Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges

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Clathrin self-assembly into a polyhedral lattice mediates membrane protein sorting during endocytosis and organelle biogenesis. Lattice formation occurs spontaneously *in vitro* at low pH and, intracellularly, is triggered by adaptors at physiological pH. To begin to understand the cellular regulation of clathrin polymerization, we analyzed molecular interactions during the spontaneous assembly of recombinant hub fragments of the clathrin heavy chain, which bind clathrin light-chain subunits and mimic the self-assembly of intact clathrin. Reconstitution of hubs using deletion and substitution mutants of the light-chain subunits revealed that the pH dependence of clathrin self-assembly is controlled by only three acidic residues in the clathrin light-chain subunits. Salt inhibition of hub assembly identified two classes of salt bridges which are involved and deletion analysis mapped the clathrin heavy-chain regions participating in their formation. These combined observations indicated that the negatively charged regulatory residues, identified in the light-chain subunits, inhibit the formation of high-affinity salt bridges which would otherwise induce clathrin heavy chains to assemble at physiological pH. In the presence of light chains, clathrin self-assembly depends on salt bridges that form only at low pH, but is exquisitely sensitive to regulation. We propose that cellular clathrin assembly is controlled via the simple biochemical mechanism of reversing the inhibitory effect of the light-chain regulatory sequence, thereby promoting high-affinity salt bridge formation.

Keywords: clathrin/coated vesicles/endocytosis/pH regulation/self-assembly

Introduction

Many cellular processes depend on regulating the spontaneous self-assembly of proteins into macromolecular structures. Such self-assembly reactions include the polymerization of cytoskeletal elements (Egelman and Orlova, 1995), the oligomerization of transcription factors (Wu, 1995) and the assembly of membrane vesicle coats (Pearse and Crowther, 1987). A prerequisite for understanding the

cellular control of these processes is to define the molecular basis for the spontaneous protein–protein interactions involved. We therefore set out to establish the biochemical mechanism of spontaneous clathrin polymerization in order to understand the cellular regulation of clathrin-coated vesicle formation. These membrane transport vesicles are composed of a polymerized clathrin coat surrounding a layer of adaptor molecules which interact with membrane proteins. They control receptor-mediated endocytosis at the plasma membrane and the biogenesis of lysosomes and secretory granules in the *trans*-Golgi network (Schmid, 1997).

Clathrin is a triskelion-shaped molecule comprising three heavy chains, each with a bound light-chain subunit. Below pH 6.5, purified clathrin triskelions self-assemble *in vitro* into a polyhedral lattice (basket) (Brodsky, 1988), but will only form baskets at physiological pH in the presence of stoichiometric quantities of purified AP-1 or AP-2 adaptor molecules or the neuron-specific assembly proteins AP-180 and auxilin (Ahle and Ungewickell, 1986; Keen, 1990; Pearse and Robinson, 1990; Lindner and Ungewickell, 1992). AP-1 and AP-2 adaptors localize clathrin lattice formation to their specific membrane-binding sites in the *trans*-Golgi network or at the plasma membrane and mediate the interaction of the clathrin lattice with the vesicle cargo (Ohno *et al.*, 1995), so that clathrin polymerization causes receptor sorting by concentrating receptors which are recognized by adaptors. The cellular role of the neuron-specific assembly factors is not yet established, but *in vitro* studies suggest that they influence the efficiency of assembly (Lindner and Ungewickell, 1992) and disassembly (Ungewickell *et al.*, 1995), a specialized level of regulation that may be required for the extensive membrane traffic at synapses (Morris and Schmid, 1995). To begin to understand how clathrin self-assembly can be controlled so tightly in the cell, we analyzed the self-assembly reaction of a bacterially expressed recombinant hub fragment of the triskelion, which has previously been shown to mimic self-assembly of the whole clathrin molecule, both biochemically and morphologically (Liu *et al.*, 1995).

Recombinant hubs, formed from residues 1074–1675 of the clathrin heavy chain, are trimeric structures that reproduce the central portion of the three-legged clathrin triskelion, extending from the vertex to the bend in each leg, comprising the binding sites for clathrin light-chain subunits. Without light-chain subunits, recombinant hubs self-assemble reversibly at physiological pH, while hubs with bound light chains only self-assemble below pH 6.5, similar to purified clathrin (Liu *et al.*, 1995). This inhibition of hub assembly by light-chain subunits appeared to be the key to controlling spontaneous clathrin self-assembly at physiological pH, so we decided to map the light-chain residues responsible for inhibition. In this paper we report

Fig. 1. Clathrin light-chain mutants and their binding to hub fragments. **(A)** Schematic alignment of sequences of the wild-type neuronal clathrin light chains (nLC_a and nLC_b) and the nLC_a mutants used for reconstitution of hub molecules. The numbers after Δ in the name of each mutant indicate the segment deleted to create the mutant protein [residue numbers are according to the bovine and human light-chain sequences (Jackson and Parham, 1988)]. The GQQN mutant has nLC_a residues 23–25 changed from EED to QQN. In wild-type nLC_b, the G at position 22 is A and positions 22–25 are EED, as in nLC_a. **(B)** Reconstituted hub molecules from the assembled fraction of the pH 6.2 experiments shown in Figure 2 were analyzed by SDS–PAGE and immunoblotted with antibodies to detect the hub fragments and associated wild-type or mutant light chain: (1) hub alone, (2) hub + nLC_b, (3) hub + nLC_a $\Delta 224-243$, (4) hub + nLC_a $\Delta 22-25$, (5) hub + nLC_a, (6) hub + GQQN. The arrowheads indicate the light chains and the stars indicate the hub fragments. Note that mutants nLC_a $\Delta 22-56$ and nLC_a $\Delta 22-96$ also bind hub molecules (data not shown) and that some of the LC_a mutants have heterogeneous migration patterns due to the formation of internal disulfide bonds (Parham *et al.*, 1989). The migration of the molecular mass marker proteins (in kDa) is indicated by the bars on the left of each panel.

that: (i) a conserved negatively charged sequence of three residues (23–25) in the clathrin light-chain subunits regulates the pH dependence of hub assembly; and (ii) two classes of salt bridge play a dominant role in driving clathrin assembly. These findings suggest a simple model for how clathrin light chains prevent non-productive clathrin assembly at physiological pH, making the process dependent on adaptors and assembly proteins within the cell.

Results

Light-chain residues 23–25 regulate hub assembly

Recombinant bovine clathrin hub fragments were reconstituted with wild-type or mutant bovine light chains (Figure 1) and their assembly analyzed at pH 6.2 and 6.7 (Figure 2). Reconstitution was confirmed by Ni²⁺-affinity purification of the assembled histidine-tagged hubs, and

Fig. 2. Identification of clathrin light-chain residues regulating assembly at pH 6.7. Hub molecules were reconstituted with the wild-type or mutant light chain indicated and assembly was measured by light scatter at A_{320} . The value of the light scatter signal, 5 min after changing to the indicated pH from pH 7.9, is plotted. The ratios of this light scatter signal at each pH for the reconstituted hubs are tabulated.

analysis for bound light chains (no polyhistidine tag) by immunoblotting following SDS–PAGE (Figure 1B). All the mutant light chains bound to hub molecules and were reactive with anti-light-chain antibodies, indicating that there were no extreme alterations in their folding or binding properties. Mammalian clathrin has two types of light chain, LC_a and LC_b, each of which has a neuronal form (nLC_a and nLC_b) including a short hydrophobic sequence near the C-terminus (Brodsky *et al.*, 1991), which is spliced out in non-neuronal tissue (Wong *et al.*, 1990). It was shown previously that full occupancy of hubs by nLC_b strongly inhibited self-assembly at physiological pH (Liu *et al.*, 1995). In initial experiments (Figure 2), a deletion mutant of nLC_a ($\Delta 26-56$) was found to have a similar effect on pH sensitivity, while another nLC_a deletion mutant ($\Delta 22-96$) was unable to inhibit assembly at physiological pH, although it bound equally well to the hub. Examination of the differences between these two deletion mutants revealed that one patch of difference,

Fig. 3. Effect of salt on hub assembly at (A) pH 6.2 and (B) pH 6.7 and (C) on Δ hub assembly at pH 6.2 (▲) compared with hub assembly at pH 6.2 (□). Recombinant clathrin hubs without light chains were purified (Liu *et al.*, 1995) and suspended in assembly buffer with the indicated salt concentration. Assembly was measured by light scatter and the signal of each assembly reaction, 5 min after reducing the pH to 6.2 or 6.7, is plotted. Note that the higher light scatter signal produced by hub assembly at pH 6.7, as seen in (B), has been observed previously (Liu *et al.*, 1995) and reflects larger particle formation, probably due to enhanced hydrophobic interactions at higher pH. This effect is also observed in Figures 2 and 4.

residues 22–25, overlapped with a conserved sequence (23–44) that is shared by all mammalian light chains (LC_a and LC_b). The sequence of the patch for all mammalian clathrin light chains is GEED in LC_a and AEED in LC_b (Jackson and Parham, 1988). In addition, in yeast and *Aplysia*, which have only one clathrin light chain each, the corresponding residues are KDDD and GEVD, respectively (Silveira *et al.*, 1990; Hu *et al.*, 1993). We hypothesized that residues 22–25 might comprise the operative pH control, due to their conserved negative charges. Therefore we generated the nLC_a deletion mutant $\Delta 22$ –25 and tested its ability to regulate the pH sensitivity of hub assembly. Reconstitution of hubs with this mutant demonstrated that the GEED sequence is essential for the light-chain inhibitory activity (Figure 2). To confirm that the negative charge of this sequence was important, a mutant (GQQN) was generated with all three charges neutralized. At pH 6.7, this GQQN mutant bound the hub fragments but did not inhibit hub assembly (Figures 1 and 2). These results established that a minimal acidic patch in the light chains controls the hub self-assembly reaction.

Two classes of salt bridges mediate hub assembly

Identification of the minimal light-chain sequence regulating the pH dependence of clathrin self-assembly raises the question of how this sequence exerts control. To address this, the biochemical nature of the spontaneous interaction between clathrin heavy chains was investigated. Earlier biochemical studies suggested that salt bridges (Keen *et al.*, 1979) and electrostatic interactions dominate clathrin assembly (Van Jaarsveld *et al.*, 1981; Nandi and Edelhoch, 1984). Therefore, this study investigated the effect of NaCl on the assembly of recombinant hubs that lacked light chain subunits (Figure 3). Recombinant hub molecules were suspended in increasing concentrations of

Fig. 4. Assembly of Δ hub recombinant protein compared with hub assembly at pH 6.2 and 6.7. Recombinant hub (residues 1074–1675) and Δ hub (residues 1213–1675) proteins were purified and induced to assemble at pH 6.2 or 6.7. Assembly was measured by light scatter over time after addition of concentrated MES buffer at the indicated pH. The value plotted is the light scatter signal for each sample 5 min after the induction of assembly.

NaCl and self-assembly was then initiated at pH 6.2 (Figure 3A) or pH 6.7 (Figure 3B). Salt inhibition of hub assembly at either pH occurred with two cooperative transitions, indicating that there are two major classes of salt-bridge interaction which mediate hub assembly. At pH 6.2, the transition midpoint for the inhibition of the lower affinity salt bridges was at ~175 mM NaCl, and for the higher affinity salt bridges was at ~275 mM NaCl. At pH 6.7, less salt was required to inhibit both classes of salt bridge to their midpoint of assembly, but the decrease was more dramatic for inhibition of the lower affinity salt bridges, whose inhibition midpoint shifted from ~175 to ~50 mM NaCl. The considerable weakening of the low-affinity salt bridges at pH 6.7 suggests the possible involvement of histidine residues, whose pK_a may be close to this pH.

Critical salt bridges map to distinct subdomains

To map which subdomains of the hub sequence contain residues that participate in the salt bridges controlling hub self-assembly, a hub deletion mutant was generated that was missing residues 1074–1212 (Δ hub, comprising residues 1213–1675). This deletion removes one-third of the residues that contribute to the interaction with another hub during assembly, and the deleted sequence is predicted to span the length of the proximal leg segment of clathrin (Liu *et al.*, 1995). The Δ hub fragment was soluble during bacterial expression and purified readily. Comparing the assembly of purified Δ hub with that of hub showed that deletion of the 1074–1212 segment removed residues that are critical for self-assembly at either pH 6.2 or 6.7 (Figure 4). Δ hub did not assemble at pH 6.7, while a reduced level of assembly was detected at pH 6.2 compared with the full-length hub. Deletion of residues 1074–1212 apparently eliminated the high-affinity salt bridges that can form at either pH but the low-affinity salt bridges, dependent on low pH for formation, were still present in Δ hub. To confirm this, the effect of salt on Δ hub assembly

Fig. 5. Sequence analysis predicting the residues involved in the formation of salt bridges during clathrin assembly. The clathrin heavy-chain sequences listed are derived from *Homo sapiens* (Nomura *et al.*, 1994), *Rattus norvegicus* (Kirchhausen *et al.*, 1987), *Bos taurus* (Liu *et al.*, 1995), *Drosophila melanogaster* (Bazin *et al.*, 1993), *Caenorhabditis elegans* (Wilson *et al.*, 1994), *Dictyostelium discoideum* (O'Halloran and Anderson, 1992), *Glycine max* (Blackbourn *et al.*, 1996) and *Saccharomyces cerevisiae* (Lemmon *et al.*, 1991). Residue numbering is based on the bovine clathrin heavy-chain sequence. The salt bridge partners of predicted high-affinity salt bridges (**A**) are labeled a and b, while those of the low-affinity salt bridges (**B**) are labeled c and d. Acidic residues are shaded and conserved predicted partners are boxed.

at pH 6.2 (Figure 3C) was studied. Indeed, Δ hub assembly was inhibited to baseline by 150 mM NaCl, which lies in the same range as the titration midpoint of the low-affinity salt bridges controlling assembly of full-length hub. While these low-affinity salt bridges were detected at pH 6.7 in the salt inhibition study (Figure 3B), they are evidently not sufficient to support hub assembly at physiological pH in the absence of high-affinity salt bridges.

The assembly properties of the Δ hub construct indicated that the two classes of salt bridge are located in two distinct subdomains of the hub molecule. The deleted hub protein sequence from 1074 to 1212 was therefore inspected for conserved, charged residues that could potentially form high-affinity salt bridges at physiological pH (Figure 5). Patches of conserved acidic and basic residues were observed in all eight diverse species whose clathrin heavy-chain sequences are known, in positions equivalent to DD (1132 and 1133) and RKKAR (1161–1165), in the bovine heavy-chain sequence (Figure 5A). The involvement of positively charged residues in clathrin self-assembly had also been suggested previously by the demonstration that the succinylation of arginines and lysines inhibits assembly at low pH (Nandi and Edelhoch, 1984). The Δ hub sequence retained residues that formed low-affinity salt bridges at pH 6.2 but not pH 6.7, implicating histidine as a possible partner in the low-affinity salt bridges, since its pK_a in a protein can be in this pH range (Hendsch and Tidor, 1994). In addition, the salt titration experiments in Figure 3 were suggestive of histidine involvement in these salt bridges. We therefore searched the Δ hub sequence for conserved histidines. Three histidines at positions 1279, 1313 and 1335 of the bovine heavy-chain sequence are present in equivalent positions in seven of the eight known clathrin heavy-chain sequences, and the histidines at 1313 and 1335 are present in all the known sequences (Figure 5B). Close to these histidine residues, all eight heavy-chain sequences have

conserved negatively charged residues that are potential salt bridge partners for the histidine residues in the opposing Δ hub sequence. This sequence analysis thus revealed that within the heavy-chain domains predicted to form the high- or low-affinity salt bridges, there are conserved residues with properties that are consistent with their participation in such salt bridges.

Discussion

Light-chain residues 23–25 inhibit the formation of high-affinity salt bridges between hub molecules

The deletion mutagenesis and biochemical experiments reported in this paper, combined with sequence analysis, suggest a remarkably simple salt-bridge formation model (Figure 6) for: (i) how clathrin heavy chains self-assemble spontaneously; (ii) how clathrin light chains inhibit spontaneous assembly at physiological pH; and (iii) how adaptors might reverse the light-chain effect in cells. This assembly model is based on our previous demonstration that the light-chain-occupied hub fragments comprise heavy- and light-chain sequences that are the primary determinants of the clathrin self-assembly reaction (Näthke *et al.*, 1992; Liu *et al.*, 1995). First we suggest that spontaneous self-assembly of the hub fragments depends on high- (Figure 6A) and low-affinity (Figure 6B) salt bridges (Figure 3), formed between conserved residues (Figure 5). These residues are oriented appropriately, i.e. opposite each other, if the sequence forming the hub spans the leg three times, according to our current model (Näthke *et al.*, 1992; Liu *et al.*, 1995), and the hubs are aligned anti-parallel as they would be during self-assembly (Pearse and Crowther, 1987).

The assembly properties of the Δ hub fragment (Figure 4) localize the high-affinity salt bridges to the 1074–1212 hub subdomain deleted from Δ hub. The low-affinity salt bridges involving histidine (Figure 6B) are the ones

predicted to induce assembly of the Δ hub protein at low pH. Since Δ hub and hub with bound light chains have a similar pH dependence of assembly, the light chains must interfere with the high-affinity salt bridges that form between the light-chain-free hubs at physiological pH. The predicted localization of the bound light chain (Figure 6C) within the hub sequence (Kirchhausen *et al.*, 1983; Ungewickell, 1983; Näthke *et al.*, 1992) is consistent with the possibility that the negatively charged residues within the 23–25 sequence could neutralize the conserved positively charged patch on the hub sequence, which, in the absence of a light chain, would form a salt bridge to a conserved negatively charged patch on another hub mole-

cule. The folded orientation of the light chains in Figure 6C has been proposed previously, based on antibody inhibition data and structure predictions (Näthke *et al.*, 1992). Studies of yeast clathrin heavy-chain mutants suggest that light-chain interactions are influenced by, and may extend into, the heavy-chain trimerization region (Pishvaei *et al.*, 1997) beyond the minimal domain that independently binds light chains (Liu *et al.*, 1995). There is also some evidence that the extreme N-terminus of the light chains could extend away from the vertex (Kirchhausen and Toyoda, 1993). These additional hypotheses about light-chain disposition which, for simplicity, are left out of Figure 6C are still consistent with our placement of the residue 23–25 regulatory region and in keeping with the idea that light chains prevent the formation of high-affinity salt bridges at physiological pH.

This model of light-chain regulation could also explain why calcium binding by residues 85–96 in the clathrin light chains (Näthke *et al.*, 1990) dramatically enhances clathrin assembly *in vitro* (Ungewickell and Ungewickell, 1991). If the calcium-binding sequence is oriented appropriately, bound calcium cations could potentially neutralize the negative regulatory sequence in the light chains, freeing up the heavy-chain residues that form the high-affinity salt bridges. While this *in vitro* effect of calcium on clathrin assembly is explained by our model, the affinity of light chains for calcium is low (25–50 μ M) and the calcium concentration required to enhance clathrin assembly is much higher (3 mM) (Liu *et al.*, 1995) than that in the cell (0.1–1 μ M) (Näthke *et al.*, 1990). Thus, at physiological concentrations, calcium may instead act to signal clathrin uncoating by low-level binding to the E-F hand motif of residues 85–96 to reveal a binding site for hsc70 (DeLuca-Flaherty *et al.*, 1990; Näthke *et al.*, 1990). In addition, our model also explains the observation that myelin basic protein (MBP) can accelerate clathrin basket formation (Prasad *et al.*, 1995) (also probably non-physiological). The 3-fold stoichiometry of MBP/hub binding necessary for the MBP effect correlates with a

Fig. 6. Model of hub alignment during assembly showing the predicted high- and low-affinity salt bridges and the role of light chains in regulating clathrin assembly. The assembled hub fragments are drawn to be compatible with our earlier folding model predicting that the heavy-chain sequences from 1074 to 1550 span the hub segment three times (Näthke *et al.*, 1992; Liu *et al.*, 1995) and with the fact that hubs self-assemble in an anti-parallel orientation (Pearse and Crowther, 1987). (A) The arrows indicate the predicted interaction between the conserved positively and negatively charged partners of the high-affinity salt bridges, which form at low or physiological pH (missing in the Δ hub construct). (B) The dashed arrows are located between patches containing conserved histidines and negatively charged residues that are potential partners for the low-affinity salt bridges, which occur only at low pH (present in the Δ hub construct). The asterisks represent variable residues. (C) The central portions of the light chains (striped) are shown in their predicted binding orientation (Näthke *et al.*, 1992; Liu *et al.*, 1995), which would locate the regulatory EED residues (of bovine nLC_a and nLC_b) in a position to neutralize the electrostatic interactions needed to form the high-affinity salt bridges, depicted in (A). In the presence of light chains, hub interactions are mediated only by the low-affinity salt bridges in (B) and assembly can occur only at low pH. The arrowheads at the N- and C-termini of the light chain indicate ambiguity in the placement of the extreme termini of the light chain (Kirchhausen and Toyoda, 1993; Pishvaei *et al.*, 1997), as discussed in the text. T denotes the trimerization domain. The hub sequences shown and the residue numbering correspond to the bovine clathrin heavy chain (Liu *et al.*, 1995).

single regulatory switch region per light chain. The positively charged MBP may tie up the negative charges in the light-chain switch, allowing high-affinity heavy-chain salt bridges to form.

Possible mechanisms for the regulation of clathrin assembly by adaptor proteins

Identification of the minimal regulatory switch in clathrin light chains that 'turns off' spontaneous salt bridge formation between clathrin heavy chains suggests a very sensitive mechanism for how adaptors could control clathrin assembly in the cell. We predict that adaptors introduce sequences that reverse the light-chain effect and suggest two possible mechanisms by which this could be achieved. First, adaptors could themselves have positively charged residues that interact with the light-chain regulatory sequence and thereby free up the heavy chains to form high-affinity salt bridges. Alternatively, adaptors could align the distal or terminal domain of the triskelion legs with the proximal hub segments to provide the competing residues needed to reverse light-chain inhibition. Adaptors do not directly induce the assembly of hubs with light chains at physiological pH (Liu *et al.*, 1995). Thus, without the distal and terminal domains of the clathrin heavy chain, they are incapable of flipping the assembly switch and allowing salt bridge formation. However, the distal and terminal domains of clathrin could be required for adaptor function either because they determine the correct orientation of regulatory sequences in the adaptors or because they contain regulatory sequences that are held in place by the adaptors. Electron microscopy shows that the AP-2 adaptor binds to the terminal domain of free triskelions (Heuser and Keen, 1988) and biochemical evidence suggests a secondary binding site for adaptors in the hub (Murphy and Keen, 1992; Liu *et al.*, 1995). These physical interactions are compatible with both proposed mechanisms for how adaptors could reverse the light-chain regulatory switch to cause cellular clathrin assembly.

Materials and methods

Clathrin light chain and heavy chain DNA constructs

The construction of nLC_a light-chain deletion mutants $\Delta 26$ –56, $\Delta 22$ –96 and $\Delta 224$ –243 has been described previously (Näthke *et al.*, 1990; Pley *et al.*, 1995). The nLC_a mutant $\Delta 22$ –25 was made by digestion of wild-type nLC_a cDNA with *NaeI* and *SacII*, re-ligation using a double-stranded linker (5'CCTGCCGC3') to preserve the reading frame and insertion into pET-15b (Novagen). The $\Delta 22$ –25 deletion construct was verified by sequencing and identity of the expressed protein was verified by immunoblotting with anti-LC_a monoclonal antibodies (mAb) CVC.6 and X16 (Brodsky *et al.*, 1987).

The GQQN mutant was made by PCR using pET LC_a-hub as template with mutant primer GQQN (5'AGAAGGCCGCGGCAGGATTCTGTTGGCC3') and the T7 promoter primer. The PCR product was digested with *XbaI* and *SacII*, gel purified and ligated back into pET LC_a-hub, predigested with the same restriction enzymes. The point substitutions were verified by sequencing. The neuronal LC_a gene was inserted in front of the hub construct in pET-15b in a manner similar to that described previously (Liu *et al.*, 1995).

The Δ hub sequence ($\Delta 1074$ –1212) was amplified out of bovine clathrin heavy-chain clone SK991-1675 by PCR using primer 1213N (5'GAAATGCATATGGCTGCTGCTAAGTTG3') and the T-7 promoter primer. The PCR product was cut with *NdeI* and *XhoI*, gel purified and ligated into pET-15b. The deletion construct was confirmed by sequencing.

Bacterial strains, protein expression and purification

Light- and heavy-chain deletion mutants were transformed into *Escherichia coli* strains DH5 α (cloning strain) and BL21/DE3 (F⁺, *hsdS*, *gal*, *r*⁺, *m*⁺) (expressing strain). Bacteria harboring plasmids for wild-type nLC_b and mutant nLC_a, hub, Δ hub and LC_a-hub were grown at 37°C in Luria broth supplemented with 100 μ g/ml of ampicillin. Protein expression was induced at 30°C with IPTG (100 μ g/ml) and the cells were lysed by sonication in ice-cold 10 mM Tris–HCl, pH 7.9, with protease inhibitors. Wild-type and mutant light chains were partially purified from bacterial lysate by boiling and centrifugation (Näthke *et al.*, 1990) and further purified by anion exchange on a POROS 20 HQ column (Perseptive Biosystems) equilibrated with 10 mM Bis–Tris, pH 6, buffer and eluted with a 2–3% gradient of 10 mM Bis–Tris, 1 M NaCl at pH 6, followed by size exclusion chromatography on a Superose 6 column (Pharmacia) in 10 mM Tris–HCl at pH 7.9. Bacterially expressed Δ hub, full length hub and LC_a-hub (all with polyhistidine tags) were purified by nickel-affinity chromatography (Chelating Sepharose Fast Flow, Pharmacia), followed by size-exclusion chromatography, as described previously (Liu *et al.*, 1995).

Hub reconstitution with light chains

Wild-type nLC_b and nLC_a deletion mutants were added in 2-fold molar excess to purified recombinant hub and incubated on ice for 10 min in 10 mM Tris–HCl, pH 7.9. For the experiments in Figure 2, these samples were either used directly for assembly assays (see below) or after reconstitution, were passed through a Superose 6 column (in 10 mM Tris–HCl, pH 7.9) to remove excess unbound light chain, prior to use in assembly assays. Reconstitution of hubs with or without the sizing column step to remove unbound light chains yielded similar assembly assay results. The sizing step for the samples in Figure 2 was included so that reconstitution could be confirmed by analyzing the assembled samples for the presence of bound light chains. The light chains present in the assembled pellet would have had to co-purify with the hubs at pH 7.9, as well as co-assemble at pH 6.2. The assembled protein from the pH 6.2 samples in Figure 2 was isolated by Ni²⁺-affinity batch chromatography and analyzed by SDS–PAGE (Laemmli, 1970). hub was detected in the upper part of the 12% acrylamide gel by immunoblotting with hub-specific mAb HUB.1. The presence of nLC_b, nLC_a and the nLC_a mutants were detected in the lower part of the gel by immunoblotting with a mixture of the anti-light-chain mAbs CON.1 and X16 (Brodsky *et al.*, 1987; Näthke *et al.*, 1992). The apparent higher molecular weight extra bands in lanes 3 and 4, which are recognized by the anti-light chain mAbs, represent fully reduced forms of nLC_a. There are cysteines at positions 182 and 213 in nLC_a (present in both mutant constructs) that readily form an internal disulfide bond, even when electrophoresed in reducing buffer, so most of the recombinant protein is in oxidized form which does not affect function or antigenicity (Parham *et al.*, 1989).

Light-scattering assembly assay

Purified hubs and light-chain-reconstituted hubs were suspended at 0.3 mg/ml in 10 mM Tris, pH 7.9, and assembly induced by the addition of 1/25 volume of 1 M morpholino-ethane-sulfonic acid (MES) buffer at pH 6.2 or 6.7 (Liu *et al.*, 1995). Purified hubs used in the salt titration experiments were suspended in 10 mM Tris–HCl, pH 7.9, with the indicated salt concentration. Purified Δ hub was dissolved in the assembly buffer given above and also in buffer containing 1 mM DTT and 0.01% sodium azide. Similar results were obtained in either buffer. Assembly was measured by following the increase in light scatter at A_{320} , over time, after reducing the pH. The plateau value for the assembly signal, reached 5 min after reducing the pH, is plotted in Figures 2, 3 and 4.

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References

- Ahle, S. and Ungewickell, E. (1986) Purification and properties of a new clathrin assembly protein. *EMBO J.*, **5**, 3143–3149.
- Bazin, C., Katzen, A.L., Morgan, M., Mahowald, A.P. and Lemmon, S.K. (1993) The *Drosophila* clathrin heavy chain gene: clathrin function is essential in a multicellular organism. *Genetics*, **134**, 1119–1134.
- Blackbourn, M.E., Anderton, B.H. and Jackson, A.P. (1996) Plant clathrin heavy-chain: sequence analysis and restricted distribution in rapidly growing pollen tubes. *J. Cell Sci.*, **109**, 777–787.

- Brodsky, F.M. (1988) Living with clathrin: Its role in intracellular membrane traffic. *Science*, **242**, 1396–1402.
- Brodsky, F.M., Galloway, C.J., Blank, G.S., Jackson, A.P., Seow, H.-F., Drickamer, K. and Parham, P. (1987) Localization of clathrin light-chain sequences mediating heavy-chain binding and coated vesicle diversity. *Nature*, **326**, 203–205.
- Brodsky, F.M., Hill, B.L., Acton, S.L., Näthke, I., Wong, D.H., Ponnambalam, S. and Parham, P. (1991) Clathrin light chains: Arrays of protein motifs that regulate coated vesicle dynamics. *Trends Biol. Sci.*, **16**, 208–213.
- DeLuca-Flaherty, C., McKay, D.B., Parham, P. and Hill, B.L. (1990) Uncoating protein (hsc70) binds a conformationally labile domain of clathrin light chain LC₃ to stimulate ATP hydrolysis. *Cell*, **62**, 875–887.
- Egelman, E.H. and Orlova, A. (1995) New insights into actin filament dynamics. *Curr. Opin. Struc. Biol.*, **5**, 172–180.
- Hendsch, Z.S. and Tidor, B. (1994) Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Sci.*, **3**, 211–226.
- Heuser, J.E. and Keen, J. (1988) Deep-etch visualization of proteins involved in clathrin assembly. *J. Cell Biol.*, **107**, 877–886.
- Hu, Y., Barzilai, A., Chen, M., Bailey, C.H. and Kandel, E.R. (1993) 5-HT and cAMP induce the formation of coated pits and vesicles and increase the expression of clathrin light chain in sensory neurons of aplysia. *Neuron*, **10**, 921–929.
- Jackson, A.P. and Parham, P. (1988) Structure of human clathrin light chains. *J. Biol. Chem.*, **263**, 16688–16695.
- Keen, J.H. (1990) Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.*, **59**, 415–438.
- Keen, J.H., Willingham, M.C. and Pastan, I.H. (1979) Clathrin-coated vesicles: Isolation, dissociation, and factor-dependent reassociation of clathrin baskets. *Cell*, **16**, 303–312.
- Kirchhausen, T. and Toyoda, T. (1993) Immunoelectron microscopic evidence for the extended conformation of light chains in clathrin trimers. *J. Biol. Chem.*, **268**, 10268–10273.
- Kirchhausen, T., Harrison, S.C., Parham, P. and Brodsky, F.M. (1983) Location and distribution of the light chains in clathrin trimers. *Proc. Natl Acad. Sci. USA*, **80**, 2481–2485.
- Kirchhausen, T., Harrison, S.C., Chow, E.P., Mattaliano, R.J., Ramachandran, K.L., Smart, J. and Brosius, J. (1987) Clathrin heavy chain: Molecular cloning and complete primary sequence. *Proc. Natl Acad. Sci. USA*, **84**, 8805–8809.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lemmon, S.K., Pellicena-Palle, A., Conley, K. and Freund, C.L. (1991) Sequence of the clathrin heavy chain from *Saccharomyces cerevisiae* and requirement of the COOH terminus for clathrin function. *J. Cell Biol.*, **112**, 65–80.
- Lindner, R. and Ungewickell, E. (1992) Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. *J. Biol. Chem.*, **267**, 16567–16573.
- Liu, S.-H., Wong, M.L., Craik, C.S. and Brodsky, F.M. (1995) Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. *Cell*, **83**, 257–267.
- Morris, S.A. and Schmid, S.L. (1995) Synaptic vesicle recycling. The Ferrari of endocytosis? *Curr. Biol.*, **5**, 113–115.
- Murphy, J.E. and Keen, J.H. (1992) Recognition sites for clathrin-associated proteins AP-2 and AP-3 on clathrin triskelia. *J. Biol. Chem.*, **267**, 10850–10855.
- Nandi, P.K. and Edelhoch, H. (1984) The effects of lyotropic (Hofmeister) salts on the stability of clathrin coat structure in coated vesicles and baskets. *J. Biol. Chem.*, **259**, 11290–11296.
- Näthke, I., Hill, B.L., Parham, P. and Brodsky, F.M. (1990) The calcium binding site of clathrin light chains. *J. Biol. Chem.*, **265**, 18621–18627.
- Näthke, I.S., Heuser, J., Lupas, A., Stock, J., Turck, C.W. and Brodsky, F.M. (1992) Folding and trimerization of clathrin subunits at the triskelion hub. *Cell*, **68**, 899–910.
- Nomura, M. *et al.* (1994) Prediction of the coding sequences of unidentified human genes. *DNA Res.*, **1**, 47–56.
- O'Halloran, T.J. and Anderson, R.G.W. (1992) Characterization of the clathrin heavy chain from *Dictyostelium discoideum*. *DNA Cell Biol.*, **11**, 321–330.
- Ohno, H. *et al.* (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science*, **269**, 1872–1875.
- Parham, P., Brodsky, F.M. and Drickamer, K. (1989) The occurrence of disulfide bonds in purified clathrin light chains. *Biochem. J.*, **257**, 775–781.
- Pearse, B.M.F. and Crowther, R.A. (1987) Structure and assembly of coated vesicles. *Annu. Rev. Biophys. Biophys. Chem.*, **16**, 49–68.
- Pearse, B.M.F. and Robinson, M.S. (1990) Clathrin, adaptors and sorting. *Annu. Rev. Cell Biol.*, **6**, 151–171.
- Pishvae, B., Munn, A. and Payne, G.S. (1997) A novel structural model for regulation of clathrin function. *EMBO J.*, **16**, 2227–2239.
- Pley, U.M., Hill, B.L., Alibert, C., Brodsky, F.M. and Parham, P. (1995) The interaction of calmodulin with clathrin-coated vesicles, triskelions, and light chains. *J. Biol. Chem.*, **270**, 2395–2402.
- Prasad, K., Barouch, W., Martin, B.M., Greene, L.E. and Eisenberg, E. (1995) Purification of a new clathrin assembly protein from bovine brain coated vesicles and its identification as myelin basic protein. *J. Biol. Chem.*, **270**, 30551–30556.
- Schmid, S.L. (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.*, **66**, 511–548.
- Silveira, L.A., Wong, D.H., Masiarz, F.R. and Schekman, R. (1990) Yeast clathrin has a distinctive light chain that is important for cell growth. *J. Cell Biol.*, **111**, 1437–1449.
- Ungewickell, E. (1983) Biochemical and immunological studies on clathrin light chains and their binding sites on clathrin triskelions. *EMBO J.*, **2**, 1401–1408.
- Ungewickell, E. and Ungewickell, H. (1991) Bovine brain clathrin light chains impede heavy chain assembly *in vitro*. *J. Biol. Chem.*, **266**, 12710–12714.
- Ungewickell, E., Ungewickell, H., Holstein, S.E.H., Lindner, R., Prasad, K., Barouch, W., Martin, B., Greene, L.E. and Eisenberg, E. (1995) Role of auxilin in uncoating clathrin-coated vesicles. *Nature*, **378**, 632–635.
- Van Jaarsveld, P.P., Nandi, P.K., Lippoldt, R.E., Saroff, H. and Edelhoch, H. (1981) Polymerization of clathrin promoters into basket structures. *Biochemistry*, **20**, 4129–4135.
- Wilson, R. *et al.* (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C.elegans*. *Nature*, **368**, 32–38.
- Wong, D.H., Ignatius, M.J., Parosky, G., Parham, P., Trojanowski, J.Q. and Brodsky, F.M. (1990) Neuron-specific expression of high molecular weight clathrin light chain. *J. Neurosci.*, **10**, 3025–3031.
- Wu, C. (1995) Heat shock transcription factors: structure and regulation. *Annu. Rev. Cell Dev. Biol.*, **11**, 441–469.

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