Abstract. The two forms of clathrin light chains (LC_1 and LC_3) or clathrin-associated proteins (CAP_1 and CAP_2) have presented an immunochemical paradox. Biochemically similar, both possess two known functional parameters: binding the clathrin heavy chain and mediating the action of an uncoating ATPase. All previously reported anti-CAP mAbs, however, react specifically with only CAP_1 (Brodsky, F. M., 1985, J. Cell Biol., 101:2047-2054; Kirchhausen, T., S. C. Harrison, P. Parham, and F. M. Brodsky, 1983, Proc. Natl. Acad. Sci. USA, 80:2481-2485). Four new anti-CAP mAbs are reported here: two, C-7H12 and C-6C1, react with both forms; two others, C-10B2 and C-4E5, react only with the lower form. Sandwich ELISAs indicated that C-10B2, C-4E5, C-6C1, and C-7H12 react with distinct epitopes. Monoclonal antibodies C-10B2 and C-4E5 immunoprecipitate clathrin-coated vesicles (CCVs) and react with CAP_2 epitopes accessible to chymotrypsin on the vesicle. These mAbs inhibit phosphorylation of CAP_2 by endogenous CCV casein kinase II. In contrast, C-6C1 and C-7H12 react with epitopes that are relatively insensitive to chymotrypsin. CAP peptide fragments containing these epitopes remain bound to reassembled cages or CCVs after digestion. Immunoprecipitation and ELISAs demonstrate that C-7H12 and C-6C1 react with unbound CAPs but not with CAPs bound to triskelions or CCVs. The data indicate that the CAPs consist of at least two discernible structural domains: a nonconserved, accessible domain that is relevant to the phosphorylation of CAP_2 and a conserved, inaccessible domain that mediates the binding of CAPs to CCVs.

Materials and Methods

Preparation of Hybridomas

Female BALB/c mice (6-8 wk of age) were initially immunized intraperitoneally with 100 μg of CAPs purified by heating at 100°C for 5 min as previously reported (9) and emulsified in complete Freund's adjuvant (day 0). Mice were boosted intraperitoneally on day 32 with 100 μg of antigen emulsified in incomplete Freund's adjuvant. Mice were boosted a final time with intraperitoneal and intravenous injections of 50 μg of antigen on days 4 and 3 before fusion on day 1.

Sp 2/0-Ag4 myeloma cells were cultured in RPMI 1640 supplemented with 15% fetal calf serum, 1 mM l-glutamine, 1 mM sodium pyruvate, 100 μM minimal essential medium nonessential amino acids, minimal essential medium vitamin solution, 100 μM 8-azaguanine, and antibiotics. Spleen cells from immunized animals were fused at a 5:1 ratio to Sp 2/0-Ag4 myeloma cells using deionized 44% polyethylene glycol 1,000 in RPMI 1640. Cells were plated into 96-well plates 24 h after fusion at a density of 10^4 myeloma cells per well (200 μl) and selected by adding 15 μg/ml hypoxanthine, 76 μg/ml thymidine, and 0.18 μg/ml aminopterin. This procedure usually yields at least one clone per well by the end of 10 d. Clones were passed to 1-ml wells before screening.

Hybridoma supernatants were screened by an ELISA. Briefly, antigens were coated onto polyvinyl chloride (PVC) plates (Linbro, Flow Laboratories, Hamden, CT) at a concentration of 5 μg/ml in 5 mM Tris, pH 8.0. Binding was performed at 37°C for 1 h followed by a 4°C incubation over-
night. Free binding sites were saturated using 1% BSA in Tris-buffered saline (TBS), pH 7.5. Wells were incubated with culture supernatants for 2 h at 37°C, washed three times with PBS containing 0.5% Tween 20. Wells were incubated with goat anti–mouse IgG/IgM conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 37°C, washed three times, then incubated for 1 h at 37°C with p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO). Absorbance at 405 nm was quantitated with a Titertek Multiskan microELISA reader.

Positive hybridomas were subcloned by limiting dilution. Ascites fluid was generated in pristane (Aldrich Chemical Co., Milwaukee, WI) primed BALB/c mice. All mAbs reported here are IgG1; mAbs C-4E5 and C-10B2 have kappa light chains while C-6C1 and C-7H12 have lambda light chains. Antibodies were purified from ascites fluid by hydroxyapatite HPLC (Bio-Rad Laboratories, Richmond, CA). Antibodies were coupled to cyagen bromide-activated Sepharose 4B as described by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ). mAb CVC-1 (6) was acquired from the American Type Culture Collection, Rockville, MD (TIB 135).

**Sandwich ELISAs**

HPLC-purified mAbs were cross-linked to alkaline phosphatase in the following manner. Antibody and alkaline phosphatase (bovine intestinal mucosa [Sigma Chemical Co.]) were combined in PBS, pH 7.4, at 0.5 mg/ml each. The mixture was placed in a dialysis bag and dialyzed against a 500-fold excess volume of PBS containing 0.04% glutaraldehyde. After 8 h at 4°C, the bag was moved to 1,000-fold excess volume PBS without glutaraldehyde and dialyzed overnight at 4°C. Samples were run through a Bio-Gel P-200 column equilibrated with PBS containing 0.1% BSA. Column fractions were assayed directly by ELISA for CAP binding and enzyme activity.

PVC 96-well plates were incubated overnight at 4°C with 50 µg/ml purified mAb in 5 mM Tris, pH 7.0. Plates were incubated the next day for 1 h at room temperature with 1% BSA in 20 mM normal TBS, pH 7.5. The plates were washed with TBS containing 0.5% Tween (TBS-Tween) and incubated overnight at 4°C with 25 µg/ml CAPs in TBS-Tween. The next day the plates were washed twice with TBS-Tween and incubated for 2 h at 37°C with 5 µg/ml alkaline phosphatase–conjugated mAbs in 1% BSA in TBS. The plates were washed twice with TBS-Tween and once with distilled H2O. Reactions were developed with p-nitrophenyl phosphate in 100 mM sodium chloride, and 0.01% sodium azide. A typical experiment consisted of 50 µl of 5% Sepharose 4B incubated with either 100 µg of radiiodinated CAPs or CCVs. After incubating overnight at 4°C with agitation, the beads were pelleted briefly in a centrifuge (3200; Brinkmann Instruments Co., Westbury, NY), washed three times with 500 µl of 20 mM Heps, pH 7.0, 1% BSA, then twice with 500 µl of 20 mM Heps, pH 7.0, containing 100 mM sodium chloride. Samples were dissociated in Laemmli sample buffer (0.1% SDS, 50 mM Tris, pH 6.5, 100 mM dithiothreitol, 10% glycerol) and counted.

**Gel Electrophoresis and Immunoblotting**

SDS PAGE was performed as described by Laemmli (7). Gels were stained with Coomassie Brilliant Blue and/or dried and autoradiographed using a Cronex long lasting plus intensifying screen and Cronex X-ray film (DuPont Co., Wilmington, DE).

Proteins were electrophoretically transferred to nitrocellulose using a transfer apparatus (Bio-Rad Laboratories) and the procedure described by the manufacturer. Nitrocellulose filters were fixed, then saturated with 0.1% BSA and 0.05% Tween 20 in PBS, pH 7.5. Filters were incubated at 4°C overnight with primary antibodies suspended in PBS, pH 7.5, containing 0.1% BSA and 0.05% Tween 20. Filters were washed three times with PBS containing 0.05% Tween, then incubated 1 h at 4°C with peroxidase-conjugated goat anti–mouse IgG/IgM (Boehringer Mannheim Biochemicals) diluted in PBS containing 0.1% BSA and 0.05% Tween 20. After three washes with TBS containing 0.05% Tween 20 and one wash with 20 mM Tris, 0.5 M NaCl, pH 7.5, reactive bands were visualized with 4-chloro-1-naphthol (Bio-Rad Laboratories) and 0.05% hydrogen peroxide.

**Results**

**Four Epitopes of CAPs**

Four unique mAbs to CAPs were eventually generated from the fusion of immune BALB/c splenocytes to Sp2/0 myeloma cells. Two of them, mAbs C-7H12 and C-6C1, were produced from a BALB/c mouse immunized with CAPs isolated from CCVs by heating at 100°C for 5 min, while mAbs C-10B2 and C-4E5 were produced from a mouse immunized with whole cell. All four mAbs reacted with CAPs from both neuronal and nonneuronal sources (Figs. 1 and 2). To localize reactive sites of the mAbs, sandwich ELISAs were performed with purified mAbs coupled to alkaline phosphatase. PVC plates were coated with a purified mAb, incubated with 100 mM sodium chloride, and 0.01% sodium azide. A typical experiment consisted of 50 µl of 5% Sepharose 4B incubated with either 100 µg of radiiodinated CAPs or CCVs. After incubating overnight at 4°C with agitation, the beads were pelleted briefly in a centrifuge (3200; Brinkmann Instruments Co., Westbury, NY), washed three times with 500 µl of 20 mM Heps, pH 7.0, 1% BSA, then twice with 500 µl of 20 mM Heps, pH 7.0, containing 100 mM sodium chloride. Samples were dissociated in Laemmli sample buffer (0.1% SDS, 50 mM Tris, pH 6.5, 100 mM dithiothreitol, 10% glycerol) and counted.

**Phosphorylation of CAPs**

Phosphorylation of CCVs was conducted in 100 mM Tris, 10 mM MgCl₂, 100 mM morpholinoethanesulfonic acid, pH 5.7. Reaction mixtures (100 µl) contained 0.1 mg of CCVs, 20 µM ATP, and 1 µCi γ-32P-labeled ATP. Some samples were supplemented by 50 µg/ml poly-L-lysine (14 kD, Sigma Chemical Co.). Incubations were performed for 60 min at room temperature. Samples were heated at 100°C for 5 min and spun at 100,000 g for 1 h before immunoprecipitation.

**Immunoprecipitation of CCVs and CAPs**

Radiiodinated CCVs, CAPs, and phosphorylated CAPs were incubated with Sepharose 4B–coupled mAbs in Heps, pH 7.0, containing 1% BSA, 898
Figure 2. Reactivity of mAb C-7H12 with chymotryptic fragments of CAPs bound to clathrin heavy chains. CCVs, reassembled clathrin cages, reassembled cages containing only CAP2, and purified CAPs (total protein concentrations: 1 mg/ml in 100 mM morpholinoethanesulfonic acid buffer, pH 6.5) were incubated with 1.7 μg/ml chymotrypsin for various time periods, treated with phenylmethylsulfonyl fluoride, pelleted at 100,000 g in an airfuge (except the purified CAPs) and analyzed by SDS PAGE and immunoblotting with mAb C-7H12. (a) Coomassie stain of digested CCVs; (b) immunoblot of gel loaded as in a, using mAb C-7H12; (c) immunoblot using mAb C-7H12 of chymotryptically digested cages containing only CAP2; (d) immunoblot using mAb C-7H12 of chymotryptically digested cages containing both CAP and CAP2; (e) immunoblot using mAb C-7H12 of chymotryptically digested free CAPs. In d and e CAP1 was obscured by CAP2 reactivity. a and b were run using 7-15% gradient gels, which allowed greater separation of the CAPs than the 8-16% gels used in c–e.

Assigning mAb Binding Sites to Chymotryptic Fragments of CAPs

To characterize the reactive sites of each mAb on the CAP molecule, CCVs were chymotryptically digested and the vesicle-bound proteolytic fragments were isolated by centrifugation and subsequently analyzed by SDS PAGE and immunoblot. By varying the digestion times, CAP fragments were generated that were trimmed increasingly close to the heavy chain binding site. Immunoblotting experiments using these fragments and the anti-CAP mAbs facilitated construction of a cleavage map of the CAPs using the clathrin binding site as a reference point.

Table I. Sandwich ELISA of Anti-CAP mAbs

| Coated mAb | Soluble alkaline phosphatase-conjugated mAbs |
|------------|---------------------------------------------|
|            | C-7H12 | C-10B2 | C-6C1 | C-4E5 | CAPs |
| C-7H12     | -      | ++     | +     | ++    | ++++ |
| C-10B2     | +++    | -      | ++++  | -     | +    |
| C-6C1      | +      | ++++   | -     | +     | +    |
| C-4E5      | ++++   | +      | ++++  | -     | +    |
| CAPs       | ++++   | ++++   | ++++  | ++++  | ++++ |

Purified mAbs were coated on PVC plates, incubated with CAPs, and subsequently incubated with alkaline phosphatase-conjugated mAbs. Reactivity was quantitated colorimetrically using p-nitrophenyl phosphate. Results represent the mean of three data points in a typical experiment. The concentration of plate-bound CAPs (last row) was adjusted stoichiometrically to 40% of the amount of plate-bound antibody. Coupled antibodies were titered to produce an OD at 405 nm of 1–1.5 after 1 h. A control value using an unrelated alkaline phosphatase-conjugated IgG1 murine mAb was determined for each row, and subtracted to give the reported results.

Figure 2 shows chymotryptic fragments that were generated when CCVs were used as substrate. Fig. 2 b is an immunoblot of an identically loaded gel using mAb C-7H12. For convenience, we have divided the CAP fragments into two categories: fragments common to CCVs and reassembled cages (CFs) and unique fragments. The CFs consist of 30-kD (CF1), 18-kD (CF2), and 15-kD (CF3) fragments. Previous experiments (8, 9) have shown that CF2 and CF3 were pro-
duced by chymotryptic digestion of either CCVs or reassembled clathrin cages. We show in Fig. 2 that CF1 can also be generated from either structure.

To determine which fragments are generated from CAP1 and which are generated from CAP2, clathrin cages were stripped of CAPs with sodium isothiocyanate as described (14), and reassociated with mAb C-4E5 affinity-purified CAP2. The reassociated cages were digested with chymotrypsin as before and blotted with mAb C-7H12. As shown in Fig. 2, c and d, CF1 and CF3 are produced by cages containing only CAP2; hence these fragments must be generated by proteolysis of CAP2, while CF2 must be generated from CAP1. These results support the notion that CAP1 and CAP2 bind clathrin with structurally similar domains shared by both proteins. Interestingly, CVC-1 (6) also binds CF2 (Fig. 1), confirming its identity as a fragment of CAP1. CVC-1 (6) does not react with CAP2, CF3, or CF1, but it does react with clathrin-bound CAP1. These data indicate that CF2 contains at least two distinct epitopes: one is shared by CAP2 and appears to be involved in clathrin binding; the other is unique to CAP1.

Several CAP fragments are generated with molecular masses <10 kD using either CCVs or reassembled cages as substrates. The common fragments in this range are difficult to identify, although CCVs appear to produce a smaller set of fragments than reassembled cages. This implies that CAPs are less accessible in CCVs than they are in reassembled cages, which is not surprising considering the more complex structure of the vesicle. The small size of unique fragments generated from either reassembled cages or CCVs has led us to suggest that mAb C-7H12 binds an epitope involved in clathrin heavy chain binding. Unless this epitope constitutes a linear string of amino acids, structural conservation of this region of the molecules may not be obvious from primary sequence analysis (3).

Anti-CAP reactivity was not detected in supernatants of chymotryptic digestion experiments using C-10B2, C-4E5, or anti-CAP1 mAb CVC-1 (6; data not shown). Either the released fragments are digested beyond recognition by these mAbs or the reactive epitopes span residues on both released and bound fragments. CAPs isolated by boiling and digested with chymotrypsin produce a plethora of new fragments in addition to CF1, CF2, and CF3 (Fig. 2 e). Hence, binding by clathrin affords considerable protection against proteolysis by chymotrypsin.

Monoclonal Antibody 7H12 Reacts with the Clathrin-binding region of both CAPs

The immunoblot in Fig. 2 b shows that mAb C-7H12 reacts with both CAPs. Until now, all reported mAbs have reacted specifically with one of the CAPs (6). The existence of cross-reactive epitopes may be predicted, however, from the biochemical characteristics of the proteins. In particular, both CAPs appear to bind a common site on the clathrin heavy chain (14). This implies that the clathrin-binding regions of both CAPs share structural homology, and antibodies to this region of the molecules should cross-react with both forms. With these considerations in mind, an ELISA was used to determine the relative reactivity of each of the mAbs with free CAPs and clathrin-bound CAPs. Fig. 3 shows mAbs CVC-1 (6), C-4E5, C-10B2, C-6C1, and C-7H12 reacting with free CAPs. On the other hand, only mAbs CVC-1 (6), C-4E5, and C-10B2 react with the clathrin-bound CAPs present in purified preparations of triskelions. mAb C-6C1 reacts poorly with clathrin-bound CAPs, while mAb C-7H12 does not react with clathrin-bound CAPs. These results were obtained only when the clathrin-bound CAPs were pre-spun as cages or CCVs before coating on PVC plates. CAPs apparently dissociate from clathrin at a slow rate during storage at 4°C.

To document further the lack of reactivity of mAb C-7H12 with clathrin-bound CAPs, immunoprecipitation experiments were performed using radioiodinated CCVs and radioiodinated free CAPs. Purified mAbs C-7H12, C-10B2, CVC-1 (6), and C-4E5 covalently coupled to Sepharose 4B were
used for these experiments. These mAbs precipitate radioiodinated free CAPs with varying efficiencies (Fig. 4). mAb C-7H12, however, does not precipitate detectable counts of radioiodinated CAPs. This indicates that in the conformation present on the CCVs, CAPs are not recognized by mAb C-7H12, but are recognized by mAbs CVC-1, C-10B2, and C-6E5. These results differ from those reported recently (2) in which mAbs to CAP1 were only shown to be hindered from binding the clathrin-CAP complex. In total, our data suggest that mAb C-7H12 reacts directly with the clathrin-binding sites shared by both CAPs.

Mapping the CAP Phosphorylation Site to Accessible End of CAP2

A kinase activity recently has been associated with CAP2 (12, 13). This activity was recently confirmed in another report and attributed to an endogenous CCV casein kinase II (1). Although this activity is intensified by polybasic compounds such as polylysine or histone 1 (12), phosphorylation can be detected in the absence of these compounds by immunoprecipitation and long-term autoradiography (Kohtz, D. S., and S. Puszkin, unpublished results). A significant feature of this phosphorylation is its substrate preference for CAP2 (12). Nevertheless, phosphorylation of CAP1, was detected in the presence of polylysine after radioimmune precipitation with anti-CAP1 mAb CVC-1 (6) and long-term autoradiography (Fig. 5). Whether phosphorylation sites are partially conserved between the two forms of CAP is unclear at present.

An ELISA was devised to determine how efficiently phosphorylated CAPs react with each of the mAbs in comparison to unphosphorylated CAPs. PVC plates were coated with unphosphorylated CAPs. The titer of purified mAb was adjusted in preliminary experiments to produce an optical density of 2 at 405 nm after 2 h of incubation (using a 1:1000 dilution of alkaline phosphatase-conjugated second antibody; Boehringer Mannheim Biochemicals). Purified mAbs at this dilution were preincubated with varying amounts of either phosphorylated or unphosphorylated CAPs. These preparations were subsequently incubated with the PVC plate-bound CAPs, and bound antibody was quantitated with the alkaline phosphatase–conjugated anti–mouse secondary antibody. As shown in Table II, phosphorylated and unphosphorylated CAPs bind almost equally well to all of the mAbs. Phosphorylated CAPs do not appear to compete as well for mAb C-10B2 as unphosphorylated CAPs, although the difference is small. The similar affinities of phosphorylated and unphosphorylated CAPs for all the mAbs is also reflected in the efficiency of immunoprecipitation of phosphorylated CAPs. CAP2 was phosphorylated by the CCV-associated kinase activity in the presence of polylysine. The samples were heated, denatured proteins removed by centrifugation, and the CAPs precipitated with mAbs coupled to Sepharose 4B. Since the beads were coupled to equal amounts of purified antibody, we assumed that their specific binding activities were equivalent. The autoradiograph in Fig. 8 shows that mAbs C-10B2, C-4E5, and C-7H12 all precipitate phosphorylated CAPs in a quantitative manner. Interestingly, a doublet was precipitated by C-10B2. Using a limited concentration of CAPs and varying the proportion of phosphorylated or unphosphorylated CAPs for all the mAbs is also reflected in the efficiency of immunoprecipitation of phosphorylated CAPs. CAP2 was phosphorylated by the CCV-associated kinase activity in the presence of polylysine. The samples were heated, denatured proteins removed by centrifugation, and the CAPs precipitated with mAbs coupled to Sepharose 4B. Since the beads were coupled to equal amounts of purified antibody, we assumed that their specific binding activities were equivalent. The autoradiograph in Fig. 8 shows that mAbs C-10B2, C-4E5, and C-7H12 all precipitate phosphorylated CAPs in a quantitative manner. Interestingly, a doublet was precipitated by C-10B2. Using a limited concentration of CAPs and varying the proportion of phosphorylated CAP2 also does not appear to quantitatively affect the efficiency of CAP2 immunoprecipitation by any of the mAbs (Fig. 6).

To determine whether any of the mAbs reacted with epitopes relevant to the phosphorylation process, kinase inhibition experiments were performed (Fig. 7). For this, mAbs at 3.5-fold molar excess to CAP2 were incubated with CCVs in phosphorylation buffer overnight at 4°C. Polylysine and gamma radiolabeled ATP were added and the reactions were stopped at various time points with EDTA. Samples were heated to 100°C for 10 min and pelleted. The supernatants were analyzed by SDS PAGE and autoradiography. None of the mAbs inhibited phosphorylation of casein by the endogenous CCV casein kinase II (data not shown). Both mAbs C-4E5 and C-10B2 inhibited phosphorylation of CAP2 for as long as 5 min after the addition of ATP. The inhibition profiles of these mAbs appear to differ slightly. CCVs preincubated with C-10B2 rapidly acquire a small amount of

**Table II. Competitive ELISA Using Phosphorylated and Unphosphorylated CAPs**

| Antibody (absorbance 405 nm) | C-4E5 | C-10B2 | C-7H12 | C-6E5 |
|------------------------------|-------|--------|--------|-------|
| µg                           |       |        |        |       |
| 50                           | 0.1/0.1 | 0.3/0.2 | 0.2/0.1 | 0.2/0.1 |
| 25                           | 0.2/0.2 | 0.6/0.5 | 0.5/0.5 | 0.5/0.5 |
| 10                           | 0.5/0.4 | 1.2/1.0 | 0.7/0.7 | 0.8/0.7 |
| 5                            | 1.1/1.0 | 1.5/1.3 | 1.1/1.1 | 1.2/1.1 |
| 1                            | 1.6/1.5 | 1.8/1.6 | 1.3/1.2 | 1.4/1.3 |
| 0.5                          | 1.7/1.6 | 1.8/1.8 | 1.6/1.6 | 1.7/1.6 |
| 0.1                          | 1.9/1.9 | 1.9/1.9+ | 1.8/1.8 | 1.9/1.8 |
| 0                            | 1.8/1.9+ | 1.9/1.9+ | 1.9/1.9 | 1.9+1.9 |

PVC plates were coated with unphosphorylated CAPs, then incubated with mAbs in the presence of the indicated amount of phosphorylated or unphosphorylated CAPs (phosphorylated/unphosphorylated). Binding was quantitated using an alkaline phosphatase–conjugated anti–mouse second antibody. Results represent the means of three values from a typical experiment. A control value using an unrelated IgG1 murine mAb was determined for each row, and subtracted to give the reported results.

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**Figure 5.** Phosphorylation of CAP2 and CAP1 in CCVs. CCVs were suspended in phosphorylation buffer containing 50 µg/ml polylysine and 1 µCi γ-32P-labeled ATP in 20 µM ATP. After a 10-min incubation at room temperature, samples were heated at 100°C for 5 min, denatured proteins pelleted, and supernatants either analyzed directly by SDS PAGE (lane A), or immunoprecipitated with anti-CAP1 mAb CVC-1 coupled to Sepharose 4B beads. (lane B) 5 µl of a 10% solution of coupled beads; (lane C) 10 µl; (lane D) 20 µl. The bands of lower molecular mass represent the kappa light chains of the immunoglobulins. (Lanes A–D) Coomassie Blue stains; (lanes A'–D') autoradiography.
Figure 6. Relative efficiency of immunoprecipitation of phosphorylated and unphosphorylated CAPs by mAbs C-10B2, C-4E5, and C-7H12. A limiting titer of coupled antibody was used to precipitate a total of 20 μg of CAP2. The various amounts of phosphorylated CAP2 in the total amount are indicated in micrograms. Were phosphorylated CAP2 precipitated less efficiently than unphosphorylated CAP2, the total precipitated protein would be reduced as the proportion of phosphorylated CAP2 increased. The lower molecular mass bands are antibody light chains, which apparently do not couple efficiently and are released in Laemmli sample buffer containing dithiothreitol. Arrow indicates CAP2 precipitated by mAb C-10B2.

CAP2-bound phosphate before being completely inhibited, while CCVs preincubated with C-4E5 slowly but progressively acquire CAP2-bound phosphate. The pattern of inhibition produced by mAb C-4E5 could be interpreted as less complete than that produced by mAb C-10B2. This would result from differences in affinity between the two mAbs, or differences in the proximity of the antigenic sites to the phosphorylation site. Alternatively, since it has been suggested that CAP2 contains more than one phosphorylation site (12), the antibodies may be inhibiting the phosphorylation of different residues. Further experiments are in progress to determine which of these possibilities is valid (Kohtz, D. S., W. J. Schook, and S. Puszkin, manuscript in preparation).

A 32-kD Phosphoprotein Cross-reacts with Anti-CAP2 mAb C-10B2

Curiously, mAb C-10B2 coprecipitates another heat-stable, 32-kD phosphoprotein from the CCV preparation. As indicated by the open arrow on the autoradiography in Fig. 8, the coprecipitated phosphoprotein migrates immediately below the phosphorylated CAP2. This phosphoprotein apparently competes very effectively with CAP2 for mAb C-10B2, since it is not detectable in preparations of total CAPs (e.g., Fig. 5, lanes A and A'). In C-10B2 precipitate, the 32-kD phosphoprotein is substantially enriched, indicating an affinity for mAb C-10B2 at least as high as that of CAP2; this is apparent in both the autoradiograph in Fig. 8 and in the Coomassie stain in Fig. 6 (indicated by arrow).

The 32-kD protein does not react with mAbs reactive with CAP1 nor with mAbs reactive with DARPP 32 (kindly provided by Dr. P. Greengard, Rockefeller University, New York). Also, it only reacts with one of the four mAbs recognizing CAP2 (although traces may be present in mAb C-4E5 precipitates, not shown), implying that it is not a proteolytic fragment of this protein. We have tentatively labeled this phosphoprotein CAPx (or LCx), and are presently determining whether it is a CCV component or a soluble contaminant of our preparations. Its stability during heat denaturation (the vesicles are heated and centrifuged before precipitation), phosphorylation properties (it is phosphorylated in the presence of polylysine), and cross-reactivity with an anti-CAP2 mAb (C-10B2) indicate that it may be structurally related to CAP2.

Discussion

The characterization of mAbs to CAPs by our laboratory and others (2, 6) has revealed considerable structural diversity between the two forms (CAP1 and CAP2). The functional significance of this diversity is probably relevant to the evolution of higher organisms, since yeast appear to possess only one form of CAP. At present, the explicit functions associated with this diversity have evaded elucidation. Instead, two properties mediated equally well by both forms of CAPs have been described: (a) an ATP-dependent uncoating enzyme works by initially binding to either of the CAP molecules (11), and (b) a single site on the clathrin heavy chain binds equally well to either form of CAP (14). Hence, mAbs that distinguish the two forms of CAPs would not be as likely to recognize epitopes involved in these conserved functions as mAbs reacting with both CAPs.

In this report, two mAbs (C-6C1 and C-7H12) are presented that react with epitopes shared by both CAPs. The term "conserved domain" may be used to describe the structural region of the CAPs reactive with these mAbs. The significance of the epitope recognized by C-6C1 has not been detailed, although chymotryptic mapping experiments re-
Figure 8. Immunoprecipitation of phosphorylated CAP2 and CAPx by mAbs coupled to Sepharose 4B. CCVs were phosphorylated in the presence of polylysine, heated at 100°C for 5 min and pelleted. Aliquots of supernatant from a single phosphorylation reaction were immunoprecipitated by various volumes (in microliters) of a 10% solution of Sepharose 4B-coupled mAbs. Approximately 1 μg of mAb is bound per μl of 10% Sepharose 4B suspension. Precipitates were dissociated with Laemmli sample buffer and analyzed by SDS PAGE and autoradiography.

revealed that it is near the clathrin-binding region of the CAPs. The mAb C-7H12 appears to react with an epitope directly on the clathrin-binding domain as evidenced by chymotryptic mapping, ELISAs using clathrin-bound CAPs and free CAPs, and immunoprecipitation experiments using radiiodinated CCVs and CAPs. Sandwich ELISAs indicate that, despite their proximity, the epitopes recognized by mAbs C-6C1 and C-7H12 are distinct.

We describe elsewhere two mAbs (C-10B2 and C-4E5) reactive specifically with CAP2 (Kohtz, D. S., J. D. Kohtz, W. J. Schook, and S. Puszkin, manuscript submitted for publication). Sandwich ELISAs indicate that mAbs C-10B2, C-4E5, C-7H12 and C-6C1 react with distinct epitopes on CAP2. As may be inferred from the degree of sterically induced inhibition observed in these assays, mAbs C-10B2 and C-4E5 appear to react with different epitopes in the same region of CAP2. As judged by its sensitivity to chymotryptic digestion, this region of CAP2 is on an accessible end of the molecule. The term “nonconserved domain” may be used to refer to this accessible edge of the CAP2 and its putative counterpart on CAP1.

Two kinase activities are associated with CCVs. The first is Ca2+/calmodulin- and cAMP-independent, and uses the 50-kD assembly complex polypeptide as a substrate; the second is accentuated by the presence of polybasic compounds and uses CAP2 as a substrate. Direct inhibition experiments indicate that mAb C-10B2 and C-4E5 react with an epitope relevant to the phosphorylation process. In particular, C-4E5 inhibits phosphorylation better in the short run while C-10B2 inhibits better in the long run. Inhibition of CAP2 phosphorylation by these mAbs could be produced by several mechanisms: steric interference of the bound antibody and the enzyme; allosteric structural alterations produced in CAPs by bound antibody; direct competition for the enzyme binding site by the antibody; and, if it is distinct from the enzyme-binding site, direct blocking of the phosphorylation site by bound antibody. Further work is necessary to determine the mechanisms responsible for inhibition of CAP2 phosphorylation by each mAb.

Immunoprecipitation experiments using mAb C-10B2 revealed a cross-reactive phosphoprotein with a molecular mass of 32 kD. We referred to this phosphoprotein as CAPx, or LCx, since it coprecipitates with CAP2. This polypeptide is not coprecipitated by C-4E5, CVC-1 (6), C-7H12, or C-6C1, indicating that it is not a CAP fragment. Since CAPx is a phosphoprotein and C-10B2 does inhibit CAP2 phosphorylation, it appears that C-10B2 cross-reacts with the phosphorylated epitopes on both polypeptides. Similarities between the polypeptides suggest that it is a member of the CAP family. In particular, CAPx is heat stable, it can be isolated from CCV preparations, and it is phosphorylated under the same conditions as CAP2. When a CAPx-specific antibody is produced it may be possible to ascertain if the polypeptide is an integral vesicle protein.

Immunoprecipitation experiments using C-4E5 and CVC-1 (6) revealed two other properties of CAP phosphorylation. CAP2 is phosphorylated in the absence of effectors, but to a considerably lesser degree; and CAP1 is also phosphorylated in the presence of polylysine, but not to the same extent as CAP2. The latter observation implies that the phosphorylation process is not absolutely specific for CAP2, and in the presence of other mediators or enzymes, CAP1 may be equally well phosphorylated. Alternatively, Usami et al. (13), have indicated that CAP1 bears three sites of phosphorylation; CAP1 may have conserved only a fraction of these sites. Further work should elucidate the physiological significance of each site of phosphorylation and their distribution on each CAP.

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