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A domain of clathrin that forms coats

(coated vesicles/limited proteolysis/clathrin light chains)

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ABSTRACT **Triskelions, trimeric complexes of clathrin and associated light chains, are the assembly units of clathrin coats [Ungewickell, E. & Branton, D. (1981) *Nature (London)* 289, 420–422; Kirchhausen, T. & Harrison, S. C. (1981) *Cell* 23, 755–761]. We report here that triskelions whose outer arms have been removed by trypsin digestion retain the ability to be assembled into coats. These digested trimers contain a 110,000 molecular weight domain of clathrin and lack intact light chains.**

Coated vesicles transport selected sets of proteins between membrane-bound compartments (1, 2). Clathrin (3), the principal constituent of the basket-like coat structure, is a multifunctional protein that must be capable of assembly into coats (4–9) as well as binding to membranes. Considering clathrin's large size (180,000 M_r), it might be anticipated that some of these distinct functions would reside in separate domains of the polypeptide chain. We report here the proteolytic dissection from clathrin of a domain (of about 110,000 M_r) that has the capacity to be assembled into cages in the absence of intact clathrin-associated light chains (33,000 and 36,000 M_r), a property not possessed by native clathrin (7).

MATERIALS AND METHODS

Purification of Clathrin. Coat proteins were purified from calf brain as described by Kirchhausen and Harrison (7) with slight modifications. All procedures were carried out at 0–4°C. Coated vesicles (approximately 25 mg) obtained from the second sucrose gradient [as described by Pearse (3)] were pelleted and resuspended in 15 ml of buffer I [0.75 M Tris/25 mM 4-morpholineethanesulfonic acid (Mes), pH 6.2/0.25 mM EGTA/0.12 mM $MgCl_2$/0.02% NaN_3] by using a Dounce homogenizer, incubated on ice for 30 min, and then centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm for 90 min. The supernatant was precipitated with ammonium sulfate (50% of saturation). The precipitate was dissolved in 3 ml of buffer II (0.5 M Tris/50 mM Mes, pH 6.2/0.5 mM EGTA/0.25 mM $MgCl_2$/0.02% NaN_3) and chromatographed on a Bio-Gel A-1.5m column (3 × 80 cm) equilibrated with buffer II. The void volume (containing clathrin) was precipitated with ammonium sulfate (80% of saturation) and dissolved in 10 ml of buffer II. Remaining membrane contaminants were removed by a second centrifugation (SW 50.1 rotor, 35,000 rpm, for 1 hr). The resulting supernatant (referred to as "column-purified clathrin") was frozen in liquid nitrogen and stored at −70°C until use. Typically, 7–9 mg of clathrin was purified from 1.5 kg of brain. As reported (7), these preparations consist almost entirely of clathrin (180,000 M_r) and light chains (33,000 and 36,000 M_r). The molar ratio of light chains (33,000 and 36,000 M_r species considered together) to clathrin was 0.78 ± 0.02 in four independent preparations.

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Reconstitution of Empty Cages and Mild Proteolysis. To form cages (7), column-purified clathrin (typically 0.5–1.0 mg/ml) was dialyzed against buffer III (20 mM Mes, pH 6.2/1 mM EDTA/2 mM $CaCl_2$) for 10–15 hr at 4°C. For routine tryptic digestions, these reconstituted cages in buffer III (typically, 0.5–0.8 mg/ml) were incubated with 1% of their weight of trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Worthington) at 37°C for 45 min, followed by the addition of soybean trypsin inhibitor (Sigma) at 2 mg per mg of trypsin. For elastase digestions (7), reconstituted cages were incubated with 0.1% of their weight of pancreatic elastase (Worthington) at room temperature for 3 hr, followed by the addition of phenylmethanesulfonyl fluoride to 1 mM (from a 200 mM stock solution in dimethyl sulfoxide). Some experiments employed cages reconstituted from the cruder preparations of clathrin that had been extracted from third gradient coated vesicles (3) with buffer I, but not further purified.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed as described by Laemmli (10) with 10% polyacrylamide slab gels. Gels were run for various lengths of time and stained with Coomassie blue. Molecular weight standards used to identify protein bands and their molecular weights were: β-galactosidase, 116,000; bovine serum albumin, 68,000; ovalbumin, 45,000; and soybean trypsin inhibitor, 20,000; the standards were included in every gel shown.

Protein concentration was determined as described by Lowry *et al.* (11), using bovine serum albumin as a standard.

RESULTS

Fragments of Clathrin Are Retained in Cages. When cages were incubated with trypsin (1% trypsin by weight, for 30–45 min at 37°C) clathrin (Fig. 1, lane a) was almost completely digested and a number of distinct cleavage products were generated (Fig. 1, lane b), including a major species of about 110,000 M_r, whose yield was 0.6 mol per mol of clathrin as determined from densitometer tracings of the stained gels. This species (as well as several minor products of similar molecular weight) could be sedimented (Fig. 1, lane c). Other products of proteolysis, including species of 53,000 and 41,000 M_r, remained in the supernatant (Fig. 1, lane d).

Electron microscopy revealed that even though only a trace of clathrin remained, the pellet contained empty cages (Fig. 2*B*) indistinguishable from those present before digestion (Fig. 2*A*). The supernatant did not contain cages (data not shown). Together, these observations suggested that the 110,000 M_r fragment of clathrin had remained stably associated as cages, while smaller fragments had been released.

Agarose gel electrophoresis (12) demonstrated that cages with the 110,000 M_r fragment as their major constituent accounted for almost all of the protein sedimentable after trypsin digestion. Before trypsin digestion, all of the protein present

Abbreviation: Mes, 4-morpholineethanesulfonic acid.

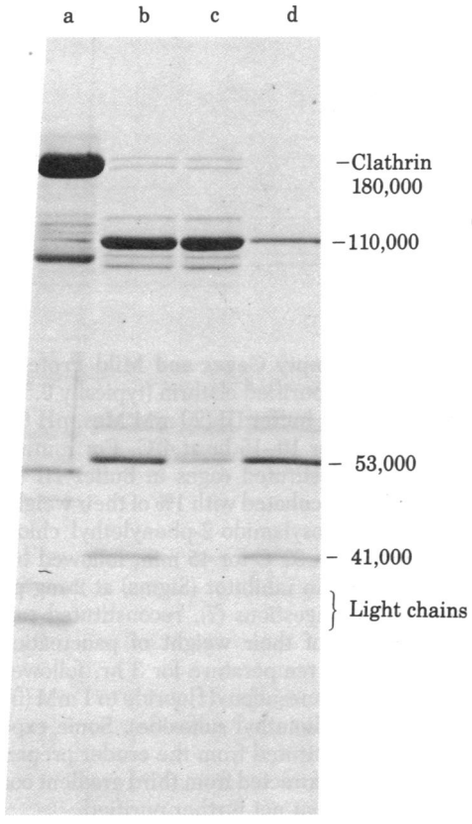

FIG. 1. $NaDodSO_4$/polyacrylamide gel of major tryptic cleavage products. Lane a, untreated cages; lane b, trypsin-digested cages; lane c, cleavage products in the pellet (after centrifugation for 90 min at 25,000 rpm in a SW 50.1 rotor); lane d, cleavage products in supernatant. Clathrin (0.4–0.8 mg/ml) was extracted from coated vesicles with buffer I and was dialyzed overnight against buffer III to form empty cages. The cages were digested with trypsin for 30 min at 37°C.

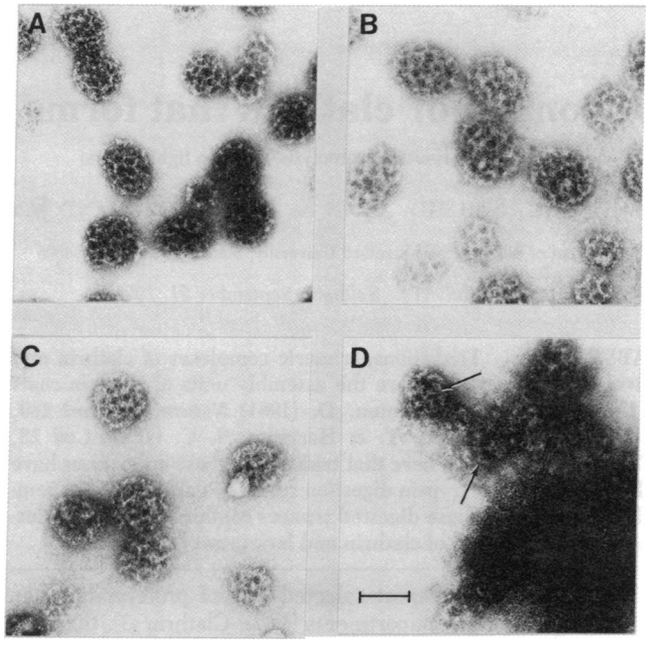

FIG. 2. Electron micrographs. (*A*) Cages re-formed by dialysis of column-purified clathrin (0.4–0.8 mg/ml) against buffer III. (*B*) Pellet of trypsin-digested cages, prepared as in Fig. 1. (*C*) Cages re-formed from isolated 110,000 M_r fragment. Trypsin-digested cages were dissociated with buffer I and passed over a Bio-Gel A-1.5m column (96-ml bed volume) equilibrated with buffer II. Fractions containing the 110,000 M_r fragment (eluting near the void volume) were collected and precipitated with ammonium sulfate (80% of saturation). The precipitated protein was made 0.6 mg/ml in buffer II and dialyzed overnight at 4°C against buffer III. (*D*) Aggregate re-formed by clathrin derived from elastase-digested cages. Cages re-formed from column-purified clathrin were treated with elastase and pelleted by centrifugation at 25,000 rpm for 90 min in an SW 50.1 rotor. The pellet was resuspended in buffer I to give a protein concentration of 0.6 mg/ml and dialyzed overnight at 4°C against buffer III. The arrows indicate some polygonal lattice structure. All samples were adsorbed onto Formvar- and carbon-coated grids and negatively stained with 1% uranyl acetate. The bar denotes 0.1 μm.

electrophoresed (12) as a distinct band (Fig. 3, lane b). After trypsin digestion, this band disappeared and was replaced by a new band of distinctly slower mobility (Fig. 3, lane a). Electron microscopy of material eluted from these bands revealed that both consisted of empty cages indistinguishable from those shown in Fig. 2 *A* and *B*. Fractions of these agarose gels were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis to reveal the distribution in agarose gels of intact clathrin before trypsin digestion and of the 110,000 M_r fragment after trypsin digestion (Fig. 3 *Lower*). Each coelectrophoresed with the major band in the agarose gel and accounted for most of the protein present in the band. The trace of intact clathrin remaining after trypsin treatment (Fig. 1, lane b) electrophoresed in the position of intact cages, not that of trypsin-digested cages (data not shown). The entire family of fragments of $\approx$110,000 M_r as well as one of 125,000 M_r coelectrophoresed with the trypsin-digested cages in the agarose gel. The limited proteolysis described here was also observed when whole coated vesicles were employed (data not shown).

Pattern of Tryptic Cleavage. Fig. 4*A* presents the time course of proteolysis at 37°C. Two major families of fragments are generated: one set of larger fragments that includes the 110,000 M_r and a 125,000 M_r species, and another set of smaller products that includes species of 53,000 and 41,000 M_r. All of these fragments appear to derive from the 180,000 M_r clathrin species, in part because no other polypeptides are present in sufficient amounts to produce them, and also because of the kinetic evidence that follows.

The molar amounts of these species were estimated from densitometer tracings of the stained polyacrylamide gel and their apparent molecular weights. The two major polypeptides (110,000 and 125,000 M_r) in the larger family together account for 1.0, 0.9, 1.0, and 0.7 mol per mol of clathrin hydrolyzed at 2, 5, 10, and 30 min of incubation at 37°C; under these conditions, the 125,000 M_r species is present only in small amounts and at early times. The two major species in the smaller family (53,000 and 41,000 M_r), considered together, account for 0.7, 0.6, 0.7, and 0.6 mol per mol of clathrin hydrolyzed at 2, 5, 10, and 30 min of incubation at 37°C.

To examine possible precursor–product relationships within each family, digestion was slowed by incubation at ice temperature (Fig. 4*B*). Under these conditions, the 125,000 M_r fragment was the major species in the large family at the earliest times, but it disappeared progressively as the 110,000 M_r species appeared and predominated. The sum of these two species accounted for all of the clathrin hydrolyzed (mole per mole) at every time point. Significant amounts of a 61,000 M_r species that had been observed at 37°C were not found when digestion was carried out at the lower temperature, suggesting that this species does not result from a primary cleavage event. For reasons that are unclear, small amounts of a 165,000 M_r species were also observed. The data in Fig. 4*B* do not permit resolution of the issue of whether the 53,000 M_r species is a precursor to

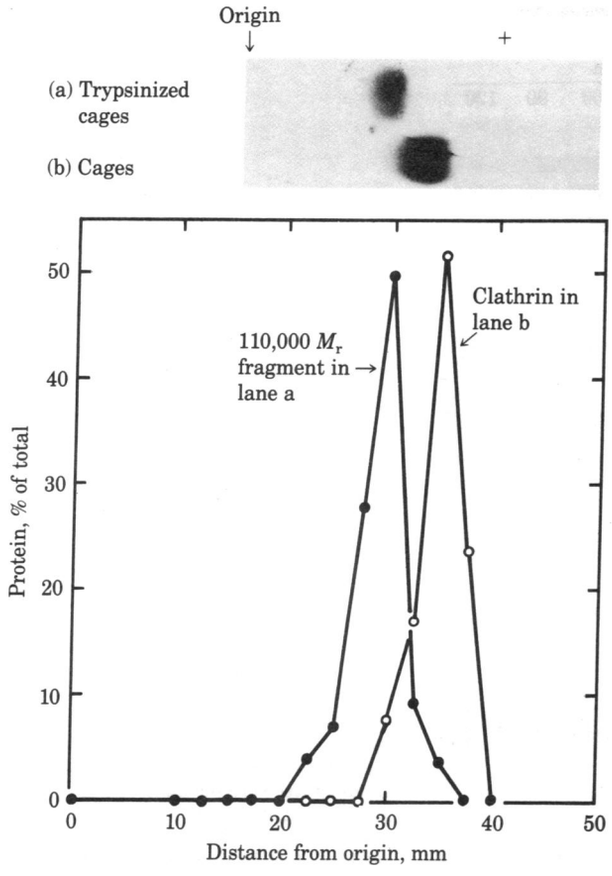

FIG. 3. (*Upper*) Agarose gels of trypsin-digested (lane a) and untreated (lane b) cage preparations. Trypsin-digested cages were prepared as in Fig. 1. Both preparations (0.6 mg/ml) were pelleted by centrifugation as described for Fig. 1. Samples (25 μg of protein each) in buffer III were electrophoresed in a 0.15% agarose gel as described (12). After electrophoresis for 24 hr at 15 mV, the gel was dried and then stained with Coomassie blue (12). (*Lower*) Parallel lanes containing 50 μg protein each of untreated or trypsin-treated cages were sliced into 2.5-mm fractions, each of which was electrophoresed in a $NaDodSO_4$/10% polyacrylamide slab gel (12). The distributions of intact clathrin (○) in the agarose gel of untreated cages and of the 110,000 M_r fragment (●) in the agarose gel of the trypsin-digested cages were determined from densitometer tracings of the appropriate bands in the Coomassie blue-stained $NaDodSO_4$ gels.

the 41,000 M_r species, but it is consistent with this possibility. Cleavage products of lower molecular weight than the light chains were also observed (Fig. 4*A*), the most prominent migrating at 19,000 M_r, just below trypsin inhibitor. This peptide pelleted with the trypsin-treated cages, coeluted with the large cleavage products in the void volume of a Bio-Gel A-1.5m column (see below), was retained in cages after reconstitution, but could be removed by elastase under conditions that selectively cleave light chains (not shown). However, it is not yet clear whether these smaller fragments derive from light chains or instead from clathrin or other polypeptides.

The simplest interpretation of these data is that the families of large and small fragments derive from different portions of clathrin. The family of larger fragments would be derived from a domain of 125,000 M_r. The family of smaller fragments, whose largest member is 53,000 M_r, would account for essentially all of the rest of the 180,000 M_r clathrin molecule.

Fragments of Clathrin Can Form Cages. Trypsin-digested cages could be disrupted by treatment with 0.75 M Tris buffer (buffer I), as evidenced by the inability to sediment the 110,000 M_r species after treatment and the concomitant loss of cages, as judged by electron microscopy (not shown). When the disrupted, trypsin-digested cages were dialyzed against the same low ionic strength buffer used to reconstitute intact clathrin (buffer III) cages formed (Fig. 2*C*). All of the protein now electrophoresed in the agarose gel as reconstituted cages (Fig. 5, lane d) in the same position as the original trypsin-digested cages (Fig. 5, lane c). $NaDodSO_4$ gel analysis showed the gel band to contain the 110,000 M_r fragment and its family of polypeptides.

The capacity to re-form cages was retained by these fragments after gel filtration. Fractions of the Tris-disrupted trypsin-digested cages eluting near the void volume of a Bio-Gel A-1.5m column were pooled, concentrated by ammonium sulfate precipitation, and dialyzed against buffer III. The resulting cages were indistinguishable in appearance from other empty cages (Fig. 2*C*), and again all the dialyzed protein electrophoresed in the position characteristic of trypsin-digested cages (Fig. 5, lane e).

Electron microscopy employing rotary shadowing (Fig. 6, lane a) confirmed that clathrin released from undigested coats exists as triskelions, as reported (7, 8). Trypsin-digested cages containing principally the 110,000 M_r fragment were similarly disrupted and then examined (Fig. 6*B*). The trimeric structure was retained, but the length of each arm was reduced, and the characteristic central bend was, in most cases, no longer evident. Before trypsin digestion (Fig. 6*A*), the mean (±SEM) contour length of 100 arms counted was 504 ± 36 Å. After digestion, the mean arm length (of 30 counted) was reduced to 315 ± 28 Å. It seems unlikely that the outer portions of the arms were still present but had folded back upon the inner portions of the arms. Therefore, the inner portion of each arm of the trimer appears to be formed by a domain of clathrin that is preserved in the 110,000 M_r and related tryptic fragments.

Selective removal of light chains by elastase without a significant cleavage of clathrin has been reported to markedly reduce the ability of clathrin to assemble into regular cages (7). Therefore, it was surprising that a 110,000 M_r tryptic fragment of clathrin could assemble very efficiently into cages (Fig. 5, lane e) when no intact light chains remained (Fig. 1, lane b).

We have confirmed the effects of elastase (7) upon the reconstitution of coats. When empty cages were incubated with elastase, the light chains were selectively removed (Fig. 7, lanes a and b) but cage structures remained. A doublet of cleavage products at 25,000 and 24,000 M_r was present in the total elastase digest but the peptides remained in the supernatant after the cages had been pelleted (data not shown). The digested cages (Fig. 7, lane d) electrophoresed more slowly in an agarose gel than before elastase treatment (Fig. 7, lane c). Elastase-digested cages were disrupted by adding the 0.75 M Tris buffer (buffer I) and then dialyzed against the low ionic strength buffer III to attempt reconstitution under these standard conditions. Electron microscopy (Fig. 2*D*) revealed that large aggregates had formed, as well as a small proportion of distinct cages. Almost all of this material (Fig. 7, lane e) electrophoresed through an agarose gel more slowly than did the original elastase-digested cages (Fig. 7, lane d), providing quantitative confirmation of the earlier evidence (7) that very few cages had reformed.

Triskelions released from undigested cages generally exhibit a unique handedness in which all three arms are bent or curved in the same direction (7, 8). In our experience, 61 ± 2% of trimers (1025 counted) released from undigested cages have all three arms bent in the same sense. But when trimers released from elastase-digested cages were examined (Fig. 6*C*), a consistent handedness was observed in only 31 ± 1% of the cases

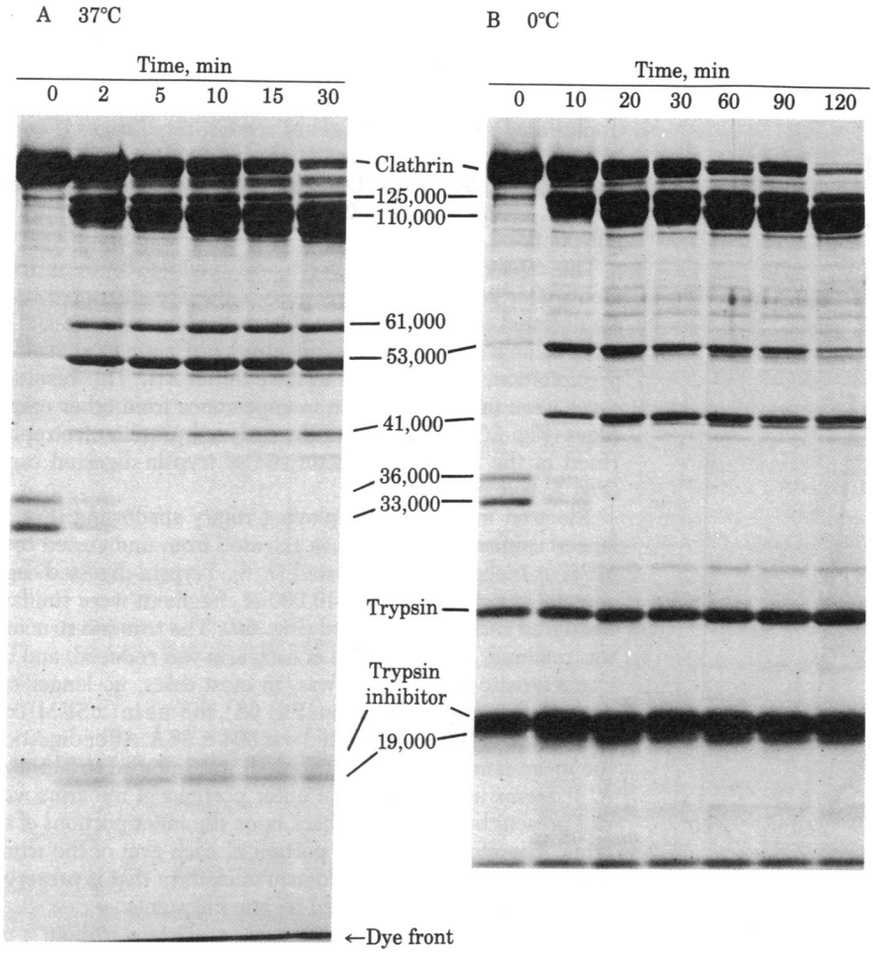

FIG. 4. Time course of tryptic hydrolysis. (*A*) Samples (0.6 mg/ml in buffer III) were incubated with 1% of their weight of trypsin at 37°C for the times indicated above each lane. (*B*) Samples (0.6 mg/ml in buffer III) were incubated with 25% of their weight of trypsin on ice for the times indicated. All incubations were stopped by addition of soybean trypsin inhibitor at 2 mg per mg of trypsin. Gels were electrophoresed until the dye front was 0.25–0.5 cm from the bottom.

(980 counted). This corresponds to a nearly random pattern.* Qualitatively, it also appeared that the normally threefold geometry at the vertex was often lost after the elastase digestion. The average arm length was 513 ± 38 Å (45 counted), indistinguishable from the control (Fig. 6*A*).

DISCUSSION

Mild tryptic digestion of cages has been shown to cleave clathrin into two major families of fragments that together can account for most and possibly all of the molecule. The family of larger molecular weight, the limit digestion product of which is 110,000 M_r, remains in the form of cages. The family of smaller fragments (including 53,000 and 41,000 M_r species) dissociates from coats. Trimers released from trypsin-digested cages, and composed almost entirely of the larger fragments, appear to lack outer arms. Therefore, the large domain may compose the inner portion of an arm, and by implication the smaller domain (giving rise to the 53,000 and 41,000 M_r species) would form the outer arm. The principal site of tryptic attack would then be at the joint between these two domains, near the characteristic bend in the arm, a region that might be expected to be especially sensitive to proteolysis. The large domain not only is retained in digested cages but also can be reconstituted into cages with high efficiency after dissociation, showing that this is the only domain of clathrin needed for this purpose. (Of course, other nonclathrin proteins may participate in this assembly process.)

Most likely, the smaller, outer arm domain also forms part of the surface lattice of the coat (7), even though fragments of this domain dissociate from the coat after cleavage.

Digestion of cages with elastase results in trimers having full-length arms that contain apparently intact clathrin but lack light chains (7). These trimers also lack the capacity to assemble into

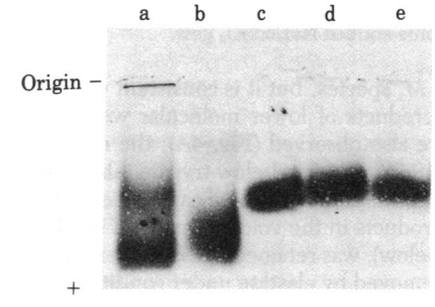

FIG. 5. Analysis of cage assembly by agarose gel electrophoresis. Lane a, coated vesicle marker. Third gradient (3) coated vesicles were used. The slower migrating band consists of contaminating smooth vesicles, and the faster migrating band consists of coated vesicles (12). Lane b, cages reconstituted by dialysis of column-purified clathrin. Lane c, cages digested with trypsin and pelleted as described for Fig. 1, lane c. Lane d, cages re-formed from the large cleavage products of trypsin-digested cages. Cages digested with trypsin and pelleted as indicated for Fig. 1, lane c, were dissociated by resuspension in buffer I to give a protein concentration of 0.5–0.8 mg/ml and dialyzed overnight at 4°C against buffer III. Lane e, cages re-formed from purified 110,000 M_r fragment and prepared as described for Fig. 2*C*. Each lane contained 25 μg of protein, which was electrophoresed as described for Fig. 3.

* If the sense of the bend at each arm were established randomly and independently, then the fraction of triskelions with the same sense at all three arms (be it clockwise or counterclockwise) would be $2(1/2)^3 = 0.25$.

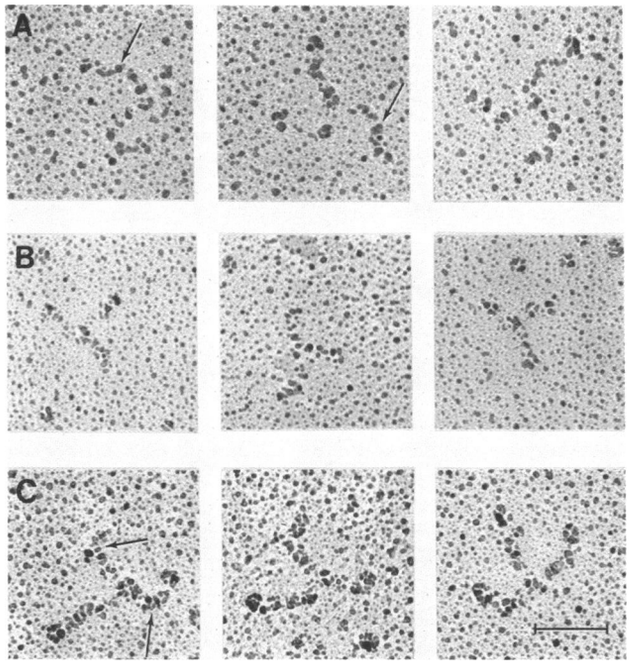

FIG. 6. Electron micrographs of platinum-shadowed clathrin trimers. (*A*) Material released by buffer I from untreated cages (formed from column-purified clathrin). (*B*) Material released by buffer I from the pellet of trypsin-digested cages. (*C*) Material dissociated from the pellet of elastase-digested cages with buffer I. Rotary-shadow replicas were obtained by following the method of Tyler and Branton (13) with some minor modifications. Thirty-microliter samples (50–70 μg of protein) in buffer I containing 65% (vol/vol) glycerol were sprayed onto freshly cleaved mica and dried under reduced pressure. Samples were shadowed with platinum/tungsten in a Denton DV-502 rotary shadowing device at a shadowing angle of 7°, floated onto copper grids, and viewed in a Philips 300 electron microscope at 60 kV. The arrows mark the positions of bends in representative arms. The examples shown are structures representative of the entire population of particles observed. The trimers were too dilute on the grid for the reproduction of an entire field to be useful. This was the case for both control (*A*) and protease-digested (*B* and *C*) preparations. Bar denotes 50 nm.

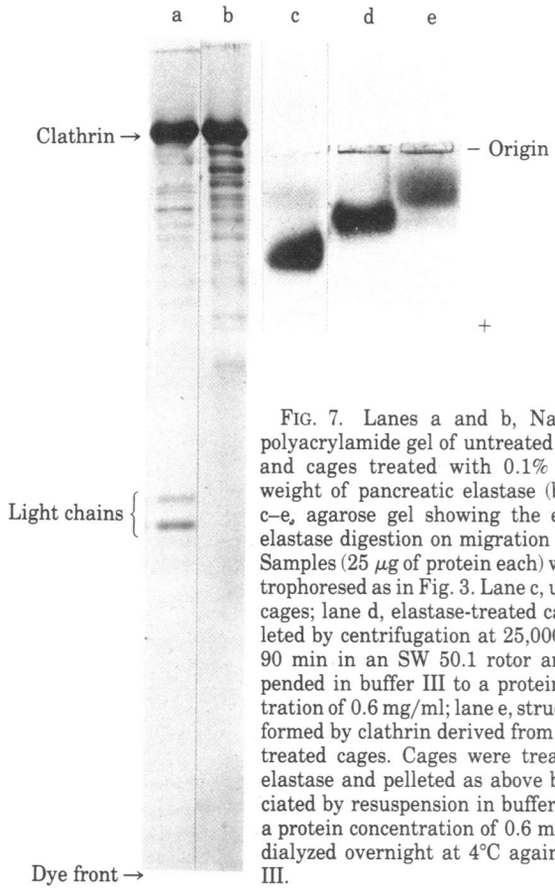

FIG. 7. Lanes a and b, $NaDodSO_4$/polyacrylamide gel of untreated cages (a) and cages treated with 0.1% of their weight of pancreatic elastase (b). Lanes c–e, agarose gel showing the effects of elastase digestion on migration of cages. Samples (25 μg of protein each) were electrophoresed as in Fig. 3. Lane c, untreated cages; lane d, elastase-treated cages, pelleted by centrifugation at 25,000 rpm for 90 min in an SW 50.1 rotor and resuspended in buffer III to a protein concentration of 0.6 mg/ml; lane e, structures reformed by clathrin derived from elastase-treated cages. Cages were treated with elastase and pelleted as above but dissociated by resuspension in buffer I to give a protein concentration of 0.6 mg/ml and dialyzed overnight at 4°C against buffer III.

regular coats under standard conditions (7), as we confirm here. We have gone on to show that the arms of these trimers are disposed nearly at random, providing a possible explanation of why elastase treatment interferes with the formation of closed cages. Thus, given that the outer arms normally form portions of the edges of cages, randomly disposed arms would be expected to permit the assembly of edges that radiate in multiple directions from the same vertex, causing large aggregates to form, as observed (Fig. 2*D*). The ability of trimers lacking outer arms to be assembled into cages could then be explained in either of two ways. In one view, simply removing the randomized outer arms (by trypsin cleavage) would now permit proper coats to form. Alternatively, trypsin digestion may spare a functional fragment of light chains that elastase removes. The retention of this fragment would then be the key to proper assembly. However, this second possibility is rendered unlikely by the finding (our unpublished data) that trypsin-digested cages treated subsequently with elastase (1 μg of enzyme per mg of clathrin, 20°C, for 1 hr) quantitatively retain the ability to reassemble into cages after dissociation. This elastase digestion is sufficient to remove light chains from native coats, and also results in the complete loss of the 19,000 M_r fragment prominent in Fig. 4*A*.

In either case, our observations raise the possibility that light chains might aid the assembly of clathrin into regular cages by fixing the geometry and handedness of the triskelion's arms. It is conceivable that light chains are not always present or active throughout the clathrin-coated vesicle cycle of assembly and disassembly that presumably takes place in cells as coated vesicles bud, uncoat, and fuse. Therefore, the light chains provide a possible point at which a clathrin-coated vesicle cycle (14) could be regulated.

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