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Title: Self-association of the *Shigella flexneri* IcsA autotransporter protein

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IcsA self-association

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The IcsA autotransporter protein is a major virulence factor of the human intracellular pathogen *Shigella flexneri*. IcsA is polarly distributed in the outer membrane of *S. flexneri* and interacts with components of the host actin-polymerization machinery to facilitate intracellular actin-based motility and subsequent cell-to-cell spreading of the bacterium. We sought to characterize the biochemical properties of IcsA in the bacterial outer membrane. Chemical cross-linking data suggested that IcsA exists in a complex in the outer membrane. Furthermore, reciprocal co-immunoprecipitation of differentially epitope-tagged IcsA proteins indicated that IcsA is able to self-associate. The identification of IcsA linker-insertion mutants that were negatively dominant provided genetic evidence of IcsA-IcsA interactions. From these results, we propose a model whereby IcsA self-association facilitates efficient actin-based motility.
INTRODUCTION

*Shigella* spp. are highly adapted human pathogens that cause bacillary dysentery and extensive global morbidity and mortality (Levine *et al.*, 2007). Ingested *Shigellae* invade colonic epithelial cells, where they multiply within the host cell cytoplasm and become motile via the polymerization of host actin in a process termed actin-based motility (ABM) (Suzuki *et al.*, 1996; 1998; 2002). Motile bacteria are able to infect adjacent cells, enabling the lateral spread of the focus of infection throughout the epithelium. Actin polymerization is initiated by the polarly distributed *Shigella* IcsA (VirG) protein (Bernardini *et al.*, 1989; Lett *et al.*, 1988), which recruits the host neural Wiskott-Aldrich syndrome protein (N-WASP), a key regulator of the actin cytoskeleton (Goldberg, 2001; Snapper *et al.*, 2001; Suzuki *et al.*, 1998). N-WASP and other members of the Wiskott-Aldrich syndrome protein (WASP) family function as a link between signalling pathways and *de novo* actin polymerization by recruiting the actin polymerizing complex Arp2/3, initiating actin polymerization-driven host cell motility and morphological changes (Miki & Takenawa, 2003; Yarar *et al.*, 1999). IcsA is essential for ABM and thus *S. flexneri* virulence (Kotloff *et al.*, 1996; 2002; Lett *et al.*, 1988; Makino *et al.*, 1986; Sansonetti *et al.*, 1991).

IcsA is a member of the autotransporter (AT) family of proteins, which is the largest family of extracellular proteins in Gram-negative bacteria (Pallen *et al.*, 2003). A prototypical AT protein consists of an N-terminal signal sequence that facilitates export across the inner-membrane; an internal passenger domain that exerts the effector function; and a C-terminal translocation domain required to direct export of the protein across the outer-membrane (OM) via the Bam complex, and to form a β-barrel OM anchor for the extracellular passenger domain (Henderson *et al.*, 2004; Jain & Goldberg, 2007; Peterson *et al.*, 2010).

A distinct sub-family of ATs has been shown to be trimeric and IcsA is related to a subgroup of self associating ATs (SAATs) that mediate bacterial aggregation and biofilm formation (Cotter *et al.*, 2005; Klemm *et al.*, 2006; Meng *et al.*, 2011). Emerging evidence suggests that a subset of conventional ATs are capable of oligomerisation in the OM. In these proteins, oligomerization is facilitated either through interactions between adjacent effector
domains (Gangwer et al., 2007; Swanson et al., 2009; Xicohtencatl-Cortes et al., 2010), or
translocation domains (Marín et al., 2010; Müller et al., 2005; Veiga et al., 2002). How
broadly applicable oligomerisation might be within the large AT family of proteins remains
to be determined. Indeed, some ATs have specifically been shown to exist as monomers
(Hritonenko et al., 2006; Marín et al., 2010), and crystal structures of translocation domains
obtained from EspP and NalP suggest a monomeric existence (Barnard et al., 2007; Oomen et
al., 2004). The biological significance of AT oligomerization remains unclear. IcsA has not
been previously investigated in this regard.

The aim of our study was to investigate the state of IcsA in the OM environment,
which is fundamental to understanding the interaction of IcsA with host proteins such as N-
WASP, and to S. flexneri virulence. In this study, we have shown that IcsA is present within a
complex in the OM, and detected the existence of direct IcsA-IcsA interactions within this
complex. The identification of negative-dominant IcsA mutants that influenced S. flexneri
plaque formation, and hence intercellular spreading, provided genetic evidence of direct
IcsA-IcsA interaction, and demonstrated the functional relevance of IcsA self-association.

METHODS

Bacterial strains and Plasmids. The strains and plasmids used in this study are listed in
Table 1.

Growth media and growth conditions. S. flexneri strains were grown from a Congo-Red-
positive colony as previously described (Morona et al., 2003). All bacterial strains were
routinely cultured in Luria Bertani (LB) media. Bacteria were grown in media with
antibiotics for 16 h with aeration then sub-cultured 1:50 and grown to mid exponential-phase
by incubation with aeration for 2 h at 37°C. Where appropriate, media were supplemented
with ampicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), kanamycin (50 µg ml⁻¹),
tetracycline (50 µg ml⁻¹). Mueller-Hinton broth and agar were used to culture strains in the
presence of trimethoprim (10 µg ml⁻¹).
DNA methods. *Escherichia coli* K-12 strain DH5α was used for routine cloning and general cloning techniques, and PCR and DNA sequencing were performed as described previously (May & Morona, 2008).

Chemical cross-linking. Cross-linking with dithio-bis(succinimidylpropionate) (DSP; Pierce) was performed as described previously (Thanabalu *et al.*, 1998). Mid exponential-phase cultures were washed in buffer (120 mM NaCl, 20 mM sodium phosphate pH 7.2) and DSP was added to each sample at a final concentration of 0.2 mM in the same buffer. Samples were incubated for 30 min at 37°C. Cross-linking was then quenched with 20 mM Tris pH 7.5, samples were washed in buffer, resuspended in SDS-PAGE sample buffer (Lugtenberg *et al.*, 1975) either with or without β-mercaptoethanol. Samples were then heated to 60°C for 5 min, prior to being resolved by SDS-PAGE. Cross-linking of cells from strains RMA2205, RMA2208, RMA2209, was performed as above, except that after quenching, the cells were lysed by passage through a French pressure cell; the lysate was centrifuged at 100,000×g for 1 h, and the pelleted whole membranes resuspended in SDS-PAGE sample buffer either with or without β-mercaptoethanol. Formaldehyde cross-linking was performed as described previously (Prossnitz *et al.*, 1988). Mid-exponential phase bacteria were washed in 10 mM K2HPO4/KH2PO4 buffer and resuspended and formaldehyde added at a final concentration 0.5% and incubated for 1 h at RT. Samples were then washed once again as above and resuspended in SDS-PAGE sample buffer. Aliquots of each sample were heated at either 60°C for 10 min or 100°C for 20 min, prior to SDS-PAGE.

Construction of plasmids

pKMRM252 was constructed by sub-cloning the *EcoRI-SalI* fragment of pIcsA encoding IcsAWT into likewise digested pBBR1MCS-2. FLAG and BIO tagged derivatives of IcsA were constructed as follows. Complementary oligonucleotides (Table 2) encoding either the FLAG epitope (IcsA-FLAG-F1, IcsA-FLAG-R1; DYKDDDDK) or the BIO sequence (KM1-BIO-F, KM1-BIO-R; GLNDIFEAQKIEWH) were annealed as described previously (Enninga *et al.*, 2005). The resultant dsDNA possessed NotI compatible 5’ overhangs, and was ligated into the unique NotI site within the linker-insertion of pKMRM1, producing
plasmids pKMRM250 (encoding icsA\textsubscript{i87::FLAG}) and pMG55 (encoding icsA\textsubscript{i87::BIO}). To enable co-expression with pMG55, the EcoRI-SalI fragment of pKMRM250 encoding icsA\textsubscript{i87::FLAG} was sub-cloned between the EcoRI and SalI sites of the compatible plasmid pBBR1MCS-2, producing plasmid pKMRM270. We confirmed that IcsA\textsubscript{i87::FLAG} and IcsA\textsubscript{i87::BIO} were functionally comparable to IcsA\textsubscript{WT} by introducing either pKRM250 or pMG55 into RMA2041 (Table 1) and performing plaque assays (data not shown).

**Reciprocal co-purification of FLAG- and BIO-tagged IcsA.**

*E. coli* UT5600 was transformed with pKMRM270 and pMG55, enabling co-expression of IcsA\textsubscript{i87::FLAG} and IcsA\textsubscript{i87::BIO} resulting in in strain MG157. Control strains were also generated that expressed untagged IcsA\textsubscript{i87} [pKRM1] with either IcsA\textsubscript{i87::FLAG} [pKRM270] (MG250) or IcsA\textsubscript{i87::BIO} [pMG55] (MG251). Strains MG157, MG250, and MG251 additionally carried pCY216 (Chapman-Smith *et al.*, 1994).

Cultures (5 L) of MG157, MG250, and MG251 were grown 16 h at 30\(^\circ\)C and extraction of outer membrane proteins from each strain was performed at 4\(^\circ\)C as previously described (Veiga *et al.*, 2002). Briefly, bacteria were pelleted, resuspended in TN buffer (20 mM Tris-HCl pH 8.0, 10 mM NaCl), lysed by passage in a French pressure cell at 12,000 psi, and centrifuged at 100,000\(\times\)g for 1 h. The pellet was solubilised in TN buffer supplemented with 1.5\% (v/v) Triton X-100 (Sigma) for 30 min, and centrifuged at 100,000\(\times\)g for 1 h. The resulting pellet was solubilised in TN buffer supplemented with 1\% (w/v) Zwittergent 3-14 (Calbiochem) for 30 min, and centrifuged at 100,000\(\times\)g for 1 h. The supernatant containing solubilised outer-membrane proteins was collected, and diluted to 0.1\% (w/v) Zwittergent 3-14 with TN buffer. Solubilised material was then used in affinity purification using FLAG M2 resin (Sigma) or MyOne streptavidin T1 Dynabeads (Invitrogen). Samples and beads or resin were incubated overnight at 4\(^\circ\)C, washed six times for 1.5 h in 8 ml TN buffer containing 0.1\% (w/v) Zwittergent 3-14. Proteins were eluted in SDS-PAGE sample buffer from FLAG M2 resin; streptavidin-Dynabeads were heated at 100\(^\circ\)C for 5 min to release bound protein, pelleted at 16,000\(\times\)g for 5 min, and the supernatant then diluted in SDS-PAGE sample buffer for electrophoresis.
**SDS-PAGE and Western blotting.** Samples were separated on 7.5% or 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in TTBS (Tris-buffered saline, 0.05% Tween-20) containing 5% skim milk and incubated with either rabbit anti-IcsA polyclonal antibody (Van Den Bosch et al., 1997), streptavidin-HRP (Chemicon), or with rabbit anti-FLAG M2 (Sigma) in TTBS overnight. After three 10 min washes in TTBS the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or a HRP-conjugated goat anti-mouse secondary antibodies (Biomediq DPC) for 2 h, washed three times in TTBS, then three times in Tris-buffered saline. The membrane was incubated with Chemiluminescence Substrate (Sigma) for 1 min. Chemiluminescence was detected by exposure of the membrane to X-ray film (AGFA) and the film was developed using a Curix 60 automatic X-ray film processor (AGFA).

**Plaque assays.** Plaque assays were performed with HeLa cells as described previously (May & Morona, 2008). Briefly, HeLa cells were maintained in MEM, 10% FCS with penicillin and streptomycin and grown to confluence overnight in 6 well trays. HeLa cells were washed twice with Dulbecco's PBS (D-PBS) and once in DMEM prior to inoculation with mid exponential-phase bacteria diluted in DMEM. At 90 min post-infection the inoculum was aspirated and an overlay (DMEM, 5% FCS, 20 µg gentamicin ml⁻¹, 0.5% agarose [Seakem ME]) was added to each well. The second overlay (DMEM, 5% FCS, 20 µg gentamicin ml⁻¹, 0.5% agarose, 0.1% Neutral Red solution [Gibco BRL]) was added at either 24 h or 48 h post-infection and plaque formation observed 6-8 h later. Plaques were in general visible without staining at 48 h.

**Indirect immunofluorescence of whole bacteria.** Indirect immunofluorescence labelling of bacteria was performed as described previously (May & Morona, 2008). Briefly, equivalent numbers of mid exponential-phase bacteria were fixed in formalin (3.7% (v/v) paraformaldehyde in 0.85% saline) and either centrifuged onto poly-L-lysine-coated coverslips for microscopic analysis or kept in suspension for flow cytometric analysis. Bacteria were incubated with the desired primary antibody diluted 1:100 in PBS with 10% foetal calf serum (FCS) prior to washing with PBS and labelling with either an Alexa 488-
conjugated donkey anti-rabbit or Alexa 488-conjugated donkey anti-mouse secondary antibody (Molecular Probes). Microscopy was performed as described previously (May & Morona, 2008), using an Olympus IX-70 microscope with phase-contrast optics using a 100× oil immersion objective and a 1.5× enlarger as required. Fluorescence and phase-contrast images were false colour merged using Metamorph (Version 6.3r7, Molecular devices).

Alternatively, after labeling, cells were washed three times in PBS and then diluted to 5 ml in PBS for analysis by flow cytometry (BD FACSCanto, BD Biosciences, San Jose, CA, USA).

Infection of tissue culture monolayers with *S. flexneri* and immunofluorescence labelling. Infection of HeLa cells and immunofluorescence labelling were performed as recently described (May & Morona, 2008). Briefly, HeLa cells were inoculated with mid exponential-phase bacteria and incubated for 1 h. The monolayers were washed, incubated with media containing gentamicin for a further 1.5 h. The monolayer was washed, formalin-fixed, and permeabilized with 0.1% Triton X-100. After blocking in 10% FCS the infected cells were incubated with polyclonal anti-*Shigella* LPS-Oag (Denka Seiken Co., Japan) to label bacteria. After subsequent washing, cells were incubated with Alexa 594-conjugated donkey anti-rabbit antibodies (Molecular probes). F-actin was visualised by staining with FITC phalloidin (0.1 μg ml⁻¹, Sigma).

**RESULTS**

*In situ chemical cross-linking of IcsA.* In order to determine if IcsA is present within a protein complex, *in situ* chemical cross-linking was performed. Mid exponential-phase cultures of *S. flexneri ΔicsA [pIcsA] (RMA2090, Table 1) were treated with membrane-permeable cross-linking agents dithio-bis(succinimidylpropionate) (DSP), and whole-cell lysates were subjected to SDS-PAGE and Western blotting with an anti-IcsA antibody. We reproducibly detected the presence of a high molecular weight (HMW) complex following cross-linking (Fig. 1, lane 2). This complex had an apparent molecular mass greater than 460 kDa, as determined by comparison to HighMark protein standard (Invitrogen). Formation of
this HMW product was reversed by the addition of β-mercaptoethanol, which can cleave the disulphide bond in the spacer arm of DSP to allow separation of the cross-linked products (Fig. 1, lane 1). This HMW complex could also be detected following cross-linking with formaldehyde (Fig. S1 in the supplemental material).

Oligomerization of the N. gonorrhea IgA protease and E. coli AIDA has been suggested to be mediated through interaction of individual translocation domains (Müller et al., 2005; Veiga et al., 2002). We examined previously characterized IcsA effector domain deletion mutants (Suzuki et al., 1996) for formation of HMW complexes. DSP crosslinking of IcsAΔ568-730 (RMA2208) or IcsAΔ105-507 (RMA2209) produced HMW products, comparable to IcsAWT (RMA2205) (Fig. 1, lanes 3-6). These data indicated that no individual region within IcsA103-730 is alone responsible for formation of the HMW complex; we cannot exclude that multiple interactions within this region are involved. Alternately, the data indicate that IcsA HMW complex formation may be mediated by a region outside of the studied deletions, namely by the translocation domain (IcsA758-1102).

Lipopolysaccharide (LPS) is a major constituent of the OM of Gram-negative bacteria consisting of three major parts - the lipid A, the core polysaccharide, and the O antigen (Oag) polysaccharide chain. The Oag component of LPS has been shown to influence both IcsA function and surface localization, restricting the protein to the cell pole (Hong & Payne, 1997; Morona & Van Den Bosch, 2003a, b; Sandlin et al., 1994). Rough LPS (R-LPS) lacking the Oag component, was reported to allow IcsA diffusion away from the cell pole (Robbins et al., 2001). We hypothesized that cross-linking of IcsA into a HMW product may be dependent on the spatial confinement of IcsA proteins to the pole or be facilitated by lateral LPS-IcsA interactions. Hence, we examined the IcsA complex formation in the absence of LPS-Oag. HMW IcsA-related complexes were observed following DSP cross-linked of IcsA expressed in the rough LPS strain S. flexneri ΔicsA ΔrmlD [pIcsA] (RMA2107), indicating that complex formation occurred independent of LPS-Oag (Fig. S1).
Co-immunoprecipitation of epitope-tagged IcsA proteins. Chemical cross-linking had indicated the presence of IcsA within a HMW complex and we were particularly interested in determining if IcsA is able to self-associate. We applied reciprocal co-purification of differentially epitope-tagged IcsA proteins to determine whether IcsA-IcsA interactions were taking place within this putative complex.

Sites permissive for epitope insertion within the IcsA passenger domain had been previously identified (May & Morona, 2008). The IcsA\textsubscript{i87} protein has a 5 amino acid linker-insertion at amino acid 87 and when expressed in S. flexneri, was found to be comparable to wild-type (IcsA\textsubscript{WT}) with respect to: (i) levels of production; (ii) polar localization at the bacterial surface; and (iii) function, determined by assaying plaque formation on HeLa cell monolayers as a measure of intercellular spreading ability (May & Morona, 2008).

Consequently, icsA\textsubscript{i87} was chosen for epitope tagging by exploiting a unique NotI restriction site within the linker. Either a synthetic FLAG epitope (DYKDDDDK), or BIO epitope (GLNDIFEAQKIEWH; a substrate for metabolic biotinylation by the BirA biotin-protein ligase; (Cull & Schatz, 1999), were introduced into icsA\textsubscript{i87} as described in the Methods.

E. coli UT5600 strains expressing both epitope-tagged IcsA proteins together (IcsA\textsubscript{i87}::FLAG and IcsA\textsubscript{i87}::BIO; MG157, Table 1), or tagged proteins individually with control IcsA\textsubscript{WT}, were generated (MG250 and MG251, Table 1). Overexpression of BirA increased the levels of biotinylated IcsA\textsubscript{i87}::BIO, while retaining the high specificity of the biotinylation reaction (data not shown). Hence, all co-expression strains additionally carried plasmid pCY216 that expressed the birA enzyme (Chapman-Smith \textit{et al.}, 1994). Insertion of neither the FLAG nor the BIO epitope affected the function of IcsA\textsubscript{i87} (data not shown).

The OM fractions of the co-expression strains were isolated and purification strategies directed against either epitope were performed independently: FLAG M2 resin was used for purification of IcsA\textsubscript{i87}::FLAG; and streptavidin-Dynabeads were used for purification of biotinyl-IcsA\textsubscript{i87}::BIO. Co-purification of tagged IcsA proteins was assessed by Western blotting using either an antibody conjugate (FLAG epitope) or streptavidin conjugates (biotin-modified BIO epitope) in each of the eluents. Following purification of IcsA\textsubscript{i87}::FLAG,
with FLAG M2 resin from OM of strain MG157, biotinyl-\textit{IcsA}_{87::\text{BIO}} was found to co-purify with it (Fig. 2a, lane 2). Taking into consideration that any \textit{IcsA}_{87::\text{BIO}} that remained unbiotinylated could not be detected with streptavidin, our data suggests the amount of co-purified biotinyl-\textit{IcsA}_{87::\text{BIO}} we observed was significant. Under the same conditions, when OM of strain MG251 that expressed \textit{IcsA}_{\text{WT}} were used, biotinyl-\textit{IcsA}_{87::\text{BIO}} was not detected in the eluted sample (Fig. 2a, lane 1) showing that the FLAG M2 resin was specific for the FLAG epitope. Similarly, when biotinyl-\textit{IcsA}_{87::\text{BIO}} was purified from OM of strain MG157 using streptavidin-Dynabeads, \textit{IcsA}_{87::\text{FLAG}} was found to co-purify with it (Fig. 2b, lane 2). However, under the same conditions, \textit{IcsA}_{87::\text{FLAG}} did not purify from OM of strain MG250 that co-expressed the untagged control protein \textit{IcsA}_{\text{WT}} (Fig. 2b, lane 3), showing that the streptavidin-Dynabeads were specific for the biotin-modified BIO epitope. Based on these results we conclude that the \textit{IcsA} self-association occurs in the OM and this self-association is resistant to disruption by the detergent Zwittergent 3-14.

**Identification of negative-dominant \textit{IcsA}_i mutants.** Having detected the existence of \textit{IcsA}-\textit{IcsA} interactions by co-purification, we investigated if \textit{IcsA} self-association impacted on its function in intracellular motility. We hypothesized the existence of \textit{IcsA} mutations that would exert negative dominance on \textit{IcsA} function in ABM and intercellular spread when co-expressed with \textit{IcsA}_{\text{WT}}. A collection of \textit{IcsA}_i insertion mutants has been previously identified (May & Morona, 2008) and some of these were screened for negative dominance in intercellular spreading when co-expressed with \textit{IcsA}_{\text{WT}} in a \textit{S. flexneri} KMRM254 background (data not shown). Two mutants (\textit{IcsA}_{563}, and \textit{IcsA}_{677}) exerted a clear negative dominant phenotype when expressed with either \textit{IcsA}_{\text{WT}} (Fig. 3a) or the functionally equivalent \textit{IcsA}_{87::\text{FLAG}} (Fig. S2). Individually, \textit{IcsA}_{563} and \textit{IcsA}_{677} are unable to recruit N-WASP (Fig. S3) or efficiently generate F-actin tails and promote plaque formation on HeLa cell monolayers (May & Morona, 2008). When either mutant was co-expressed with \textit{IcsA}_{\text{WT}}, F-actin tails could still detected by FITC-phalloidin staining of fixed monolayers infected with the various \textit{Shigella} strains (Fig. 3b). However, efficient F-actin tail formation is
required for efficient intercellular spreading. As a sensitive measure of the proficiency of the detected F-actin tails to drive intercellular spreading, the ability of the co-expression strains to form plaques on HeLa cell monolayers was assessed. Plaque formation could not be detected when IcsA_{i563} was co-expressed with IcsA_{WT} and co-expression of IcsA_{i677} with IcsA_{WT} resulted in only a few small plaques (<1 mm diameter) that were markedly smaller than those formed by the control strain KMRM255 (which typically formed between 50-100 plaques/well, averaging 6.4 mm in diameter) (Fig. 3a). The defective plaque formation clearly indicated that the F-actin tails formed by these strains were not comparable to wild-type, as they were not able to facilitate efficient ABM and intercellular spreading.

**Negative dominance of IcsA_{i} mutants on IcsA_{WT} does not arise due to defects in production, export or polar localization of the wild-type protein.** The observation of negative dominance when IcsA_{WT} was expressed with IcsA_{i} mutants could have arisen by titration by the mutant proteins of factors required for IcsA synthesis, polar localisation and surface presentation, resulting in a decrease-in-function of IcsA_{WT}. We sought to scrutinise these possibilities by using a tagged version of IcsA_{WT} (IcsA_{i87::FLAG}) in *S. flexneri* ΔicsA strains additionally expressing IcsA_{i563} or IcsA_{i677} mutants. IcsA_{i87::FLAG} retains wild-type equivalent function in plaque formation (Fig. S2 in the supplemental material). We confirmed that the IcsA_{i563} and IcsA_{i677} mutants had a negative dominant effect on IcsA_{i87::FLAG} with respect to plaque formation (Fig. S2).

The levels of IcsA_{i87::FLAG} protein during co-expression with IcsA_{i563} and IcsA_{i677}, were then assessed by Western blotting with anti-FLAG M2 antibodies. IcsA_{i87::FLAG} production was equivalent when expressed alone from either plasmid pKMRM250 (pBR233-derivative) or plasmid pKMRM270 (pBRR1MCS2-derivative), confirming the suitability of the plasmids for use in co-expression studies (not shown). Production of IcsA_{i87::FLAG} when co-expressed with IcsA_{WT} in KMRM275 was comparable to production of IcsA_{i87::FLAG} when expressed alone in KMRM273 (Table 1; Fig. 4a). Despite being unable to form detectable plaques, the strain co-expressing IcsA_{i677} with IcsA_{i87::FLAG} expressed comparable levels of
FLAG-tagged functional IcsA; co-expression of IcsA<sub>i563</sub> and IcsA<sub>i87::FLAG</sub> resulted in a slight
decrease in FLAG-tagged IcsA (Fig. 4a). Additionally, we quantitated the level of surface
expressed IcsA<sub>i87::FLAG</sub> by flow cytometry. Co-expression of IcsA<sub>i87::FLAG</sub> with either mutant did
not result in a detectable reduction in the amount of functional protein at the bacterial surface
(Fig. 4b). Moreover, the surface distribution of IcsA<sub>i87::FLAG</sub> was seen by immunofluorescence
to be polar when co-expressed either with IcsA<sub>WT</sub>, IcsA<sub>i563</sub>, or IcsA<sub>i677</sub> (Fig. 4c).

Taken together, these data suggest that the negative dominant phenotype conferred by
IcsA<sub>i563</sub> and IcsA<sub>i677</sub> during co-expression with active IcsA results from a net functional
defect at the cell surface, and was not due to these mutants affecting the expression, export or
localisation of functional IcsA proteins.

DISCUSSION

Subversion of the host cell actin regulatory network enables *Shigellae* infection to spread
throughout the human intestinal epithelium. IcsA is both necessary and sufficient to
potentiate actin-based motility through activation of N-WASP. The nature of IcsA interaction
with N-WASP remains poorly understood. In light of data demonstrating that the
conventional AT, IgA protease from *N. gonorrhoea*, forms an oligomer in the OM, we sought
to characterize the properties of IcsA in the OM. In this study, we tested the hypothesis that
IcsA is able to self-associate.

The self-association of IcsA was supported by the presence of HMW IcsA-related
complexes that were detected following cross-linking of whole cells with DSP or
formaldehyde. While IgA1 protease *in vitro* oligomerization was reported as homo-
pentameric (Veiga *et al.*, 2002), our findings do not exclude the possibility that IcsA
oligomerization is hetero-oligomeric. The reciprocal co-purification and genetic interaction
data we have presented in this study strongly suggests the existence of IcsA-IcsA interactions
within the HMW oligomer.

Support for the existence of direct IcsA-IcsA interactions within HMW complexes
was provided by observation of negative dominant genetic interactions of IcsA<sub>i</sub> linker-
insertion mutants (IcsA<sub>i563</sub> and IcsA<sub>i677</sub>) on IcsA function in intercellular spreading (assessed with both IcsA<sub>WT</sub> and IcsA<sub>i87::FLAG</sub>). One of two explanations can account for negative dominant phenotypes (Herskowitz, 1987). The first is titration of factors away from the functional IcsA protein by co-expression of a mutants IcsA<sub>i</sub> protein. Since no defects were observed in functional IcsA whole-cell protein levels, in cell surface expression, or in polar targeting, the factors that underpin the respective processes were not titrated by IcsA<sub>i</sub> mutants. Similarly, the examined IcsA<sub>i</sub> mutants cannot recruit N-WASP (May & Morona, 2008), and titration of this host cell ligand from functional IcsA was improbable. These findings indicated that titration could not explain the negative dominant phenotype. Instead, we favour the alternate explanation of negative dominance: that the products of the functional icsA and mutant icsA<sub>i</sub> alleles functionally interact in vivo. We suggest that during co-expression, IcsA<sub>i</sub> mutants are included into mixed complexes with functional IcsA proteins. The inclusion of IcsA<sub>i</sub> proteins renders these complexes defective for efficient F-actin tail formation, as evidenced by defective intercellular spreading. This deficiency in the negative dominant co-expression strains occurs despite levels of functional IcsA that, when expressed alone, can efficiently potentiate intercellular spreading. Clearly, IcsA-IcsA interactions are functionally important.

We have demonstrated for the first time biochemical and genetic evidence of IcsA self-association. Furthermore, the observed negative dominance of certain inactive IcsA<sub>i</sub> mutant proteins is consistent with self-association being important for the biological function of the protein. Although N-WASP and WASP family members are activated by an array of different molecules, these activators act by allosteric relief of N-WASP/WASP auto-inhibition (Kim <i>et al.</i>, 2000), and by facilitating clustering and multimerization of active N-WASP (Padrick <i>et al.</i>, 2008; Padrick & Rosen, 2010), often by their own oligomerization. Therefore, we hypothesise the self-association of IcsA may play a role in N-WASP clustering. In our study the incorporation of defective IcsA<sub>i563</sub> or IcsA<sub>i677</sub> proteins into IcsA<sub>WT</sub>-containing complexes could interfere with efficient spatial clustering of activated N-WASP, thereby accounting for the reduced efficiency of intercellular spreading. In this way,
IcsA would appear analogous to other bacterial and host proteins that either interact with or functionally mimic WASP family members. Most of these have been shown to either directly self-associate (Enterohaemorrhagic *Escherichia coli* intimin-tir), to spatially cluster (*Listeria monocytogenes* ActA) or are inherently multivalent with internal repeats (Enterohaemorrhagic *Escherichia coli* EspF, EspFu and Tccp) (Alto *et al.*, 2007; Campellone *et al.*, 2008; Footer *et al.*, 2008; Sallee *et al.*, 2008; Touzé *et al.*, 2004). Significantly, our data demonstrates self-association of yet another protein known to activate a WASP family protein, which has emerged as an important feature in the regulation of the host actin-cytoskeleton.
ACKNOWLEDGEMENTS

We thank Chihiro Sasakawa for virG deletion constructs. Luisa Van Den Bosch is thanked for technical support. This work is supported by a Program Grant from the National Health and Medical Research Council (NHMRC) of Australia. K.L.M was the recipient of a Faculty of Science Postgraduate Scholarship from the University of Adelaide. M.G. was the recipient of an Australian Postgraduate Award (NHMRC).

Author contributions: K.L.M. M.G. and L.V.D.B performed research; S.P. provided reagents and expertise on biotinylation; and K.L.M., M.G. and R.M. designed research, analyzed data, and wrote the manuscript.

REFERENCES


type III effectors into host cells in real time. Nat Methods 2, 959–965.


** Morona, R., Daniels, C. & Van Den Bosch, L. (2003). Genetic modulation of Shigella flexneri 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence. Microbiol 149, 925–939.


### TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant characteristics *</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli K-12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>endA</em> <em>hsdR</em> <em>supE44</em> <em>thi-1</em> <em>recA1</em> <em>gyrA</em> <em>relAΔ</em> <em>(lacZYA-argF)</em> U169 [φ80 <em>dlacΔ</em> <em>(lacZ)</em> M15] <em>phoA</em></td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>UT5600</td>
<td>Δ*(ompT-fepC)*266 <em>entA403</em> <em>trpE38</em> <em>rbd1</em> <em>rpsL109</em> <em>xyl-5</em> <em>mtl-1</em> <em>thi-1</em></td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>MG157</td>
<td>UT5600 [pKMRM270, pMG55, pCY216]</td>
<td>This study</td>
</tr>
<tr>
<td>MG250</td>
<td>UT5600 [pKMRM270, pKMRM1, pCY216]</td>
<td>This study</td>
</tr>
<tr>
<td>MG251</td>
<td>UT5600 [pKMRM252, pMG55, pCY216]</td>
<td>This study</td>
</tr>
<tr>
<td><strong>S. flexneri</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2457T</td>
<td><em>S. flexneri</em> 2a wild-type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>RMA2041</td>
<td>2457T Δ<em>icsA::Te</em>R</td>
<td>(Van Den Bosch &amp; Morona, 2003)</td>
</tr>
<tr>
<td>RMA2090</td>
<td>RMA2041 [pIcsA]</td>
<td>(Van Den Bosch &amp; Morona, 2003)</td>
</tr>
<tr>
<td>RMA2107</td>
<td>RMA2041 Δ<em>armLD::KmR</em> [pIcsA]</td>
<td>(Van Den Bosch &amp; Morona, 2003)</td>
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<td>RMA2205</td>
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<td>This study</td>
</tr>
<tr>
<td>RMA2208</td>
<td>RMA2041 [pD10-1 <em>virG3</em>]</td>
<td>This study</td>
</tr>
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<td>RMA2209</td>
<td>RMA2041 [pD10-1 <em>virG4</em>]</td>
<td>This study</td>
</tr>
<tr>
<td>KMRM111</td>
<td>RMA2041 [pKMRM11]</td>
<td>(May &amp; Morona, 2008)</td>
</tr>
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<td>KMRM134</td>
<td>RMA2041 [pKMRM34]</td>
<td>(May &amp; Morona, 2008)</td>
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<td>RMA2041 [pKMRM252]</td>
<td>This study</td>
</tr>
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<tr>
<td>KMRM277</td>
<td>RMA2041 [pKMRM34] [pKMRM270]</td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-2</td>
<td>Broad-host-range vector; KmR; medium copy no.; ori compatible with CoEI plasmids</td>
<td>(Kovach et al., 1995)</td>
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<tr>
<td>pCY216</td>
<td>encodes BirA; CmR</td>
<td>(Chapman-Smith et al., 1994)</td>
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<tr>
<td>pD10-1</td>
<td>encodes VirG (same as IcsA&lt;sub&gt;WT&lt;/sub&gt;); TpR</td>
<td>(Suzuki et al., 1996)</td>
</tr>
<tr>
<td>pD10-1&lt;sub&gt;virG3&lt;/sub&gt;</td>
<td>encodes VirG&lt;sub&gt;-508-730&lt;/sub&gt;; TpR</td>
<td>(Suzuki et al., 1996)</td>
</tr>
<tr>
<td>pD10-1&lt;sub&gt;virG4&lt;/sub&gt;</td>
<td>encodes VirG&lt;sub&gt;-103-507&lt;/sub&gt;; TpR</td>
<td>(Suzuki et al., 1996)</td>
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<tr>
<td>pBR322</td>
<td>pBR322 encoding IcsA&lt;sub&gt;187&lt;/sub&gt;; ApR</td>
<td>(May &amp; Morona, 2008)</td>
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<tr>
<td>pKMRM1</td>
<td>pBR322 encoding IcsA&lt;sub&gt;563&lt;/sub&gt;; ApK</td>
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<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Resistance</td>
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<td>---------</td>
<td>-------------</td>
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</tr>
<tr>
<td>pKRM34</td>
<td>pBR322 encoding IcsA&lt;sub&gt;i677&lt;/sub&gt;; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(May &amp; Morona, 2008)</td>
</tr>
<tr>
<td>pKRM250</td>
<td>pBR322 encoding IcsA&lt;sub&gt;i87&lt;/sub&gt;·FLAG; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pKRM252</td>
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<tr>
<td>pMG55</td>
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<td>This study</td>
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* Cm<sup>R</sup>, chloramphenicol-resistant; Te<sup>R</sup>, tetracyline-resistant; Km<sup>R</sup>, kanamycin-resistant; Ap<sup>R</sup>, ampicillin-resistant; Tp<sup>R</sup>, trimethroprim-resistant.
TABLE 2. Oligonucleotides

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<thead>
<tr>
<th>Oligo</th>
<th>Sequence (5' - 3')*</th>
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<tr>
<td>KM1 BIO F</td>
<td>GCCCTGAACGACATCTTCTGAGCTCAGAAATCGAATGGCAC</td>
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<tr>
<td>KM1 BIO R</td>
<td>GCCCTTGCCCATTCGATTTTCTAGCTTCGAGATGCTGTTCA</td>
</tr>
<tr>
<td>IcsA_FLAG F1</td>
<td>GCCCGCGACTACAAGGACGATGACGACAAG</td>
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<tr>
<td>IcsA_FLAG R1</td>
<td>GCCCCTTTGTCGTCTACGTCGTCCTTGTAGTCGC</td>
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* Bold nucleotides comprise the Not1 overhang of the annealed epitopes.
FIG. 1. DSP cross-linking of *S. flexneri*. Mid-exponential phase cultures of indicated *S. flexneri* strains were treated with 0.2 mM DSP as described in the Methods. Whole membranes were isolated and resuspended in sample buffer with or without β-mercaptoethanol (β-ME) and heated to 60°C. Cross-linked samples were resolved by SDS-PAGE (7.5% polyacrylamide) and analysed by Western blotting with anti-IcsA antibody. Sizes were approximated from the electrophoretic mobility of HighMark (Invitrogen) protein standard. Expected electrophoretic migration of monomeric IcsA truncation mutants is indicated. IcsA* indicates HMW complexes containing IcsA. The resolving gel is shown.

FIG. 2. Co-purification analysis. *E. coli* K-12 strains (MG157, MG250, MG251) were grown, then Zwittergent 3-14-solubilised OM fractions were prepared and purified through FLAG-agarose resin (a) or streptavidin-Dynabeads (b), as described in the Methods. Samples were subjected to SDS-PAGE, and Western blotting with either a rabbit anti-FLAG M2 antibody followed with an anti-rabbit Ig HRP conjugate, or streptavidin-HRP, as indicated.

FIG. 3. Plaque and F-actin tail formation by complemented strains. (a) Relative size and frequency of plaque formation on HeLa cell monolayers by *S. flexneri* strains expressing either IcsA<sub>WT</sub> (RMA2090), or IcsA<sub>i563</sub> (KMRM111), or IcsA<sub>i677</sub> (KMRM134) alone, and *S. flexneri* co-expressing IcsA<sub>WT</sub> with IcsA<sub>i563</sub> (KMRM256), IcsA<sub>i677</sub> (KMRM258), or IcsA<sub>WT</sub> control (KMRM255). Plaque assay was performed as described in detail in the materials and methods. (b) IF microscopy of F-actin tail formation by intracellular *S. flexneri* strains expressing either IcsA<sub>WT</sub> (RMA2090), or IcsA<sub>i563</sub> (KMRM111), or IcsA<sub>i677</sub> (KMRM134) alone, and *S. flexneri* co-expressing IcsA<sub>WT</sub> with IcsA<sub>i563</sub> (KMRM256), IcsA<sub>i677</sub> (KMRM258), or IcsA<sub>WT</sub> control (KMRM255). HeLa cells infected with *S. flexneri* were labelled with anti-LPS antibodies and Alexa 594-conjugated donkey anti-rabbit antibodies, and F-actin was labelled with FITC phalloidin as described in detail in the Methods. Strains were assessed in three independent experiments. Arrows indicate F-actin tails. Scale bar = 10 μm.
FIG. 4. Protein levels and distribution of IcsA$_{i87::\text{FLAG}}$ during co-expression with IcsA$_{i563}$ and IcsA$_{i677}$ mutants. (A) Whole-cell lysates from mid exponential-phase cultures of indicated S. flexneri strains expressing IcsA$_{i87::\text{FLAG}}$ with IcsA$_{i563}$ (KMRM276), IcsA$_{i677}$ (KMRM277), or control IcsA$_{\text{WT}}$ (KMRM275) were subjected to Western blotting with anti-FLAG antibodies. The samples represent $1 \times 10^8$ cells. IcsA'$_{i87::\text{FLAG}}$ is a proteolytic fragment also observed in IcsA$_{\text{WT}}$. (B) FACS analysis of the indicated S. flexneri strains. Equivalent numbers of bacteria were labelled with rabbit anti-FLAG and then anti-rabbit-Alexa 488 antibodies, and fluorescence intensity assessed by flow cytometry as described in the Material and Methods. Grey area denotes fluorescence intensities of the negative control strain (KMRM250). For all strains, $n = 50,000$ cells. (C) IF microscopy of IcsA$_{i87::\text{FLAG}}$ surface distribution. Mid exponential-phase cultures of indicated S. flexneri strains were formalin fixed and labelled with anti-FLAG antibodies and then goat anti-rabbit Alexa 488 secondary antibodies. Scale bar = 3 µm.
FIG. 1. DSP cross-linking of *S. flexneri*. Mid-exponential phase cultures of indicated *S. flexneri* strains were treated with 0.2 mM DSP as described in the Methods. Whole membranes were isolated and resuspended in sample buffer with or without β-mercaptoethanol (β-ME) and heated to 60°C. Cross-linked samples were resolved by SDS PAGE (7.5% polyacrylamide) and analyzed by Western blotting with anti-IcsA antibody. Sizes were approximated from the electrophoretic mobility of HighMark (Invitrogen) protein standard. Expected electrophoretic migration of monomeric IcsA truncation mutants is indicated. IcsA* indicates HMW complexes containing IcsA.

<table>
<thead>
<tr>
<th></th>
<th>RMA2205</th>
<th>RMA2208</th>
<th>RMA2209</th>
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<tbody>
<tr>
<td>DSP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-ME</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

IcsA* (>460kDa)
IcsA_{1-1102}
IcsA_{Δ508-730}
IcsA_{Δ103-507}
FIG. 2. Co-purification analysis.

E. coli K-12 strains (MG157, MG250, MG251) were grown, then Zwittergent 3-14-solubilised OM fractions were prepared and purified through FLAG-agarose resin (a) or streptavidin-Dynabeads (b), as described in the Methods. Samples were subjected to SDS-PAGE, and Western blotting with either a rabbit anti-FLAG M2 antibody followed with an anti-rabbit Ig HRP conjugate, or streptavidin-HRP, as indicated.
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