

Analysis of Clathrin Light Chain-Heavy Chain Interactions Using Truncated Mutants of Rat Liver Light Chain LCB3*

(Received for publication, September 21, 1989)

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Clathrin light chains are extended molecules located along the proximal segment of each of the three heavy chain legs of a clathrin trimer. All mammalian light chains share a central segment with 10 repeated heptad motifs believed to mediate the interaction with clathrin heavy chains. In order to test this model in more detail, we have expressed intact rat liver clathrin light chain LCB3 in *Escherichia coli* and find that it binds tightly to calf clathrin heavy chains. Using a set of expressed truncated mutants of LCB3, we show that the presence of seven to eight heptads is indeed necessary for a successful interaction. More extensive deletions of the central segment completely abolish the ability to bind to heavy chains. Neither the amino- nor the carboxyl-terminal domain is essential for binding, but competition experiments show that the presence of the carboxyl-terminal domain does enhance the interaction with heavy chains.

Clathrin-coated pits and coated vesicles are organelles found in association with the plasma membrane and Golgi complex of eukaryotic cells that mediate the selective entrapment of macromolecules destined for vesicular traffic (reviewed in Refs. 1-4). It is believed that self-assembly of clathrin, the main structural unit of the cytoplasmic lattice surrounding the membrane, generates the driving force for vesiculation (5). Clathrin has a pinwheel-shaped three-legged structure (triskelion) (6, 7), and each leg is composed of a heavy and a light chain (6, 7). The heavy chain appears to be the invariant scaffold of the leg, as heavy chains from rat (1675 residues) and yeast (1653 residues) are encoded by single genes, and their mRNA shows no evidence of differential processing (8, 9).¹ However, mammalian light chains are heterogeneous, and they fall into two related classes, known as LCA and LCB (6, 7, 11-14). Members of each class are encoded by a different gene with an overall 60% identity among their primary structures (11, 14). Their mRNAs are differentially spliced in a tissue-specific manner, giving rise to several light chain types of different size (11, 14). In rat, for example, LCA1 and LCB2 are exclusively found in brain, whereas LCA3 and LCB3 are common to all tissues (11). Only one light chain is tightly and noncovalently bound to the proximal segment of each heavy chain leg (15, 16), and the

distribution of the different types on an individual clathrin trimer is nearly random (15). The function of the light chains is at present uncertain, although their tissue variability suggests possible regulatory roles in the formation of coated vesicles.

We have recently demonstrated that the polypeptide chains of all light chains share a central segment of 10 repeated heptad motifs, characteristic of α -helical coiled coils surrounded by the amino- and carboxyl-terminal segments of 92 and 48 residues, respectively (11). We have proposed a model in which this segment mediates binding to clathrin heavy chains while the surrounding regions mediate interactions with other proteins. This model is supported by the observation that several monoclonal antibodies directed against epitopes located within the heptad region inhibit the light chain-heavy chain interaction (17). However, no comparable data is available for the outer segments. The binding properties of the amino-terminal segment could not be directly tested because there were no antibodies directed against epitopes located in this region. Nevertheless, an indication that at least part of this segment interacts with other proteins is the fact that type II casein kinase, found in association with coated vesicles, can in solution phosphorylate serine residues at positions 11 and 13 located at the amino-terminal segment of LCB2 and LCB3 (18). Unlike the epitopes located in the central segment, the antibodies directed against epitopes in the carboxyl-terminal segment did not block light chain-heavy chain binding. However, these antibodies also react with native light chain-containing clathrin trimers indicating that the epitopes are accessible because they are oriented toward the aqueous environment. Since the lack of blocking activity by the antibodies could be explained by a relative orientation of epitopes away from the surface of light chain-heavy chain interaction it is not possible to conclude with certainty that the carboxyl-terminal segment of the light chains does not interact with the heavy chain.

We now report experiments that test part of our model in more detail. We have expressed rat liver clathrin light LCB3 in *Escherichia coli* and shown that it binds specifically to calf clathrin heavy chains. We have then constructed LCB3 truncation mutants lacking the amino- or carboxyl-terminal segments and found that these mutants still bind to heavy chains. However, the presence of seven to eight heptad motifs is essential for binding demonstrating that the central segment is directly involved in the surface contacts that stabilize the interactions with the heavy chain. In addition, we find that removal of the carboxyl-terminal segment of LCB3 decreases the strength of the light chain-heavy chain interaction suggesting that this region may be required for initial anchorage of light chains to heavy chains followed by the more extensive interaction mediated by assembly of the helical segment.

* This work was supported in part by Grant R01 GM36548-01 from the National Institutes of Health (to T. K.), a Grant-in-Aid from the American Heart Association (to T. K.) and by a contribution from the M. Peckett Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ S. Lemon, personal communication.

MATERIALS AND METHODS

Purification of Bovine Clathrin Heavy and Light Chains—Clathrin trimers containing heavy and light chains were obtained by Tris extraction from calf brain coated vesicles (19, 20). Heavy chain trimers were subsequently separated from their bound light chains by dialysis against a solution containing 1.35 M KSCN, 50 mM triethanolamine, pH 8.0, 2 mM EDTA, 0.5 mM DTT,² 0.5 mM PMSF, 0.02% NaN₃, followed by sizing chromatography on a Sephacryl S-300 (Pharmacia LKB Biotechnology Inc.) column (1 × 60 cm) equilibrated with the same buffer (21, 22).

A pool of all classes and types of bovine light chains was isolated from a boiled solution of brain clathrin trimers (13, 23). Briefly, clathrin (5–10 mg/ml in 50 mM triethanolamine, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.02% NaN₃) was incubated at 95–98 °C for 5 min. Soluble light chains were recovered in the supernatant of the sample following centrifugation at maximum speed (Eppendorf microcentrifuge) for 20 min at 4 °C.

Recombinant Plasmids—Conventional DNA recombinant technology was used to create the recombinant plasmids (24), and the constructs were verified by DNA chemical sequencing (25) and restriction mapping. Below follows the detailed description for the transfer of the protein coding sequence of rat liver light chain LCB3 from the plasmid clone ppb5-2 (11) into the secretion vector pINIII-ompA3/lpp/p-5 (26, 27) and for the generation of truncation mutants.

The full-length cDNA for rat LCB3 was first inserted into the *Bam*HI site of the plasmid Bluescript (Stratagene). A blunt *Eco*RI linker (GGAATTCC, New England Biolabs) was introduced in the unique light chain *Hpa*I site, 250 base pairs downstream from the stop codon of LCB3. The first 45 bases, starting with the initiation codon of LCB3 and up to the unique light chain *Apa*I site were replaced by two complementary synthetic oligonucleotides allowing the creation of an *Nco*I site (CCATGG) at the start codon. The sequences of these oligonucleotides are 5-CATGGCTGAGACTT-CGGCTTCTTCTCGTTCGTCGGAGAGCGGGGCC and its complement 5-CCGCTCTCCGACGACGAGAAGAAGCCGAAGTCCTCAGC. When these annealed oligonucleotides, the *Apa*I-*Eco*RI LCB3 insert, and the vector pkk-394-5 (28) linearized with *Nco*I and *Eco*RI are ligated, they create pkk-394-5/rLCB3. The protruding ends of the *Nco*I site were filled in with Klenow fragment (DNA polymerase I) and ligated to the *Eco*RI linker. The *Eco*RI-*Eco*RI insert containing the coding segment for LCB3 was ligated to the secretion expression vector pINIII/ompA3 (26), linearized at the *Eco*RI site of the polylinker. This site is downstream of the secretion and cleavage signal sequences of ompA. This construct introduces three amino acids (glycine, proline, and isoleucine) after the cleavage sequence and before the first translated methionine of LCB3. The expression levels were improved by exchange of the *Lpp* promoter of this vector with the modified *Lpp* p-5 promoter of the vector PINIII-ompA3/lpp/p-5 (27). This was accomplished by exchange of the *Pst*I-*Xba*I vector fragment upstream of the light chain insert. The new plasmid is referred to as rLCB3.

The truncation mutants N29, N36, N78, and N92 with deletions of amino acids 1–29, 1–36, 1–78, and 1–92 from the amino-terminal domain were obtained in two steps. First we eliminated the *Eco*RI site located downstream of the LCB3 insert by partial *Eco*RI digestion of rLCB3 followed by a fill-in reaction with Klenow fragment and religation. This creates the plasmid rLCB3/RI bearing the remaining 5' *Eco*RI site upstream of the LCB3 insert. Second, rLCB3/RI was linearized with *Eco*RI, treated with *Bal*31 nuclease followed by a fill-in reaction with Klenow fragment, and recircularized in the presence of the *Eco*RI linker. A miniprep was digested to completion with *Eco*RI and *Bam*HI, and the resulting deletion fragments were ligated to the vector portion of rLCB3 previously linearized with *Eco*RI and *Bam*HI.

N122 with a deletion of amino acids 1–122 (the complete amino-terminal domain and 4.5 adjoining heptads) was generated by complete *Eco*RI digestion of rLCB3/RI followed by partial digestion with *Bst*EII, filled-in with Klenow fragment, and religated.

Truncated light chains C87 and C32 with deletions of amino acids 127–211 and 182–211 from the carboxyl-terminal domain were obtained from the plasmid rLCB3. The plasmid was digested either with *Bst*EII followed by a fill-in reaction with Klenow fragment or with

*Pvu*II. After *Eco*RI digestion, the *Eco*RI blunt end fragments containing the initiation codon were ligated to the vector ompA3/lpp p-5/*Xba*I-stop previously linearized with *Hind*III, filled-in with Klenow fragment, and digested with *Eco*RI. This vector contains instead of the unique *Bam*HI site at the 3' end of the polylinker, a *Xba*I-stop linker (CTAGTCTAGACTAG, New England Biolabs) with stop codons in the three reading frames. Upon religation the resulting plasmids C87 and C32 introduce 5 and 7 amino acids before the corresponding stop codons.

N108, C47, and C69 missing amino acids 1–108, 163–211, and 143–211 were obtained by site-directed mutagenesis (29) of the plasmid pTZ18/rLCB3 using the mutagenesis kit of Bio-Rad. This plasmid is obtained by insertion of the *Xba*I-*Hind*III portion of rLCB3 containing the coding sequence for LCB3 into the vector pTZ19 (Bio-Rad) linearized at its polylinker with *Xba*I and *Hind*III. Synthetic oligonucleotides of sequence 5-ATCCGCAAGTGAATTTCAGGAGCA-GAA, 5-GGCTTTTGTGTAAGGATCCAAGGAG, and 5-GAAC-CAGCTCTAAGCTTACAGGTTGA were annealed to single-stranded pTZ19/rLCB3 and used as primers for second strand synthesis of the mutants N108, C49, and C69, respectively. The first oligonucleotide sequence replaces Arg-107 and Glu-108 for Ile and Gln and includes an *Eco*RI site. N108 was generated by complete *Eco*RI and *Hind*III digestion of the mutagenized plasmid followed by religation of the insert to the corresponding sites of the PINIII-ompA3/lpp/p-5 vector. The second oligonucleotide replaces Lys-163 with a translational stop followed by a *Bam*HI site. C49 was generated by insertion of the *Eco*RI-*Bam*HI fragment of the mutagenized plasmid into the corresponding sites of the vector PINIII-ompA3/lpp/p-5. The sequence of the third oligonucleotide introduces a translational stop instead of Gln-143 and includes a *Hind*III restriction site. C69 was generated by insertion of the *Xba*I-*Hind*III fragment from the mutagenized plasmid into the corresponding sites of the vector PINIII-ompA3/lpp/p-5.

Expression of Rat Liver Clathrin Light Chains in *E. coli*—*E. coli* cells (strains JM101 or DH5 α) were transformed with the secretion plasmid PINIII-ompA3/lpp/p-5 carrying the appropriate constructs of the rat liver clathrin light chain LCB3. One ml of cells was grown overnight at 37 °C to saturation and used to inoculate 120–150 ml of fresh medium (YT or 2YT) supplemented with 100 μ g/ml ampicillin and 2.5 mM isopropyl-1-thio- β -D-galactopyranoside. Cultures were allowed to grow at 37 °C to an optical density at 550 nm of 1.2. Cells were cooled in ice and pelleted by low speed centrifugation in a SA-14 rotor (Sorvall) at 7000 rpm for 5 min at 4 °C. The pellet was resuspended with 20 ml of an iced chilled solution containing 10 mM Tris, pH 8.0, 0.1% β -mercaptoethanol and recentrifuged in a SA-31 rotor (Sorvall) at 7000 rpm for 5 min at 4 °C. The pellet was resuspended in 20 ml of 20% (w/v) sucrose, 10 mM Tris, pH 8.0, 0.1% β -mercaptoethanol and incubated for 10 min at room temperature. After centrifugation, the cells were osmotically shocked (30) by rapid resuspension in 20 ml of iced chilled water followed by centrifugation at 10,000 rpm for 40 min at 4 °C. The supernatant containing periplasmic proteins was lyophilized, resuspended in 1 ml of 100 mM NaCl, 10 mM Tris, pH 7.4–8, 0.5 mM EDTA, 0.5 mM PMSF, 0.02% NaN₃ and boiled for 10 min. The sample became turbid and was clarified by centrifugation in a microcentrifuge (Eppendorf) at maximum speed for 20 min at 4 °C. The supernatant, significantly enriched with recombinant light chain products, was stored at –20 °C and directly used for all the experiments.

Amino-terminal Sequence Determination—The amino acid terminal sequence of the two major expression products of rLCB3 generated in *E. coli* were verified by protein sequencing. About 50 μ g of these products were purified, by preparative SDS-13% polyacrylamide gel electrophoresis (SDS-PAGE) (8), from other minor protein components of a periplasmic preparation of *E. coli* transformed with rLCB3. The proteins were isolated by electroblotting into an Immobilon (Millipore) membrane and subjected to automated amino-terminal sequencing in a sequenator (Applied Biosystems) (10, 31).

Cage Assembly—Cages were assembled from calf brain clathrin trimers containing heavy and light chains or from heavy chain trimers by dialysis at 4 °C against assembly buffer (20 mM NaMES, pH 6.2, 2 mM CaCl₂, 0.5 mM DTT, 0.5 mM PMSF, 0.02% NaN₃) (6). Cages were separated from unassembled clathrin trimers by high speed centrifugation in a 45 Ti rotor (Beckman) at 40,000 rpm for 35 min at 4 °C and resuspended in assembly buffer. Quality of cage assembly was assayed by negative staining electron microscopy (20). The presence or absence of light chains had no effect on the size distribution of cages nor in the yield of cage assembly.

Binding of Intact Recombinant Light Chain rLCB3 to Clathrin

² The abbreviations used are: DTT, dithiothreitol; PMSF phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

Trimers—Heavy chain clathrin trimers (0.3 mg/ml in 100 mM NaCl, 10 mM Tris, 25 mM triethanolamine, pH 8.0, 1 mM EDTA, 0.5 mM PMSF, 0.02% Na₂S₂O₈) were mixed with a molar excess of the recombinant products generated by the plasmid rLCB3 containing the full-length rat liver LCB3 light chain. The mixture, with a total volume of 200 μ l, was incubated for 3 h in ice. At the end of the incubation, the mixture was applied to a size exclusion Superose-6 column (HR10/30, Pharmacia) and fractionated by fast protein liquid chromatography (FPLC, Pharmacia) at room temperature. The samples containing heavy chains (void fractions) were pooled, concentrated (Centricron microconcentrator, Millipore), and analyzed by SDS-PAGE. The elution profile of the column was calibrated with intact calf brain clathrin trimers, calf brain light chains, ferritin, catalase, and aldolase.

Binding of Intact and Truncated Recombinant Light Chains to Clathrin Cages—Binding experiments between light chains and cages were conducted with samples in a total volume of 180 μ l in 25 mM NaCl, 30 mM NaMES, 4–5 mM Tris, pH 6.2, 1 mM CaCl₂, 0.5 mM EDTA. They normally contained 25–50 μ g of calf clathrin heavy chain cages and up to 60 μ l of native (calf) or recombinant (rat) light chains (0.1–0.5 mg/ml). When necessary, cages assembled with intact calf brain clathrin containing their endogenous light chains were used as nonbinding controls. Unless otherwise stated, the mixtures were incubated in ice for 3 h followed by high speed centrifugation (Beckman Airfuge, 20 p.s.i.g. for 45 min at room temperature) and analysis of supernatants and pellets by SDS-PAGE. Protein concentrations were estimated by Coomassie Blue staining of the SDS gels.

RESULTS

Expression of Full-length Rat Liver Clathrin Light Chain LCB3 in *E. coli*—The plasmid rLCB3 directs the synthesis of two principal protein products of similar size that accumulate in the periplasm of *E. coli* (Fig. 1, lane 3). They were purified from other periplasmic proteins by a procedure, described under "Materials and Methods," that takes advantage of the last resistance of clathrin light chains (13, 23). Amino-terminal sequence determination of both proteins showed that the ompA signal peptide was correctly removed, resulting in the production of LCB3 with the expected three extra amino acids from the linker sequence (glycine, isoleucine, and proline) at

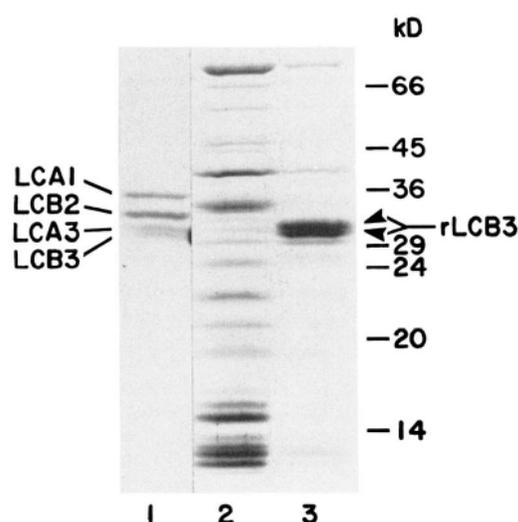


FIG. 1. Expression of rat liver LCB3 clathrin light chain in *E. coli*. The heat-resistant soluble fraction of calf brain clathrin or of the periplasmic proteins from *E. coli* were fractionated by SDS-13% PAGE and stained with Coomassie Blue. Lane 1, the most abundant classes and types of calf brain clathrin light chains: LCA1, LCB2, LCA3, and LCB3; lane 2, *E. coli* transformed with the expression vector pINIII-ompA3/lpp/p-5; lane 3, *E. coli* transformed with rLCB3, a plasmid containing the protein coding sequence for rat liver light chain LCB3 under control of the expression vector pINIII-ompA3/lpp/p-5. Arrows indicate the two main secretion products. The samples in lanes 2 and 3 derive from 7 ml of an *E. coli* culture. Size of calibration proteins is indicated.

the amino terminus. These residues were followed by methionine, alanine, and glutamic acid, corresponding to the first three residues at the amino terminus of authentic rat LCB3.

The larger of the two expression products of rLCB3 had a predicted size of 214 residues and displayed an electrophoretic mobility similar to native bovine light chain LCA3 (213 residues (14); Fig. 1, lane 1). Therefore, this recombinant product probably corresponds to the full-length rat liver LCB3. The smaller expression product, with a mobility similar to that of bovine LCB3 (210 residues (14); Fig. 1, lane 1), has apparently been subject to an additional proteolytic cleavage close to its carboxyl terminus. Measurement of its decreased ability to bind heavy chain clathrin cages relative either to the full-length rLCB3 or to other truncation products is consistent with this interpretation (see below). Based on the DNA sequence of the expression product of rLCB3, the molecular size of the full-length species is 23.5 kDa. However, its electrophoretic mobility corresponded to a calibration protein of 33 kDa. This result indicates that earlier determinations of molecular weight values based on electrophoretic mobilities on SDS gels (6, 7, 12, 13) or on ultracentrifugation measurements (16) have been overestimated by about 30%. Experiments described below show the sequences responsible for this anomaly. Two additional plasmid constructs containing the cDNA sequences for rat liver LCA3 (218 residues (11)) and rat brain LCB2 (229 residues (11)) light chains were also tested for expression in *E. coli*. The SDS gel patterns indicated the presence of several bands that can be attributed to a more extensive proteolytic degradation. These protein products bound to cages but were not characterized further (data not shown).

Rat Liver Light Chain LCB3 Expressed in *E. coli* Binds to Bovine Clathrin Heavy Chains—We have used two different approaches to demonstrate that clathrin light chains expressed in *E. coli* maintain their ability to bind to clathrin heavy chains. In the first approach, calf heavy chain trimers depleted of their endogenous light chains (Fig. 2, lane 1) were incubated with molar excess amounts of the expression products of rLCB3 (Fig. 2, lane 4). After 3 h of incubation, the mixture was fractionated by sizing chromatography using a Superose-6 column that allows for the complete separation of clathrin trimers from unbound light chains. SDS-PAGE analysis of the pooled void fractions showed that clathrin heavy chain trimers primarily coeluted with the larger of the two main expression products of rLCB3 (Fig. 2, lanes 2 and 3). Certain preparations of expressed LCB3 contained variable amounts of smaller fragments, in this case of less than 14 kDa (Fig. 2, lane 4), which also coelute with clathrin trimers. These fragments probably result from the proteolytic degradation of the expressed LCB3 by proteases present in *E. coli*. Inspection of the gel also suggests that the binding was specific, since none of the other proteins also present in the *E. coli* sample transformed with the vector alone coelute with clathrin heavy chains (Fig. 1, lane 2).

The second and more convenient approach involved incubation of expressed proteins with heavy chain cages, followed by high speed centrifugation and SDS-PAGE analysis of pellets and supernatants. Fig. 3 shows the results of an experiment designed to demonstrate the specificity of the interaction between the products of rLCB3 and clathrin cages. Calf brain heavy chain cages depleted of more than 95% of their endogenous light chains (Fig. 3, lane 1) were competent to rebound light chains. A mixture of calf brain light chains (LCA1, LCB2, LCA3, and LCB3) co-sedimented with the cages (in this case there is a preferential binding of LCB2, Fig. 3, lane 2), as did a similar amount of the full-length

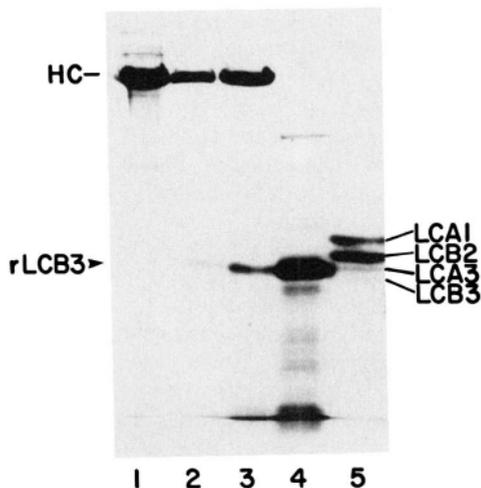


FIG. 2. Binding of expressed rat liver LCB3 clathrin light chain to clathrin heavy chain trimers. The rat light chain expression products generated in *E. coli* by the plasmid rLCB3 were mixed with calf brain clathrin heavy chain trimers applied to a sizing Superose-6 gel filtration column and void fractions analyzed by SDS-12% PAGE. Lane 1, sample of heavy chain trimers used in the binding experiment showing depletion of their endogenous light chains; lanes 2-3, void fractions pooled into two consecutive samples and concentrated by ultrafiltration (Centricon microconcentrator). The samples contain heavy chains (HC) coeluting with the larger of the two major expression products of rLCB3. Lane 4, polypeptide composition of the expression products generated by rLCB3 used in the experiment; the mobility of the two main recombinant rat liver products overlap due to overloading of the gel sample. Lane 5, calf brain clathrin light chains included for comparison. The precise identity of the small polypeptide chains running with the front of the samples in lanes 3 and 4 is not known. They are absent in *E. coli* preparations transformed solely with the expression vector pINIII-ompA3/lpp/p-5 (see Fig. 1, lane 2), and their relative yield varies with different preparations of *E. coli* transformed with rLCB3. These fragments may reflect the activity of proteases present in *E. coli* since clathrin light chains are very susceptible to enzymatic degradation (6).

recombinant product of rLCB3 (Fig. 3, lane 3). In contrast, the contaminant protein products from *E. coli* did not bind to cages and remain in the supernatant (Fig. 3, lane 9).

The results shown in Fig. 3 also demonstrate that under the experimental conditions the displacement of prebound light chains was negligible and that the interaction between light chain and heavy chains required specific binding sites in both proteins. The observations can be summarized as follows. First, the rat light chain recombinant products from rLCB3 were essentially unable to bind and sediment with cages which still contained their original set of endogenous calf light chains (Fig. 3, lane 4). Second, the products of rLCB3 were similarly unable to interact with heavy chain cages previously replenished with calf light chains (Fig. 3, lane 5). Third, prebound LCB3 is effective in blocking most of the binding of all types and classes of calf light chains (Fig. 3, lane 6).

Further support for the specificity of interaction between the recombinant rat light chain product of rLCB3 and calf heavy chain cages was the demonstration that their binding was saturable. In one type of experiment, cages were incubated with a constant molar excess of rLCB3 expression products and then analyzed for the amount of bound LCB3 that co-sedimented with cages after different incubation periods (Fig. 4). We found that within 44 min of incubation (Fig. 4, lane 3) a maximal amount of LCB3 is bound and that this amount did not increase during further incubation for up to 6 h (Fig. 4, lane 7). In order to ensure that the interactions between the heavy and light chains had reached saturation, most of

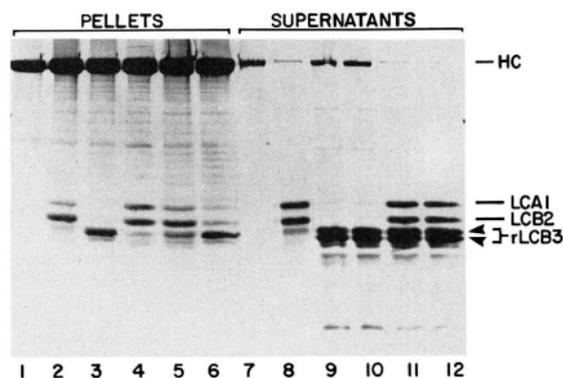


FIG. 3. Binding of expressed rat liver LCB3 clathrin light chain to clathrin cages. 60 μ l of a solution containing approximately 30 μ g of rat light chain expression products generated by rLCB3 in *E. coli* or authentic calf brain light chains were incubated with 40 μ g of cages assembled from calf brain clathrin heavy chains (HC). The samples (180 μ l) were centrifuged in the Airfuge, and pellets (lanes 1-6) and one-half of the supernatants (lanes 7-12) were analyzed by SDS-13% PAGE. Lanes 1 and 7, control of heavy chain cages showing absence of endogenous light chains; lanes 2 and 8, heavy chain cages mixed for 6 h with authentic calf brain light chains; lanes 3 and 9, heavy chain cages mixed for 6 h with the rat light chain expression products of rLCB3; lanes 4 and 10, clathrin cages containing their intact set of calf brain light chains mixed for 6 h with the expression products of rLCB3; lanes 5 and 11, heavy chain cages, first mixed with calf brain light chains for 3 h followed by the addition of the expression products of rLCB3 for another 3 h; lanes 6 and 12, heavy chain cages, first mixed with the expression products of rLCB3 for 3 h followed by the addition of calf brain light chains for another 3 h. Labels are as in Fig. 2.

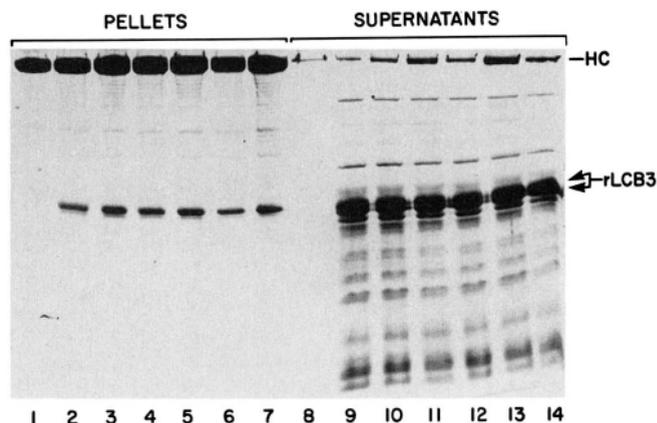


FIG. 4. Effect of the time of incubation on the amount of expressed rat liver light chain LCB3 bound to clathrin cages. Calf brain clathrin heavy chain cages (20 μ g) were mixed with 60 μ l of a solution containing a molar excess of the two main expression products of the plasmid rLCB3 in a total volume of 200 μ l. The proteins were allowed to interact for various extents of time followed by centrifugation in the Airfuge (elapsed time, 15 min). Pellets (lanes 1-7) and one-half of the supernatants (lanes 8-14) were analyzed by SDS-13% PAGE at the indicated final times. Lanes 1 and 8, control with heavy chain cages alone showing absence of endogenous light chains; lanes 2 and 9, 19 min; lanes 3 and 10, 44 min; lanes 4 and 11, 76 min; lanes 5 and 12, 135 min; lanes 6 and 13, 195 min; lanes 7 and 14, 375 min. Clathrin heavy chains (HC) and the main expression products of rLCB3 are indicated.

the binding experiments described in this article have been performed with an incubation period of 3 h.

Further evidence that the binding between the recombinant rat LCB3 and the calf heavy chain cages was saturable derives from a second type of titration experiment. In this case we used increasing amounts of the expression products of rLCB3

and a constant concentration of heavy chain cages (Fig. 5). At low concentrations of rLCB3 products, both expressed species bound to the cages (Fig. 5, lanes 2 and 3), although there was preferential binding of the larger species as the total amount of light chains was increased (lanes 4–6). At saturation, the full-length rLCB3 bound 4–6 times more than the smaller fragment (Fig. 5, lane 6). Such binding behavior would be expected if we consider that at low concentrations of light chain enough binding sites were available on the heavy chains to incorporate all expressed forms of rLCB3. At saturation, the light chain with the higher binding constant (in this case the full-length LCB3) competed for the available sites and preferentially bound to cages. Under this condition, the ratio of bound species was proportional to their relative binding constants. This observation is used below to estimate the effect on the binding constant of specific deletions from the amino- or carboxyl-terminal ends of rLCB3.

Expression of Deletion Mutants of rLCB3 and Their Binding to Clathrin Heavy Chain Cages—In order to map those regions of the light chain necessary to recognize and bind to the proximal segment of the heavy chain leg, we created deletion mutants of rat liver LCB3 missing sections of various lengths from either the amino or carboxyl terminus of the protein.

SDS-PAGE analysis performed on periplasmic preparations from *E. coli* expressed with the various constructs indicated that each plasmid directs the generation of several protein products. By analogy with the protein pattern obtained with the full-length rLCB3, we assumed that the predominant (and usually the larger) species from each construct corresponded to the primary expression product, while the smaller fragments derived from proteolytic degradations at unknown sites. We noted that the electrophoretic mobilities of intact rLCB3 and of all the carboxyl end truncation mutants containing the intact amino-terminal segment (C32 through C87) predict proteins of incorrectly large apparent sizes (Fig. 6). However, removal of the first 36 amino acid residues from the amino-terminal segment of LCB3 (N36) effectively eliminates the otherwise anomalous behavior in SDS gels and allows reasonable size estimates (Fig. 6).

Our data show that despite extensive deletions from the amino or carboxyl ends, the truncated light chains still bound to and sedimented with cages. These deletions included the removal at either end of the complete amino (N92)- or carboxyl (C49)-terminal segments (Fig. 7, lanes 5 and 11; see

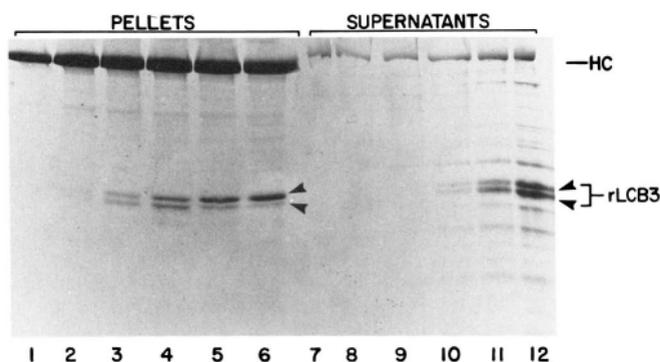


FIG. 5. Preferential binding of the full-length expression product of rat light chain LCB3 to heavy chain cages. Samples of calf brain heavy chain cages (20 μ g) were incubated for 3 h with increasing volumes (0, 3.5, 7, 15, 30, and 60 μ l) of a stock solution containing the two main expression products of rLCB3 (arrows). The final volume was kept at 200 μ l. Cages with bound light chains were separated from unbound proteins by centrifugation in the Airfuge, and the pellets (lanes 1–6) and one-half of the supernatants (lanes 7–12) were analyzed by SDS-13% PAGE.

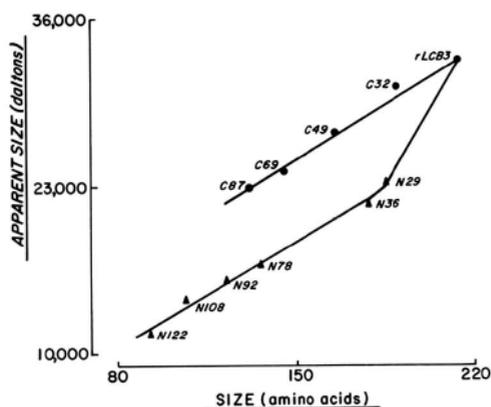


FIG. 6. Effect of removal of amino- or carboxyl-terminal segments from rat LCB3 on the apparent molecular size of the truncation mutants. The plot illustrates the relationship for each construct between the number of expected amino acid residues as determined by DNA sequence and restriction map analysis and the apparent molecular sizes deduced from electrophoretic mobilities on SDS gels. Electrophoretic mobilities were determined for the main expressed species by SDS-18% PAGE (N122 and C87) or SDS-13% PAGE (all the other recombinant products). Notation for the mutants indicates number of residues removed from the amino (N) or carboxyl (C) terminus of rat LCB3.

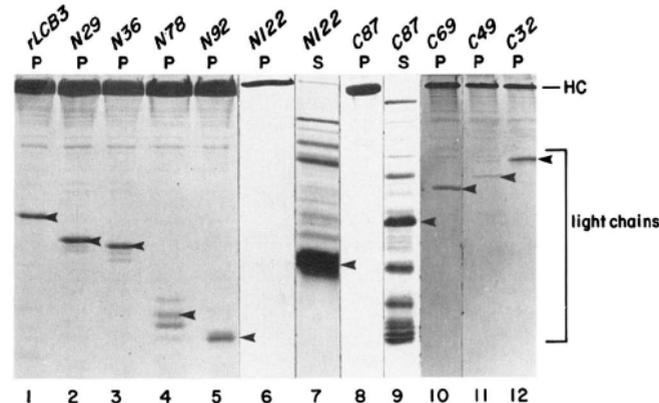


FIG. 7. Binding of truncation mutants of rat clathrin light chain LCB3 to heavy chain cages. Main recombinant products of rat clathrin light chain LCB3 (indicated by arrowheads) bearing deletions from the amino or carboxyl terminus were tested for their capability to co-sediment with clathrin heavy chain (HC) cages. The mixtures were centrifuged in the Airfuge and analyzed by SDS-PAGE. Lanes 1–5, pellets (P) of incubations with the full-length product of rLCB3 and with a series of amino-terminal truncation mutants that bind to cages. They correspond to rLCB3, N29, N36, N78, and N92, missing 0, 29, 36, 78, and 92 residues from the amino-terminal end. Lanes 6–9, pellets (P) and supernatants (S) of incubations with two truncation mutants that fail to sediment with cages. They are N122 and C87, missing 122 and 87 amino acids from the amino or carboxyl terminus of LCB3, respectively. Lanes 10–12, pellets (P) of incubations with a series of carboxyl-terminal truncation mutants that bind to cages. They are C69, C49, and C32 missing 69, 49, and 32 amino acids from the carboxyl terminus of LCB3.

also legend to Fig. 9). The additional elimination of two to three neighboring heptads from the central segment (C69 and N108; see Figs. 7 and 9) generated light chains that were also competent to bind heavy chain cages. In contrast, N122 and C87 with deletions including the amino- or carboxyl-terminal segments together with four to five neighboring heptads completely lost their ability to bind cages (compare absence of bound light chains in pellets in Fig. 7, lanes 6 and 8, with the corresponding supernatants, lanes 7 and 9). Taken together, these results show that seven to eight heptads from the central segment are necessary for binding to clathrin heavy chains.

The binding strength of the deletion mutants is affected by the extent of the truncation. We have obtained an estimate of their relative binding constants by competition experiments in which mixtures containing different light chain constructs were co-incubated with heavy chain cages. Relative binding was then deduced from the amounts of light chain co-sedimenting with cages. Results of such experiments are given in Fig. 8. For example, in lanes 1–4 and 5–8 we show a titration experiment in which cages were incubated with increasing volumes of a mixture containing similar amounts of full-length rLCB3 and the deletion mutants N36 or N78, missing 36 and 78 residues from the amino terminus, respectively. Both deletion products bound and co-sedimented with cages, maintaining a constant molar ratio to full-length rLCB3, regardless of their absolute concentration in the incubation mixture. Similar results were obtained in another titration experiment using the full-length rLCB3 and the deletion mutant N92, which lacks the entire 92 residues of the amino-terminal domain (not shown). Only when two heptads were also deleted, in mutant N108, did we observe diminished binding relative to the intact chains (see legend to Fig. 9). From these experiments we infer that the amino-terminal domain of LCB3 does not strongly influence the heavy chain interaction.

In contrast, we found that small deletions from the carboxyl-terminal end of LCB3 did significantly affect its ability to bind heavy chains. In Fig. 8, lanes 13–16 show an example of a competition experiment of cages co-incubated with increasing amounts of C32 and N78, mutants lacking 32 and 78 residues from the carboxyl- and amino-terminal ends of LCB3. The gel shows the preferential binding of N78 with the concomitant exclusion of C32. We can rule out the trivial explanation that C32 does not bind to heavy chains, since in the absence of competing species it bound to a similar extent as full-length rLCB3 (compare Fig. 8, lane 12, with Fig. 5, lane 6). A cumulative decrease on the ability of a truncated light chain to interact with heavy chains is also observed with the more extensive deletions C69 and C49, missing 69 and 49 residues from the carboxyl-terminal end. Although the products of these constructs still bound to cages (Fig. 7, lanes 10 and 11), the relative binding of C49 diminished in the presence of C32 and that of C69 in the presence of C49 (see legend to Fig. 9 for a summary of the data). A decrease in relative binding was also detected for the smaller of the two expression

products of rLCB3 (see Fig. 5, lanes 2–6 and Fig. 8, lanes 1–4). The carboxyl-terminal end of this protein must be truncated at a site very close to that of C32 since its electrophoretic mobility was similar to that of C32 and since it had the same amino-terminal sequence as the full-length rLCB3.

DISCUSSION

To obtain adequate amounts of protein for *in vitro* binding studies, we used a very efficient secretion vector to express rat clathrin light chains in the periplasm of *E. coli*. This approach is perhaps unorthodox, since it is not clear whether proteins like the light chains normally found in the cytoplasm of eukaryotic cells will fold properly upon synthesis and membrane translocation and therefore be useful in these types of binding studies. However, it is known that light chains isolated from mammalian clathrin retain their ability to bind back to clathrin heavy chain even after exposure to temperatures higher than 95 °C (21). We therefore reasoned that even if the rat light chains secreted into the periplasm of *E. coli* were misfolded, upon heat denaturation they would refold and could recognize their binding site in the clathrin heavy chains. Indeed, our binding experiments indicate that this was the case.

We have previously found that mammalian clathrin light chains contain a central segment with 10 heptad repeats and have proposed that this heptad region forms a helix mediating binding of light chains to heavy chains (11). Partial support for this model is the observation that most light chain lysines are in the central segment, and their efficient labeling with Bolton-Hunter reagent is only obtained with free light chains (16). The model is also consistent with the finding that several monoclonal antibodies directed against epitopes located within the heptad region inhibit the light chain-heavy chain interaction (17). However, no comparable information is available for the amino-terminal domain since no epitopes were located in this region. Based on the same epitope mapping study, it has been proposed that the carboxyl-terminal domain does not interact with clathrin heavy chains because antibodies directed against two epitopes located in this segment do not block binding to heavy chains. The certainty of this conclusion is limited because the antibodies also recognize light chains bound to intact clathrin trimers, therefore raising the possibility that lack of blocking activity is due to the

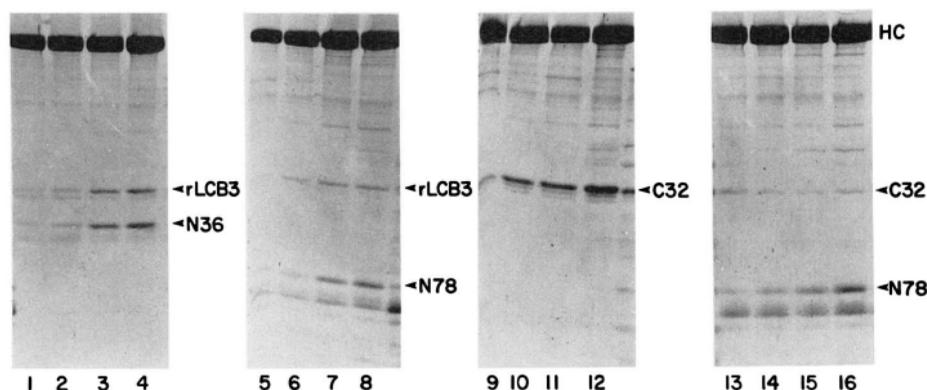


FIG. 8. Analysis of relative binding strength of several expressed LCB3 mutants to clathrin cages by competition tests. Heavy chain cages (HC, 20 μ g) were incubated with increasing volumes (7, 15, 30, and 60 μ l) of a stock solution containing a 1:1 (v/v) mixture of the expression products of rLCB3 and N36 (lanes 1–4), of rLCB3 and N78 (lanes 5–8), of C32 alone (lanes 9–12), or of C32 and N78 (lanes 13–16). After the incubation, samples were centrifuged in the Airfuge, and the pellets were subjected to SDS-13% PAGE followed by staining with Coomassie Brilliant Blue. A gel scanner was used with lanes 1–8, to confirm that the staining ratio of bound light chains remained constant (data not shown).

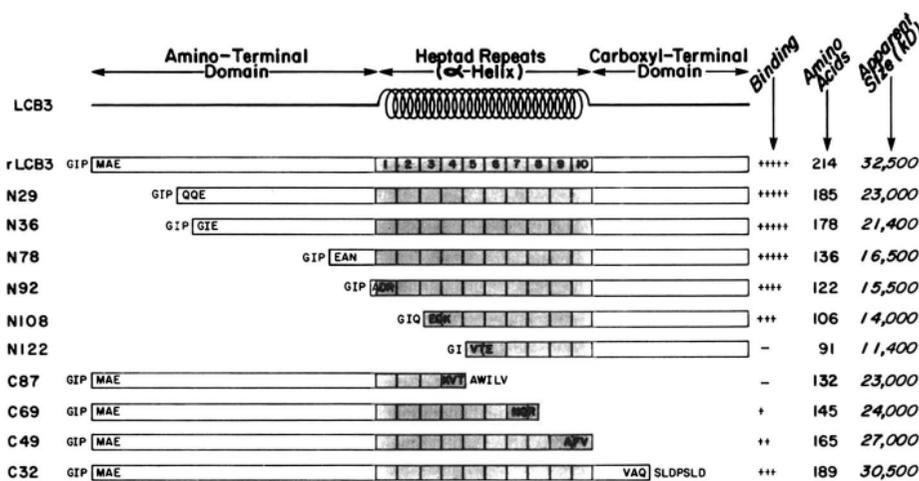


FIG. 9. Schematic representation of the full-length rat LCB3 and the truncation mutants used. The drawings are aligned in reference to the primary structure of rat clathrin light chain LCB3 as deduced from its cDNA sequence. The central domain of the light chain has 70 amino acids organized in 10 repeats of the heptad motif. This segment is surrounded by amino- and carboxyl-terminal domains, each with 92 and 48 residues. Predicted amino acid sequences of the amino or carboxyl terminus of the constructs are indicated. The amino-terminal sequences of the two major expression products of rLCB3 were identical as determined by automated Edman degradation. The number of amino acid residues of each construct was determined by partial DNA sequences and restriction map analysis, and their apparent sizes were deduced from electrophoretic mobilities on SDS gels (shown in Fig. 6). The relative abilities of the mutants to bind heavy chain cages were obtained with experiments similar to those shown in Fig. 8.

relative orientation of the epitopes away from the light chain-heavy chain interface.

The binding experiments using deletions of LCB3 circumvent these limitations and are consistent with the prediction of our structural model (see legend to Fig. 9). Removal of the complete amino-terminal segment (N92) does not prevent light chain binding, but additional removal of the first two heptads (N108) does partially impair the interaction. The more extensive deletion mutant N122, including the amino-terminal segment and 4.5 heptads, completely destroys the ability to bind heavy chains. A similar analysis using truncation mutants from the carboxyl end shows that removal of the complete carboxyl-terminal segment together with three neighboring heptads (C69) still allows light chain binding. The first non-binding mutant in a carboxyl-terminal deletion series is C87, which lacks the entire carboxyl-terminal segment as well as the adjoining 5.5 heptads. Since these mutants represent sequential deletions from either end of the light chain, we conclude that seven to eight contiguous heptads, from either end of the central segment, are essential for the association with heavy chains. A smaller number of contiguous heptads, more centrally located within the helical region, could also be sufficient to mediate the binding of light chains to heavy chains. However, our experiments do not test this possibility since we have not generated double mutants with simultaneous deletions from both ends of the heptad region.

Our inability to observe displacement of native light chains by either intact or truncated species shows that the dissociation rate from heavy chains is very slow. Therefore, the experiments involving competition of intact and truncated LCB3 are really measuring relative rates of association under these experimental conditions. The properties of truncated light chains C32 and C49 show that the carboxyl-terminal domain can influence the rate of binding. What might be the mechanism for such an effect? In solution and in the absence of clathrin heavy chains, the light chains appear to be extended (15, 16) and loosely folded molecules (16). The 20 α -helical runs of the central segment probably coil up during

binding. It may be that the tight binding of light to heavy chains, with reported dissociation constants in the nanomolar to picomolar range (16, 21), is obtained through a zipper-type mechanism. Initial anchorage of light to heavy chains could be provided by a short segment at one end of the light chain, followed by a more extensive interaction mediated by assembly of the helical central segment. We suggest that the carboxyl-terminal domain of the light chains fulfills the anchoring role, since the competition experiments show that the rate of binding of LCB3 is sensitive to the extent of the deletions from its carboxyl-terminal end. However, this role in assembly need not exhaust the activities of the carboxyl-terminal region. Indeed, we believe that the principal functions of both the amino- and carboxyl-terminal domains are likely to involve interactions with other proteins during cycles of coated vesicle assembly and disassembly.

Acknowledgments—We thank W. Matsui for help in clathrin purification, K. Tucker for assistance in plasmid preparations, and S. C. Harrison, J. Brosius, and J. Wilson for helpful discussions.

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