Role of c-Cbl in invasion of mammalian cells by *Rickettsia conorii*

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Submitted by

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Specific Aims

Rickettsia conorii is an intracellular bacterium that causes Mediterranean spotted fever. *R. conorii* is transmitted from ticks to humans and invades vascular endothelial cells. A recent publication (Martinez et al., 2005) has identified Ku70 as a host cell receptor that binds to rOmpB, an *R. conorii* surface ligand. The engagement of Ku70 by rOmpB leads to a rapid ubiquitination of Ku70 by c-Cbl, followed by the entry of *R. conorii* into the host cell. However, the role of c-Cbl in ubiquitinating Ku70 and communicating with the invading bacterium remain to be clarified. Based on the report of Martinez *et al* and other groups on the roles played by *R. conorii* surface ligands rOmpA and rOmpB, we propose the following hypothesis:

Hypothesis

c-Cbl ubiquitinates Ku70 in response to signals from a *Rickettsia conorii* cell surface protein called rOmpA. This ubiquitination event enables Ku70 and the attached bacteria to be endocytosed into the cell.

Specific Aims

1. **To analyse the effect of c-Cbl – mediated ubiquitination on Ku70 localisation.** We will investigate if ubiquitination of Ku70 by c-Cbl is necessary for Ku70 to localize to the bacterial entry foci. We will culture c-Cbl deficient cells and observe the effects of *R. conorii* infection on them.
2. **To map the putative signal(s) involved in the communication between *R. conorii* rOmpA outside the cell and c-Cbl inside the cell.** We will identify the putative rOmpA receptor on host cells and protein(s) that may act as bridges of communication between rOmpA and c-Cbl.
3. **To investigate the mechanism of how ubiquitination of Ku70 enables *R. conorii* to enter a host cell.** We will determine if any c-Cbl interacting proteins and/or endocytic proteins are recruited to the cell surface by ubiquitinated Ku70 to form a phagosome. If endocytic proteins are recruited, we will proceed to determine whether the mechanism of endocytosis is clathrin dependent or independent.

Background and Significance

Rickettsia conorii : a pathogenic micro organism

Bacteria of the genus *Rickettsia* cause a variety of human diseases. They are gram negative, obligate intracellular parasites. *Rickettsiae* are broadly classified as belonging to either the Typhus group or Spotted Fever group based on the type of disease they cause. The Spotted Fever group includes the species *Rickettsia conorii* that causes Mediterranean Spotted fever. *R. conorii* is transmitted from arthropods like ticks, fleas and lice to humans. It enters human blood vessels at the site of the arthropod vector's bite and invades vascular endothelial cells by inducing phagocytosis. Once inside the cell, it escapes from the phagosome and grows in the host cell's nucleus and cytoplasm. The bacteria then multiply and spread rapidly to adjacent cells by means of an actin-based motility mechanism. This leads to vasculitis and increased vascular permeability (Hackstadt, 1996), which manifest in clinical symptoms like hypovolemia and edema of the skin, lungs and brain (Walker, 2006). Mediterranean Spotted fever, also called Boutonneuse fever, is endemic to Africa, southern Europe and south Asia. It is usually transmitted to humans in these places by the bite of a dog tick of the *Rhipicephalus* genus. Although the mortality rate in Mediterranean fever patients is not more than 5%(Yagupsky and Wolach, 1993), the fever could be fatal if undiagnosed. Also, the severity of the disease is vastly exacerbated in patients who are deficient in Glucose-6-phosphate dehydrogenase (Whelton et al., 1968).

Importance of studying *R. conorii* pathogenesis

Though not occurring naturally in the Americas, the threat of bioterrorism and increased numbers of travellers venturing into the endemic areas have brought the dangers of Rickettsial diseases into focus in the USA. The National Institute for Allergy and Infectious Diseases (NIAID) has classified *Rickettsia spp*. as potential biological weapons (http://www3.niaid.nih.gov/Biodefense/bandc_priority.htm). This assumes significance in light of the fact that some accidental laboratory infections have occurred by inhaling aerosols containing

Rickettsia. As a terror weapon, Rickettsial infections could be spread among a large number of people by dispersing infectious aerosols in the atmosphere (Walker, 2003). Therefore, there is a pressing need to understand more about Rickettsial diseases like Mediterranean Spotted fever. Currently, Mediterranean fever is treated with a limited range of antibiotics (Rolain et al., 1998). Specific information on how the bacteria enter host cells, if available, could be utilised to develop a wider range of drugs as well as vaccines.

Entry of *R. conorii* into host cells

R. conorii infects mainly vascular endothelial cells. Although these cells are normally non-phagocytic, *R. conorii* induces the formation of phagosomes and enters the host cell by being engulfed and internalised in the phagosomes (Teysseire et al., 1995). In order to do so, *R. conorii* first has to attach itself to the host cell membrane and then send signal(s) to the host cell to cause the latter to modify its cytoskeleton and form phagosomes. Until recently, the putative host cell receptor(s) facilitating the attachment and entry of the *Rickettsiae* was unknown. A recent publication (Martinez et al., 2005) threw some light on this question by identifying Ku70 as a host cell receptor for *R. conorii* and rOmpB as the bacterial ligand for this receptor.

Receptor and Ligand involved in *R. conorii* entry

Ku70 is one of the subunits of the DNA dependant Protein Kinase (DNA-PK) with known functions in DNA repair, transcription and replication (Tuteja and Tuteja, 2000) as well as in apoptosis prevention (Sawada et al., 2003a; Sawada et al., 2003b). Although predominantly a nuclear protein, the Ku heterodimer (consisting of Ku70 and Ku80) has been found on cell membranes, facing the extracellular environment (Muller et al., 2005). In this context, the Ku heterodimer has been suggested to be involved in cell adhesion (Monferran et al., 2004) and proteolysis (Muller et al., 2005) via its interaction with Fibronectin and Matrix Metalloprotease 9 respectively.

rOmpB is the most abundant *R. conorii* cell surface protein and is made from a large precursor by proteolytic cleavage. It is believed to remain associated at the Rickettsial outer membrane with the smaller product of the cleavage reaction. Mutant *Rickettsiae* that harbour a cleavage-deficient full length precursor of rOmpB are avirulent (Hackstadt et al., 1992). Also, rOmpB from a related strain of *Rickettsia* called *R. japonica* has been shown to mediate infection of mammalian cells by non-invasive *E. coli* by inducing phagocytosis (Uchiyama, 2003). There is also evidence that rOmpB is essential for the escape of internalized *R. conorii* from phagosomes (Feng et al., 2004; Hackstadt et al., 1992).

Insights obtained from the work of Martinez *et al*

Martinez *et al* used a cellular model of infection: Vero and HeLa cells exposed to purified *R. conorii*.They used a pull-down assay consisting of an incubation of mammalian cellular lysates with bacterial pellets followed by elution of bound mammalian proteins by high salt treatment. The recovered mammalian proteins were analysed by mass spectroscopy. It was found that Ku70 is the major host cell protein that interacts with *R. conorii*. They went on to show that Ku70 localised to plasma membrane lipid rafts, moves to bacterial adhesion foci within 15 minutes of infection. This was important for *R. conorii* entry. They also found that Ku70 was rapidly ubiquitinated within a minute of *R. conorii* infection and c-Cbl was the major ubiquitin ligase responsible for this ubiquitination event. There was no change in the total amount of Ku70 after ubiquitination. Loss of Cbl protein expression in host cells inhibited *R. conorii* entry. In order to identify the bacterial ligand(s) interacting with mammalian Ku70, Martinez *et al* expressed FLAG –tagged Ku70 in HEK293 cells, lysed the cells and bound the tagged Ku70 to a FLAG affinity matrix. They then incubated *R. conorii* protein extracts with the FLAG-Ku70 matrix and eluted the Ku70 along with all proteins binding to it. Mass Spectroscopy identified a single binding partner for Ku70 from the *R. conorii* lysate. This was the bacterial cell surface protein, rOmpB.

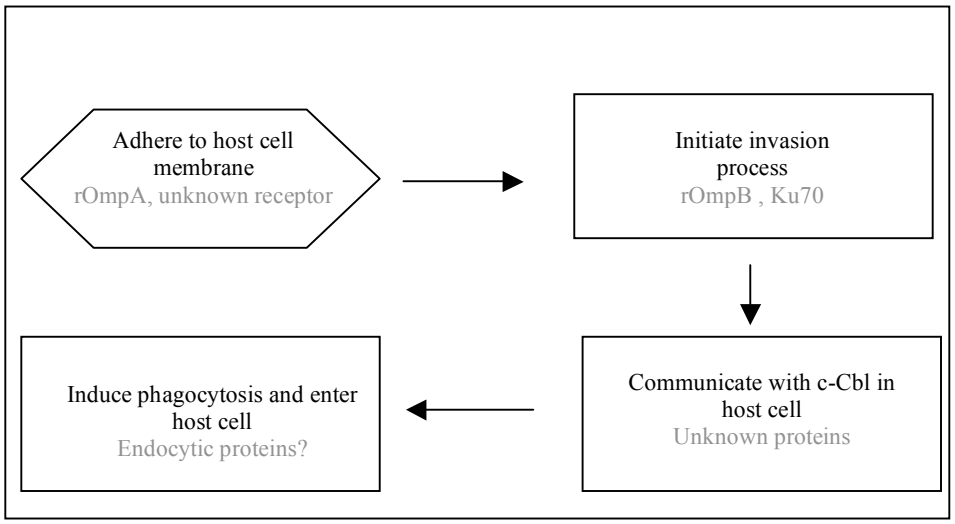
c-Cbl and endocytosis

c-Cbl is an E3 ubiquitin ligase that is known to monoubiquitinate several membrane receptors like Epidermal Growth Factor receptor (Soubeyran et al., 2002). Unlike polyubiquitination, multiple monoubiquitination of cell membrane receptors enables their endocytosis. Ubiquitination of the receptor enables it to be recognised and bound by ubiquitin-binding proteins like Epsin, which facilitates caveolar endocytosis. c-Cbl can also initiate clathrin-mediated endocytosis by recruiting proteins like CIN85, AP2 and endophilin. c-Cbl maintains the ubiquitinated status of the endocytosed receptors in order to prevent their being recycled to the plasma membrane (Schmidt and Dikic, 2005). This is one of the mechanisms used by normal cells to attenuate responses to extracellular signals. Pathogens like *R. conorii* appear to use this process to their advantage.

Adherence vs Invasion

It is evident from the report of Martinez *et al* that adherence of *R. conorii* to host cells did not require Ku70 or rOmpB. This is in agreement with an earlier report (Li and Walker, 1998) that established another *R. conorii* membrane protein, rOmpA, as being important for adhesion to host cells, but not entry. In other words, rOmpA is critical for *R. conorii* to attach itself to a host cell, but it cannot enable the bacteria to enter the intracellular environment of the host cell. On the other hand, rOmpB appears to be a minor contributor to bacterial adherence to host cells (Uchiyama et al., 2006), but is essential for the invasion of host cells. Therefore, the initial events of the infection process may be summed up as follows: rOmpA is important for *R. conorii* to adhere to host cells, and rOmpB is a lesser contributor. The next step in the infection process is to induce the host cell to take up the adhering bacterium. rOmpB seems to be the principal bacterial protein in this step and accomplishes this through its interaction with Ku70 at the host cell membrane (Figure-1).

Figure-1: Schematic of early events in R. conorii infection



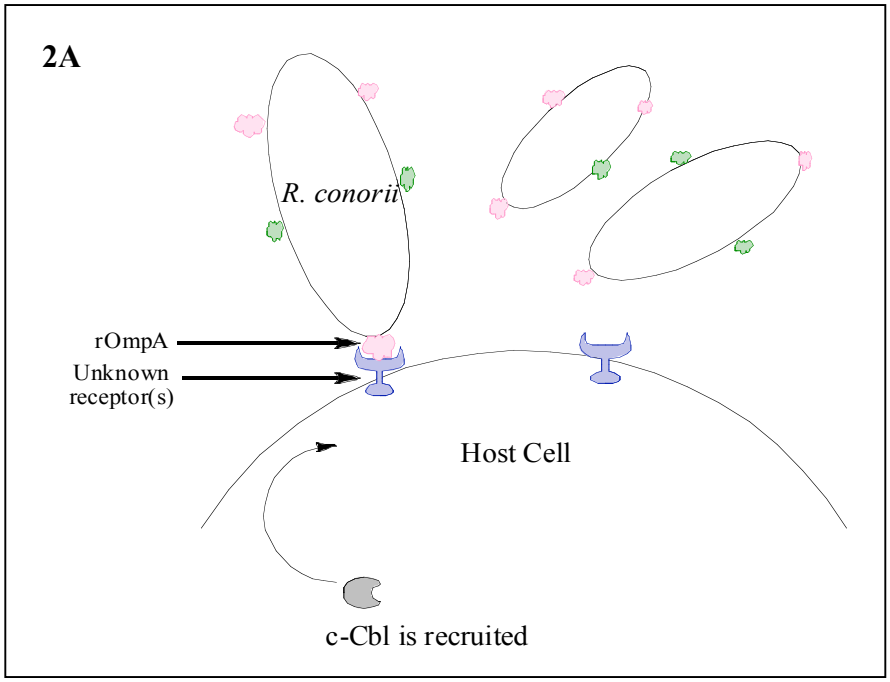
To summarize the results reported by Martinez *et al* (as shown in Fig.2), *R. conorii* adheres to the surface of mammalian host cells. This leads to the recruitment of c-Cbl to the cell membrane, where it ubiquitinates endogenous Ku70. Ku70 is then localised to lipid microdomains at the cell surface, where it comes into contact with a bacterial cell surface protein called rOmpB.

Unanswered questions

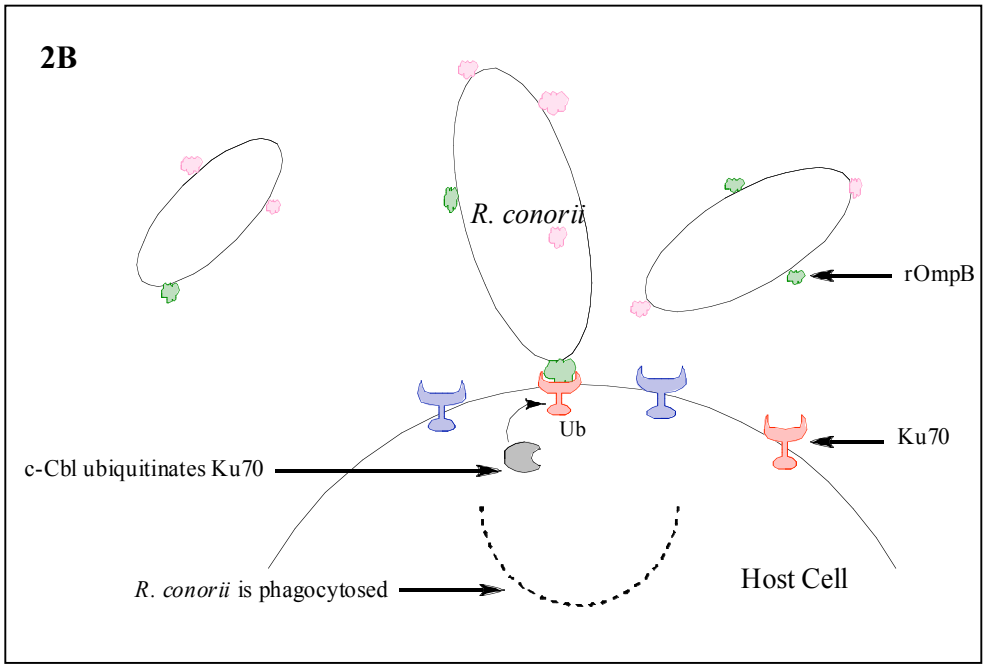
The report by Martinez *et al* has revealed that Ku70 and rOmpB are the major factors involved in enabling *R. conorii* to invade a cell. However, many important questions about the infection process still remain. What is the purpose of ubiquitinating Ku70? What is the role played by c-Cbl? How is c-Cbl recruited to Ku70? c-Cbl is known to play an important role in the internalization of another intracellular bacterium, *Listeria monocytogenes*. An *L. monocytogenes* cell surface protein called InlB engages the Hepatocyte Growth Factor receptor, Met, on the host cell membrane. c-Cbl then monoubiquitinates Met, causing the latter to be endocytosed. This facilitates the entry of *L. monocytogenes* into the host cell (Veiga and Cossart, 2005). It needs to be explored if c-Cbl acts similarly during *R. conorii* infection. Researching these questions will lead to

a more detailed picture of the infection process of *R. conorii* and may enable the development of new vaccine and/or drug candidates to combat Rickettsial diseases.

Figure-2: Putative mechanism of *R. conorii* entry into mammalian cells based on the experiments reported by Martinez *et al*, 2005.



R. conorii first attaches itself to a host cell by means of rOmpA and c-Cbl is recruited to the cell periphery.



c-Cbl ubiquitinates cell surface Ku70, to which bacterial rOmpB is bound. This enables the bacteria to enter the cell by induced phagocytosis.

Experimental Methods

Specific Aims

1. **To analyse the effect of c-Cbl – mediated ubiquitination on Ku70 localisation.**

c-Cbl is essential for the invasion of mammalian cells by *Listeria monocytogenes,* another intracellular bacterium. c-Cbl monoubiquitinates the *L. monocytogenes* receptor and causes the latter to be endocytosed. The bacteria enter the cell by including themselves in the endocytic vesicles (Veiga and Cossart, 2005). Martinez *et al* found that c-Cbl co-localises with *R. conorii* attachment sites in Vero cells within 5 minutes of infection. This coincides with ubiquitination of Ku70. They went on to show, by loss-of-function experiments, that inhibition of c-Cbl expression in mammalian cells had no effect on the adherence of *R. conorii* to the host cell membranes, but suppressed bacterial invasion and entry. The inhibition of c-Cbl also correlated with a loss of Ku70 ubiquitination. These data indicate that c-Cbl may play a major role in *R. conorii* infection as it does in *L. monocytogenes* infection. Therefore, we propose to analyze the c-Cbl-Ku70 interaction as follows:

<u>To determine whether ubiquitination of Ku70 by c-Cbl is necessary for Ku70 to localize to the bacterial entry foci.</u>

We hypothesize that c-Cbl ubiquitinates Ku70 in response to signals from an *R. conorii* cell surface protein called rOmpA. This ubiquitination event is necessary for Ku70 and the attached bacteria to be internalized into the cell (internalisation hypothesis). This is consistent with the well-documented functions of c-Cbl as an endocytosis-promoting factor and adaptor protein (Swaminathan and Tsygankov, 2006). However, it could be that c-Cbl is involved in a much earlier step of the *R. conorii* infection process; an alternate hypothesis would be that Ku70 is recruited to rOmpB at the bacterial adhesion sites through the ubiquitinating activity of c-Cbl (localisation hypothesis). The published data of Martinez *et al* would support both the above hypotheses. We will test the localisation hypothesis first. We have obtained primary vascular endothelial cells from c-Cbl

deficient mice (a kind gift of the group that generated the mice)(Murphy et al., 1998). We will culture these cells for our experiments. In the meantime *R. conorii* will be grown, purified and stored according to previously published methods (Gouin et al., 1999). We will then infect control and c-Cbl deficient cells with *R. conorii* as per established procedures (Martinez and Cossart, 2004). At different time points after incubation with *R. conorii* (for instance, 1 minute, 2 minutes, 5 minutes etc), coverslips will be removed from the infected cell cultures, fixed and processed for immunofluorescence with an anti- *R. conorii* antibody named R47 (which has been kindly provided to us by the group that raised it) and commercially available anti-Ku70 antibodies. Using this approach we will determine whether Ku70 localises to the bacterial entry sites (visible as the sites stained by the R47 antibody) or not. We will also perform 'rescue' experiments where we will express c-Cbl in the c-Cbl deficient cells in order to confirm that the effects seen are indeed due to the absence of c-Cbl.

To further differentiate between the two hypotheses, we will overexpress Ku70 at the plasma membrane in c-Cbl deficient cells and then analyse the ability of *R. conorii* to invade the cells. By doing this experiment, we will test if the presence of Ku70 at the plasma membrane is sufficient to enable *R. conorii* to invade the cells despite c-Cbl being absent. We will make a Ku70 plasmid construct that has an N-terminal Signal Peptide for import into the Endoplasmic Reticulum and subsequent secretion to the plasma membrane. The Signal Peptide (N-MMSFVSLLLVGILFWATEAENLTKCEVFN-) will target a cytosolic protein to the plasma membrane when fused to the latter. We will stably transfect this plasmid into c-Cbl deficient cells (Murphy et al., 1998) and confirm the enforced plasma membrane localisation of Ku70 by immunofluorescence. We will confirm that the Ku70-Signal Peptide fusion protein expressed at the plasma membrane is functional by testing if it can support the invasion of *R. conorii* in c-Cbl expressing cells. We will then analyze whether the absence of c-Cbl affects the entry of *R. conorii*. In this experiment, the endogenous Ku70 is likely to be a confounding factor.

Ku70 deficient mice are available - however we are precluded from using cells from these mice in our experiments because Ku86 also disappears when Ku70 expression is ablated (Ouyang et al., 1997).

Expected result The above experiments are designed to distinguish between two possibilities: Does c-Cbl enable recruitment of Ku70 to bacterial entry foci or is c-Cbl required only to endocytose the bacterium? If the localisation hypothesis is true, we should see Ku70 co-localized with *R. conorii* in control cells, but not in c-Cbl deficient cells. This would show that ubiquitination of Ku70 by c-Cbl is necessary for Ku70 to localize to the bacterial entry foci. In the second experiment, if we find that *R. conorii* is unable to enter the host cells despite the enforced presence of Ku70 at the plasma membrane, it would suggest that c-Cbl was needed for endocytosis.

Potential Pifalls

1. In the case of primary cells from c-Cbl deficient mice, it is possible that they may not be amenable to maintenance in culture. They might not grow well and consequently, only a limited number of cells may be available for experiments. Also, there might be technical difficulties in transfecting primary cells.
2. c-Cbl has two highly homologous family members named Cbl-b and Cbl-3 (Swaminathan and Tsygankov, 2006). It is conceivable that in c-Cbl deficient cells, the c-Cbl homologues can functionally replace c-Cbl, although such a scenario has not been observed so far.
3. The Signal Peptide-Ku70 fusion protein may be improperly folded.

Alternate Approaches

1. We will inhibit the expression of endogenous c-Cbl in Vero and Hela cells by RNA interference. We will confirm the extent of residual expression (if any) by doing a western blot on lysates of control and RNAi –treated cells with commercially available antibodies against c-Cbl. We will resort to

microinjection of plasmid DNA into primary cell cultures or viral transduction of the primary cells if transfection techniques with different transfection reagents are not efficient.

2. In order to confirm that c-Cbl homologues do not functionally replace c-Cbl, we will infect c-Cbl knockdown cells with *R. conorii* and verify that Ku70 is not ubiquitinated in these cells by immunoprecipitating Ku70 from these cells and doing a Western blot on the IP product with commercially available anti-Ubiquitin antibodies.
3. In order to enhance the folding efficiency of the Signal Peptide-Ku70 fusion protein, we will insert a a string of four to five glutamine residues between the C terminus of the Signal peptide and the N terminus of Ku70. Glutamine is used because it has the lowest ability to form secondary structures (Minor and Kim, 1994). This technique has been used and found to be successful for correctly folding and localising a Ku70-GFP fusion protein (Rodgers et al., 2002). Since the polyglutamine chain is able to cause correct folding of a protein with a complex secondary structure like GFP, it is reasonable to expect it to work well with a short Signal peptide.

2. To map the putative signal(s) involved in the communication between *R. conorii* rOmpA outside the cell and c-Cbl inside the cell.

It is unknown at present how c-Cbl in the host cell is recruited to the Ku70-bacteria foci within 1 minute of infection. Since Martinez *et al* showed that *R. conorii* adhesion and entry seem to be separate processes, it is conceivable that the initial adhesion may be the event that recruits c-Cbl to Ku70. We will test this idea and try to delineate the molecular link between the bacterial attachment ligand, rOmpA and c-Cbl.

<u>Subaim 2A: To determine if the presence of rOmpA is required for c-Cbl recruitment to Ku70</u>

We will first test if c-Cbl is capable of localising to bacterial entry foci and ubiquitinating Ku70 in the absence of rOmpA. This should tell us if bacterial adhesion is indeed the triggering factor for c-Cbl dynamics. In our

experiment, we will make use of the fact that rOmpA on the bacterial surface is sensitive to proteases, but rOmpB is only partially so (Li and Walker, 1998). Purified *R. conorii* will be treated with trypsin for different lengths of time. Bacterial lysates will be subjected to Western immunodetection with antibodies against rOmpA (kindly provided to us by the lab that made them) to check that rOmpA is cleaved while rOmpB is intact. The rOmpA-deficient bacteria will then be used to infect tissue culture cells. Using immunofluoresence, we will observe the localization of c-Cbl. We will also track Ku70 ubiquitination by immunoprecipitations and immunodetection of Ku70 and ubiquitin respectively in whole cell lysates. As a complementary approach, we will try to block rOmpA on the bacterial surface by incubation with a range of monoclonal antibodies (obtained from the Walker group) (Li and Walker, 1998). The antibodies should bind rOmpA and prevent it from engaging its receptor. We will then use immunofluorescence to observe the localisation of c-Cbl. Both the above techniques have been successfully utilised by Li and Walker and have been shown to inhibit Rickettsial adhesion to varying degrees (Li and Walker, 1998).

Expected results We expect to see that c-Cbl is not localized to bacterial entry foci and does not ubiquitinate Ku70 when host cells are infected with rOmpA-deficient bacteria.

Potential pitfalls It is possible that the trypsinized rOmpA is still capable of engaging its putative receptor and communicating with c-Cbl, as observed by Li and Walker (Li and Walker, 1998). Owing to the virtual impossibility of genetically modifying *Rickettsiae* (Wood and Azad, 2000), it is not feasible to produce rOmpA mutant bacteria by any other technique.

Alternate Approach The *R. conorii* genome has been sequenced and is available in a public database (http://www.genome.jp/dbget-bin/www_bfind?R.conorii). The rOmpB ORF could be isolated from *R. conorii* by PCR cloning and inserted into a bacterial expression vector. Then *E. coli* bacteria can be transformed with this expression plasmid. When mammalian cells are incubated with the recombinant *E. coli*, the rOmpB should

be sufficient to confer on the *E. coli*, the ability to induce phagocytosis and invade the host cells (Uchiyama et al., 2006). However, since the recombinant E. coli do not express rOmpA, the c-Cbl should not be localised to bacterial entry foci and should not ubiquitinate Ku70 under these conditions. This can be monitored by immunofluorescence against c-Cbl and immunodetection of ubiquitinated Ku70.

Subaim 2B: To identify the putative rOmpA receptor on host cells

It is clear from some early studies done with Typhus group Rickettsiae (Walker and Winkler, 1978; Wisseman et al., 1976) as well as more recent work done with Spotted Fever group Rickettsiae (Li and Walker, 1998; Martinez et al., 2005) that there is a protein component of the host cell membrane that is required for adhesion of *R. conorii*. The first port of communication of bacterial rOmpA with mammalian cells is this putative receptor(s) on the host cell membrane. The identity of this receptor(s) has to be found before the mechanism of communication with c-Cbl can be investigated. We propose to identify this unknown receptor(s) essentially taking the same approach as Martinez *et al*, 2006. We will prepare mammalian whole cell lysates and incubate them with *R. conorii* bacterial lysate. Since some reports support the existence of *R. conorii* receptors in cholesterol-rich membrane domains (Martinez et al., 2005; Ramm and Winkler, 1976), we will use strong detergents like Octylglucoside to extract lipid raft proteins into our mammalian cellular lysates. Since a receptor for a bacterial ligand is likely to be present on the cell membrane, it may be more efficient to use membrane-enriched fractions of the cellular lysates instead of the whole cell lysate. Therefore, we will prepare cell membrane fractions using a commercially available bead-based isolation kit (Qproteome Plasma Membrane Protein Kit from Qiagen Inc). Then we will immunoprecipitate rOmpA and analyse co-immunoprecipitated proteins by SDS-PAGE and silver staining. We will proceed to use mass spectroscopy to identify mammalian proteins interacting with rOmpA. As controls, we will immunoprecipitate rOmpA from bacteria lysate in the absence of mammalian lysate to be able to exclude bacterial proteins that are pulled down and do

immunoprecipitations with unrelated isotypic antibodies. In the event that this method is not productive, we will resort to a screening approach: we will use the yeast two-hybrid system. We will use a commercially available two-hybrid kit (CytoTrap from Stratagene Inc.) and a Human Umbilical Vein Endothelial Cell (HUVEC) cDNA library from the same company. We will subclone the cDNA sequence of rOmpA from *R. conorii* into the bait vector supplied with the Kit and use the HUVEC cDNA library as prey. We will validate our findings by performing loss of function studies in cultured cells. We will do this by RNA interference and short hairpin RNA (shRNA) based knockdown approaches. We will inhibit the expression of the putative receptors and observe whether *R. conorii* are able to adhere to the host cell membrane. As a complementary approach, we will also do blocking experiments where we will 'block' the putative receptors by exposing them to partial blocking peptides and/or antibodies. We will then observe the effect of these treatments on *R. conorii* adhesion.

Expected results We expect to identify host protein(s) interacting with rOmpA by the above experiment. Once we do so, based on the identity of the protein(s), we will perform further experiments to validate our result. By analogy to *L. monocytogenes*, we expect to find that the receptor(s) are integrins or similar adhesion molecules (Pizarro-Cerda and Cossart, 2006).

Potential Pitfall It is possible that there is more than one receptor for rOmpA. Therefore, inhibiting the expression of only one candidate protein at a time may not cause an obvious difference in *R. conorii* adhesion ability.

Alternate Approach Apart from using immunofluorescence as a standard approach to observe the interaction of *R. conorii* with host cells, we will also use flow cytometry as per established procedures (Li and Walker, 1992) to quantitate the numbers of *R. conorii* adhering to host cells after any given treatment condition. A quantitative method should enable even small differences in adhesive ability to be discerned.

Subaim 2C: To identify any protein(s) that may act as bridges of communication between rOmpA and c-Cbl

Once we identify the rOmpA receptor, we will use that information as a starting point to find its links with c-Cbl. In the meantime, we will use a candidate protein approach to identify c-Cbl interacting proteins: we will do co-immunoprecipitation studies of c-Cbl to identify proteins interacting with it at different time points before and after infection. Comparison of the interacting protein profiles in the presence and absence of infection should give some clues as to the major proteins involved. Then we will follow up on candidate proteins in order of their probability of being involved. We will perform *in vitro* binding studies in cell free systems to confirm the binding. Then we will perform loss of function studies in cultured cells to determine the function(s) of the identified proteins in communicating with c-Cbl. We will do this by RNA interference and short hairpin RNA (shRNA) based knockdown approaches.

Expected results Based on previously published information (Martinez and Cossart, 2004), we know that Focal Adhesion Kinase, PI3-kinase, c-Src and several actin-interacting proteins like Cdc42 and Cortactin are involved in the signaling pathway that arises when mammalian cells are parasitized by *R. conorii*. It has also been reported that c-Cbl responds to integrin mediated signalling by interacting with Src and p85 (Ojaniemi et al., 1997). We therefore expect to see these proteins in our experiments.

Potential Pitfall c-Cbl may have more interacting partners than is reasonably feasible to identify by mass spectrometry. c-Cbl is involved in many cellular processes and has functions distinct from its ubiquitin ligase activity (Swaminathan and Tsygankov, 2006). There could potentially be noise from unrelated protein interactions that can cause ambiguity in our experiments.

Alternate Approach We will use a candidate approach based on our knowledge of known c-Cbl interacting proteins. A compendium of c-Cbl interactions (an interactome) has been published (Schmidt and Dikic, 2005). c-Cbl functions in integrin-mediated adhesion by acting as an adaptor protein. It binds to several cytoskeletal

interacting proteins like paxillin, Rac, Rho, Vav, CrkL and so on (Schmidt and Dikic, 2005). We will focus on these proteins since they are known to be recruited to and interact with c-Cbl in the context of adhesion.

3. **To investigate the mechanism of how ubiquitination of Ku70 enables *R. conorii* to enter a host cell.**

We will test our hypothesis that ubiquitination of Ku70 by c-Cbl leads to endocytosis of the bacterium into the cell. We will first employ immunofluorescence to identify if any endocytic proteins are recruited to the bacterial entry foci during *R. conorii* infection of mammalian cells. We will investigate the presence of clathrin-dependent endocytic proteins (Clathrin, AP2, EEA1 and Dynamin) as well as a caveolar endocytic protein, Caveolin 1. We will also repeat the above experiment in c-Cbl deficient cells. This should tell us if the recruitment of endocytic proteins (if any) is a Cbl-dependent process. In performing the above experiments, we will always use the uptake of fluorescently-labelled Transferrin (for clathrin-mediated endocytosis), Albumin (for caveolar endocytosis) and fluorescently-labelled Dextran (for non-caveolar lipid raft mediated endocytosis) (Choudhury et al., 2006) as positive controls to ensure that the effects we observe are not due to unrelated defects in the endocytosis machinery. In another approach, we will inhibit clathrin-mediated endocytosis with commercially available pharmacological inhibitors like Concanavalin A (Smith et al., 2001) and Phenylarsine Oxide (Boudin et al., 2000). We will also express dominant negative Eps15 (to inhibit clathrin-mediated endocytosis) and dominant negative Dynamin 2 (to inhibit caveolar endocytosis) (Parton and Richards, 2003). We will also disrupt lipid rafts in living cells by treating them with methyl-β- Cyclodextrin, which extracts the cell membrane cholesterol (Ohtani et al., 1989). Then we will investigate if *R. conorii* is capable of being internalised or not. In all the above experiments, apart from using immunofluorescence, we will use transmission electron microscopy as a tool to monitor the endocytic process. The distinct morphological differences between clathrin dependent endocytic vesicles and caveolar endocytosis should be clear in electron

micrographs. Therefore, electron microscopy will help us distinguish between the type(s) of endocytosis occurring (if any) during Rickettsial entry.

We will also transfect and overexpress mutant versions of c-Cbl that have been shown to be defective in ubiquitin ligase activity *in vitro* and *in vivo* (Joazeiro et al., 1999; Thien et al., 2001). Mammalian expression constructs of c-Cbl harbouring the RING domain mutations C381A and W408A have been kindly provided to us by the group that generated them. We will perform immunodetection of immunoprecipitated Ku70 in the mutant Cbl overexpressing cells both before and after infection. This will tell us if Ku70 is capable of being ubiquitylated in the absence of c-Cbl activity. We will also perform immunofluorescence experiments to monitor the recruitment of endocytic proteins and the entry of *R. conorii* in the mutant overexpressing cells.

Expected Result If our hypothesis is true, we expect to see the recruitment of essential proteins of the endocytic machinery to the sites of bacterial entry on a time-scale compatible with the bacterial invasion. This recruitment should be abolished when c-Cbl is absent and when the mutant c-Cbl is overexpressed. The absence of endocytic proteins at the bacterial entry sites should correspond to decreased number of *R. conorii* inside the host cells, as seen with immunofluorescence and electron microscopy techniques. When endocytosis is inhibited with drugs, *R. conorii* should be unable to enter the host cells. Thus, the above experiments will test both the necessity and sufficiency of endocytosis for bacterial invasion.

Conclusion

We plan to do a series of experiments to identify the molecular players involved in the *R. conorii* infection process. Understanding the mechanism of infection of *R. conorii* will not only provide us with better tools to combat Rickettsial Spotted fevers, but will also enlarge our current understanding of cellular endocytosis and cytoskeletal dynamics and their exploitation by pathogenic organisms.

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