A novel structural model for regulation of clathrin function

Babak Pishvaee, Alan Munn¹ and Gregory S.Payne²

Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90095, USA

¹Present address: Yeast Cell Biology, Institute of Molecular Agrobiology, 59A, The Fleming, Singapore, 118240, Republic of Singapore

²Corresponding author

The distinctive triskelion shape of clathrin allows assembly into polyhedral lattices during the process of clathrin-coated vesicle formation. We have used random and site-directed mutagenesis of the yeast clathrin heavy chain gene (CHC1) to characterize regions which determine Chc trimerization and binding to the clathrin light chain (Clc) subunit. Analysis of the mutants indicates that mutations in the trimerization domain at the triskelion vertex, as well as mutations in the adjacent leg domain, frequently influence Clc binding. Surprisingly, one mutation in the trimerization domain enhances the association of Clc with Chc. Additional mutations in the trimerization domain, in combination with mutations in the adjacent leg domain, exhibit severe defects in Clc binding while maintaining near normal trimerization properties. The position of these trimerization domain mutations on one face of a putative α-helix defines a region on the trimer surface that interacts directly with Clc. These results suggest that Clc extends into the Chc trimerization domain from the adjacent leg, thereby bridging the two domains. On the basis of this conclusion, we propose a new model for the organization of the triskelion vertex which provides a structural basis for regulatory effects of Clc on clathrin function.

Keywords: clathrin/light chain/mutants/structure/trimerization

Introduction

In eukaryotic cells, transfer of proteins between membrane-delineated compartments is executed efficiently and accurately by small transport vesicles. One well-described class of transport vesicles is characterized by a polyhedral coat composed of the multimeric protein clathrin (Pearse, 1975). Clathrin-coated vesicles participate in selective protein transport processes from the plasma membrane and the Golgi complex, including endocytosis, sorting of newly made lysosomal proteins, secretory granule formation and localization of certain Golgi membrane proteins (reviewed in Brodsky, 1988; Pearse and Robinson, 1990; Wilsbach and Payne, 1993).

Clathrin is composed of three heavy chains (Chc) and three light chains (Clc) that are organized into an extended, trimeric structure termed a triskelion (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Figure 1A). Assembly of triskelions onto the cytoplasmic surface of an organelle membrane, mediated by adaptor (AP) complexes, results in a polyhedral clathrin lattice bridged to the membrane by AP complexes (reviewed in Keen, 1990; Robinson, 1992; Kirchhausen, 1993). The resulting clathrin coat collects specific integral membrane proteins through interactions between the AP complexes and the cytoplasmic domains of the membrane proteins. Thus, polymerization of triskelions into a lattice provides a scaffold which concentrates the AP complexes and associated cargo molecules, thereby selectively incorporating the cargo into a nascent vesicle. Once a clathrin-coated vesicle is formed, the clathrin coat is disassembled to allow fusion with the target organelle membrane (Rothman and Schmid, 1986). Released triskelions are then available for further rounds of transport vesicle biogenesis.

Deciphering the structural and functional organization of the triskelion is key to understanding the molecular basis of the assembly–disassembly cycle that is central to clathrin function in vesicle biogenesis. Chc comprises the principal structural component of the triskelion and assembled lattices (Winkler and Stanley, 1983). Each Chc appendage carries an associated Clc (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Kirchhausen et al., 1983; Ungewickell, 1983). The Clcs exhibit properties that suggest a role in regulating clathrin dynamics: they are phosphorylated and bind to the uncoating ATPase (Hsc70), calcium and calmodulin (reviewed in Brodsky et al., 1991). A number of studies have addressed triskelion properties in vitro using clathrin obtained from purified coated vesicles. Triskelions isolated in this manner can assemble into polyhedral cages that resemble the lattice coats on vesicles (Woodward and Roth, 1978; Keen et al., 1979). In support of a regulatory role for Clc, triskelions lacking Clc no longer depend on calcium or APs for cage assembly in vitro (Ungewickell and Ungewickell, 1991; Liu et al., 1995). Hence, it has been suggested that Clc may act to prevent premature triskelion association in vivo. Also, studies of clathrin cage disassembly have suggested that Clc is necessary for cage depolymerization by the Hsc70 ATPase and binds the ATPase in a calcium-regulated manner (Schmid et al., 1984; Flaherty et al., 1990). However, more recent studies have challenged the requirement for Clc in cage depolymerization (Ungewickell et al., 1995).

Biochemical and electron microscopic (EM) analyses have provided insight into the structural organization of the triskelion (Kirchhausen and Harrison, 1984; Blank and Brodsky, 1986, 1987; Kirchhausen et al., 1986, 1987; Näthke et al., 1992; Liu et al., 1995). The Chc polypeptides are joined near the C-termini to form the triskelion vertex...
Fig. 1. Position of mutations in a temperature-sensitive clathrin heavy chain, Chc1-521. (A) Diagram of a clathrin triskelion. Heavy chains (solid lines) join at their C-termini to form the vertex. Clathrin light chains (open bars) associate with the vertex-proximal domain of Chc. The N-terminal region of Chc forms a distinct globular domain. (B) Position of the unique SstI site used to map the mutations responsible for the temperature sensitivity of Chc1-521. (C) The position of the mutations in Chc1-521. Regions in bovine Chc which are proposed to be involved in Clc binding (Liu et al., 1995) are shown as open bars (LCB) and span residues 1213–1313, 1438–1481 and 1513–1522. The trimerization domain (TD) is shown as a solid bar and encompasses residues 1550–1615 (Liu et al., 1995). Although the yeast Chc is 22 amino acids shorter than bovine and rat Chc (Lemmon et al., 1991; Liu et al., 1995), gaps introduced to optimize the alignment of yeast and rat Chc are mostly located in the N-terminal regions. As a consequence, there is only a six amino acid difference in the residue numbering within the designated region from 1318 to 1653 (Lemmon et al., 1991). That is, residue 1590 in yeast Chc corresponds to residue 1584 in rat Chc. (Figure 1A). The vertex-proximal third (=600 amino acids) of Chc forms the proximal leg domain which extends to the bend in the leg (Figure 1A). The middle third of Chc forms the vertex-distal leg domain that extends from the bend to the N-terminal globular domain (Figure 1A). It is the ‘hub’ region (vertex and the proximal leg) which is responsible for three features that are key to clathrin function: trimerization, Clc binding and clathrin assembly. Combining proteolytic fragmentation of purified clathrin triskelions with ultrastructural mapping of monoclonal antibody epitopes, Näthke et al. (1992) characterized the structure of the C-terminal hub region and proposed a low resolution model of Chc and Clc folding in this domain. In this model, the trimerization and Clc-binding domains lie adjacent to each other in the primary sequence (Figure 1C). Recent experiments assessing trimerization, Clc binding and lattice assembly by Chc fragments produced in Escherichia coli have led to a refined model which delineates the trimerization and Clc-binding segments more precisely as independent domains (Liu et al., 1995).

As a complement to the in vitro approaches described above, we have analyzed the structure of clathrin in yeast cells expressing mutated forms of yeast Chc. By generating both random and site-directed amino acid changes in Chc and introducing the mutants into cells lacking the wild-type Chc, it was possible to probe the role of different domains in Chc trimerization and Clc binding in a native context. While the results generally conform to the hub model, we provide evidence that the trimerization domain interacts with Clc. This observation suggests that trimerization and Clc binding are not as distinct as previously proposed and leads to an alternative view of the triskelion hub with functional consequences.

**Results**

**A temperature-sensitive Chc exhibits trimerization and Clc-binding defects**

As an approach to studying clathrin function in yeast, we isolated a temperature-sensitive allele of the clathrin heavy chain gene, *CHC1* (Seeger and Payne, 1992a; see Materials and methods). Cells containing this allele *chc1-521* display temperature-sensitive defects in growth, endocytosis, vacuolar protein sorting and localization of Golgi membrane proteins (Seeger and Payne, 1992a,b; Tan et al., 1993). In order to define the molecular basis of the defects caused by the *chc1-521*-encoded Chc, we sought to identify the causative mutations. Since the mutant allele was generated by hydroxylamine mutagenesis of plasmid DNA carrying the complete *CHC1* gene, we first identified the region of the mutant gene that was responsible for the sensitivity to elevated temperature. Recombinant genes were generated between the mutant and wild-type alleles by taking advantage of a unique SstI recognition site at a position in *CHC1* corresponding to amino acid 1318 (Figure 1B). The recombinant genes, inserted into a centromere-containing single copy plasmid, and under
Fig. 2. Chc1-521 is defective in Clc binding and trimerization. (A) A co-immunoprecipitation assay was used to monitor Chc binding to Clc in cells expressing chc1-521 (521; lanes 1 and 2) or wild-type Chc (WT; lanes 3 and 4). Cell extracts were incubated with antibodies directed against Clc, and the resulting precipitates were collected and subjected to SDS–PAGE (Ip; lanes 1 and 3). Untreated extracts (20% of the amount used in the immunoprecipitations) were also analyzed (E; lanes 2 and 4). Chc (upper panel) and Clc (lower panel) were visualized by immunoblotting with specific antibodies. The intense signal labeled 'Ig' results from binding of the enzyme-conjugated secondary antibodies to the rabbit immunoglobulin heavy chains present in the immunoprecipitation samples. (B) Extracts prepared from cells expressing wild-type (WT; panels 1 and 3) or chc1-521 (521; panels 2 and 4) were fractionated by chromatography through Sepharose CL-4B. Cells analyzed in panels 1 and 2 were grown at 24°C. Cells analyzed in panels 3 and 4 were shifted to 37°C for 30 min prior to lysis. Selected fractions were precipitated with trichloroacetic acid and analyzed by SDS–PAGE and immunoblotting with antibodies directed against Chc or Clc. The resulting images were quantified by densitometry and the levels of Chc and Clc in each lane were plotted as a fraction of the total detected in all lanes.

control of the native CHC1 promoter, were introduced into cells in which the chromosomal CHC1 gene was disrupted. The resulting strains were tested for temperature-sensitive growth defects (data not shown). In this way, the region of the gene responsible for the temperaturesensitive phenotype was mapped to the 3’ 1 kb of coding sequences corresponding to amino acids 1318–1653. This region of the gene was sequenced and found to harbor five different base changes that produce four amino acid replacements: S1331 was converted to F; S1347 to F; P1553 to L and E1590 to K (Figure 1C). Because previous work identified the C-terminal region of Chc as an important contributor to both Clc binding and Chc trimer formation (Kirchhausen et al., 1983; Ungewickell, 1983; Näthke et al., 1993; Liu et al., 1995; Figure 1C), we reasoned that these properties could be affected in the mutant protein. To measure Clc binding, we utilized a co-immunoprecipitation assay. Cell extracts were prepared under non-denaturing conditions, Clc was immunoprecipitated with affinity-purified polyclonal antibodies, and the precipitates were analyzed by SDS–PAGE and immunoblotting for both Clc and Chc (Figure 2A).

When wild-type cell extracts were subjected to precipitation with the anti-Clc antibodies, the association of Clc and Chc in triskelions resulted in co-precipitation of Chc (Figure 2A, lane 3). The observation that more Clc is present in the immunoprecipitate lane than the extract lane is due to the fact that the amount of extract loaded in lane 4 represents only 20% of the total extract used in co-precipitation. More importantly, comparison of the levels of Chc and Clc in the immunoprecipitated sample (Figure 2A, lane 3) with the levels of the two proteins in the cell extract (Figure 2A, lane 4) indicates that, whereas the level of Clc increases, that of Chc decreases, resulting in a lower Chc to Clc ratio in the immunoprecipitate. Thus, it appears that binding of the antibodies to Clc leads to some dissociation of Chc in wild-type clathrin. Nevertheless, co-immunoprecipitation represents a convenient and sensitive assay for Clc binding by Chc. When Clc was immunoprecipitated from mutant cell extracts, mutant Chc was not co-immunoprecipitated (Figure 2A, lanes 1 and 2). This result argues that the mutations in Chc1-521p cause a defect in Clc binding. It should be noted that the immunoprecipitation was carried out on
extracts prepared from cells grown at the permissive temperature of 24°C to avoid degradation of mutant Chc which frequently was observed in cells shifted to 37°C. Thus, a Clc-binding defect was observed in extracts of cells grown at a temperature where defects are not apparent in vivo (Seeger and Payne, 1992a,b; Tan et al., 1993). This difference between in vitro and in vivo properties is not an uncommon characteristic of temperature-sensitive mutants and can be attributed to exacerbation of the defect which occurs upon exposure of the protein to non-physiological conditions during lysis and subsequent manipulations. Because defects were apparent when cells were grown at 24°C, subsequent experiments were carried out with cells grown at this temperature, unless otherwise noted.

Trimerization of mutant Chc and binding to Clc were also analyzed by gel filtration chromatography. Because of the extended, oligomeric structure of intact clathrin triskelions, chromatography through Sephacryl CL-4B results in co-elution of Chc and Clc in fractions just following the void volume (Payne and Schekman, 1985). Cell extracts prepared from wild-type and mutant cells were applied to a Sephacryl CL-4B column and the elution profiles of Chc and Clc were determined by immunoblotting column fractions (Figure 2B). When extracts from wild-type cells grown at either 24 or 37°C were analyzed, Chc and Clc exhibited an elution pattern characteristic of triskelions, with levels peaking together in fractions 12–14 (Figure 2B, panels 1 and 3; fraction 9 represents the column void volume). The elution profiles were quantified by densitometry and the results illustrate the co-elution of Chc and Clc (Figure 2B, panels 1 and 3). When extracts of chc1-521 cells grown at 24°C were analyzed, there was a clear and dramatic shift in the elution profiles of both Chc and Clc (Figure 2B, panel 2). The bulk of Chc eluted in fractions 16–20, and Clc levels peaked in fractions 18–20. If the cells were first shifted to 37°C for 30 min prior to extract preparation, a similar elution pattern was observed (Figure 2B, panel 4); however, Chc and Clc were more clearly separated under these conditions. The shift in the elution position of mutant Chc from fraction 12 to fractions 16–18 is consistent with a defect in trimerization. This interpretation is strongly supported by analysis of a mutant Chc which lacks the C-terminal 169 amino acids encompassing the trimerization region. Like the Chc1-521 protein, the trimerization-defective Chc levels peak in fractions 16 and 18 (see Figure 3E, ‘Trimer-minus Chc’ panel). The elution position of unbound Clc was established by analyzing an extract from a strain carrying a deletion of Chc (see Figure 3E, ‘Free Clc’ panel). Free Clc elutes predominantly in fraction 20. Based on this pattern, the elution profile of Chc from chc1-521 cells grown at 24°C suggests a degree of binding to Chc which results in a shift of the Clc peak to fraction 18 (Figure 2B, panel 2). This residual Chc–Clc interaction was abolished in the cells grown at 37°C (Figure 2B, panel 4). Taken together, the results of the gel filtration chromatography and the co-immunoprecipitation experiments indicate that the Chc1-521 protein is defective in both trimerization and Clc binding.

The contribution of individual mutations in chc1-521
The amino acid changes encoded by chc1-521 were clustered as pairs in two regions (Figure 1C); one pair was located in the trimerization region and the second pair was located in a more amino-terminal region close to sequences implicated in Clc binding (Näthke et al., 1992; Liu et al., 1995). To dissect the relative contributions of each domain to the defects in trimerization and Clc binding, we created a series of genes encoding proteins with various combinations of the four amino acid changes. For the sake of convenience, the four changes will be referred to as A (S1331F), B (S1347F), C (P1553L) and D (E1590K).

Clc binding was first monitored using the co-immunoprecipitation assay (Figure 3A). All mutants which contained the D (E1590K) mutation displayed defects in Clc binding. Mutants ABCD, BCD and CD were not co-precipitated with Clc (Figure 3A, lanes 3, 5 and 13). In contrast, single (not shown), double and triple mutants without the D (E1590K) change were co-precipitated with Clc at levels comparable with wild-type (Figure 3A, lanes 1, 7, 9 and 11). In each case, the levels of Chc in the cell extracts were similar, indicating that the lack of co-precipitation was not due to degradation of mutant Chc (Figure 3A, even numbered lanes). These results suggested that the trimerization domain mutation E1590K (D) could be the major contributor to the Clc-binding defect. In confirmation of this view, analysis of the ‘D’ single mutant showed an almost complete defect in Clc binding (Figure 3B, lane 1).

Gel filtration was used to analyze mutant Chc trimerization, as well as Clc binding in the absence of the destabilizing effects of Clc antibodies used in the co-precipitation experiments. The D mutant Chc eluted as monomers peaking in fractions 16–18 (Figure 3E, D panel). This trimerization defect is consistent with the position of the D mutation within the previously mapped trimerization domain (Liu et al., 1995). Clc binding was more difficult to assess in the gel filtration assay because the elution profile of the trimerization-defective Chc was shifted towards the position of free Clc. However, Clc from the D mutant peaked in fractions 16 and 18 coincident with the Chc peak, and distinct from the elution position of free Clc (Figure 3E, compare ‘Free Clc’ and D panels). Hence, by this assay, the D mutation did not appear to affect Clc binding significantly. The difference between the results from co-precipitation and gel filtration are not unexpected since, as was discussed above, the anti-Clc antibodies appear to destabilize the Chc–Clc interaction and consequently provide a more sensitive measure of Clc-binding defects. The gel filtration analysis also showed no significant defect in Clc binding by the ABC mutant, in accord with the results of the more stringent co-precipitation assay (Figure 3E, ABC panel). Hence the results of the gel filtration analysis suggest that neither the D mutation alone, nor the combined A, B and C mutations, are sufficient to disrupt Clc binding. In contrast, the combination of all four mutations in Chc1-521p caused a substantial Clc-binding defect based on distinct Chc and Clc profiles with a Clc peak in fractions 18 and 20 (Figure 3E, ABCD panel). In summary, the analysis presented above demonstrated that while the trimerization domain mutation E1590K is chiefly responsible for the trimerization and Clc-binding defects of Chc1-521p, however, there is also a contribution from the ABC mutations to the Clc-binding defect.
Clathrin triskelion structure

Fig. 3. Analysis of the contribution of individual Chc1-521 mutations to defects in trimerization and Clc binding. (A, B and C) Results of co-immunoprecipitation of Chc with antibodies directed against Clc. Samples were prepared and analyzed as described in the legend to Figure 2A. Odd-numbered lanes show co-immunoprecipitated samples and even-numbered lanes show untreated extract. The S1331F mutation is designated as A, the S1347F mutation as B, the P1553L mutation as C, the E1590K mutation as D and the E1590A mutation as D*. Clc signals were detected in all extract samples in the original immunoblot although the signals in some lanes are not apparent in the photographic reproduction. (D) Location of the four Chc1-521 mutations as shown in Figure 1. (E and F) Sepharose CL-4B column elution profiles of Chc and Clc from mutant strains grown at 24°C. Samples were analyzed as described in the legend to Figure 2B. The WT and ABCD panels are the same as shown in Figure 2 and are included to facilitate comparison.

To characterize the importance of position 1590 in Clc binding and trimerization in more detail, we replaced E1590 with an uncharged residue, alanine. The E1590A mutant (designated D*) exhibited a defect in Clc binding by co-precipitation which was not as severe as that of the E1590K mutant (Figure 3C, lane 3), and gel filtration analysis showed a wild-type trimerization and Clc-binding profile (Figure 3F, D* panel). The less severe phenotype of the D* trimerization domain mutation in both of the Clc-binding assays suggested that this mutation could be useful in further probing the contributions of the other Chc1-521 mutations to Clc binding. We focused on the A
B. Pishvaee, A. Munn and G. S. Payne

and B mutations since they are situated in the proximal leg domain within a region previously shown to bind Clc. Although the A and B mutations did not cause Clc-binding defects by co-precipitation (Figure 3A, lane 9), Clc binding was abolished when A and B were combined with D* (Figure 3C, lane 1). Gel filtration profiles of AB, D* and ABD* mutants also demonstrated that combining AB with D* causes defects in both Clc binding and Chc trimerization (Figure 3F). These results indicate that the A and B proximal leg mutations, which do not disturb Clc binding or Chc trimerization by themselves, interfere with both properties when combined with the D* trimerization domain mutation.

Taken together, the results shown in Figure 3 indicate that mutations in two separate regions, the trimerization and proximal leg domains, can destabilize Clc binding. The effect on Clc binding of the trimerization domain mutations at residue 1590 raised the possibility that Clc may extend into, and interact with, the trimerization domain.

**A trimerization domain mutation enhances the affinity of Chc for Clc**

A second hydroxylamine-induced, temperature-sensitive allele of *CHC1, chc1-523*, was analyzed. This mutant carries two mutations which convert E1366 to K and L1560 to F. Surprisingly, when the co-precipitation assay was applied to mutant cell extracts, higher levels of Clc were precipitated compared with Clc co-precipitated from wild-type cell extracts (Figure 4A, lanes 3 and 4; note the relative intensities of Clc and Chc bands in the two lanes). This result was not due to differences in Chc levels in the extracts (Figure 4A, lanes 1 and 2). Nor was it due to non-specific precipitation (or aggregation) of the mutant Chc during the Clc immunoprecipitation procedure since immunoprecipitation of glucose-6-phosphate dehydrogenase from mutant cell extracts did not result in any detectable co-precipitation of Chc (data not shown). The results of the Chc co-precipitation shown in Figure 4A were quantified as the ratio of Clc to Chc precipitated and normalized to the ratio observed in the wild-type extract lane (Figure 4B, lane 2). Comparison of the relative ratios of Chc/Clc in the immunoprecipitate lanes (Figure 4B, lanes 3 and 4) indicates that there was three times more Chc1-523 protein precipitated than wild-type Chc. When averaged over three experiments, the amount of Chc1-523 co-precipitated with Clc was 3.1 ± 0.5 times greater than the wild-type Chc, while this ratio was nearly equal in the extracts (1.2 ± 0.1 times wild-type). A mutant carrying only the L1560F mutation also displayed the unusual co-precipitation behavior (data not shown; this mutant did not result in a temperature-sensitive growth defect).

**Fig. 4.** A mutation at position 1560 within the trimerization domain enhances the affinity of Chc for Clc. (A) Co-immunoprecipitation of Chc1-523. E: cell extracts. Ip: samples precipitated with anti-Clc antibodies. (B) The relative levels of Chc and Clc were quantified by densitometry of the immunoblot in (A). The Chc/Clc ratio in the WT extract was set as unity and the other ratios were expressed relative to the WT extract value.

**Fig. 5.** A mutation at position 1560 within the trimerization domain disrupts Clc binding without affecting trimerization. Sepharose CL-4B fractionation of clathrin from cells expressing the AB mutant Chc (A, taken from Figure 3F) and clathrin from cells expressing the AB L1560R mutant Chc (B). AB designates the S1331F and S1347F mutations.
Because the anti-Clc antibodies destabilize Chc–Clc interactions, the increased level of co-precipitated mutant Chc suggests that the mutation actually stabilizes the binding of Clc. Thus, a change of leucine to phenylalanine at position 1560 within the trimerization domain enhances the affinity of Chc for Clc. This result lends considerable support to the possibility of a direct interaction between the trimerization domain and Clc.

**Site-directed mutagenesis of the trimerization domain**

To explore further the possibility that Clc interacts with the trimerization domain, site-directed mutagenesis was used to engineer additional mutations within the domain. Since the trimerization domain is predicted to form a predominantly α-helical structure (Garnier et al. 1978; Näthke et al. 1992; Rost and Sander, 1993), we chose sites on different faces of the putative helix. To increase the probability of causing binding defects, selected neutral or acidic amino acids were converted to arginine, thereby introducing a positive charge. Also, to increase the sensitivity of the assays, we combined the site-directed mutations with the vertex-distal S1331F and S1347F changes identified in chc1-521 (referred to as A and B). The strategy of combining mutations arose from characterization of the Chc1-521 mutant, which indicated that mutations distributed in two separate domains contribute to the full extent of the Clc-binding and trimerization defects. In particular, the two S→F mutations, located in the vertex-proximal leg segment implicated in Clc binding by Liu et al. (1995), did not cause defects by themselves but accentuated mild defects resulting from the trimerization domain mutation E1590A. These results suggested that extensive interactions of Clc with both the proximal leg and the trimerization domain might obscure the effects of individual mutations. Hence we generated the site-directed trimerization domain mutations in a template that included the two S→F mutations.

The first site chosen for mutagenesis was L1560, since the enhanced Clc binding by the L1560F mutant suggested a possible interaction of this residue with Clc. Co-precipitation with anti-Clc antibodies indicated that the AB L1560R mutant was defective in Clc binding (see Figure 6, row 2; data not shown). Figure 5 presents the gel filtration elution profiles of Chc and Clc from cells expressing the AB L1560R mutant. For comparison, the gel filtration profiles from cells expressing the AB mutant are also presented (Figure 5A). The presence of the L1560R mutation causes a dramatic shift in the Clc elution peak while leaving the Chc elution pattern undisturbed (Figure 5B). Thus, the L1560R mutation, in combination with the two S→F changes, causes a striking defect in Clc binding without affecting trimerization. When the L1560R mutation was present alone, the Clc-binding defect was not evident in the gel filtration analysis and was only partially evident in the co-precipitation assay (Figure 6, row 8; data not shown). This marginal effect of the L1560R mutation, similar to the effect of the D* mutation, validates the strategy of using multiple mutations to provide a more sensitive means of measuring Clc-binding or trimerization defects.

Mutations introduced at other sites in the trimerization domain could be classified into three groups: (i) those which primarily disrupt Clc binding; (ii) those which disrupt both trimerization and Clc binding; and (iii) those with no dramatic effects on either property. In the first class is the AB L1575R mutant (Figure 7, panel AB L1575R; Figure 6, row 4). The elution profile of this mutant Chc shows a partial defect in trimerization but a severe Clc-binding defect, similar to the AB L1560R mutant. AB L1582R may also be classified as a less extreme member of this class, exhibiting a weaker Clc-binding defect (Figure 7, panel AB L1582R; Figure 6, row 5). In the second class, AB F1572R and AB L1559R display defects in both trimerization and Clc binding (Figure 7, panels AB L1559R and AB F1572R; Figure 6, rows 1 and 3). Class 3 mutant AB I1591R does not show drastic effects on either property (Figure 7, AB I1591R; Figure 6, row 6).

The trimerization domain mutations lie within sequences...
Mutations that preferentially affect Clc binding lie on one face of a putative α-helix. (A) The position of mutations within the trimerization domain. The large bold letters indicate positions where a mutation alters both Clc binding and trimerization. The medium-sized bold letters indicate positions where a mutation preferentially affects Clc binding. The small bold letter (I1591) indicates a position where mutation has no significant effect. (B) The region of the trimerization domain encompassing the mutations is likely to be α-helical (Garnier et al., 1978; Rost and Sander, 1993). A helical wheel representation of this region is presented with mutations which affect both Clc binding and Chc trimerization (thick arrows) and mutations preferentially disrupting Clc binding (designated by thin arrows).

Discussion

The structural organization of the clathrin triskelion hub region has been probed through the analysis of random and site-directed yeast Chc mutants. The results generally offer independent support for the low resolution structural model of the triskelion hub proposed by Näthke et al. (1992) and Liu et al. (1995). However, while that model positions Clc entirely within the Chc vertex-proximal segment, our analyses of Chc mutants provide strong evidence that Clc also interacts with the trimerization domain. Establishing the position of Clc in the trimer structure is important because Clc exhibits properties which suggest that it plays a role in regulating clathrin dynamics, including phosphorylation, and binding to calcium, calmodulin and the Hsc70-uncoating ATPase (Brodsky et al., 1991).

Fig. 7. Gel filtration chromatography of clathrin from strains expressing Chc trimerization domain mutants. Elution profiles of Chc and Clc from site-directed trimerization domain mutants were quantified as described in the legend to Figure 2B. AB and AB L1560R panels are taken from earlier figures and are included for comparison. AB designates the S1331F and S1347F mutations.

Clc spans the Chc trimerization domain and the vertex-proximal leg domain

Characterization of Chc1-521 and derivatives revealed the possible involvement of both the trimerization and proximal leg domains in Clc binding. The E1590K mutation by itself abolished trimerization, a result which is consistent with its position within the trimerization domain defined in bovine Chc (Liu et al., 1995; Figure 9A) and which establishes this residue as critical for trimer interactions or for proper folding of the trimerization domain.
Fig. 9. Model for the organization of the triskelion hub. (A) Clc interacts with the trimerization domain and the vertex-proximal leg segment of Chc. Cylinders represent the putative helices involved in Chc-Clc binding. The globular Chc domain, visible under EM at the trimer vertex, is represented as a sphere. This organization is based on the yeast Chc mutations (marked by Xs) described in this study and characterization of domains within the triskelion hub of bovine Chc (Näthke et al., 1992; Liu et al., 1995). The general folding pattern of Chc and the amino acid positions of the boundaries of each domain are taken from the model proposed by Liu et al. (1995). Mutations that disrupt yeast Chc trimers define a region involved in trimerization, consistent with the boundaries of the trimerization domain (residues 1550–1615) recently defined by Liu et al. (1995). A subset of mutations within this trimerization domain destabilizes Clc binding without dramatic effects on trimerization. Based on these mutations, we propose that Clc interacts directly with the trimerization domain. Proximal leg mutations fall within the Clc-binding region in bovine Chc (residues 1213–1313 and 1438–1522) defined by Liu et al. (1995). Residues 1151–1178 are predicted to form coiled-coils and hence might be involved in a Chc helix bundle in the proximal domain. The C-terminus of Clc (C) is shown interacting with the trimerization domain based on antibody mapping of rat Clc within trimers which positions the Clc C-terminus at the trimer vertex (Kirchhausen et al., 1983; Kirchhausen and Toyota, 1993). Conflicting evidence has been presented concerning the orientation of the Clc N-terminus (N) (Näthke et al., 1992; Kirchhausen and Toyoda, 1993). We show an orientation consistent with the data of Kirchhausen and Toyoda (1993), but our experiments do not address this issue. (B) Three-dimensional model of the triskelion vertex region involved in Clc binding. The central feature of this model is the positioning of Clc as a molecular hinge connecting the Chc proximal leg to the trimerization domain. By extending into the trimerization domain, Clc is strategically positioned to influence the flexibility around the trimerization domain–proximal leg joint. Clc-mediated changes in the angles at the trimer vertex could affect the pucker of the triskelion and consequently the curvature and/or stability of clathrin lattices. The N-terminal region of Clc which is not involved in Clc binding has been omitted for clarity.
a role for Clc in trimer stabilization, analysis of Chc in yeast cells lacking Clc indicates that trimerization is partially compromised (Chu et al., 1996; Huang et al., 1997). Furthermore, in experiments involving proteolytic fragmentation of triskelions, Clc was only associated with trimerized Chc, consistent with a role for Clc in stabilizing trimers (Näthke et al., 1992). This role could also account for the partial loss of trimerization in the AB L1575R mutant which exhibits a severe Clc-binding defect. However, trimers can be formed, in the absence of Clc, by high level expression of Chc fragments in bacteria, indicating that the contribution of Clc to trimer stabilization is not essential.

**Clc interacts with the Chc trimerization domain**

The specific effects of several trimerization domain mutations on Clc binding argue that Clc contacts the trimerization domain. The L1560F mutation in the trimerization domain enhanced the level of Chc co-precipitated with Clc, indicating that the mutation stabilizes the Chc–Clc association. Conversion of the same L1560 residue to R abolished Chc co-precipitation and, in combination with the S→F proximal segment mutations, severely reduced Clc binding without significantly affecting trimerization in the gel filtration assay. The effect of L1560R on Clc binding is remarkably specific given the observation that mutation of the adjacent residue, L1559 to R, affects trimerization as well as Clc binding. Finally, L1575R or L1582R, in combination with the S→F mutations, also resulted in strong defects in Clc binding and less severe trimerization defects. Based on these observations, and the evidence that Clc also binds to the proximal leg, we propose that Clc extends from the proximal leg into the trimerization domain as shown in Figure 9A.

An alternative, more complicated model to account for the specific effects of trimerization domain mutations on Clc binding would place Clc entirely in the proximal leg domain while invoking an interaction between the trimerization domain and proximal leg which stabilizes Clc binding. Given this interaction, specific mutations in the trimerization domain could influence Clc binding to the proximal leg segment without disturbing trimerization. While this possibility cannot be excluded, we prefer the more straightforward interpretation that the trimerization domain directly contacts Clc.

The mutations that affect Clc binding more severely than trimerization identify residues in the trimerization domain which are candidates to interact directly with Clc. Secondary structure prediction programs reveal that the sequences encompassing the trimerization domain mutations (residues 1555–1591) may form a continuous α-helix (Garnier et al., 1978; Rost and Sander, 1993). The location of L1560, L1575 and L1582 on one face of this helix potentially defines a region that contacts Clc (Figure 8B). Even though we represent this region as a continuous α-helix, the glycine at position 1567 has the potential to break the helix into two segments (Garnier et al., 1978; Rost and Sander, 1993). In this case, L1575 and L1582 would identify a Clc-binding surface on one of the helices while L1560 would identify a Clc-binding site on the other helix.

In contrast to the mutations which preferentially affect Clc binding, E1590K, E1590A, F1572R and L1559R have strong effects on trimerization. E1590K/A and F1572R fall within the same helix in either the one or two helix models, and their position on one face of this helix defines a putative Chc–Chc binding interface (Figure 9B). L1559R displays a more severe Clc-binding defect, suggesting that this residue, consistent with its position on the continuous helix between the trimerization and Clc-binding faces, might be involved in Clc binding as well. If Clc and Chc helices are arranged as shown in Figure 9B, then L1559 could be located in or near a second Clc-binding face on the trimerization helix.

Our conclusion that Clc interacts with the trimerization domain is at variance with positioning of Clc in the bovine clathrin hub model (Näthke et al., 1992; Liu et al., 1995). In this model, the Clc-binding and trimerization domains are proposed to be physically and functionally distinct (Figure 1C). This aspect of the model is based largely on studies of trimerization and Clc binding by bacterially expressed fragments of Chc (Liu et al., 1995). The contribution required for binding to Clc is contained in the vertex-proximal segment of the triskelion leg, between residues 1213 and 1522. This region, produced in bacteria, binds Clc but lacks sequences necessary for trimerization. In agreement with this observation, our results indicate that the trimerization-defective E1590K mutant retains Clc binding (Figure 3E, panel D). In contrast, C-terminal fragments beginning at residue 1550 trimerize in the absence of Clc when expressed in bacteria. Thus, the two domains were proposed to function independently. However, the C-terminal trimerization domains were not tested for Clc binding. Thus, while these studies show that the proximal leg can bind to Clc in the absence of the trimerization domain, they do not preclude interactions between the trimerization domain and Clc.

The orientation of Clc in the triskelion hub model (Figure 9A) is based on antibody mapping studies which located the Clc C-terminus to the triskelion vertex (Kirchhausen et al., 1983; Brodsky et al., 1987; Kirchhausen and Toga, 1993). Analysis of Chc binding sequences in Clc have mapped the principal binding sequences to a region of heptad repeats predicted to form an α-helical coiled-coil within the core of Clc (Scarmato and Kirchhausen, 1990). However, deletion of 25 amino acids from the C-terminus of Clc resulted in a slight decrease in binding, leading to the suggestion that the Clc C-terminus might provide an initial anchor to Chc (Scarmato and Kirchhausen, 1990). Our model suggests that this interaction occurs with the Chc trimerization domain. However, the relatively small effect of the Clc C-terminal deletion on binding (Scarmato and Kirchhausen, 1990) and the binding of Clc by the triskelion proximal leg segment lacking the trimerization domain (Liu et al., 1995) indicate that the interaction between the trimerization domain and the Clc C-terminus provides only limited contributions to the overall Chc–Clc binding energy. This point further demonstrates the utility of probing the structure of clathrin using site-directed mutagenesis. Being able to introduce and combine mutations in two independently folding domains of Chc has allowed us to demonstrate interactions of Clc with the trimerization domain which otherwise would have been obscured by
the extensive association of Clc with the proximal leg segment.

The folding pattern of Clc in the triskelion proximal leg is currently unresolved. Competitive binding of triskelia by antibodies directed against the two ends of the central coiled-coil domain of Clc which is essential for Clc binding, along with secondary structure predictions, has led to the proposal that the central Clc domain bends so that the N- and C-termini are in proximity (Näthke et al., 1992). In contrast, EM mapping of anti-Clc antibody epitopes and the more recent mapping of an epitope tag, placed immediately N-terminal to the Clc coiled-coil domain, suggest that Clc is extended along the Chc proximal leg with the N-terminus positioned near the Chc proximal–distal leg boundary (Ungewickell, 1983; Kirchhausen and Toyoda, 1993). Since both proposals orient the Clc C-terminus towards the trimer vertex, our results are compatible with either view.

A new model of the triskelion vertex

If the three-dimensional structure of the clathrin hub region is considered, positioning the Clc C-terminus in contact with the trimerization domain, as shown in Figure 9B, has significant functional consequences. The sequences containing the trimerization domain and extending to the C-terminus are thought to form a protuberance, visualized in the EM, that extends from the trimer vertex (Kirchhausen et al., 1986; Figure 9B). The proximal segments splay out from the vertex in a downward direction to give the hub region a concave surface or pucker. If Clc extends from the proximal segment into the trimerization domain, then it could act as a molecular hinge to influence the angle between the trimerization domain and the proximal segments, which in turn would influence the concavity or pucker of the triskelion. In this way, the trimer hub can be viewed as a molecular tripod with adjustable leg angles. Regulatory parameters such as calcium binding, calmodulin binding or phosphorylation could modulate the tripod angles by altering Clc conformation or positioning on Chc. Hence, Clc-mediated changes in the angles at the trimer vertex could affect the concavity of the triskelion and consequently the curvature and/or stability of clathrin lattices.

It is intriguing that the Clc C-terminus, which we propose to associate with the Chc trimerization domain, also binds calmodulin (Pley et al., 1995). This calmodulin binding to the Clc C-terminus has the potential to modulate the Clc binding to the trimerization domain and in turn influence the pucker of the trimer in a calcium/calmodulin-dependent way. For example, calcium-dependent calmodulin binding could simply displace the Clc carboxy end from the trimerization domain. Partial or complete displacement of the Clc C-terminus from the trimerization domain would allow the hinge to acquire the flexibility needed to accommodate curvature changes required during coated vesicle biogenesis or uncoating reactions. In contrast, in the absence of the destabilizing regulatory parameters like calcium/calmodulin, the stable binding of Clc to the trimerization domain could restrict the hinge flexibility and hence trap the pucker of the trimer at a conformation incompatible with premature empty cage assembly in the cell cytosol. This hypothesis is consistent with the observation that Clc prevents empty cage assembly in the absence of regulatory parameters like calcium (Ungewickell and Ungewickell, 1991; Liu et al., 1995).

As discussed earlier, our results can also be accommodated by a model in which the trimerization and proximal leg domains interact but Clc binds only to the proximal leg segment. However, even from a position entirely within the proximal leg, Clc would still be able to mediate changes in the triskelion pucker by altering interactions between the two Chc domains.

Regardless of the specific regulatory mechanisms involved in triskelion conformational dynamics, our mutational analysis of triskelion organization suggests that Clc is strategically positioned to influence the triskelion pucker. This novel view of the triskelion vertex offers a mechanism by which Clc could alter the propensity of clathrin to form planar versus caged lattices, as well as a means for Clc to regulate lattice assembly and/or disassembly. Thus, our model of the triskelion provides a structural basis for Clc-mediated regulation of the cycle of lattice assembly and disassembly that is fundamental to clathrin function.

Materials and methods

Materials

Unless noted, all reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Strains, genetic methods and media

Yeast strains used in this study are derived from GPY 68.1 (MATα leu2 3.112 ura3-52 his4-519 trp1-289 prb1 gal2 chc1-ΔH-1:LEU2). Yeast centromere plasmids harboring different alleles of the CHCI gene were introduced into GPY 68.1 by lithium acetate-mediated transformation (Ito et al., 1983). SD medium is 0.67% yeast nitrogen base (Difco Laboratories, Inc, Detroit, MI), 2% dextrose, with 20 μg/ml each of histidine, methionine, uracil and tryptophan and 30 μg/ml of adenine, leucine and lysine. SD CAA medium is SD containing 5 mg/ml vitamin assay casamino acid mix (Difco), with 15 μg/ml adenine and 10 μg/ml histidine, methionine, uracil and tryptophan. To select for cells carrying plasmids, SD CAA or SD medium was used which lacked the appropriate supplement, either uracil or tryptophan. YP medium is 1% yeast extract, 2% Bacto-Peptone (Difco). YPD is YP plus 2% dextrose. All solid media contained 2% agar (Difco). Cell densities in liquid culture were measured in a 1 cm plastic cuvette using a Beckman Instruments DU-62 spectrophotometer. One A600 unit is equivalent to a density of 10^7 cells/ml.

Plasmid construction and mutagenesis

The temperature-sensitive mutants were isolated by random hydroxylamine mutagenesis as described (Seeger and Payne, 1992). Single mutations were introduced, by oligonucleotide-primed, site-directed mutagenesis (Kunkel et al., 1987). The Srf1–SalI fragment of the CHCI gene was cloned into the pBKs vector (Stratagene, La Jolla, CA). Single-stranded DNA was produced by infecting a Dut ung– phage M13K07 as described in Vieira and Messing (1987).

Double mutations were constructed by taking advantage of two HindIII sites within the Srf1–SalI fragment. One map downstream of the two proximal leg mutations (S1331F, S1347F) but 5’ of the trimerization domain mutations. The other HindIII site maps 3’ of the trimerization domain and outside the CHCI reading frame. To separate the trimerization domain and proximal leg domain mutations, HindIII fragments between wild-type and mutant pBKs-Srf1–SalI plasmids were exchanged. The triple mutants were constructed by introducing the third mutation(s) by site-directed mutagenesis on a double mutant template. Hence all the trimerization domain mutations were introduced as single mutations on a single-stranded template that contained the S1331F and S1347F mutations. All mutations were confirmed by dideoxy DNA sequencing of the constructs, using the method of Sanger et al. (1977). Finally, the mutant Srf1–SalI fragment was subcloned in place of a wild-type Srf1–SalI fragment in the yeast centromere plasmid

2237
The page contains a section discussing the extraction preparation of a protein sample, immunoblotting procedures, and references. It also includes a table and some mathematical equations. The text is a mixture of scientific terminology and technical descriptions. The references are cited throughout the document. The page is part of a larger scientific manuscript, likely from a journal, discussing the isolation and characterization of certain proteins using various biochemical techniques. The text is dense with scientific content and includes detailed experimental setups and results. The references at the end cite a variety of scientific journals and books, indicating the manuscript’s reliance on established scientific literature. The overall structure of the page suggests it is part of a research paper or a scientific review article.


Received on October 14, 1996; revised on December 11, 1996