Clathrin-mediated endocytosis of a lipid-raft-associated protein is mediated through a dual tyrosine motif

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Summary

We have previously shown that the integral membrane protein CD317 has both a conventional transmembrane domain near its N-terminus and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor. With the possible exception of a minor topological variant of the prion protein, there remain no other convincing examples of a mammalian protein with such a topology. CD317 is localised to cholesterol-rich lipid microdomains ('lipid rafts') in the plasma membrane and is internalised from the cell surface for delivery to a juxta-nuclear compartment (most probably the TGN). We have now investigated the mechanism by which CD317 is internalised and find that this raft-associated integral membrane protein is internalised through a clathrin-dependent pathway, internalisation is dependent upon a novel dual-tyrosine-based motif in the cytosolic domain of CD317, the cytosolic domain of CD317 can interact with the μ subunits of the AP2 and AP1 adaptor complexes, interaction with AP1 is required for delivery of CD317 back to the TGN, and removal of the GPI anchor from CD317 reduces the efficiency of CD317 internalisation. Collectively, these data indicate that CD317 is internalised and delivered back to the TGN by the sequential action of AP2 and AP1 adaptor complexes and that, surprisingly, the clathrin-mediated internalisation of CD317 occurs more efficiently if CD317 is localised to lipid rafts.

Supplementary material available online at
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Introduction

CD317 (also known as BST2 or HM1.24 antigen) (Goto et al., 1994; Vidal-Laliena et al., 2005) is an integral membrane protein that has been shown to play a role in regulating the growth and development of B cells, and to be highly expressed in human myeloma cells (Goto et al., 1994; Ishikawa et al., 1995; Ohtomo et al., 1999). The precise function of CD317 is, however, unclear. It was initially described as a being present at the cell surface (Goto et al., 1994; Ishikawa et al., 1995; Ohtomo et al., 1999), but we subsequently demonstrated that it also resides in an intracellular pool (most probably the TGN) and that it cycles between this intracellular pool and the cell surface. Furthermore, we showed that CD317 has an unusual topology, having both a conventional transmembrane domain near the N-terminus and a glycosyl-phosphatidylinositol (GPI) anchor at the C-terminus (Kupzig et al., 2003). CD317 resides – at least at the cell surface – in cholesterol-rich lipid microdomains ('lipid rafts') with the transmembrane domain apparently lying outside the lipid raft and with the raft localisation being dependent upon the GPI anchor at the C-terminus of the protein (Kupzig et al., 2003). There has been much debate concerning the size and longevity (even existence) of lipid rafts (sphingolipid- and cholesterol-rich microdomains in cell membranes), however, there is considerable evidence that lipid rafts are small and highly dynamic (Simons and Toomre, 2000). They have been implicated as being important in membrane trafficking, as helping to segregate proteins for delivery to specific locations and in cell signalling, by providing a platform for the assembly of signalling complexes (Simons and Toomre, 2000). In the case of B cells, lipid rafts appear to have a role in B cell activation as platforms for B cell receptor (BCR) signalling (reviewed in Dykstra et al., 2001). This is particularly pertinent given the fact that human CD317 is expressed on terminally differentiated B cells and at high levels on multiple myeloma cell lines (Ohtomo et al., 1999). These observations led us to hypothesise that CD317 plays a role in both trafficking and signalling events. We previously speculated that the lumenal domains of CD317 molecules would be in a position to bind cargo proteins for delivery to a specific location, whereas their cytosolic domains would be positioned to bind cytosolic proteins involved in trafficking events (e.g. adaptor or coat proteins) and considered that, because CD317 is present in the apical membrane of polarised cells – consistent with the fact that it possesses both a GPI anchor and two N-linked glycans, both implicated in the targetting of proteins to apical membranes of polarised cells (Gut et al., 1998; Lisanti et al., 1989; Lisanti et al., 1991; Scheiffele et al., 1995) – CD317 might play a role in apical trafficking pathways. We now show that the cytosolic domain of CD317 does bind adaptor proteins

and that, intriguingly for a lipid-raft-associated membrane protein, it is internalised through a clathrin-mediated pathway. Indeed, clathrin-mediated endocytosis of CD317 is more efficient if it is raft associated than if it is not. Furthermore, interaction between the cytosolic domain of CD317 and the μ2 subunit of the AP2 adaptor is dependent upon a new motif that contains two tyrosine residues, whereas efficient delivery of CD317 to the TGN following internalisation is dependent upon the sequential action of AP2 and AP1 adaptor complexes.

Results

FRAP analysis of CD317-GFP

We previously demonstrated in antibody uptake experiments that CD317 is internalised from the cell surface and delivered to an intracellular compartment where it colocalises with TGN38, a marker of the trans Golgi network (TGN) (Kupzig et al., 2003). To study this process in living cells, and as a first step in discerning the molecular mechanisms involved, we made use of H4IIE cells that stably expressed GFP-tagged CD317. The GFP fluorescence signal emanating from the TGN was photobleached and the recovery of fluorescence (fluorescence recovery after photobleaching, FRAP) in the photobleached region recorded. As can be seen from the images presented in Fig. 1A and supplementary material Movie 1, the fluorescence signal in the photobleached region recovered within minutes. The data presented are representative of several experiments performed with cells that had been pre-incubated in the presence of 200 μg/ml cycloheximide for 1 hour prior to imaging (and in the continued presence of cycloheximide during imaging) to ensure that any recovery of the TGN signal is not because of re-population of the TGN by newly synthesised CD317. Rather, the re-population of the intracellular pool of CD317 is due to internalisation of CD317 from the cell surface, a result consistent with antibody-uptake data (Kupzig et al., 2003). In fact, structures that may be trafficking intermediates transporting CD317 from the cell surface to the intracellular pool can be observed in supplementary material Movie 1.

CD317 is internalised through a clathrin-dependent pathway

We considered it unlikely that CD317 is internalised through clathrin-mediated endocytosis (CME) given its localisation to lipid rafts, but felt that it was important to exclude this pathway before considering other routes by which CD317 might be internalised. We therefore performed antibody-uptake experiments on cells that had been transiently transfected with a Myc-epitope-tagged, dominant-negative form of AP180 (AP180-C-myc) that

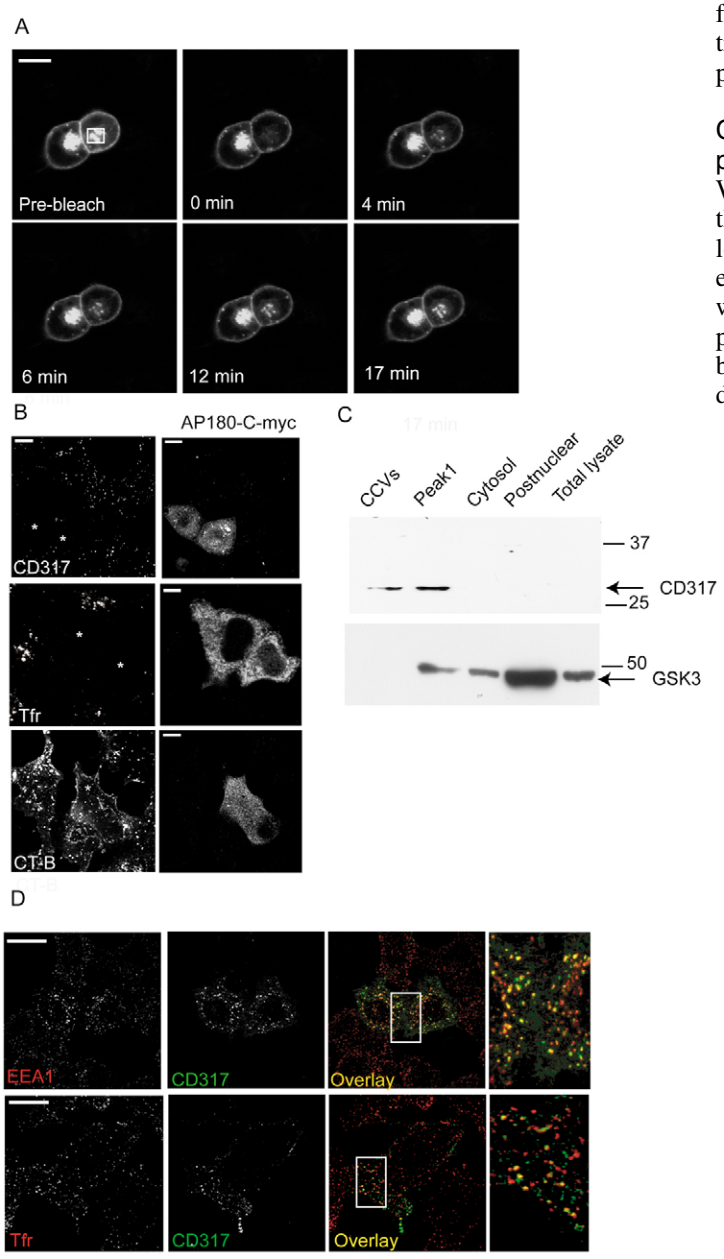

Fig. 1. CD317 is internalised through a clathrin-dependant pathway. (A) H4IIE cells transiently expressing CD317-GFP were imaged and the highlighted area was photo-bleached and allowed to recover for 17 minutes. Images were taken every minute, Cells were incubated in the presence of 200 μg/ml cycloheximide for 1 hour prior to imaging (and in the continued presence of cycloheximide during imaging). Bar, 10 μm. (B) Transiently transfected H4IIE cells expressing dominant-negative AP180-C-myc were subjected to uptake of transferrin (Tfr), cholera-toxin-B subunit (CT-B) or CD317 antibody uptake for 15 minutes: cells were then acid washed to remove any non-internalised material. Asterisks denote transfected cells. Bars, 10 μm. (C) Immunoblot of fractions obtained during the isolation of purified clathrin-coated vesicles (CCVs) from rat brain, which had been separated by SDS-PAGE (12% gel), using anti-CD317 antibody (upper panel), or anti-GSK3 antibody (lower panel). Lane 1, purified CCVs; lane 2, peak 1 from the purification of CCVs (containing vesicles depleted in CCVs); lane 3, cytosol; lane 4, post-nuclear fraction; lane 5, total rat brain lysate. The same blot was initially probed with anti-GSK3 antibody, stripped and re-probed with anti-CD317 antibody. The fractions are the same as those previously described and characterised (Korolchuk and Banting, 2002). (D) HeLA cells transiently transfected with a construct encoding wild-type CD317. After 24-hour- expression, cells were subjected to antibody uptake (using a CD317 antibody) and Tfr uptake for 2 minutes, or just antibody uptake for 6 minutes. Images show colocalisation between EEA1 or Tfr and CD317 as indicated. Indicated areas have been enlarged. Colocalisation between EEA1 and CD317 is 64.1% (n=310) and between Tfr and CD317 40% (n=233). Bars, 10 μm.

consists of the clathrin-binding domain of the adapator protein AP180. This construct has been shown to be an effective competitor for clathrin binding and, so, significantly slows down CME (Ford et al., 2001; Zhao, 2001). Expression of AP180-C-myc had no detectable effect on the uptake of fluorescently labelled cholera toxin B (CTB) subunit (Fig. 1B, lower panels), a protein that is internalised through a clathrin-independent pathway (Orlandi and Fishman, 1998). By contrast, and as expected, cells expressing AP180-C-myc failed to internalise fluorescently labelled transferrin [a molecule internalised through CME (Hanover et al., 1984)] (Fig. 1B, middle panels). They also failed to internalise anti-CD317 antibodies (Fig. 1B, upper panels), indicating that CD317 is internalised through a clathrin-dependent pathway. Consistent with this, immunoblot analysis showed CD317 to be present in purified clathrin-coated vesicles (Fig. 1C, upper panel) whereas a control protein, glycogen synthase kinase 3 (GSK3), was not (Fig. 1C, lower panel). These vesicles were purified as previously shown, are enriched in clathrin and exclude PRK (Korolchuk and Banting, 2002). GSK3 is a cytosolic protein that can associate with a host of cellular proteins to give the enzyme diverse subcellular localisations (Jope and Johnson, 2004); however, we are unaware of any reports of it being associated with clathrin-coated vesicles.

Furthermore, internalised CD317 colocalises with both internalised transferrin (40%, after 2 minutes) and EEA1 (64.1% after 6 minutes) (Fig. 1D). These are both well characterised markers of the CME pathway. Internalised anti-CD317 antibody was not detected in LAMP-1/CD63 positive late endosomes at any time post internalisation (data not shown). These data suggest that CD317 is delivered back to the TGN through early endosomes and not through late endosomes.

Role of the cytosolic domain of CD317 in internalisation

Having shown that internalisation of CD317 occurs through a clathrin-dependent pathway, we asked which region of CD317 is required for that internalisation. To address this question, constructs encoding N-terminally truncated forms of GFP-tagged CD317 were generated and expressed transiently in COS7 cells (Fig. 2A). The most extreme truncation, lacking the entire N-terminal cytosolic domain of CD317, was efficiently expressed but the protein failed to exit the ER (presumably due to misfolding; data not shown). CD317-GFP that lacks only the N-terminal 12 amino acids was efficiently expressed and delivered to the cell surface (Fig. 2A, MDER); however, in antibody-uptake experiments it was observed that this truncated protein is inefficiently internalised from the cell surface (Fig. 2A, MDER).

The N-terminal 12 amino acids of human, rhesus monkey, mouse and rat CD317 contain two conserved tyrosine residues (Y6 and Y8). We considered that either of these residues might serve as the core of a tyrosine-based internalisation motif (Boll et al., 1996; Kirchhausen, 1997; Marks et al., 1997) and thereby constructed mutant forms of CD317-GFP in which each of these tyrosine residues was individually mutated to alanine. These constructs were then expressed transiently in COS7 cells and antibody-uptake experiments performed. Antibody was efficiently internalised in cells expressing either the Y6A mutant or the Y8A mutant (Fig. 2A, MAPSF*A*HY and MAPSFYH*A*). Therefore we mutated both Y residues to A and expressed the construct transiently in COS7 cells. There was no uptake of antibody in these cells (Fig. 2A, MAPSF*AHA*). Thus, there is either a redundant tyrosine-based internalisation motif in the cytosolic domain of CD317 or the internalisation motif is one that requires two Y residues. The latter would be a new scenario but analogous to the situation that has been described for P-selectin, where the Y3 residue is important for the interaction with μ2 (Owen et al., 2001). In either case, the cytosolic domain of CD317 would be expected to bind to the μ2 subunit of AP2. To test this possibility, we performed pull-down assays of His-tagged thioredoxin (His-TRX)–μ2 fusion proteins using streptavidin-coated

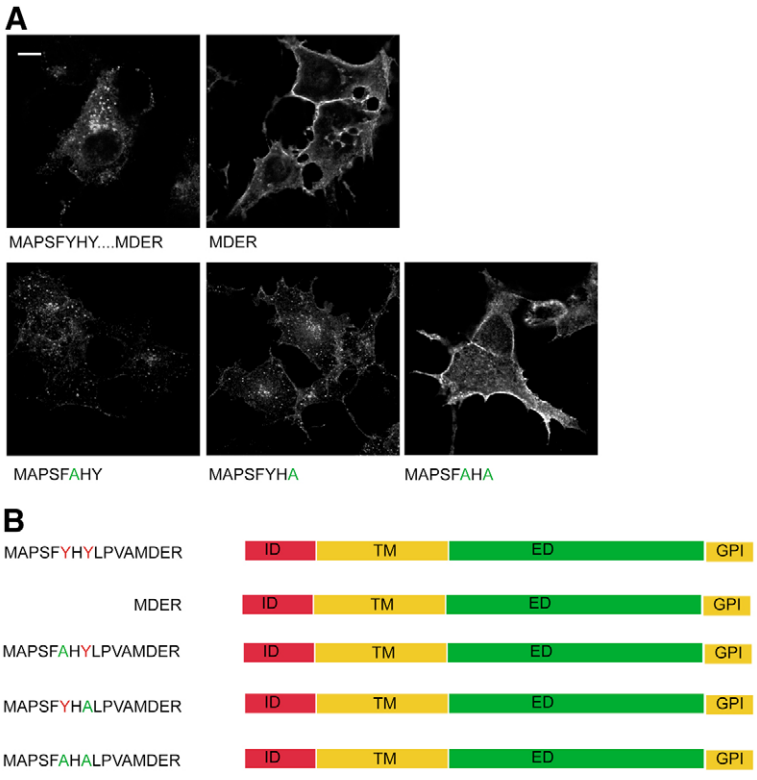

Fig. 2. The role of the cytosolic domain in the internalisation of CD317. (A,B) COS cells were transiently transfected with constructs encoding either full-length, truncated or mutated CD317 as indicated and subjected to anti-CD317 antibody uptake for 15 minutes. CD317-antibody uptake is clearly inhibited in cells expressing either truncated CD317 (MDER panel) or CD317 with both Y6A and Y8A mutations (MAPSFAHA panel), as shown by the accumulation of antibody at the plasma membrane in these cells. By contrast, the punctate intracellular fluorescent signal shows that there is efficient internalization of CD317 in cells expressing either the Y6A (MAPSFAHY) or Y8A (MAPSFYHA) mutations. The cytosolic N-terminal sequence of full-length rat CD317 is MAPSFYHYLPVAMDER. The MDER construct lacks the N-terminal 12 amino acids of the cytosolic domain, the MAPSF*A*HY construct has a Y6A mutation, the MAPSFYH*A* construct has a Y8A mutation and the MAPSF*AHA* construct has the point mutations Y6A and Y8A. All mutations are in the context of the full-length cytosolic domain. Bar, 10 μm. ID, intracellular domain; TM, transmembrane domain; ED, extracellular domain and GPI; glycosylphosphatidylinositol anchor.

sepharose beads linked to a biotin-tagged peptide encoding the entire cytosolic domain of CD317. Beads coated with the CD317 peptide pulled down both the His-TRX–μ1 and the His-TX–μ2 fusion proteins but failed to pull down a control fusion protein (His-TRX–lactate-dehydrogenase), whereas biotin-coated control beads failed to pull down any of the fusion proteins (Fig. 3A). The interactions between the peptide and fusion proteins were abolished by pre-incubation with a peptide containing the CD317 tyrosine motif (see Materials and Methods) but left intact by an unrelated peptide, thereby demonstrating the specificity of the interactions.

Internalisation of CD317 is dependent on μ2

The results of the preceding pull-down experiments suggested that AP2 is required for the internalisation of CD317. To test this hypothesis we used a previously described small interfering RNA (siRNA) (Motley et al., 2003) to knock down expression of μ2 in HeLa cells (Fig. 3B, μ2 siRNA lane); a control siRNA targeting lamin A/C (Elbashir et al., 2001) had no effect on expression of μ2 (Fig. 3B, lamin A/C siRNA lane). Fluorescently labelled transferrin, fluorescently labelled EGF and antibody-labelled CD317 were efficiently internalised in HeLa cells transfected with siRNA targeting lamin A/C (Fig. 3C, top panels), but knock down in expression of μ2 following transfection of HeLa cells with siRNA targeting μ2 led to an inhibition of the internalisation of fluorescently labelled transferrin and of antibody-labelled CD317 but had no effect on EGF uptake (Fig. 3C, bottom panels). Previous work has shown that μ2 knock-down has no effect on EGF internalisation (Motley et al., 2003). These data were quantified in Fig. 3D. This result is entirely consistent with the clathrin-mediated endocytosis of CD317 being dependent upon the cytosolic domain of CD317 interacting with the μ2 subunit of AP2.

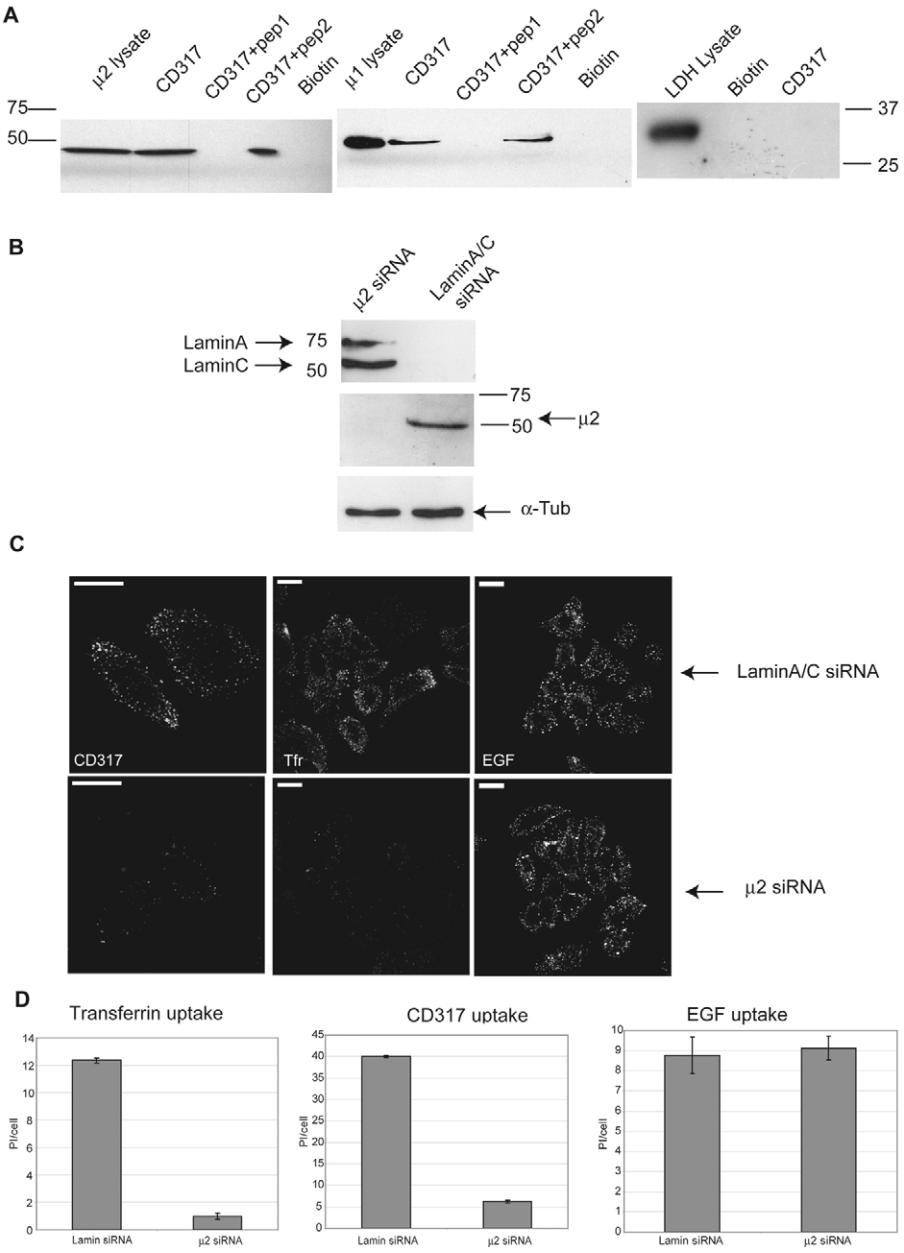

Fig. 3. The cytosolic domain of CD317 binds the AP1 and AP2 adaptor-complex subunits μ1 and μ2. (A) Immunoblots, probed with an anti-His tag antibody, of pull-down assays between a biotinylated N-terminal peptide of CD317 and His-tagged thioredoxin (His-TRX)-fusion proteins of the μ subunits of the AP1 and AP2 adaptor complexes or LDH. Lanes are labelled according to the loaded lysate (10% of input for pull-down) ; biotin-labelled lanes represent material isolated using biotin-coated beads and CD317-labelled lanes material isolated using beads coated with biotinylated CD317 peptide, pep1 and pep2 refer to specific and non-specific competing peptides. Markers indicate molecular mass in kDa. (B) Immunoblots of lysates from HeLa cells that had been transfected with siRNA targeting μ2 (μ2 siRNA) or siRNA targeting lamin A/C (lamin A/C siRNA) and probed with anti-μ2 or anti-lamin antibodies as indicated. Blots were then stripped and reprobed with an anti-tubulin antibody as a loading control. (C) HeLa cells that had been transfected with siRNA targeting μ2 or siRNA targeting lamin A/C (as indicated) were used for uptake of transferrin, EGF or CD317 antibody for 15 minutes and then acid-washed to remove any non-internalised material. Bars, 10 μm. (D) Quantification of the data presented in C. PI/cell, pixel intensity per cell. Transferrin uptake in μ2-knockdown cells is 8% of control (n=106 and 168), CD317 uptake in μ2-knockdown cells is 15.2% of control (n=20 and 21), EGF uptake is not affected (n=118 and 92).

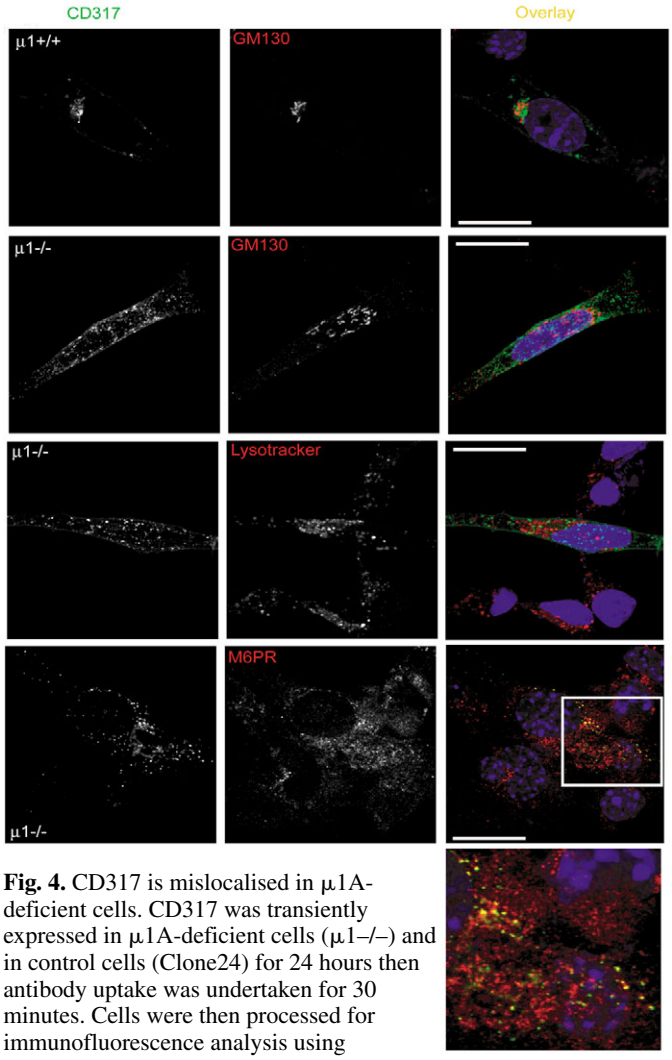

Fig. 4. CD317 is mislocalised in μ1A-deficient cells. CD317 was transiently expressed in μ1A-deficient cells (μ1–/–) and in control cells (Clone24) for 24 hours then antibody uptake was undertaken for 30 minutes. Cells were then processed for immunofluorescence analysis using antibodies against either GM130 or M6PR, or processed for fluorescence analysis using Lysotracker. Colocalisation between CD317 and M6PR is 43.9% n=129. Bars, 10 μm.

Delivery of CD317 from the early endosome to the TGN is dependent on μ1A

The results of the pull-down experiments also led us to ask whether depletion of μ1A from cells expressing CD317 has any effect on CD317 localisation. A cell line derived from μ1A knockout mice (μ1A$^{-/-}$), in which AP1 is ablated, has been described previously (Meyer et al., 2000). We made use of this cell line and of a control cell line (24A clone 4) in which AP1 expression has been re-constituted by expression of μ1A in the μ1A$^{-/-}$ cells (Meyer et al., 2000), to study the effects of μ1A knock out on CD317 localisation. Rat CD317 (Kupzig et al., 2003) was transiently expressed in both μ1A$^{-/-}$ cells and 24A clone 4 cells and antibody uptake experiments were carried out for 30 minutes. As expected, and consistent with previous observations in other cell types (Kupzig et al., 2003), after 30 minutes uptake the CD317 molecules were detected in a juxtanuclear position (adjacent to GM130, a marker of the Golgi complex) in control 24A clone 4 cells (Fig. 4, first row of panels). Colocalisation with the TGN marker TGN38 was not possible because endogenous levels of this protein are depleted in the knockout cells (our unpublished data). However, a significant proportion of the CD317 signal in μ1A$^{-/-}$ cells came from dispersed punctate structures that were separate from the Golgi complex (Fig. 4, second row of panels). This population of CD317 showed minimal colocalisation with markers of either early (EEA1) or late (LAMP2) endosomes (data not shown) or with lysosomal markers (e.g. lysostracker, Fig. 4, third row of panels). The subcellular distribution of M6PR, which normally follows a retrograde pathway from early endosome to TGN (Carlton et al., 2004; Megideshi and Schu, 2003), has previously been shown to be affected in μ1A$^{-/-}$ cells (Meyer et al., 2000). We therefore considered that internalised CD317 and M6PR might colocalise in μ1A$^{-/-}$ cells – which indeed was the case (Fig. 4, row 4).

Removal of the GPI anchor impedes CD317 internalisation

Since CD317 possesses both a conventional transmembrane domain and a GPI anchor, with the GPI anchor residing within a 'lipid raft' domain and the transmembrane domain lying outside the raft (Kupzig et al., 2003), we reasoned that removal of the GPI anchor (by incubation of cells in the presence of phosphoinositol-dependent phospolipase C, PI-PLC) would release the protein from the raft and thereby affect its internalisation. We hypothesised that this release from raft domains would allow CD317 to be internalised more readily. To address this hypothesis, H4IIE cells were incubated in the presence or absence of 5 units/ml PI-PLC for 1 hour – conditions, which we have previously demonstrated, efficiently release GPI-anchored proteins from their GPI anchor at the plasma membrane (Kupzig et al., 2003) – prior to CD317-antibody-uptake analysis. CD317 was more efficiently internalised in control cells than in PI-PLC-treated cells (Fig. 5A, upper panels). The majority of surface-bound CD317 antibody is internalised during a 20 minute incubation with control cells, whereas a significant amount of CD317 antibody remains at the surface of PI-PLC-treated cells (Fig. 5A, upper panels). By contrast, PI-PLC treatment had no effect on the uptake of fluorescently labelled transferrin (Fig. 5A, lower panels).

Lipid-raft-associated membranes are found near the top of sucrose gradients following centrifugation of membranes that have been solubilised in ice-cold Triton X-100 (Brown and London, 1998). So, in order to complement our cell-imaging studies (Kupzig et al., 2003) and to provide further evidence that incubation of cells in the presence of 5 units/ml PI-PLC for 1 hour does lead to the release of CD317 from lipid rafts, ice-cold Triton-X-100-solubilised membranes from control and PI-PLC-treated H4IIE cells were subjected to centrifugation on sucrose gradients (see Materials and Methods), and fractions from those gradients were analysed by SDS-PAGE and immunoblot using an anti-CD317 antibody (Fig. 5B). As we showed previously, the majority of CD317 from control cells is detected in the lighter fractions (predominantly fraction 3 in this case) of the gradient (Fig. 5B, upper panel) (a result consistent with lipid raft association of CD317). However, CD317 from cells that have been incubated in the presence of PI-PLC is distributed across denser fractions (7-9) of the gradient (Fig. 5B, lower panel), i.e. is no longer associated with lipid rafts.

To quantify the effect of PI-PLC treatment on CD317 internalisation we took a biochemical approach. Using an adapted biotinylation method (Gampel et al., 2006) (see Materials and Methods) we quantified the amount of biotinylated CD317 and transferrin receptor that was internalised at different time points (Fig. 5C). At 8 minutes all of the surface-biotinylated CD317 had been internalised in the control cells, whereas only 19% of the bioinylated CD317 was internalised in the PI-PLC-treated cells (Fig. 5C). Thus, to our surprise, removal of the GPI anchor from CD317 decreased the rate at which CD317 was internalised and delivered to an intracellular compartment, implying that raft association of CD317 is required for its efficient internalisation. These experiments were also done using fluorescently labelled transferrin and antibody-labelled CD317, and representative images of cells showing 2-, 4- and 8-minute chases at 37°C (followed by an acid wash) are shown in the supplementary material Fig. S1. These results are in agreement with the biochemical data that removal of the GPI-anchor impedes CD317 internalisation.

Discussion

The data we present show that CD317 is internalised and delivered back to the TGN through a clathrin-dependent pathway that requires two different heterotetrameric adaptor complexes (AP2 and AP1) to act sequentially. The recognition of CD317 by AP2 is dependent upon a dual-tyrosine motif in the cytosolic domain of CD317 being recognised by the μ2 subunit of the adaptor. Internalised CD317 is then delivered to an early endosomal compartment, but fails to reach late endosomes, en route to the TGN. Thus CD317 must be sorted for delivery back to the TGN from an early endosomal compartment. This is reminiscent of the pathway followed by other molecules, such as TGN38 (Kupzig et al., 2003; Mallet and Maxfield, 1999; Roquemore and Banting, 1998), shiga toxin (Saint-Pol et al., 2004; Tai et al., 2004), GPP130 and GP73 (Puri et al., 2002), which follow what has been described as 'the late-endosome independent TGN38/46 pathway' (Puri et al., 2002). In the case of CD317, this sorting step is dependent upon recognition of the cytosolic domain of the protein by the μ1 subunit of the AP1 adaptor complex.

It is also of note that CD317, an integral membrane protein that has previously been shown to be associated with lipid rafts (Kupzig et al., 2003), is internalised through a clathrin-mediated pathway. Whether CD317 remains associated with lipid rafts during internalisation or is removed from them during internalisation has not been addressed during the current studies, but it is clear that lipid raft association is required for efficient internalisation of CD317 because removal of the GPI anchor leads to a reduction in the efficiency of internalisation of CD317. There used to be a fairly implicit assumption that clathrin-mediated endocytosis and the internalisation of proteins associated with lipid rafts were essentially independent processes (e.g. Parton and Richards, 2003). However, there is a growing body of evidence to suggest that, in certain circumstances, there is a clear link between lipid rafts and clathrin-mediated endocytosis, and that – in some cases – raft domains and the proteins they contain might actually be internalised through clathrin-mediated endocytosis. Thus, it has recently been shown that cholera-toxin entry into pig enterocytes occurs through a lipid raft and clathrin-dependent mechanism (Hansen et al., 2005), that in motor neurons tetanus toxin is

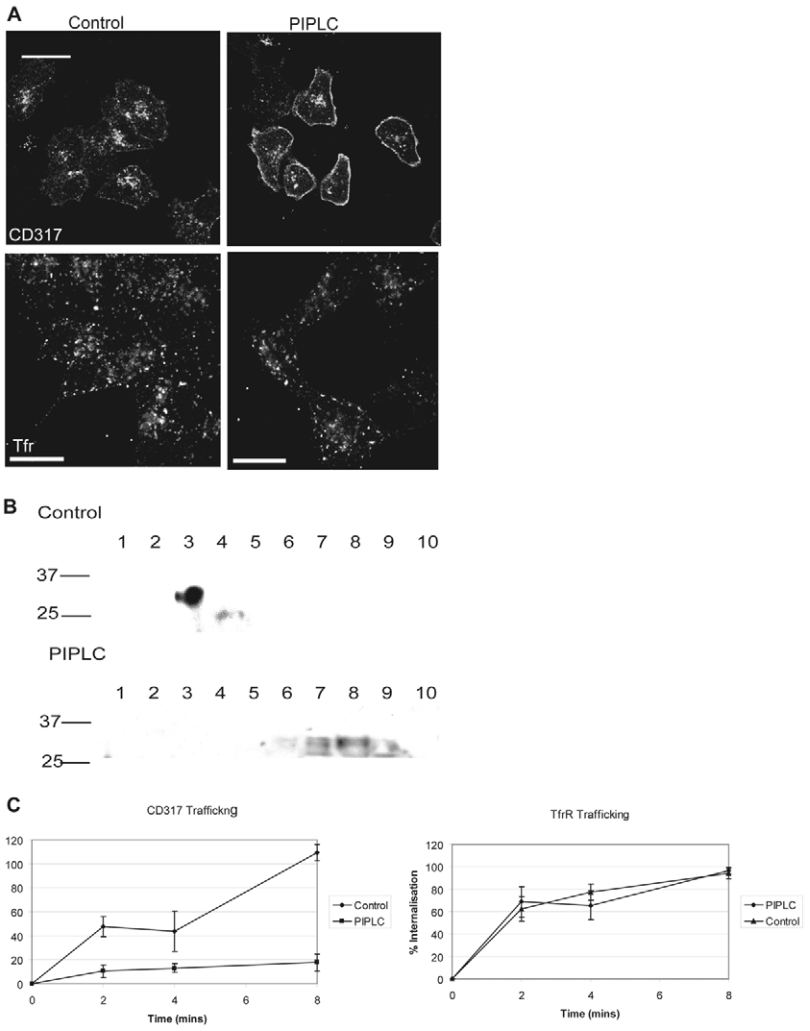

Fig. 5. Removal of the GPI anchor impedes CD317 internalisation. (A) H4IIE cells were incubated for 1 hour at 37°C in serum-free medium with or without PI-PLC as indicated, and subjected to anti-CD317 antibody uptake for 20 minutes at 37°C prior to fixation and processing for immunofluorescence analysis. Bars, 10 μm. (B) Immunoblot analysis of fractions from sucrose-density-gradient separation of H4IIE cell lysates from control cells (Control) and from cells that had been incubated in PI-PLC prior to lysis (PIPLC). Fractions were taken from the top of the gradient (i.e. fraction 1 is the most buoyant), and blots were probed with CD317 antibody. (C) Surface proteins of HeLa cells were treated with PI-PLC or serum-free medium and then biotinylated. Endocytosis was allowed to proceed for 0, 2, 4 or 8 minutes after which surface biotin was removed. Internalised proteins were pulled down with streptavidin beads and analysed by western blotting with antibodies against transferrin receptor and CD317.

internalised through a clathrin-dependent mechanism initiated within lipid rafts (Deinhardt et al., 2006), anthrax toxin is endocytosed through a receptor that is internalised through a raft-mediated and clathrin-dependent process (Abrami et al., 2003), and that the lipid raft associated apolipoprotein E receptor 2 (apoER2) is internalised through a clathrin-mediated pathway (Cuitino et al., 2005). These recent studies complement earlier work that also supports the notion that certain raft-associated proteins may be internalised through clathrin-mediated endocytosis, e.g. the B-cell receptor has been shown to cluster into lipid rafts following ligand binding (Cheng et al., 1999) but it is then internalised through a clathrin-dependent mechanism (Stoddart et al., 2002). The data we have presented on the internalisation of CD317 further emphasise the fact that association of proteins with lipid rafts and the internalisation of proteins by clathrin-mediated endocytosis should not necessarily be considered as being mutually exclusive events.

As previously reported (Kupzig et al., 2003), there are some similarities between CD317 and the prion protein PrP. One is that the only reported mammalian protein to share the topology shown by CD317 is a minor topological isoform of PrP (Hegde et al., 1998). However, the majority of PrP exists in a GPI-anchored form that, intriguingly – given that it possesses no cytosolic domain, can be internalised through a clathrin-mediated pathway (Shyng et al., 1994). The explanation for this observation appears to be that the extracellular domain of PrP associates with that of an integral membrane protein whose cytosolic domain links to the machinery required for clathrin-mediated endocytosis (Shyng et al., 1995). PrP and its integral membrane partner thus form a complex whose topology mimics that of CD317 and both PrP and CD317 are examples of proteins that are internalised from lipid rafts through clathrin-mediated endocytosis.

Materials and Methods

Strains and culture conditions

COS-7 (Gluzman, 1981) and H4IIE (Pitot et al., 1964) and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, UK), 10% foetal calf serum and 1% penicillin-streptomycin in a 5% CO_2 atmosphere at 37°C. Mouse embryonic fibroblasts (control and μ1A-deficient) have been described previously (Meyer et al., 2000). Cells were treated with 200 μg/ml cycloheximide for 1 hour or 5 units/ml phosphoinositol-dependent phospolipase C (PI-PLC) for 1 hour in serum-free medium for 60 minutes before fixing or live cell imaging.

Immunofluorescence and confocal microscopy

Cells were fixed with either 3.7% paraformaldehyde (PFA) followed by permeabilisation with 0.1% Triton X-100, or with methanol (–20°C for 2 minutes) and blocked in 3% bovine serum albumin (BSA)-PBS for 1 hour as previously described (Kupzig et al., 2003). Cells for live imaging were maintained in Krebs-Ringer solution at 37°C in custom-made holders (Kupzig et al., 1999). Transfection of COS-7 and H4IIE cells was performed on cells plated on coverslips the previous day using FuGene (Roche, Germany) according to the manufacturer's instructions. Stably transfected cells were selected by the addition of 400 μg/ml G418 (neomycin) to the culture medium. Transgene expression (driven by the cytomegalovirus, CMV, promoter) was induced by the addition of 10 μm sodium butyrate overnight as previously described (Girotti and Banting, 1996). Dual immunolabelling was done by incubating with the primary antibody (appropriately diluted in blocking buffer) for 1 hour, washing with PBS and incubating with the relevant Alexa Fluor®-594 or Alexa Fluor-488 conjugated secondary antibody (appropriately diluted in blocking buffer) for 30 minutes (Kupzig et al., 2003). Fixed cells were imaged using a Leica TCS-NT confocal laser scanning microscope equipped with a Kr-Ar laser (488 nm, 594 nm and 647 nm lines) attached to a Leica DMRBE upright epifluorescence microscope (Leica, Germany). Live cells were imaged using a Leica DM IRBE inverted epifluorescence microscope. All images were collected using a 63× oil immersion objective and processed with Leica and Adobe Photoshop software, additional movies were made using Quicktime Pro.

siRNA knockdowns

Knockdown of μ2 was done as previously described (Motley et al., 2003), using the same 5′-AACACAGCAACCUCUACUUGG-3′ sequence (MWG, Germany). The same method was used to knockdown LaminA/C using the 5′-CUGGA-CUUCCAGAAGAACA-3′ (Elbashir et al., 2001) sequence (MWG, Germany). Cells were either plated onto coverslips in six-well plates for transfection and/or uptake assays or onto 35-mm dishes for western analysis of the knockdown efficiency.

Antibodies, probes and plasmids

The following rabbit polyclonal antibodies were used: SK-2, raised against a GST-CD317 fusion protein (Kupzig et al., 2003), affinity purified and used at a dilution of 1:100 in immunofluorescence and a dilution of 1:1000 for immunoblotting; anti-BST-2 (abcam) and M6PR (a kind gift from Paul Luzio, Cambridge Institute for Medical Research, Cambridge, UK) at 1:100 for immunoflourescence; μ2 (Genway Biotech, CA) and Lamin A/C (Santa Cruz). Monoclonal antibodies used were: 9E10 (Abcam) raised against a synthetic peptide of Myc (Evan et al., 1985); anti-transferrin receptor (Zymed); anti-GSK3 (Transduction Labs); anti-EEA1 (Becton Dickinson Transduction Labs, BD transduction), a marker of early endosomes, used at 1:200 for immunofluorescence; anti-6×His-epitope-tag (ABR), used at 1:100 for immunoblotting; and anti-GFP (Clontech) used at 1:200 for antibody-uptake experiments.

Transferrin–Alexa-Fluor-594 and EGF–Alexa-Fluor-488 (Molecular Probes) were used at 100 μg/ml for uptake experiments, CT-B–Alexa-Fluor-488 (Molecular Probes) was used at 50 μg/ml for uptake experiments and LysoTracker® (Molecular Probes) was used at 75 nM for 2 minutes to label acidic compartments.

H4IIE cells were stably transfected with pCIneo- CD317-GFP, (Kupzig et al., 2003). pCIneo-MDER-GFP is a deletion mutant of pCIneo-CD317-GFP and pCIneo-A6Y8-GFP, pCIneo-Y6A8-GFP and pCIneo-A6A8-GFP were made by site-directed mutagenesis using the Quickchange kit (Stratagene).

pET32a μ1ΔN and pET32a μ2ΔN: cDNA encoding rat μ1 and μ2, and pET32aLDH have been described previously (Stephens and Banting, 1997). Truncation of the μ sequences ensured that the expressed proteins were soluble, whereas the vector contains a His tag for purification purposes. The AP180-C-Myc construct (Ford et al., 2001) was a generous gift from Harvey McMahon (MRC, LMB, Cambridge, UK).

Antibody, transferrin and CT-B uptake

Cells were grown to 50% confluency on coverslips in six-well plates and transiently transfected with the given construct. After 24-hour expression the plates were chilled on ice for 10 minutes then appropriate concentrations of GFP antibody, SK-2 antibody, CT-B–Alexa-Fluor-594 or Transferrin–Alexa Fluor-594 – all in serum-free medium – were kept on ice for a further 15 minutes. Coverslips were then immediately transferred to warmed medium and uptake was allowed to start at 37°C and continue for the time stated. Coverslips were then acid washed three times with ice-cold glycine (pH 2, 150 mM) to remove any remaining surface-bound antibody, fixed and processed for immunofluorescence.

Purification of clathrin-coated vesicles and detergent-resistant membranes.

Clathrin-coated vesicles were purified as described previously (Korolchuk and Banting, 2002). Detergent-resistant membranes were prepared and separated on sucrose-density gradients as previously described (Kupzig et al., 2003), except that cells were grown to confluency on twelve 22-mm coverslips and then incubated in serum-free medium in the presence or absence of 5 units/ml PI-PLC for 1 hour at 37°C prior to processing. 1-ml fractions were taken from the sucrose gradients, TCA (trichloroacetic acid)-precipitated, resuspended in sample buffer and separated on a 15% SDS polyacrylamide gel.

FRAP analysis

Cells were either grown to 50% confluency and transfected with the stated construct, or stably transfected cells were grown to 50% confluency and induced with 10 μM sodium butyrate. After 24-hour expression, coverslips were treated with serum-free medium with or without 5 units/ml PI-PLC for 1 hour before being transferred to the appropriate holders (Kupzig et al., 1999) and used for FRAP analysis (Bastiaens and Pepperkok, 2000). Cells were incubated in the presence of 200 μg/ml cycloheximide (Sigma) for 2 hours before imaging and throughout the imaging period to ensure that any observed fluorescence recovery was not due to protein synthesis. The highlighted areas of cells were photobleached by scanning with the Ar-Kr laser at 64× magnification and at 100% transmission for ten scans. Cells were then scanned at 25% transmission at 64× magnification for the time course given. For the FRAP analysis with and without PI-PLC, the data were taken from seven different experiments and the results were analysed using SigmaPlot.

Endocytosis assay

Endocytosis assay was adapted from the method described by Gampel et al (Gampel et al., 2006). HeLa cells, grown on 22-mm glass coverslips to confluency, were incubated with PI-PLC (5 units/ml) for 1 hour at 37°C. Cells were then transferred

to ice and washed with PBS. Surface proteins were labelled on ice with 1 mg/ml NHS-SS-biotin in borate buffer (10 mM orthoboric acid, 154 mM NaCl, 7.2 mM KCl, 7.2 mM $CaCl_2$) for 30 minutes. Excess biotin was quenched with glycine, washed with PBS and returned to ice. Cells were transferred to pre-warmed medium and left for 2, 4 or 8 minutes for endocytosis to proceed. A zero-minute time point was also taken and total surface protein determined. The cells for the zero-, 2-, 4- and 8-minute time points were then treated with 100 mM MesNa (sodium salt of 2-mercaptoethanesulphonic acid; Sigma) in 100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.6), 02% BSA for a 5-minute and 10-minute incubation on ice. The cell-impermeable MesNa removes surface-bound biotin whereas internalised biotinylated proteins are protected. Excess MesNa was quenched with 100 mM iodoacetic acid (Fluka). Cells were then lysed in buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 0.05% NP-40, 0.1% SDS, 0.5% deoxycholic acid) and biotinylated proteins were pulled down on immobilised strepdavidin. Proteins were then eluted in sample buffer containing 10% β-mercaptoethanol and 100 mM DTT, separated by 12% SDS PAGE, transferred to PVDF and probed with the appropriate antibody. Bands were quantified using the molecular analyst (Bio-rad) software and means were taken from three experiments.

Pull-down assays

Batches of LB broth (500 ml) with BL21(DE)3 bacteria, transformed with the pET32a μ1ΔN and pET32a μ2ΔN or pET32aLDH were grown for 4 hours post induction. Cells were harvested and resuspended in 10 ml buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM PMSF) and bacteria were lysed with six 15-second high-intensity bursts of sonication at 4°C. The interaction between the recombinant His-tagged μ-chain fusion proteins and a synthetic biotinylated peptide of the CD317 N-terminus, MAPSFYHYLPVAMDERWEPKGWSIRR-Biotin (synthesised by Graham Bloomberg, peptide synthesis facility, Bristol University, UK), was tested. This peptide was immobilised on streptavidin-coated agarose beads (Sigma) according to the manufacturer's instructions and the beads were blocked with biotin (Vector labs). Beads without CD317 peptide but biotin-blocked, were also prepared as a negative binding control. Beads were blocked with 3% BSA-PBS. At this point lysate was pre incubated with specific (MAPSFYHYLPC) and non-specific (GRDEYDEVAMPV) competing peptides. Bacterial whole-cell lysate (5 mg) was then incubated with 25 μl beads for 30 minutes at room temperature before the beads were washed three times for 10 minutes in 5 ml resuspension buffer (20 mM Tris-HCl pH 7.0, 100 mM NaCl). At this point, beads were either snap-frozen in liquid nitrogen for storage or heated to 70°C with SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 12% (w/v) glycerol, 2 mM EDTA, 0.01% Bromophenol Blue) to elute any bound protein. Eluted protein was then run on a 12% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose membrane for immunoblot analysis using antibody against the His-tag on the recombinant proteins.

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