The Molecular Basis for the Endocytosis of Small R-SNAREs by the Clathrin Adaptor CALM

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SUMMARY

SNAREs provide a large part of the specificity and energy needed for membrane fusion and, to do so, must be localized to their correct membranes. Here, we show that the R-SNAREs VAMP8, VAMP3, and VAMP2, which cycle between the plasma membrane and endosomes, bind directly to the ubiquitously expressed, PtdIns4,5P2-binding, endocytic clathrin adaptor CALM/PICALM. X-ray crystallography shows that the N-terminal halves of their SNARE motifs bind the CALMA(NT) domain as helices in a manner that mimics SNARE complex formation. Mutation of residues in the CALM:SNARE interface inhibits binding in vitro and prevents R-SNARE endocytosis in vivo. Thus, CALM:R-SNARE interactions ensure that R-SNAREs, required for the fusion of endocytic clathrin-coated vesicles with endosomes and also for subsequent postendosomal trafficking, are sorted into endocytic vesicles. CALM’s role in directing the endocytosis of small R-SNAREs may provide insight into the association of CALM/PICALM mutations with growth retardation, cognitive defects, and Alzheimer’s disease.

INTRODUCTION

SNAREs (Soluble NSF Attachment Protein REceptors) are small membrane-anchored proteins that lie at the heart of the vesicle:organelle and organelle:organelle membrane fusion machinery, providing much of the energy and specificity required for membrane fusion (Hong, 2005; Jahn and Scheller, 2006; Sutton et al., 1998). As with all membrane proteins, SNAREs must be positioned in their appropriate cellular location in order to function correctly. In recent years, it has become apparent that the cell possesses mechanisms for transporting SNAREs between its various membranes alongside standard (non-SNARE) cargo. Here, we investigate the molecular mechanism by which the SNAREs VAMP8, VAMP3, and VAMP2 are internalized from the plasma membrane.

There are at least 38 SNAREs in mammalian cells (Bock et al., 2001; Hong, 2005; Kloepper et al., 2007). Most contain a single conserved helical SNARE motif of 60–70 residues, although SNAP23, SNAP25, and SNAP29 contain two (Jahn and Scheller, 2006). N-terminal to their SNARE motifs, most SNAREs have a folded region that varies in length from 100–150 residues and is usually either a three helical Habc domain or a longin domain (reviewed in Hong, 2005). SNARE complexes are formed when four SNARE motifs come together as a tetrameric coiled-coil (Sutton et al., 1998). Three of these SNARE motifs are associated with one membrane and derive from the so-called Q-SNAREs, while the other SNARE motif is provided by an R-SNARE that resides in the membrane that will fuse with the first membrane (Fasshauer et al., 1998). It is this relative orientation of the (Q-) and (R-) SNAREs that draws the two membranes close enough to drive their fusion. The specificity of vesicle:organelle and organelle:organelle fusion arising from the limited combinations of SNAREs that can form complexes can only come about if the localization of SNAREs is itself controlled. For instance, SNAREs must be transported to a given organelle membrane so that they can subsequently be sorted into transport vesicles and tubules leaving that membrane since this enables these transport vesicles/tubules to fuse, ultimately, with their desired target membrane, into which the correct cognate SNAREs must have already been placed.

The active sorting of SNAREs into transport vesicles/tubules is achieved primarily by direct interaction with components of the vesicle/tubule’s protein coat, although transmembrane helix length may also play a role (Sharpe et al., 2010). Initial mechanistic descriptions of active SNARE sorting came from studies on COPII coated vesicles, which mediate ER to Golgi transport (Mancias and Goldberg, 2007; Mossessova et al., 2003). In post-Golgi trafficking, the sorting of Vti1b by EpsinR (Miller et al., 2007) and of VAMP7 by Hrb (Pyrck et al., 2008) and AP3 (Martinez-Arca et al., 2003) are mediated by the direct interactions of the folded N-terminal domains of the SNAREs with the...
VAMP2, VAMP3, and VAMP8 endocytosis. The three SNAREs (Hong et al., 1999; McPherson et al., 1999) are incorporated into transport vesicles.

VAMP8 and VAMP3 cycle between the cell’s limiting membrane and early endosomes/recycling endosomes and thus mediate the fusion of vesicles with both compartments, whereas VAMP2 drives the fusion of fast-recycling synaptic vesicles with the plasma membrane (Antonin et al., 2000; Grote et al., 1995; McMahon et al., 1993). None of these three SNAREs have a conventional cargo motif (such as the ExxLL motif found on VAMP4 [Peden et al., 2001], nor do they possess folded N-terminal domains. However, preceding their SNARE motifs there are short regions of 10–30 residues that are predicted to be unstructured (Ellena et al., 2009; Fiebig et al., 1999; Hazzard et al., 1999). The question thus arises: how are these SNAREs sorted into endocytic clathrin-coated vesicles? Based on the observations that both Vti1b and VAMP7 can be transported as part of a cis-SNARE complex (Miller et al., 2007; Pryor et al., 2008), one possibility was that VAMPs 8, 3, and 2 could be internalized as a complex with their acceptor (Q) SNAREs (a syntaxin and a SNAP) (Gordon et al., 2009). However, endocytic and synaptic vesicles contain a large excess of these small R-SNAREs over their acceptor (Q) SNAREs (Takamori et al., 2006) and in addition, the steady state localization of VAMPs 8, 3, and 2 is endosomal, whereas that of syntaxins 1–4 and SNAPs is at the plasma membrane. Together, these observations suggest that trafficking of small R-SNARE VAMPs as part of a complex is unlikely.

In this study, we provide structural, biochemical and in vivo data to show that the internalization of VAMP8, and consequently that of the highly related small R-SNAREs, VAMP3 and VAMP2, is mediated by specific, direct interactions between the N-terminal halves of their SNARE motifs and the ANTH (AP180 N-terminal Homology) domain of the endocytic clathrin adaptor CALM.

RESULTS

CALM Is Involved in the Endocytosis of VAMPs 2, 3, and 8

Recent in vivo studies (Harel et al., 2008) suggested that the ubiquitously expressed CALM and possibly its neuronal specific homolog AP180 (Dreyling et al., 1996; Lindner and Ungewickell, 1992; Yao et al., 2003), may play a role in the trafficking of VAMP2 in mammalian cells. This supported earlier work in D. melanogaster (Bao et al., 2005), C. elegans (Nonet et al., 1999), and yeast (Burston et al., 2009), suggesting that these organisms’ single ANTH domain containing clathrin adaptor proteins were involved in the endocytosis of short endocytic R-SNARE VAMPs.

To confirm a role for CALM in the trafficking of VAMP2 and to show that CALM was similarly involved in endocytosis of the related small endocytic R-SNAREs VAMP3 and VAMP8 (Hong, 2005), we investigated the effect of CALM depletion on VAMP2, VAMP3, and VAMP8 endocytosis. The three SNAREs were tagged with a double HA epitope at their (lumenal) C-termini (termed VAMP2-HA, VAMP3-HA, and VAMP8-HA) (Gordon et al., 2009) and were stably expressed in HeLa cells at relatively low levels (Figure S1A available online). Depletion of CALM by siRNA caused all three SNAREs to accumulate on the cell surface (Figure 1A). To investigate the rate of endocytosis of the three HA-tagged VAMPs, we used a flow cytometry-based antibody uptake assay (as outlined in Figure S1B). In control cells, the three SNAREs were rapidly internalized, with 40–60% of the prebound antibody already inside the cells after 5 min. However, when CALM was depleted, antibody uptake was negligible even after 30 min (Figure 1). These effects are considerably more dramatic than previously reported (Harel et al., 2008), presumably because we expressed the constructs at lower than endogenous levels so as not to saturate the intracellular sorting machinery. The inhibition of VAMP endocytosis was rescued by expression of Myc-tagged, full-length, siRNA-resistant CALM, indicating that the phenotype is specific and not an off-target effect (Figure 1B and Figure S1C). In agreement with previous studies (Harel et al., 2008; Huang et al., 2004), the endocytosis rates of standard cargoes such as EGF and TfR were not significantly altered by CALM depletion (Figure S1D).

VAMPs 2, 3, and 8 Bind Directly to CALMANTH

To investigate the possibility of a direct interaction between any of these small R-SNAREs and the ANTH domain of CALM (defined as residues 19–289 [Ford et al., 2001], we carried out “GST pull-down” binding assays using recombinantly expressed proteins. As shown in Figure 2A the three SNAREs interacted directly with the CALMANTH domain in a concentration-dependent manner. Isothermal titration calorimetry (ITC) was used to quantify the strength of binding. The interaction between GST-VAMP8 and CALMANTH is the tightest with a Kd of ~18 µM (Figure 2B), which is typical for a dynamic cargo/coat interaction (Owen et al., 2004; Pryor et al., 2008). Binding of the CALMANTH domain to GST-VAMP3 and GST-VAMP2, although similar to one another (~46 µM and ~43 µM respectively), were weaker than the binding to VAMP8 (Figure 2B). These differences in affinities are reflected in the relative rates of endocytosis of the three VAMPs (Figure 1B). No interaction could be detected between any of the three R-SNAREs and the related ENTH domain of the endocytic clathrin adaptor, epsin1.

The ANTH domains of CALM and neuronal specific AP180 share 82% sequence identity. However, in our hands, the ANTH domain of mammalian AP180 showed no significantly measurable binding to mammalian VAMPs 2, 3, or 8 by GST pull-downs or surface plasmon resonance (SPR) (Figure S2A and S2B), despite being correctly folded as indicated by circular dichroism (CD) (data not shown) and being competent to bind PtdIns(4,5)P2 similarly to CALMANTH by ITC (Figure S2C) and liposome-based SPR (data not shown). We have no simple explanation for this, but note that AP180 and CALM are not functionally redundant (Bushlin et al., 2008; Harel et al., 2008) and that the single ANTH domain containing clathrin adaptor in lower organisms is more like CALM than AP180 (Harel et al., 2008).

Taken together with the in vivo studies published by others and presented here, our biochemical data point to the direct
binding of the VAMPs 2, 3, and 8 by CALM being directly responsible for the endocytosis of these small R-SNAREs. Hence, we set out to investigate the molecular basis for this interaction using a combination of structure determination and in vitro and in vivo assays using wild-type and structure-directed mutant versions of both proteins. Because their sequence similarities (Figure 3A) and similar binding affinities indicated a conserved mechanism of interaction for the three SNAREs, for technical reasons we characterized at the structural level only the interaction between CALM ANTH and the R-SNARE to which it bound most tightly, VAMP8.

The N-Terminal Half of the VAMP8 SNARE Motif Binds to CALM ANTH

In order to delineate which portion of VAMP8 binds to CALM ANTH, a number of deletion constructs were made (Figure 3B), an approach we deemed acceptable since several studies (Fasshauer et al., 1997; Fiebig et al., 1999; Hazzard et al., 1999) have shown small VAMPs to have no secondary structure in solution.

Figure 1. CALM Depletion Abolishes Endocytosis of HA-Tagged VAMPs
(A) Effect of CALM depletion on surface expression of HA-tagged VAMPs. Control and siRNA-treated cells expressing HA-tagged VAMP2, VAMP3, or VAMP8 were mixed together, fixed without permeabilization, and labeled with anti-HA, then permeabilized and labeled with anti-CALM. Knocking down CALM increases the surface expression of all three VAMPs. The scale bar represents 20 μm.

(B) Endocytosis of anti-HA in cells expressing HA-tagged VAMPs. Antibody was bound to the cells at 4°C, then the cells were warmed to 37°C for 2–30 min and antibody remaining at the cell surface was quantified by flow cytometry. Each point is derived from at least three separate experiments; the error bars show the SEM. Knocking down CALM effectively abolishes the uptake of all three VAMPs. The specificity of the knockdown phenotype was demonstrated by stably transfecting VAMP8-HA-expressing cells with siRNA-resistant myc-tagged CALM (CALMres wt) and knocking down endogenous CALM. Expression of the CALM construct almost completely rescues the knockdown phenotype. See also Figure S1.

GST pull-down experiments using truncated versions of VAMP8 (Figure 3C) revealed that the minimal region necessary for binding was residues 10–41 i.e., the N-terminal half of the SNARE motif. ITC showed that the binding of residues 10–41 of VAMP8 was almost identical (KD ~20 μM) to that of wild-type VAMP8 (KD ~18 μM) (Figure 3D), indicating that all the determinants for CALM binding are contained within residues 10–41. Significantly, the corresponding regions of mammalian VAMP2 and of the yeast endocytic VAMP Snc1p contains two key residues, Val43 and Met46 (VAMP2 numbering), which, when mutated to alaines, blocks their endocytosis (Grote et al., 1995; Lewis et al., 2000). In agreement with these data, we found that mutation of these residues to alaines in VAMP2-HA and likewise of the analogous residues in VAMP3-HA (Val30 and Met33) and VAMP8-HA (Lys24 and Met27) blocked their internalisation from the cell surface (Figures 3E and S3).

The VAMP8 SNARE Motif Binds in Place of the CALM ANTH Final Helix

Attempts were made to crystallize a complex of CALM ANTH with a peptide corresponding to residues 10–41 of VAMP8, both with the two components free in solution and with residues 10–41 of VAMP8 appended on the C terminus of CALM ANTH. While these cocrysallizations did not yield the structure of a complex, they did yield a 1.8Å resolution structure (final R/RFree 0.182/0.216; see Table S1) of the unliganded ANTH domain with two molecules in the asymmetric unit. In both molecules the positions of
the first ten helices are identical. The C-terminal 25 residues of the ANTH domain were poorly ordered in one molecule of the asymmetric unit, as in the published CALM ANTH structure determined from crystals of a different spacegroup (1HF8; Ford et al., 2001), while in the other molecule the C terminus was constrained by crystal packing and formed a four-turn helix (Figures 4 and S4A). The CALM ANTH structures presented here in conjunction with the previously published structure (Ford et al., 2001) suggested that residues 265–289 of CALM are not integral to the ANTH domain but are flexible and associate only weakly with the domain’s core residues (19–264). Inspection of the CALMANTH surface showed that the elongated patch on which the ordered short helix 11 and preceding ten residues of CALM sat was hydrophobic in nature and of the correct dimensions to bind an α helix and was thus a good candidate for the interaction site for the hydrophobic SNARE motif of a VAMP (Figure 4B). If this hydrophobic trough were indeed the VAMP binding site on CALMANTH, then deletion of helix 11 would be expected to increase VAMP8 binding. However, a truncated CALM ANTH construct missing helix 11 and the preceding ten residues, termed CALM ANTH(1-264), unexpectedly showed no binding to VAMP8 by GST pull-down experiments and ITC (Figures 4C and 4D) while still displaying normal binding to PtdIns4,5P2 containing liposomes by SPR (data not shown). Multiangle light scattering (MALS) indicated that in solution CALMANTH(1-264) is in fact a tight dimer (Figure S4B). This was confirmed by the determination of the structure of CALM ANTH(1-264) at 1.7Å resolution (final R/R free 0.170/0.191; see Table S1). The dimeric structure explained the lack of VAMP8 binding since the dimer interface (burying 2100Å² of accessible surface area in total (Krisinel and Henrick, 2007)) was the proposed VAMP8 binding site (Figures 4E and 4F). As there were no other significant changes between the surfaces of CALM ANTH and CALM ANTH(1-264), these data strongly indicated that the hydrophobic trough in which helix 11 (when formed) sits was indeed the VAMP binding site on CALM ANTH.

### Figure 2. CALM Binds Directly to VAMP8, VAMP3, and VAMP2

(A) GST Pull downs using His6MycCALM ANTH and the GST-fusion proteins indicated. Top: Coomassie blue stained gel. Lower: western blot probed with anti-myc. In this and all subsequent experiments, the lane adjacent to the Molecular weight markers (MWM) is loaded with His6MycCALM ANTH only. The ANTH domain of CALM binds directly to VAMP2, 3, and 8 in a concentration dependent manner.

(B) ITC quantitating the binding of CALM ANTH to VAMP2, 3, and 8 (black squares). The Adaptor:SNARE interaction was tightest for VAMP8 (K_D = 17 ± 1 μM) and weaker for both VAMP3 and VAMP2, (46 ± 7 μM and 48 ± 4 μM, respectively). Epsin ENTH showed no VAMP binding (black triangles) indicating the CALM:SNARE interaction is specific. Data for epsin1 is offset by −0.3 μcal/sec for clarity. See also Figure S2.
Structure of a CALM\textsubscript{ANTH}(1-264):VAMP8\textsubscript{(11-41)} Chimeric Complex

In an attempt to obtain a structure of a complex between CALM\textsubscript{ANTH} and VAMP8, a construct was designed in which residues 11–41 of VAMP8 were fused through an artificial linker of six residues to residues 1–264 of CALM\textsubscript{ANTH}. The resulting chimeric protein (CALM\textsubscript{ANTH}(1-264):VAMP8\textsubscript{(11-41)}) crystallized and its 2.0 Å resolution structure (final R/\textit{R}_\text{free} 0.183/0.199; see Table S1) showed unambiguously (Figures 5 and 6) that residues 15–38 of VAMP8 form a single α helix with residues between Phe16 and Arg37 contacting CALM\textsubscript{ANTH}, consistent with the truncation data (Figure 3). The VAMP8 helix fitted into the spatially complementary groove of CALM\textsubscript{ANTH}, lined by residues from helix 9 and helix 10, which was blocked by helix 11 in the unliganded CALM\textsubscript{ANTH} structures and by dimerization in CALM\textsubscript{ANTH}(1-264). There was a small movement of helices 9 and 10 with respect to each other that caused a slight widening of the groove as compared with the unliganded domain structure (Figure S5). No electron density was visible for 17 residues that connect the last ordered residue of CALM with the first of VAMP8. This would allow considerable flexibility in the positioning of the VAMP8 and indeed in the crystals where residues 1–76 of the full cytoplasmic portion of VAMP8 (residues 1–76) comprises residues 10–41.

Figure 3. The N-Terminal Half of the Small R-SNARE Vamps Binds to CALM\textsubscript{ANTH}

(A) Sequence alignment of the SNARE motifs of VAMP2, VAMP3, SnC1p, and VAMP8. Conserved residues are boxed in gray. The position of mutated residues in VAMP8: L16 and V23 (open triangles), K24 and M27 (black circles), L44 and L51 (open circles) are indicated.

(b) Schematic representation of truncation mutants of VAMP8.

(C) GST pull-down experiments using His6MycCALM\textsubscript{ANTH} and the GST-fusion proteins indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. The minimal fragment of VAMP8 that is able to bind to CALM with a similar affinity as the full cytoplasmic portion of VAMP8 (residues 1–76) comprises residues 10–41.

(D) ITC quantitating the binding of CALM\textsubscript{ANTH} to truncated VAMP8. VAMP8\textsubscript{10-41} (black triangles) exhibited an essentially identical binding affinity to CALM\textsubscript{ANTH} as VAMP8\textsubscript{1-76} (black squares) (K\textsubscript{D} = 17 ± 1 μM and 20 ± 3 μM respectively). Data for VAMP8\textsubscript{10-41} is offset by −1.3 μcal/s for clarity.

(E) Localization of anti-HA in cells expressing different wild-type or mutant HA-tagged VAMPs. The cells were allowed to endocytose the antibody for 40 min, then processed for immunofluorescence. The cells expressing wild-type VAMP2-HA, VAMP3-HA and VAMP8-HA have endocytosed the antibody, but the cells expressing the VAMP2-HAV43AM46A, VAMP3-HAV30AMetA, and VAMP8-HAK24AM27A mutants have retained the antibody on the plasma membrane. The scale bar represents 20 μm. See also Figure S3.
it bound to CALM<sub>ANTH</sub>, a VAMP8 SNARE motif could reach an adjacent ANTH domain to which it was not covalently linked.

Key residues in the interaction, 13 on VAMP8 and 16 on CALM, are spread throughout the interface between the two proteins (Figure 5). This shows that the binding is not a short, linear motif-mediated interaction that is typical of standard adaptor/cargo interactions. Eleven of the thirteen key interacting residues are conserved between VAMPs 8, 3, and 2 (Figure 3A), suggesting that they bind CALM<sub>ANTH</sub> in a similar manner. The residues in the CALM<sub>ANTH</sub>-VAMP8 interface are highly conserved from human to fish. The interface is mainly hydrophobic in nature, burying a total of 1500Å² of accessible surface area (Krissinel and Henrick, 2007), and a full list of interacting residues is given in Figure 5D. The residues Lys24 and Met27 of VAMP8 play key roles in the interaction, explaining their initial identification as residues of importance for VAMP endocytosis (Grote et al., 1995) (Lewis et al., 2000). Mutation of these residues to alanines in VAMP8 abolished the interaction with CALM<sub>ANTH</sub> in vitro as shown by GST pull downs and quantified by ITC (Figures 6D and 6E), as did mutations of the analogous residues in VAMP2 and VAMP3 (data not shown). The single Met27Ala mutation when introduced into VAMP8 abolished the VAMP8-CALM interaction, whereas the Lys24Ala mutation on its own only weakened the interaction. The latter is to be expected since in VAMP2 and VAMP3, which also bind directly to CALM<sub>ANTH</sub>, albeit more weakly, the equivalent residues are valines. When transferred to the in vivo situation, the non-CALM binding Lys24AlaMet27Ala mutant of VAMP8 accumulated on the cell surface and failed to be internalized (Figures 6H and 6I).

GST pull-down experiments demonstrated that mutation of hydrophobic residues in CALM<sub>ANTH</sub> that participate in VAMP8 binding, Leu219Ser, Phe240Ala, Met244Lys, Ile247Asp, and Leu251Ser, all abolished the interaction between the two proteins in vitro (Figures 6B and 6C) without affecting the fold (as judged by expression levels and circular dichroism, data not shown). Two of these, Leu219Ser and Met244Lys, whose lack of binding was confirmed by ITC (Figures 6B and 6C), were introduced into myc-tagged, siRNA-resistant CALM. When transfected into cells expressing VAMP8HA, depletion of endogenous CALM by siRNA caused the SNARE to be retained at the plasma membrane, because it could no longer be internalized (Figures 6F and 6G). This is in contrast to cells transfected with wild-type myc-tagged siRNA-resistant CALM, which retained the ability to internalize VAMP8HA upon endogenous CALM depletion (Figures 6F and 6G, 1B, and 1C). When taken together, the data presented in this study demonstrate that VAMP8 and the similar small R-SNAREs VAMP3 and VAMP2 are internalized from the plasma membrane by clathrin-mediated endocytosis due to a direct interaction with the clathrin adaptor CALM.

SNARE Complex Formation and Binding by CALM Are Mutually Exclusive Processes for VAMP8

Superposing the structure of the N-terminal half of the VAMP8 SNARE motif with the same portion of VAMP8 from the SNARE complex formed from VAMP8, Syntaxin7, Syntaxin8, and Vti1b (Antonin et al., 2002) demonstrates that, not only does VAMP8 adopt the same gently curving conformation in both complexes, but when the complex structures are overlayed via the VAMP8 molecules they contain, helices 9 and 10 of CALM<sub>ANTH</sub> superimpose on the SNARE motifs of Syntaxin8 and Syntaxin7, respectively (Figure 7A). Comparison of the two complex structures thus demonstrates that CALM binding and SNARE complex formation by VAMP8 must be mutually exclusive processes. This is confirmed by recombinant protein binding experiments, which show that GST-VAMP8 binds CALM<sub>ANTH</sub>, but GST-VAMP8 complexed with SNAP23 and syntaxin3 (residues 195–253) does not (Figure 7B). It is important to note that the Lys24AlaMet27Ala, non-CALM binding mutant of VAMP8 cannot be internalized but, as we show in this work, is able to form SNARE complexes with its cognate plasma membrane SNARE partners. These observations show that, contrary to a previous proposal (Gordon et al., 2009), the ability of short R-SNAREs to form SNARE complexes and the ability to be endocytosed must in fact be independent of each other.

CALM<sub>ANTH</sub> Binds VAMP8 and PtdIns4,5P<sub>2</sub> Simultaneously

Since the binding sites on CALM<sub>ANTH</sub> for PtdIns4,5P<sub>2</sub> and a VAMP are on opposite ends of the ANTH domain, it is probable that both sites could bind simultaneously to their ligands. If this were indeed the case, avidity effects would result in greatly increased binding of CALM to a membrane containing both ligands when compared to the binding to membranes containing only one of the ligands, i.e., the K<sub>d</sub> of the apparent binding to liposomes containing both ligands would be much lower than the average value of the individual K<sub>d</sub> for the two ligands measured separately. To investigate whether or not simultaneous PtdIns4,5P<sub>2</sub> and VAMP binding does indeed occur, the binding of CALM to liposomes containing PC/PE/PtdIns4,5P<sub>2</sub>, PC/PE+VAMP8 and PC/PE/PtdIns4,5P<sub>2</sub>+VAMP8 was compared using lipid-based SPR. Figure 7C shows that indeed there is an almost order of magnitude decrease in K<sub>d</sub> (i.e., increase in apparent affinity) over the average of the K<sub>d</sub>s for the individual ligands when both ligands are present in the same liposome membrane. Thus CALM must be able to bind simultaneously to PtdIns4,5P<sub>2</sub> and VAMP8.

Conformation of Membrane-Embedded Vamps when Bound to CALM

Given that CALM<sub>ANTH</sub> can bind PtdIns4,5P<sub>2</sub> and a small R-SNARE VAMP simultaneously and based on the structural identification of the binding sites for a VAMP and PtdIns4,5P<sub>2</sub>, the following deductions can be made. To allow simultaneous binding to a membrane-embedded VAMP and PtdIns4,5P<sub>2</sub> to occur, the amino-terminal half of the VAMP SNARE motif must be able to reach its binding site, which is at a distance of around 50Å from the membrane surface. Thus, the 38 residues between the end of the CALM binding sequence and the start of the transmembrane helix of a VAMP8 molecule must be able to stretch at least this far. The published NMR and CD studies and our own CD measurements (data not shown) indicate that in fact the entire cytoplasmic domain of short VAMPs has no secondary structure in solution (Fasshauer et al., 1997; Fiebig et al., 1999; Hazzard et al., 1998). In such an unstructured conformation,
Figure 4. Structure of the CALM<sub>ANTH</sub> Domain
(A) Ribbon representation of the structure of the CALM<sub>ANTH</sub> domain colored from pink (residue 19) to purple (residue 288). Helices are numbered as in (Ford et al., 2001) with no α6. The insets show 2Fo–Fc electron density contoured at 1.2 σ for the well- and poorly-ordered α11 helix in the two CALM<sub>ANTH</sub> molecules in the asymmetric unit (designated chains A and B).
(B) Surface representation of helices α1–α10 of the CALM<sub>ANTH</sub> domain colored from high (dark green) to low (white) hydrophobicity oriented as in (A). The hydrophobic groove in which helix α11 and the ten preceding residues sit can be clearly seen.
(C) GST pull-down experiments using GST, GSTVAMP8, and the His<sub>6</sub>MycCALM<sub>ANTH</sub> proteins indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. Residues 1–289 but not 1–264 of CALM<sub>ANTH</sub> bound to GSTVAMP8.
(D) ITC quantitating the binding of residues 1–264 of CALM<sub>ANTH</sub> to VAMP8. CALM<sub>ANTH</sub>(1-264) (black triangles) showed no measurable binding to VAMP8 whereas WT CALM<sub>ANTH</sub>(1-289) bound with a K<sub>d</sub> of 20 ± 1 μM (black squares). Data for CALM<sub>ANTH</sub>(1-264) is offset by −0.2 μcal/sec for clarity.
(E) Structure of the CALM<sub>ANTH</sub>(19-264) dimer. One monomer is colored pink/purple and the other blue. The view is rotated by 60° around the vertical axis relative to (A).
the relevant portion of the VAMP’s cytoplasmic domain could stretch up to 130Å. This is easily sufficient to allow a single CALM molecule, to simultaneously bind a VAMP8 molecule and a PtdIns4,5P2 molecule from the same membrane.

In support of this model in which most or all of the portion of small R-SNARE VAMPs between their CALM binding helices and their transmembrane helices is unstructured, insertion of helix-disrupting proline residues in the predicted unstructured C-terminal half of the SNARE motif at positions Leu44 and Leu51 of VAMP8 does not inhibit its ability to bind CALM or to be endocytosed (Figures 7D–7F and S6). It does, however, prevent SNARE complex formation with SNAP23 and Syntaxin3 (Figure 7B), which relies on the VAMP being able to form a helix over the entire length of its SNARE motif. In contrast, introducing proline residues at positions Leu16 and Val23 in the CALM binding helix of VAMP8 blocks the interaction between the two

See also Figure S5.

(F) Surface representation of the CALMANTH(1-264):VAMP8(11-41) Chimera
(A) Overall structure of the complex with CALMANTH colored as in Figure 4 with VAMP8 colored from pale (residue 15) to dark (residue 37) green. The relative position of the membrane (gray bar) is inferred from the position of the PtdIns4,5P2 (marked as PIP2) binding site on CALMANTH as in 1HF8. The dotted line is a schematic representation of how the remainder of the cytoplasmic portion of VAMP8 domain connects its CALM binding helix to its transmembrane helix.
(B) Orthogonal views of the CALMANTH:VAMP8 complex shown in molecular surface representation colored as in (A).
(C) Spatial complementarity of the CALMANTH:VAMP8 interface with key side chains involved in the binding of CALMANTH by VAMP8 shown. Molecular details of the interactions of the key residues (i) K24 and (ii) M27 are shown.
(D) Schematic representation of VAMP8 residues 15–38. CALMANTH residues that make hydrophobic interactions with VAMP8 are labeled in black, those that make salt bridge interactions with VAMP8 are labeled in blue and those that make hydrogen bonds with VAMP8 are labeled in turquoise.
(E) The final refined VAMP8 helix (green sticks) is shown in unbiased (Fo−Fc) electron density contoured at 3.5 σ calculated before the addition of the helix to the model (left panel). The VAMP8 helix lies on the same face as, but differs significantly from, the orientation of helix α11 (purple sticks) in the unliganded CALMANTH structure (right panel).
See also Figure S5.

(G) Sequence of CALMANTH(1-289). Secondary structure is shown above the sequence representing helices α1 to α11; α6 has been omitted as per (Ford et al., 2001). See also Figure S4.
Figure 6. Mutation of Key Residues in the CALM\textsubscript{ANTH}:VAMP8 Interface Abolishes Their Interaction In Vitro and the Endocytosis of VAMP8 In Vivo

(A) Structure of the CALM\textsubscript{ANTH}:VAMP8 complex “opened out like a book” as indicated by the arrows. Residues whose mutation affect binding between CALM and VAMP8 are colored and labeled in red, while mutations that have no effect are colored and labeled in gray.

(B) Pull-down experiments using GSTVAMP8 and WT or mutant His\textsubscript{6}MycCALM\textsubscript{ANTH} as indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. The mutations L219S, F240A, M244K, I247D, and L251S in His\textsubscript{6}MycCALM\textsubscript{ANTH} abolished binding to VAMP8.

(C) ITC quantitating the binding of certain point mutant versions of CALM\textsubscript{ANTH} to VAMP8. The wild-type CALM\textsubscript{ANTH} binds with a K\textsubscript{D} = 17 ± 2 \text{mM} (black squares) whereas CALM\textsubscript{ANTH} L219S (black circles) and M244K (open triangles) showed no measurable interaction with VAMP8. Data for CALM\textsubscript{ANTH} L219S and M244K are translated by 0.3 \text{μcal/s} and −0.7 \text{μcal/s} respectively.

(D) GST pull-down experiments using His\textsubscript{6}MycCALM\textsubscript{ANTH} and WT or mutant GSTVAMP8 fusion proteins as indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. Wt GSTVAMP8 bound CALM\textsubscript{ANTH} whereas the K24AM27A VAMP8 did not. The GSTVAMP8 K24A mutant interacted weakly with CALM\textsubscript{ANTH}, however, the single M27A mutation of VAMP8 was sufficient to completely abolish the interaction with CALM\textsubscript{ANTH}.

(E) ITC quantitating the binding of K24AM27A mutant version of VAMP8 to CALM\textsubscript{ANTH}. Wt GSTVAMP8 bound wt CALM\textsubscript{ANTH} with a K\textsubscript{D} = 17 ± 1 \text{μM} (black squares) whereas GSTVAMP8 K24AM27A (black circles) showed no measurable interaction. Data for GSTVAMP8 K24AM27A is translated by −0.3 \text{μcal/s} for clarity.
proteins and prevents VAMP8 endocytosis as well as SNARE complex formation. These observations also are in line with the assertion made earlier that SNARE complex formation and the ability to be endocytosed are not linked, which disagrees with the interpretation of data in (Gordon et al., 2009).

DISCUSSION

The endocytic vesicles in most cells must incorporate the R-SNAREs that will allow them to fuse with their target endosome (likely VAMP8 and VAMP3 [Antonin et al., 2000; McMahon et al., 1993]). Alternatively, if they are to undergo immediate recycling without passing through an endosomal compartment, as has been proposed for neuronal synaptic vesicles, the vesicles need to be able to fuse with the plasma membrane, for which it is believed they use VAMP2 (Grote et al., 1995). Vesicles destined for endosomes must also return any SNAREs that have previously been used for fusion with the plasma membrane for reuse in subsequent vesicle/plasma membrane fusion events. Such SNAREs include VAMP3 and VAMP8 for recycling back to the plasma membrane; VAMP7 and VAMP8 for endosome/lysosome biogenesis (Advani et al., 1998; Antonin et al., 2000; Pryor et al., 2004); VAMP8, VAMP7 and VAMP2 for the regulated secretion of lysosomes through an endosomal compartment (reviewed in Chaineau et al., 2009); and VAMP4 for TGN-to-endosome transport (Steegmaier et al., 1999; Tran et al., 2007). VAMP7, which is active on late endocytic organelles (Advani et al., 1998; Pryor et al., 2004), has been shown to be endocytosed in an inactive cis-SNARE complex with a SNAP and a syntaxin family member (Pryor et al., 2008) through binding to the clathrin adaptor and ArfGAP Hrb. Transport in an inactive form prevents erroneous VAMP7-mediated fusion of endocytic vesicles with degradative late endosomes and lysosomes.

In contrast, we demonstrate that VAMP8, VAMP3, and VAMP2, which are required for the fusion of endocytic vesicles with their target early endosomes, are endocytosed in an uncomplexed and therefore essentially active form through their interaction with CALMANTH. However, since binding to CALM and participation in SNARE complexes are mutually exclusive processes, as both binding events utilize the same face of the VAMP, it would be more correct to state that VAMPS 8, 3, and 2 are endocytosed in only a potentially active form, as their conserved SNARE motifs are shielded by being bound to CALMANTH. Corecognition of PtdIns4,5P2 markedly increases the strength of binding between CALM and a membrane containing both a small R-SNARE VAMP and PtdIns4,5P2. In order for a small R-SNARE VAMP to bind to CALMANTH, helix 11 of the ANTH domain must be displaced. This dissociation will be facilitated by the fact that helix11 is only poorly associated with the rest of the ANTH domain (helices 1–10) (Figure 4) and may be further modulated by the C-terminal tail of CALM that follows helix11 binding to the clathrin terminal domains that are displayed on the underside of the polymeric clathrin lattice (Morgan et al., 2000). It should be noted that despite it being necessary to truncate CALMANTH at residue 264 in order to obtain the structure of the CALMANTH:VAMP8 complex, the displacement of helix11 must have occurred in our biochemical assays as all were performed on residues 1–289 of CALMANTH.

Following scission of an endocytic clathrin-coated vesicle (CCV), the vesicle will uncoat by processes that include PtdIns4,5P2 hydrolysis and clathrin cage disassembly. Once PtdIns4,5P2 is hydrolyzed, the avidity effect generated by a single small R-SNARE molecule simultaneously binding PtdIns4,5P2 and a small R-SNARE VAMP will vanish leaving only the weak, transient R-SNARE:CALM interaction. The two proteins will therefore quickly dissociate. In order to minimise the energetically unfavorable situation of the hydrophobic face of an amphipathic helix being exposed to the aqueous environment, the N-terminal half of the SNARE motif of the short R-SNAREs, while still remaining as a helix, switches to lying on the surface of the vesicle membrane (Ellena et al., 2009). In the case of other SNAREs such as the syntaxins and long domain SNAREs, the SNARE motifs can be shielded from the aqueous environment by binding back on their N-terminal regulatory domains, which are not present in the small R-SNAREs. On reaching its final destination, the VAMP will now form tight, energetically favorable complexes with its cognate Q-SNAREs on an endosome and so drive fusion between the endocytic vesicle and endosomal membranes. Thus in the cell, the SNARE motif of a small R-SNARE is never free but interacts with either CALM, a membrane surface or is part of a cognate SNARE complex (Figure 7G).

Nonsense point mutations in CALM are responsible for hematopoietic and iron metabolism abnormalities, growth retardation, and shortened life span in fit1 mice (Kiebig et al., 2003), the strongest phenotypes resulting from premature translational termination in the CALMANTH domain. The CALM/PICALM gene has also been directly implicated in alterations in cognitive function with increasing age (Mengel-From et al., 2011) in risk of developing Alzheimer’s disease (Harold et al., 2009) and in modifying the toxicity of Aβ in a yeast, C. elegans and primary rat cortical neuron models (Treich et al., 2011). These pathophysiological effects could, until now, only be explained by the role of CALM.

(F) Cells expressing either VAMP8-HA alone, or VAMP8-HA plus Myc-tagged siRNA-resistant CALM (CALMres: wt, L219S, or M244K) were mixed together and endogenous CALM was depleted by siRNA treatment. The cells were then fixed without permeabilization and labeled with anti-HA antibody, and then permeabilized and labeled with anti-CALM. The scale bar represents 20 μm.

(G) Endocytosis of anti-HA in cells coexpressing VAMP8-HA and siRNA-resistant wild-type, L219S or M244K mutant myc-tagged CALM. Antibody was bound to the cells at 4°C, then the cells were warmed to 37°C for 2–30 min and antibody remaining at the cell surface was quantified by flow cytometry. Each point is derived from at least 3 separate experiments; the error bars show the SEM. Expression of the CALM construct rescues the knockdown phenotype, but expression of the two CALM mutants does not.

(H) Localization of anti-HA in cells expressing wild-type or K24AM27A mutant VAMP8-HA. The cells were allowed to endocytose the antibody for 40 min, then processed for immunofluorescence. Unlike the cells expressing wild-type VAMP8, the cells expressing the mutant have retained the antibody on the plasma membrane. The scale bar represents 20 μm.

(I) Endocytosis of anti-HA in cells expressing wild-type or K24AM27A mutant VAMP8-HA, using the flow cytometry assay described in Figure S1B. There is negligible endocytosis of the mutant construct.
Figure 7. Binding of CALMANTH and SNARE Complex Formation by VAMP8 Are Mutually Exclusive

(A) Comparison of the mode of VAMP8 binding to CALMANTH and to Syntaxin7:Syntaxin8:Vti1b (PDB 1GL2) (Antonin et al., 2002) made by superimposing the VAMP8 residues 15–39 from the two complexes. VAMP8 adopts the same superhelical twist in both structures and helices α9 and α10 of CALMANTH correspond to the helices of Syntaxin 8 and Syntaxin 7, respectively.

(B) Formation of GSTVAMP8:SNAP23:Syntaxin3(195–253) SNARE complexes (see Supplemental Information) and their binding to CALMANTH. Pull-down experiments using His6MycCALMANTH and the GST fusion proteins indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. SNARE complexes formed with GSTVAMP8 wt and K24AM27A but not with GSTVAMP8 L16PV23P or L44PL51P. Neither the wt VAMP8 SNARE complex nor the K24AM27A SNARE complex bound CALMANTH indicating that SNARE complex formation and CALMANTH binding shown are mutually exclusive events.

(C) Avidity of CALM binding to PtdIns4,5P 2 and VAMP8. Shown are the sensorgrams for the concentration dependent binding of CALM to membranes with captured VAMP8 (top: calculated K_D = 0.9 ± 0.15 μM), membranes with PtdIns4,5P 2 (middle: calculated K_D = 1.9 ± 0.45 μM) and membranes with both, PtdIns4,5P 2 and captured VAMP8 (bottom: calculated K_D = 0.17 ± 0.03 μM). Given are the mean values and SD of four independent measurements The affinity of CALM increases by 8.5 fold over the average K_D for the two ligands when VAMP8 and PtdIns4,5P 2 are bound simultaneously.

(D) Pull-down experiments using His6MycCALMANTH and wt and mutant GSTVAMP8 fusion proteins as indicated. Top panel: Coomassie blue stained gel. Lower panel: western blot probed with anti-myc. The GSTVAMP8 mutants L16PV23P and K24AM27A (see also Figure 6) do not bind CALMANTH. However, GSTVAMP8 L44PL51P bound CALMANTH with a similar strength to wt GSTVAMP8.

(E) ITC quantitating the binding of point mutated versions of VAMP8 to CALMANTH. The binding of wt CALMANTH and GSTVAMP8 L44PL51P (open circles) was comparable to that of wt CALMANTH and wt GSTVAMP8 (black squares) (K_D of 23 ± 2 μM and 17 ± 1 μM respectively). However, GSTVAMP8 L16VP23P (open triangles) and GSTVAMP8 K24AM27A (black circles) both showed no measurable interaction with CALMANTH. Data for GSTVAMP8 L16VP23P, L44PL51P and K24AM27A are translated by −0.3 μcal/s, −1.5 μcal/s and −1.9 μcal/s respectively for clarity.

(F) Localization of anti-HA in cells expressing different VAMP8-HA constructs. The cells were allowed to endocytose the antibody for 40 min, then processed for immunofluorescence. The cells expressing wt VAMP8 and the L44PL51P mutant have endocytosed the antibody, but the cells expressing the L16VP23P mutant have mainly retained the antibody on the plasma membrane similar to the K24AM27A mutant. The scale bar represents 20 μm.

(G) Schematic representation of the model of VAMP8 trafficking from the plasma membrane. The clathrin adaptor CALM binds simultaneously to the R-SNARE VAMP8 and PtdIns4,5P 2 (labeled PIP 2) at the plasma membrane. CALM is released from the surface of an endocytosed vesicle when PtdIns4,5P 2 hydrolyzed and the clathrin cage disassembled and the hydrophobic CALM-binding helix of VAMP8 now “lies” on the vesicle’s surface (Ellena et al., 2009). Finally VAMP8 forms a trans-SNARE complex with its cognate SNAREs on an early endosome to drive vesicle fusion. Thus throughout the interaction of the hydrophobic VAMP8 SNARE motif with the aqueous environment is minimized.
in linking clathrin to the PtdIns4,5P2-containing membrane during endocytosis, a role replicated by all clathrin adaptors. The work presented here raises the possibility that such effects are related to CALM’s ability to directly drive the endocytosis of the small R-SNAREs VAMP8, VAMP3, and VAMP2, since failure to correctly transport these small R-SNARE VAMPs to early endosomes as a result of a reduction in CALM levels would perturb subsequent trafficking of a wide variety of proteins through the endocytic pathway. In the case of Alzheimer’s especially it appears that any alterations in vesicle trafficking give rise to increased levels of Aβ production, presumably by modifying the localization of Amyloid Precursor Protein (APP) or that of its β-, and γ-secretases (Burgos et al., 2010; Lee et al., 2008; Sannerud and Annaert, 2009). Further, it has been documented that a major pathway for clearance of Aβ from the brain parenchyma is endocytosis by various cell types including astrocytes, microglia, and endothelial cells. The latter may further mediate Aβ clearance from the brain through the blood-brain-barrier by transcytosis, and it is of note that these cells were shown to possess the highest levels of CALM expression in the brain (Baig et al., 2010; Bu, 2009). This provides another possible explanation for why CALM has been linked to risk of Alzheimer’s disease since CALM should play a key role in this endocytic process by selecting the R-SNAREs needed both for endocytic vesicle/endosome fusion and for subsequent fusion events along the endocytic pathway.

In summary, we have provided insight into the molecular mechanism by which the post-GoGli small R-SNAREs bind specifically to the ubiquitously and highly expressed endocytic clathrin adaptor CALM, recently confirmed in Koo et al. (2011). This ensures that the SNAREs required for fusion with the endosomal system and for subsequent trafficking steps are actively selected into endocytic CCVs. This ability to mediate the endocytosis of small R-SNARE VAMPs from the cell’s limiting membrane provides a possible explanation for the association of CALM with a variety of both neurological and other disorders.

EXPERIMENTAL PROCEDURES

For a list of the constructs and full materials and methods used in this study, please see the Extended Experimental Procedures.

Cell Biology

HeLaM cells were used for all experiments. For most experiments, the cells were stably transfected with HA-tagged wild-type or mutant VAMPs (2, 3, and 8), and cell lines were selected that expressed the construct at low levels so as not to saturate sorting machinery. For some experiments, the cells were additionally transfected with myc-tagged, siRNA-resistant wild-type or mutant CALM, and cell lines were selected that expressed the tagged CALM at similar levels to endogenous CALM. The steady state localization of the constructs and other proteins, both under control conditions and after siRNA-mediated knockdowns, was observed by immunofluorescence microscopy. Endocytosis kinetics were measured using either a flow cytometry-based antibody uptake assay for the tagged VAMPs, or a radiolabeled ligand uptake assay for other surface receptors. Details of transfection and knockdown conditions, immunolabelling, and endocytosis assays are all described in Supplemental Information.

Protein Expression and Purification

All recombinant proteins were expressed in BL21(DE3) pLysS E. coli for 16 hr at 22°C after induction with 0.2 mM IPTG at 37°C and purified by standard procedures on glutathione sepharose and/or Ni²⁺-NTA agarose as appropriate. GST-tagged proteins were eluted with free glutathione or by thrombin cleavage of the GST-tag while fusion proteins were bound to the beads. His₆-tagged proteins were eluted with buffer containing 300 mM imidazole. All proteins were subsequently purified by S200 gel filtration.

Recombinant Protein Pull Downs

SNARE:adaptor interactions were tested using varying concentrations of GST-tagged SNAREs and relevant His₆-myc-tagged prey proteins. SNARE complexes were made with a 3-fold excess of SNAP23 and syntaxin3 to GSTVAMP8 and purified by GST sepharose and gel filtration. SNARE complex:adaptor interactions were tested using 2 nmoles of 1:1:1 GST-tagged complex incubated with His₆-myc-CALMANTH. All binding experiments were carried out in 1 ml of buffer supplemented with 30 µl glutathione beads and incubated with constant agitation at 4°C. The supernatant removed and the beads washed with 1 ml of buffer, three times. Bound proteins were analyzed by SDS-PAGE and western blots probed with anti-myc antibody (9E10 Santa Cruz Biotechnologies).

Isothermal Titration Calorimetry

Due to yield and solubility issues, GST-tagged SNAREs were used. A VPITC machine (GE Healthcare) was used to titrate 37 injections of 4-8 µl 2 mM His₆-MycCALMANTH with or GST cleaved Epsin1 ENTH proteins into 0.15 mM GST SNARE proteins. Titration curves were fitted using ORIGIN software. Figures show a representative example of each experiment with the KD and associated confidence of the fit (SEM) shown; n = 0.9-1.1. KDs quoted in the text are the average of all runs.

Surface Plasmon Resonance

Liposome-based SPR was carried out using a Biacore 3000 (GE Healthcare) with GST cleaved CALMANTH and Epsin1 ENTH adaptor proteins as analytes and PC/PE liposomes supplemented with 5% PtdIns4,5P₂ and/or His₆-myc-tagged VAMP8 attached via 5% DGS-NTA(Ni). The binding was monitored during one minute injections at 50 µl/min at concentrations ranging from 10 nM to 50 µM. The kinetic parameters were calculated after background (PC/PE binding) subtraction.

Cryocrystallization, Data Collection, Structure Solution, and Refinement

Crystals of CALMANTH(1-289), CALMANTH(1-264) and CALMANTH(1-289):VAMP8(11-41) were grown in sitting drops at 16°C using GST fusion proteins form which the GST had been cleaved. CALMANTH(1-289) was equilibrated against reservoirs containing 100 mM Bis Tris propane, 200 mM sodium malonate, 20% (v/v) PEG 3350; CALMANTH(1-264) was equilibrated against reservoirs containing 15% v/v ethanol, 100 mM imidazole (pH 6.0), 200 mM MgCl₂; CALMANTH:VAMP8 was equilibrated against reservoirs containing 100 mM phosphate-citrate (pH 4.2), 200 mM NaCl, 50% (v/v) PEG3000. All structures were solved by molecular replacement using previously published ANTH domain of CALM as a starting model (1HF8). Detailed descriptions of structure determination, refinement, and structural analysis can be found in Supplemental Information. Structure factors and final refined coordinates have been deposited in the PDB with accession codes 3ZYK (CALMANTH), 3ZYL (CALMANTH(1-264)), and 3ZYM (CALMANTH(1-264):VAMP8(11-41)).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, one table, and six figures and can be found with this article online at doi:10.1016/j.cell.2011.10.038.

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