) does not produce cell rounding-this can be cited as data not shown in text. (B) Do all cells transfected w/ EspH round up?
9. Page 8, when referring to luciferase assays, the text should cite figure 2D, not 2C.
10. Page 4, "this process is aided by EspFu" might be altered to read "this process is greatly aided by EspFu".
11. Page 9, when referring to CNF-1 antagonism of EspH-induced cell rounding, the text should cite figure 2E, not figure 2D.
12. Figure 3E. The authors assert in text that when EspH is co-expressed with p115-RhoGEF in HeLa cells, the formation of enhanced, thick actin filamentous bundles is counteracted. Although we see rounded up cells when EspH is co-expressed, the phenotype doesn't appear to be strikingly different from cells transfected w/ EGFP control. Please clarify.
13. Fig. 2D. Please explain why 293T cells, not HeLa cells, were utilized. (Presumably, it is due to the efficiency of transfection.)
14. For clarity for the uninitiated, please describe briefly what an RBC pulldown is.

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15. Discussion, Page 16. Note that C. rodentium encodes an EspH, and the role of EspH on colonization in the mouse model has been investigated (see Mundy et al., 2004, Infection and Immunity).

Referee #2 (Remarks to the Author):

The manuscript "A bacterial effector targets host DH-PH domain RhoGEFs and antagonizes macrophage phagocytosis" presents evidence that the effector protein EspH inhibits actin dynamics through inhibition of Rho-GEF proteins. They demonstrate that transfection of EspH induces cell rounding similar to previous reports and show that 20% of transfected cells round up after 1 hour of EPEC infection. The authors perform a set of biological studies using an 'epistasis' type of approach. They convincingly place EspH signaling upstream of Rho GTPases and downstream of G-protein coupled receptors. Less convincing is their conclusions that EspH competitively inhibits Rho-GEFs and this activity is responsible for inhibiting macrophage phagocytosis. Nevertheless, the manuscript does advance our understanding of EspH function at the biochemical level.

Major points

(1) This reviewer found the EPEC infection experiments in Figure 1A to be quite weak in comparison to the transient transfection data. In fact, it is not clear why cells round up after 1 hour of infection with an MOI of 100. This has not been detected previously despite the plethora of data published on EPEC infection at this time range. This is likely a cell type dependent effect and not something inherent with EPEC infection.

(2) The authors should demonstrate that their EPEC complemented strains of espH- indeed secretes EspH protein.

(3) The authors attempt to induce actin stress fibers with LPA and serum in cells that were transiently transfected with the EspH. The conclusions drawn from these studies seem to be invalid given that EspH is expressed for 10 hours before LPA treatment. Are the LPA receptors surface expressed in these cells etc.? Are their other modifications that could account for these effects? This experiment should be done with infection and not with transient transfection of EspH. At the very least, the caveats of this experimental approach should be directly stated in the text since there are certainly additional explanations for this finding (i.e. rounded cells do not signal to actin due to loss of surface receptors).

(4) The authors perform pulldown studies with recombinant DH-PH domains and find EspH can be precipitated from mammalian cell lysates. While this is a good experiment suggesting that EspH indeed interacts with this complex, it does not demonstrate a direct interaction as the authors state since this is a complex mixture of proteins. This "direct binding" statement needs to be removed from the article since there is no evidence in support of this conclusion (i.e. mixing two highly purified proteins).

(5) One of the weaknesses of this study is that the conclusions are entirely based on overexpression of EspH. This could certainly force an interaction between EspH and Rho GEF proteins. The authors need to demonstrate that bacterial secreted EspH indeed inhibits Rho GEFs. One simple prediction would be that RNAi knockdown of Rho GEF would inhibit the EPEC phenotypes induced by EspH. The reviewer does recognize that this experiment may be quite difficult due to redundant Rho GEF proteins. Nevertheless, thinking about these types of experiments highlights the problems of the current data to convincingly draw the conclusion that EspH competitively inhibits Rho GTPase activation. Some of the weaknesses of this model should be discussed, particularly given the very low amounts of EspH that are likely secreted into host cells and whether this is compatible with a competitive inhibition model.

(6) There is no evidence in support of the conclusion that EspH Rho GEFs during EPEC infection of macrophage. This is a substantial missing linking in the manuscript that would significantly bolster the authors hypothesis.

Minor points:

a. In Figure 6, the espB- land has the merged image in the wrong position.

b. The Supplementary data 1 graph should be included in Figure 6. In addition, statistical significance should be assigned to the graph.

Referee #3 (Remarks to the Author):

This report by Dong et al. shows that EspH of EPEC promotes cell rounding and the disappearance of actin stress fibers. Using a combination of co-transfection experiments, it is shown that EspH inhibits signaling upstream of Rho GTPases and downstream of external stimuli ot heterotrimeric G proteins that lead to RhoA activation. In pull down assays from transfected cell lysates, EspH was shown to associate with various constructs of p115Rho-GEF that contain the DH-PH domain. Consistently, EspH transfection inhibited the association of DH-PH domain of Rho-GEFs to RhoA and subsequent RhoA activation. Finally, EspH appears to play a role in preventing bacterial phagocytosis by macrophage.

This is an interesting report with potentially important implications. From this report, EspH may represent the first bacterial effector that targets a Rho-GEF and prevents its association with a Rho-GTPase. However, I have major reservations on the work mostly dealing with the specificity of the EspH target. The fluorescent pictures could also probably be significantly improved since it is very difficult to see cytoskeletal structures.

1. The authors have used co-transfection experiments and analyzed the effects on the actin cytoskeleton or a SRF-reporter luciferase read out to pinpoint the step targeted by EspH. Although probably indicative, I do not think that these experiments can definitely establish a strict hierarchy. This would require strict control of the relative ratio of the transfected constructs, and certainly, better fluorescent pictures. Indeed, a lack of epistatic effects may simply be due to difference in expression levels, or different sub-cellular localization of the co-transfected constructs.

2. There is no clear indication that EspH directly binds to the DH-PH domain of the RhoGEFs. In all the co-transfection experiments that are shown, complex formation may still occur indirectly. I agree that the rationale use of Rho-GEF DH-PH domain point mutants suggests that the binding may be direct, but the experiments do not formally prove it. Such demonstration would require the utilization of purified EspH or EspH constructs in in vitro binding experiments. It is not entirely clear what was the problem encountered in this purification, and whether it was linked to expression or solubility of EspH. Clearly, EspH can be detected upon transfection so expression, suggesting that it is rather solubility that is problematic, which could potentially raise other concerns. The authors should try other means that have been used elsewhere to show direct interaction between GEFs and GTPases and that would be require purification such as overlay assays, or yeast two-hybrid.

3. The authors used Western blotting analysis to show that EspH pulls down p115RhoGEF when co-transfected in cells, but it is unclear whether other GEFs or even other unrelated targets are primary targets of EspH. It would be interesting to determine how specific EspH is for Rho-GEFs and whether EspH also prevents the activation of Cdc42 or Rac in PBD-pull down experiments. This is important in light of the role proposed by the authors of EspH in anti-phagocytosis. Previous reports have shown that RhoA is involved in complement receptor 3 mediated phagocytosis but not in Fc-gamma R mediated phagocytosis, while Cdc42 and Rac are involved in Fcgamma R -mediated phagocytosis.

Other points:

1. Fig. 1, a complete disruption of the filamentous actin cytoskeleton is hard to tell from Fig.1A, since cells seem to show patches of polymerized actin. This panel should be quantified. Also, these results are not new and have been previously reported by the Rosenshine's group. This should be acknowledged in the text.

2. The quality of the fluorescent pictures in Fig. 2 could be significantly improved. It is very difficult to see the star-like stress fibers that are usually associated with the expression of active ROCK, or the fusiform cells in cells expressing active mDia, or even the stress fibers. Furthermore, it is unclear to me how co-transfected cells were identified. A immunofluorescent staining of the active ROCK-I delta 3, mDia1 delta or active RhoA1 should be shown. The data in Fig. 2A-C, E should also be quantified in terms of cell rounding up.

3. The localization of GFP-EspH should be analyzed in more details in particular relative to the co-transfected constructs, to rule out a putative compartmentalization that would lead to absence of epistatic effects. Previous works from the Rosenshine 's group had shown that transfected EspH associates with host cell membranes.

4. In Fig. 2D, all the EspH co-transfectants show a decrease in luciferase activity compared to the vector control. Is this decrease significant? Also, these experiments are not very easy to interpret since to be meaningful, the transfection should be performed with a ratio in favor of EspH. This could be performing dilutions of the DNA construct corresponding to the active RhoGTPase in the presence of constant GFP-EspH, and controlled by scoring by IF the relative rates of transfection within the sample.

5. p.9, l.1: Fig. 2C should read Fig 2Ds.

6. Statistical analysis should be performed in Figs. 3A

7. It is impossible to see the thick bundles of actin filaments n Fig.3E. How were the high levels of p115RhoGEF determined?

8. For all the luciferase assays, I could not find how many independent experiments were performed, and the number of replicates per determination.

9. The anti-phagocytotic effects of EspH are not very impressive. The role of EspH in preventing phagocytosis could probably better shown in different assays. The authors could use macrophage primed with EPEC or the isogenic espH mutant to analyze the effects of EspH on FcR -mediated phagocytosis, or Cos cells expressing the FcgammaR to look at the uptake of opsonized particles in EspH transfection experiments.

1st Revision - Authors' Response — 26 January 2010

Referee #1 (Remarks to the Author)

1. Although this paper thoroughly shows that the DH-PH domain of RhoGEFs and EspH interact, it is not clear whether this interaction is direct. One difficulty is that the authors have been unable to purify recombinant EspH. By co-immunoprecipitation assays in transfected mammalian cells, they show that no other bacterial proteins are required for EspH-RhoGEF interaction. The authors could similarly test whether any mammalian factors are required for this interaction by expressing the DH-PH domain of RhoGEF in EHEC (or an E. coli K12 strain expressing EspH) and test for complex formation by co-immunoprecipitation. Interaction in this assay would show that the only mammalian factor necessary for an interaction between EspH and RhoGEF is the DH-PH domain of RhoGEF and support the contention that the interaction is direct.

We thank the reviewer for this great suggestion and have performed the suggested co-immunoprecipitation assay in EPEC. The data shown in Figure 4D confirms that no other mammalian factors are required for the interaction between EspH and the DH-PH domain.

2. The title implies that the RhoGEF interaction is essential to the antiphagocytic effect. This may well be the case, but in spite of the comprehensive biochemical data showing EspH interaction with RhoGEFs, a clear link between this interaction and inhibition of phagocytosis is lacking. Any

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evidence for this that the authors could provide would greatly strengthen the manuscript. Possible experiments include co-localization of EspH and RhoGEF in mouse macrophages, or co-immunoprecipitation of EspH and RhoGEF in mouse macrophages, although these experiments may be technically challenging.

We have performed both co-localization and co-immunoprecipitation assays of EspH and RhoGEF in mouse macrophages (RAW264.7). In addition, we have also confirmed the interaction between EPEC-secreted EspH and exogenous p115 RhoGEF in EPEC infection of mouse macrophages. All three pieces of data shown in the new Figure 6 clearly suggest that EspH could potentially target RhoGEF in macrophages.

3. Figure 3A is lacking error bars, please include. In this and other figures, although it is usually intuitively clear which differences are statistically significant, it may be useful to include asterisks depicting statistical significance.

As suggested, we have added error bars and statistical significance in Figure 3A and all other relevant figures.

4. In transfection experiments, described throughout manuscript, the efficiency of transfection might influence the interpretation of results. For example, luciferase assays were performed on entire populations of cells, so that a low transfection efficiency might be predicted to result in a much more modest effect than if all of the cells were successfully transfected. The authors should address this issue in Methods and/or Results.

We have clarified this issue in the Methods session (For 293T and HeLa cells, we have achieved a consistently high transfection efficiency of ~ 85%). As luciferase reporter plasmids are transfected together with EspH and other co-transfected plasmids, the luciferase counts only report the luciferase reporter activity from transfected cells. In Figure 2G, where we show that EspH did not inhibit Rho-stimulated SRE luciferase activation, we have now performed a series of titrations of transfected Rho plasmid and the results shown in the new Supplementary Figure S2 are consistent.

5. Fig. 3C. A prediction is that the presence of EspH should have no effect on RhoA-GTP when cells are treated with GST-CNF1, and this could be included as a specificity control.

We have included the suggested control in the new Figure 3C.

6. Figure 2A and B. The actin staining seems to be very bright in transfected cells. Is this due to bleedthrough of GFP? Please clarify

We apologize that the fluorescence pictures provided in our original submission are not that high-quality (taken on a regular microscope). We have now provided much better images taken on a confocal microscope. The bright actin staining is definitely not due to bleedthrough of GFP as the GFP staining pattern is different from that of actin staining in those cells. Instead, the actin staining in these cells are expected to be brighter due to the activity of transfected active ROCK and mDia1.

7. Figure 2C. The transfected cells appear to be somewhat rounded in comparison to untransfected cells. Please clarify.

Overexpression of constitutively active RhoA (RhoA L63) stimulates formation of much enhanced actin stress fibers. Meanwhile, it is also known that transfection of RhoA L63 leads to a slight shrinkage of the cell body (not rounded up). We have now clarified this in the revised manuscript and also included EGFP-transfected cells as the control to rule out the possibility that the cell morphology change results from the activity of EspH.

Minor Points:

8. Figure 1. (A) Presumably, the authors have shown that vector only control (EGFP) does not produce cell rounding-this can be cited as data not shown in text. (B) Do all cells transfected w/ EspH round up?

We have now included the EGFP control that shows no cell rounding (Figure 1A), and have also provided quantitative statistics showing that almost all EspH-expressing cells develop a rounding-up phenotype (Figure 1B).

9. Page 8, when referring to luciferase assays, the text should cite figure 2D, not 2C.

This mistake has been corrected.

10. Page 4, "this process is aided by EspFu" might be altered to read "this process is greatly aided by EspFu".

This sentence has been revised as suggested.

11. Page 9, when referring to CNF-1 antagonism of EspH-induced cell rounding, the text should cite figure 2E, not figure 2D.

This mistake has been corrected.

12. Figure 3E. The authors assert in text that when EspH is co-expressed with p115-RhoGEF in HeLa cells, the formation of enhanced, thick actin filamentous bundles is counteracted. Although we see rounded up cells when EspH is co-expressed, the phenotype doesn't appear to be strikingly different from cells transfected w/ EGFP control. Please clarify.

Again, this issue is due to the quality of images (overexposed) that we presented in the original submission. The newly provided high-quality images have now addressed this concern. We have done this experiment many times and it is very clear under the microscope that EspH expression can disrupt the actin fibers induced by p115-RhoGEF.

13. Fig. 2D. Please explain why 293T cells, not HeLa cells, were utilized. (Presumably, it is due to the efficiency of transfection.)

Yes. Luciferase reporter activity in HeLa cells is generally much lower than that in 293T cells due to the relatively lower transfection efficiency and more importantly the lower protein expression level. To obtain convincing data, we chose 293T cells rather than HeLa cells for this assay.

14. For clarity for the uninitiated, please describe briefly what an RBC pulldown is.

We have described the RBD pulldown assay in detail in the Methods session and also cited a reference in the main text.

15. Discussion, Page 16. Note that C. rodentium encodes an EspH, and the role of EspH on colonization in the mouse model has been investigated (see Mundy et al., 2004, Infection and

Immunity).

We have cited this reference in the appropriate place in the Discussion session.

Referee #2 (Remarks to the Author):

Major points

(1) This reviewer found the EPEC infection experiments in Figure 1A to be quite weak in comparison to the transient transfection data. In fact, it is not clear why cells round up after 1 hour of infection with an MOI of 100. This has not been detected previously despite the plethora of data published on EPEC infection at this time range. This is likely a cell type dependent effect and not something inherent with EPEC infection.

We consistently observed a small percentage of EPEC-infected HeLa cells becoming rounding up in an EspH-dependent manner. We include this data as a supporting evidence for the biochemical activity of EspH in disrupting the actin cytoskeleton structure. As this experiment was done with relatively high MOI, we did not interpretate it in any physiological context. In fact, as the reviewer pointed out, this is indeed a cell type dependent phenomenon as we did not observe this phenotype in EPEC-infected Caco-2 or T84 cells. We have noted this fact in the revised manuscript. We can also move the data into the Supplementary part if this reviewer feels that will be more appropriate.

(2) The authors should demonstrate that their EPEC complemented strains of espH- indeed secretes EspH protein.

We have now demonstrated this in the new Figure 1E.

(3) The authors attempt to induce actin stress fibers with LPA and serum in cells that were transiently transfected with the EspH. The conclusions drawn from these studies seem to be invalid given that EspH is expressed for 10 hours before LPA treatment. Are the LPA receptors surface expressed in these cells etc.? Are their other modifications that could account for these effects? This experiment should be done with infection and not with transient transfection of EspH. At the very least, the caveats of this experimental approach should be directly stated in the text since there are certainly additional explanations for this finding (i.e. rounded cells do not signal to actin due to loss of surface receptors).

First, we would like to point out that the luciferase assay was performed at the time point before EspH-transfected cells developed evident rounding-up phenotype. Second, due to the lack of antibodies suitable for staining endogenous LPA receptors, we have examined localization of exogenous LPA receptors in the presence of EspH. Although transfected LPA receptors are not exclusively on the plasma membrane, we did not find significant alterations of localization of LPA receptors as well as their expression levels by EspH (new Supplementary Figure S3). We are aware that the above two points do not directly address the reviewer's potential concern in this regard. Therefore, we have stated the caveats of this experimental approach in the revised manuscript, as suggested by the reviewer.

(4) The authors perform pulldown studies with recombinant DH-PH domains and find EspH can be precipitated from mammalian cell lysates. While this is a good experiment suggesting that EspH indeed interacts with this complex, it does not demonstrate a direct interaction as the authors state since this is a complex mixture of proteins. This "direct binding" statement needs to be removed from the article since there is no evidence in support of this conclusion (i.e. mixing two highly purified proteins).

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See responses to the 1st point of referee #1.

(5) One of the weaknesses of this study is that the conclusions are entirely based on overexpression of EspH. This could certainly force an interaction between EspH and Rho GEF proteins. The authors need to demonstrate that bacterial secreted EspH indeed inhibits Rho GEFs. One simple prediction would be that RNAi knockdown of Rho GEF would inhibit the EPEC phenotypes induced by EspH. The reviewer does recognize that this experiment may be quite difficult due to redundant Rho GEF proteins. Nevertheless, thinking about these types of experiments highlights the problems of the current data to convincingly draw the conclusion that EspH competitively inhibits Rho GTPase activation. Some of the weaknesses of this model should be discussed, particularly given the very low amounts of EspH that are likely secreted into host cells and whether this is compatible with a competitive inhibition model.

As we do not know the exact RhoGEF (among more than 50 DH-PH domain RhoGEFs) targeted by EspH during infection, we can not perform the RNAi experiment mentioned by the review. We appreciate that the reviewer recognizes the difficulties of these kinds of analyses in macrophages. However, we are now able to show that EPEC-secreted EspH co-immunoprecipitated with exogenous p115-RhoGEF during EPEC infection in the new Figure 6C. This result largely addresses the concern that the observed interaction is a result of EspH overexpression. Also as the reviewer indicated, EspH delivered by EPEC is at very low amounts, and it is not expected that it could cause a global decrease of RhoA activation during EPEC infection. Therefore, it is reasonable that EspH likely only targets a specific pool of certain RhoGEF at the endogenous level, which will be compatible with our model. As suggested, we have also discussed the potential weakness of our model in the revised manuscript.

(6) There is no evidence in support of the conclusion that EspH Rho GEFs during EPEC infection of macrophage. This is a substantial missing linking in the manuscript that would significantly bolster the authors hypothesis.

In the new Figure 6C, we have now shown that EPEC-secreted EspH coimmunoprecipitated with p115-RhoGEF during EPEC infection. This result addresses this concern and strongly suggests that EspH could target the DH-PH domain-containing RhoGEF during EPEC infection of macrophages similarly as that observed with transfection assays.

Minor points:

a. In Figure 6, the espB- land has the merged image in the wrong position.

This mistake has now been corrected.

b. The Supplementary data 1 graph should be included in Figure 6. In addition, statistical significance should be assigned to the graph.

As suggested, we have assigned statistical significance and moved the statistic data into the main Figure 7. The original Supplementary Figure 1A (enlarged fluorescence pictures) remains in the Supplementary data (Figure S5) due to the limited space in the main body for large-size images.

Referee #3 (Remarks to the Author):

1. The authors have used co-transfection experiments and analyzed the effects on the actin cytoskeleton or a SRF-reporter luciferase read out to pinpoint the step targeted by EspH. Although probably indicative, I do not think that these experiments can definitely establish a strict hierarchy. This would require strict control of the relative ratio of the transfected constructs, and certainly, better fluorescent pictures. Indeed, a lack of epistatic effects may simply be due to difference in expression levels, or different sub-cellular localization of the co-transfected constructs.

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As suggested by the reviewer, we have now added data showing the unchanged protein expression levels for epistasis analyses presented in Figure 2 and 3. We have also replaced the original fluorescence images with high-quality ones that argue against the possible compartmentalization effect. In particular, for the most important epistatic data showing that EspH does not inhibit actin stress fibers phenotype and SRE luciferase induced by constitutively active Rho, the now much complete and extensively analyses (new Figure 2D-G and Supplementary Figure S2), including better fluorescence images and a series of titrations of the ratio of transfected plasmids, confirm our previous observation. We do agree with the reviewer that these kinds of epistasis assays are indicative in nature. However, we would also like to stress that the overall conclusion, which is substantially strengthened by the newly added biochemical data demonstrating the functional interaction between EspH and the DH-PH domain of RhoGEF, was not solely based on data from these indicative assays.

2. There is no clear indication that EspH directly binds to the DH-PH domain of the RhoGEFs. In all the co-transfection experiments that are shown, complex formation may still occur indirectly. I agree that the rationale use of Rho-GEF DH-PH domain point mutants suggests that the binding may be direct, but the experiments do not formally prove it. Such demonstration would require the utilization of purified EspH or EspH constructs in in vitro binding experiments. It is not entirely clear what was the problem encountered in this purification, and whether it was linked to expression or solubility of EspH. Clearly, EspH can be detected upon transfection so expression, suggesting that it is rather solubility that is problematic, which could potentially raise other concerns. The authors should try other means that have been used elsewhere to show direct interaction between GEFs and GTPases and that would be require purification such as overlay assays, or yeast two-hybrid.

We agree with the reviewer that our previous data are not strong enough in concluding the direct interaction between EspH and the DH-PH domain. Similarly as the reviewer has been thinking of, We did put lots of efforts to purify enough high-quality recombinant EspH from *E .coli*, but the problem of low expression level prevented us from obtaining enough protein with sufficient purity and quantity for *in vitro* assays. However, we have performed and confirmed the co-immunoprecipitation between EspH and the DH-PH domain in EPEC (new Figure 4D), and this rules out the possibility that the interaction require any other eukaryotic factor and substantiates our conclusion that the interaction indeed is direct.

3. The authors used Western blotting analysis to show that EspH pulls down p115RhoGEF when co-transfected in cells, but it is unclear whether other GEFs or even other unrelated targets are primary targets of EspH. It would be interesting to determine how specific EspH is for Rho-GEFs and whether EspH also prevents the activation of Cdc42 or Rac in PBD-pull down experiments. This is important in light of the role proposed by the authors of EspH in anti-phagocytosis. Previous reports have shown that RhoA is involved in complement receptor 3 mediated phagocytosis but not in Fc-gamma R mediated phagocytosis, while Cdc42 and Rac are involved in Fcgamma R -mediated phagocytosis.

We thank the review for bringing out this question. Indeed, we did performed co-immunoprecipitations between EspH and several different DH-PH domain RhoGEFs including the three RGS-RhoGEFs we have already mentioned as well as another RhoA/Cdc42-specific DH-PH domain RhoGEF, Dbl. We did not find that there were evident preferences for EspH binding. This is consistent with the fact that the DH-PH domain is highly conserved in sequence and structure. Under transfection and *in vitro* conditions, it is not surprising that EspH can target the DH-PH domain from multiple RhoGEFs. We have made a note of this fact in the revised manuscript. There are over 50 DH-PH domain-containing RhoGEFs in human, and it is technically infeasible to test the specificity of EspH on all these RhoGEFs. Therefore, as our manuscript presents, we only conclude that the DH-PH domain is the host target of EspH and did not argue that any particular RhoGEF is the primary target of EspH. It is certainly possible that other RhoGEFs could be the primary target of EspH during infection. As suggested by the reviewer, we have employed the PBD pulldown assay and examined whether EspH could inhibit Dbl-mediated activation of Cdc42. The new result shown in Figure 5F suggests that transfected EspH indeed could inhibit DH-PH domain-

catalyzed Cdc42 activation, which is consistent with the inhibition of FcγR-mediated phagocytosis by EspH.

Other points:

1. Fig. 1, a complete disruption of the filamentous actin cytoskeleton is hard to tell from Fig.1A, since cells seem to show patches of polymerized actin. This panel should be quantified. Also, these results are not new and have been previously reported by the Rosenshine's group. This should be acknowledged in the text.

We have replaced the original images with better ones taken on a confocal microscope, which clearly shows the disruption of actin cytoskeleton structure. As suggested, we have included quantification of the phenotype and also acknowledged the Rosenshine's paper in the revised version.

2. The quality of the fluorescent pictures in Fig. 2 could be significantly improved. It is very difficult to see the star-like stress fibers that are usually associated with the expression of active ROCK, or the fusiform cells in cells expressing active mDia, or even the stress fibers. Furthermore, it is unclear to me how co-transfected cells were identified. A immunofluorescent staining of the active ROCK-I delta 3, mDia1 delta or active RhoA1 should be shown. The data in Fig. 2A-C, E should also be quantified in terms of cell rounding up.

Again the fluorescence images have been significantly improved by using the confocal images. Co-transfected cells were identified by double positive staining of EGFP and active ROCK-I/mDia1. In addition, the new figures also include requested quantification of the phenotype.

3. The localization of GFP-EspH should be analyzed in more details in particular relative to the co-transfected constructs, to rule out a putative compartmentalization that would lead to absence of epistatic effects. Previous works from the Rosenshine 's group had shown that transfected EspH associates with host cell membranes.

With the new high-quality fluorescence images provided in the revised submission, we have confirmed the Rosenshine's data and show that transfected EspH associates with host cell membrane in both HeLa (Figure 1A and Figure 3E) and macrophage cells (Figure 6A). We have also included clear fluorescence images of EGFP-EspH in our epistasis analysis, which shows that at least a significant portion of transfected EGFP-EspH remains membrane-associated.

4. In Fig. 2D, all the EspH co-transfectants show a decrease in luciferase activity compared to the vector control. Is this decrease significant? Also, these experiments are not very easy to interpret since to be meaningful, the transfection should be performed with a ratio in favor of EspH. This could be performing dilutions of the DNA construct corresponding to the active RhoGTPase in the presence of constant GFP-EspH, and controlled by scoring by IF the relative rates of transfection within the sample.

We have carefully and extensively titrated amounts of transfected Rho plasmid in relative to EspH in this experiment. There were no statistically significant difference between the luciferase activity from EspH-transfected cells and that from the vector control cells. The data shown in the new Supplementary Figure S2 clearly demonstrate that EspH can not inhibit SRE luciferase activation induced by constitutively active Rho GTPases regardless of their expression levels in relative to EspH.

5. p.9, l.1: Fig. 2C should read Fig 2Ds.

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This mistake has been corrected.

6. Statistical analysis should be performed in Figs. 3A

As suggested, the new Figure 3A includes statistical analysis.

7. It is impossible to see the thick bundles of actin filaments n Fig.3E. How were the high levels of p115RhoGEF determined?

Again, we apologize that our previous fluorescence pictures are not that high-quality. We have now provided much better images taken on a confocal microscope. Thick bundles of actin filamentous can be clearly seen in the new Figure 3E. We have replaced “the high levels of p115RhoGEF” with “transfection of p115-RhoGEF”.

8. For all the luciferase assays, I could not find how many independent experiments were performed, and the number of replicates per determination.

All the luciferase assays were repeated for three times with each in duplicate. We have included the information in the relevant figure legends and also assigned statistical significance.

9. The anti-phagocytotic effects of EspH are not very impressive. The role of EspH in preventing phagocytosis could probably better shown in different assays. The authors could use macrophage primed with EPEC or the isogenic espH mutant to analyze the effects of EspH on FcR -mediated phagocytosis, or Cos cells expressing the FcgammaR to look at the uptake of opsonized particles in EspH transfection experiments.

We have performed the requested experiment examining the effect of EspH on FcR-mediated phagocytosis of IgG-opsonized latex beads using EPEC infection of J774A.1 macrophage cells. The data shown in the new Figure 8 suggest that EspH can similarly inhibit FcR-mediated phagocytosis, consistent with the newly provided evidence that EspH can also target and inhibit DH-PH domain RhoGEFs for Cdc42 (Figure 5F).

2nd Editorial Decision 12 February 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-72778R. It has now been seen again by all three referees, who are satisfied with your responses to the previous round of review, and who all now support publication. Before we can accept your manuscript, however, there are a few minor changes requested by referees 1 and 3 - in terms of clarifying the text and also the figure labelling. I would therefore ask you to revise your manuscript accordingly, although it is not necessary for you to remove figure 2 from the main text as suggested by referee 1 - you are currently well within our space constraints and I think the information in the figure is valuable.

Once we receive this final version of your manuscript, we should then be able to accept your study for publication in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the

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revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Minor points only.

1. p. 5. This sentence is confusing. "Notably, co-expression of EGFP-EspH did not affect development of ROCK-I Δ3 or mDia1 ΔN-induced featured actin stress fibers and cell morphology change as well as their expression levels"

2. If space is an issue, Fig. 2 is all negative data and could be moved to supplementary data.

3. Fig. 4D. (1) labeling is incomplete. (2) what is the upper band in the lefthand lane?

4. Fig. 5A. In legend, indicate significance of "12" and "13". The arrangement of panels A-F is not consistent.

Referee #3 (Remarks to the Author):

P8. L8: rephrase "As ..., intact HeLa cells treated with recombinant GST-CNF1 developed expected much enhanced actin stress fibers .... "

P8L10 :« However, GST-CNF1 treatment could largely reverse the phenotype and EspH-expressing HeLa cells now showed a flat and normal cell morphology and enhanced actin stress fibers » should read : However, GST-CNF1 treatment reversed this phenotype and EspH-expressing HeLa cells showed a flat and normal cell morphology and enhanced actin stress fibers

P12L6 : remove "significantly" »

P15Last sentence : «Different from EspH, » should read « As opposed to, EspH »

P17, bottom

EspJ should probably also be mentioned for its anti-phagocytic activity.

2nd Revision - Authors' Response 19 February 2010

Many thanks for taking good care of our manuscript (2009-72778) entitled "A bacterial effector targets host DH-PH domain RhoGEFs and antagonizes macrophage phagocytosis". I am very glad to know that all the three reviewers are satisfied with our revision and your interest in publishing our manuscript as an article in *the EMBO Journal*. I am now submitting a revised manuscript for your final decision.

In response to your editorial deliberations, I have revised the manuscript accordingly and incorporated all the requested changes that are itemed below.

Referee #1 (Remarks to the Author)

1. We have rephrased the sentence in the revised submission;
2. As suggested by the editor, Fig. 2 remains in the main body of the manuscript;
3. We have fixed the labeling and explained the nature of upper band in the legends;
4. We have explained "12" and "13" and also rearranged the panels in this figure to be consistent.

Referee #3 (Remarks to the Author):

P8. L8, P8. L10 and P15Last sentence: As suggested by the reviewer, we have rephrased these three sentences in the revised submission;

P12L6: We have removed the word of 'significantly" accordingly;

P17, bottom: We have added one sentence to discuss the anti-phagocytosis function of EspJ in the revised manuscript.

Thanks again for your interest in our manuscript and I am looking forward to hearing form you. If I can be of any additional assistance, please feel free to contact me.

3rd Editorial Decision 19 February 2010

Many thanks for submitting the revised version of your manuscript. I have to say that I still find a couple of the sentences highlighted by the referees confusing. I would propose the following changes:

p7 lines 8-10:

Notably, co-expression of EGFP-EspH did not affect development of featured actin stress fibers and cell morphology change induced by ROCK-I Δ3 or mDia1 ΔN
(Figure 2A-2C; Supplementary Figure S1).

change to:

Notably, co-expression of EGFP-EspH did not affect the development of actin stress fibres or the cell morphology changes that are induced by ROCK-I Δ3 or mDia1 ΔN
(Figure 2A-2C; Supplementary Figure S1).

p8 lines 7-9:

As shown in Figure 2H, HeLa cells treated with recombinant GST-CNF1 developed expected much enhanced actin stress fibers.

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change to:

As shown in Figure 2H, HeLa cells treated with recombinant GST-CNF1 displayed significantly enhanced actin stress fiber formation, as expected.

Can you just let me know whether you are happy with these modifications; if so, we can make the appropriate changes in the word document, and will then be able to accept your manuscript.

Yours sincerely,

Editor
The EMBO Journal

Additional Correspondence 19 February 2010

Thanks for your prompt responses. Your wording seems to fit well and I am happy with it.

Regards,

Feng Shao

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