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Brain clathrin light chain 2 can be phosphorylated by a coated vesicle kinase

(protein kinase/clathrin-associated protein 2)

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ABSTRACT **A protein kinase activity was observed in coated vesicles, prepared from bovine brain, that had clathrin-associated protein 2 (CAP_2, also known as clathrin light chain 2) as its principal substrate. Coated vesicles were purified by sucrose density gradient centrifugation followed by Sephacryl S-1000 column chromatography, and all buffers utilized in these procedures contained a mixture of proteolysis inhibitors to maintain CAP_2 kinase activity. Incubation of vesicles with [γ-^{32}P]ATP in the presence of 7 μM polylysine resulted in an overall increase in the incorporation of phosphate. NaDodSO$_4$/PAGE revealed that the principal recipient of this additional phosphate was CAP_2 (M_r 33,000), the faster-migrating component of the clathrin coat-associated proteins, whereas CAP_1 (M_r 36,000) was not phosphorylated. A number of other proteins, in the M_r 140,000 and 100,000 regions, were phosphorylated to a lesser extent. Polyarginine and polyethylenimine also supported CAP_2 phosphorylation, but arginine and lysine were ineffective. The phosphorylated protein was identified as CAP_2 because addition of exogenous CAPs resulted in increased incorporation of label into M_r 33,000 polypeptides and because heat treatment of labeled vesicles followed by ultracentrifugation resulted in recovery of labeled M_r 33,000 protein in the supernatant. Phosphorylation of CAP_2 may play a regulatory role in clathrin coat/coated vesicle functions.**

Coated vesicles are known to carry a protein kinase activity that utilizes a vesicle-associated, M_r 50,000 protein (pp50) as the principal substrate (1–4). This enzyme activity is not modulated by a variety of known effectors, although it does require Mg^{2+}. It was recently suggested (5) that the kinase activity resides in the pp50 itself, since 8-azidoadenosine 5′-[α-^{32}P]triphosphate was found to associate with a doublet of proteins (M_r 53,000 and 50,000) in liver coated vesicles which appears to be related to the brain vesicle protein, a singlet at M_r 50,000. Additional support for this proposal is the copurification of kinase activity with a complex isolated from brain coated vesicles and consisting of M_r 50,000 and 100,000 proteins. These proteins are implicated as an assembly factor for the *in vitro* formation of "baskets" by the clathrin–CAPs complex (6). Furthermore, interaction of the CAPs (clathrin-associated proteins) with the kinase complex stimulates phosphate incorporation into pp50 (7). However, it is unknown what role, if any, phosphorylation of pp50 plays in the function of the assembly polypeptide complex. We report here the expression of a kinase activity associated with brain coated vesicles that utilizes CAP_2 (also known as clathrin light chain 2) as a principal substrate and therefore may modulate clathrin coat functions in coated vesicles.

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MATERIALS AND METHODS

Materials. Histones (types II-AS, III-S, V-S, and VIII-S), poly(L-lysine) (average M_r 16,000), poly(L-arginine) (M_r 40,000), poly(L-aspartate) (M_r 15,000), poly(N^{ϵ}-carbobenzoxy-L-lysine) (M_r 10,000), spermidine, lysine, arginine, leupeptin, benzamidine, egg trypsin inhibitor, phenylmethylsulfonyl fluoride, and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were from Sigma. [γ-^{32}P]ATP (10 mCi/ml, 2900 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Polyacrylamide gel electrophoresis reagents were from Bio-Rad. Scintiverse scintillation solution and other reagent-grade chemicals were from Fisher. X-Omat R film and developing chemicals were from Kodak. Sephacryl S-1000 was from Pharmacia, and enzyme-grade sucrose from Schwarz/Mann.

Methods. NaDodSO$_4$ (0.1%)/polyacrylamide (5–15%) gradient gel electrophoresis was performed according to Laemmli (8). Gels were stained with Coomassie blue, and protein was determined by the method of Lowry *et al.* (9) using bovine serum albumin as standard.

Preparation of Coated Vesicles. Four beef brains yielding ≈500 g of cortex were obtained from a local slaughterhouse and processed as described (10), with the addition of a mixture of proteolysis inhibitors [leupeptin, 1 μg/ml; egg trypsin inhibitor, 2 μg/ml; phenylmethylsulfonyl fluoride, 0.2 mM; and benzamidine, 0.5 mM (final concentrations)]. The 100,000 × *g* pellets obtained from the crude homogenate were resuspended in 0.1 M Mes buffer containing 0.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 1 mM EGTA, 0.02% sodium azide, and the proteolysis inhibitors and sedimented through two sets of sucrose gradients. The vesicle fractions were resuspended in the Mes buffer containing the inhibitors and chromatographed on a 2.5 × 100 cm Sephacryl S-1000 column to remove contaminating membranes. Purified vesicles were generally rechromatographed to increase their purity, finally yielding 35–50 mg of highly pure coated vesicles. The whole procedure took 4 days. Vesicles remained active for a month when stored at 4°C with the proteolysis inhibitors. In agreement with previous reports (1, 2), vesicles prepared in this manner did not exhibit Ca^{2+}/calmodulin-regulated protein kinase activity (3). Inclusion of the proteolysis inhibitors appeared important for preservation of proton-pump activity and CAP_2 kinase activity, because earlier preparations of coated vesicles carried out in the absence of the inhibitors yielded equivocal results in the detectable levels of these enzyme activities.

Assay for the Endogenous Coated Vesicle Kinase Activities. The standard reaction mixture contained 100 mM Tris Cl buffer (pH 7.5), 10 mM $MgCl_2$, coated vesicles (100 μg of protein, added in 10–20 μl of the Mes buffer mixture), 20 μM [γ-^{32}P]ATP (specific activity 500–1000 cpm/pmol), and various additional reagents in a total volume of 100 μl. The

Abbreviation: CAP, clathrin-associated protein.

reaction mixture was preincubated at 30°C for 5 min, and the reaction was initiated by the addition of ATP. Generally, the reaction was allowed to proceed for 3 min, at which time 50 μl of 1% $NaDodSO_4$/30% (vol/vol) glycerol/0.01% phenol red/0.1% 2-mercaptoethanol was added. An 80-μl aliquot was loaded on a $NaDodSO_4$/polyacrylamide slab-gel lane, and electrophoresis was carried out at 7 mA per gel overnight. Gels were fixed, stained, and destained following standard procedures and dried using a Hoeffer drying apparatus. Dried gels were subjected to autoradiography, generally for 15 hr at 4°C. Radioactive bands were cut out of the gel and analyzed by liquid scintillation counting in Scintiverse.

RESULTS

Phosphorylation of Histones. To characterize further the protein kinase activity present in coated vesicles, we investigated the vesicles' ability to phosphorylate exogenously added substrates. Substrates tested included a variety of histone preparations and phosvitin. Fig. 1 illustrates the changes in the coated vesicle phosphorylation pattern. Lane 1 shows the phosphorylation pattern obtained in the absence of effectors. Upon addition of histone type II-AS (lane 2), there was a decrease in the level of phosphate incorporation into pp50, with the appearance of some label in CAP_2 and histone type II-AS (M_r 14,000). Upon addition of histone type III-S, heavy phosphorylation was observed in the M_r 33,000 region of the gel, which contained both histone type III-S and CAP_2. When histone type V-S was used, the pattern obtained resembled that of lanes 2 and 3 combined. Lane 5 shows somewhat less overall phosphate incorporation and contained added histone type VIII-S, which is arginine-rich, in contrast to the other histone preparations, which are lysine-rich. Lanes 6 and 7 illustrate that phosvitin served as an effective substrate for phosphorylation only when histone type II-S was added concurrently. Since the histone fractions altered the phosphorylation pattern of endogenous vesicle proteins with varying effectiveness, it was deemed necessary to determine whether the observed changes were due to the charged nature of histones. A series of amino acid polymers were tested for their ability to alter the substrate specificity of the coated vesicle kinases. These homopolymers were chosen because they were incapable of serving as substrates for the enzyme and, consequently, would not complicate the labeling pattern observed after gel electrophoresis.

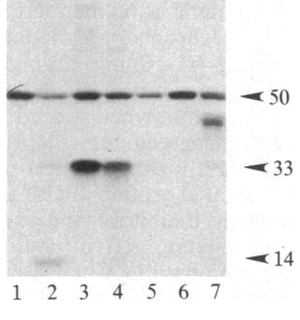

FIG. 1. Autoradiograph showing the effect of histone fractions on the phosphorylation pattern of coated vesicle proteins. All incubations were performed at 30°C with the addition of 20 μg of the indicated histone fraction (Sigma). An 80-μl aliquot from a final volume of 150 μl was loaded onto each lane of the gel. Lane 1: coated vesicles (100 μg of protein) alone. Lane 2: vesicles plus histone type II-AS. Lane 3: vesicles plus histone type III-S. Lane 4: vesicles plus histone type V-S. Lane 5: vesicles plus histone type VIII-S. Lane 6: vesicles plus phosvitin (20 μg). Lane 7: vesicles, phosvitin (20 μg), plus histone type II-AS (20 μg).

Dependence of CAP_2 Phosphorylation on the Presence of Basic Polymers. Fig. 2 illustrates the effects of a variety of agents upon phosphorylation of endogenous coated vesicle substrates. Lanes 1–3 illustrate the dose–response for polyethylenimine. The change in phosphorylation pattern that occurs when sufficient polymer has been added may be due to a second kinase activity or, alternatively, to a shift in the specificity of the pp50 kinase. Lane 4 illustrates the effect of poly(L-lysine) on this system. Note the clearly labeled doublet in the M_r 50,000 region of the gel and not detectable (or resolved) in the other lanes. Lanes 5–8 show the effect of various amounts of poly(L-arginine) on the vesicle kinase. This polymer is not as effective as the previous two, resulting in an overall decrease (≈50%) in total phosphate incorporation when compared with standard assay conditions.

The addition of poly(L-aspartate) (lane 9), an anionic polymer, did not change the pattern of phosphorylation, and it neutralized the effect of polylysine when added concurrently (data not shown). Addition of poly(N^{ε}-carbobenzoxylysine) (lane 10), a polymer in which the ε-NH_2 groups have been neutralized; of spermidine (lane 11); of lysine (lane 12); or of arginine (lane 13) resulted in no change in phosphorylation, indicating that the modulatory ability of the basic polymers is dependent upon both charge and conformation.

Table 1 lists the results of representative experiments showing the relative effectiveness of these polymers. There was considerable variation in the absolute amount of phosphate incorporated from one vesicle preparation to another, as well as decreased incorporation after longer storage times. Comparisons of relative phosphate incorporation between the polymers were made with the same batch of vesicles. Of the three polymers, the most effective at inducing phosphate incorporation into CAP_2 was poly(L-lysine).

Time-course experiments were run to determine whether phosphate was incorporated into one polypeptide in preference to others. Results of a representative experiment are shown in Fig. 3. From this autoradiograph it is apparent that phosphate was incorporated rapidly and uniformly into the various proteins, with maximal incorporation at 2 min. Liquid scintillation counting of the major radioactive bands indicated that the reaction was 85% complete after 1 min (data not shown).

To determine whether labeling of CAP_2 occurred in the absence of effectors, we increased the specific activity of the [γ-^{32}P]ATP 10-fold to ensure that even minor phosphate incorporation would be detected. Fig. 4 compares a standard

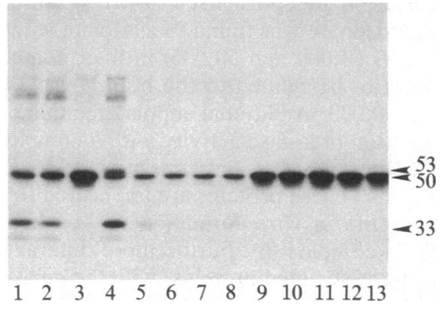

FIG. 2. Autoradiograph showing the effect of various basic polymers upon the phosphorylation pattern of coated vesicle proteins. Lanes 1–3: vesicles plus 0.01%, 0.005%, and 0.001% polyethylenimine, respectively. Lane 4: vesicles plus 20 μg of poly(L-lysine). Lanes 5–8: vesicles plus 40 μg, 30 μg, 20 μg, and 10 μg of poly(L-arginine), respectively. Lane 9: vesicles plus 20 μg of poly(L-aspartate). Lane 10: vesicles plus 20 μg of poly(N^{ε}-carbobenzoxy-L-lysine) [added in 10 μl of dimethyl sulfoxide, a reagent that has no effect upon the assay (data not shown)]. Lane 11: vesicles plus 90 μg of spermidine. Lane 12: vesicles plus 200 μM L-lysine. Lane 13: vesicles plus 200 μM L-arginine.

Table 1. Stimulation of phosphate incorporation into pp50 and CAP_2 by various effectors

Effector	Concentration	^{32}P, cpm	
		pp50	CAP_2
Polyethylenimine	1% (vol/vol)	552	828
	0.1%	859	1463
	0.01%	2870	1765
	0.005%	3181	1464
	0.001%	3676	189
Polylysine	27 μM	1177	1756
	20 μM	1061	1943
	13.5 μM	1588*	1941*
	6.8 μM	1508	1626
	1.4 μM	2872	387
Polyarginine	9 μM	1107	260
	7 μM	1060	301
	4 μM	1122	246
	2 μM	1081	287
None	—	3085	100

The lower incorporation at the higher concentrations of polyethylenimine and at all polyarginine concentrations tested is probably due to the aggregation of the vesicles that occurred at these levels of polymer. The concentrations of polylysine were calculated assuming a M_r of 15,000, whereas those for polyarginine used an M_r of 40,000. Incubations were for 3 min as described in *Materials and Methods*, with the reaction started by addition of ATP. The data show comparative values from a representative experiment. This set of experiments was repeated three times with different vesicle preparations.

*This level of polylysine was chosen as the standard for future studies and the observed decrease in incorporation of phosphate into pp50 when compared with control vesicle incubations run without polylysine addition was 55 ± 10%. To date, more than 50 determinations have been performed, using 20 different vesicle preparations, all yielding results in this range.

incubation in the presence of polylysine with one in which the specific activity of the ATP had been increased and no effector added. Where no effector had been added, no detectable incorporation of labeled phosphate in the CAP_2 region was observed. Also, there was greater labeling of the pp50 region and appearance of label in a broad band that migrated to the bottom of the gel and in a narrow band around M_r 70,000. This experiment suggests that phosphate is not incorporated into CAP_2 by coated vesicles unless a modulator is present.

Identification of the M_r 33,000 Protein Labeled by the Polylysine-Stimulated Coated Vesicle Kinase as CAP_2. Two-dimensional PAGE of coated vesicles has shown no evidence for the presence of a polypeptide other than CAP_2 in the M_r 33,000 region, either on the basis of Coomassie blue staining (11) or on the basis of autoradiography after electrophoresis

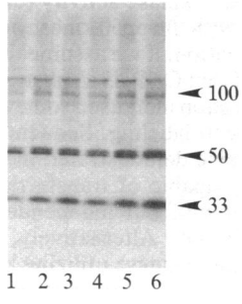

FIG. 3. Autoradiograph showing the incorporation of phosphate into various coated vesicle proteins in the presence of 20 μg of poly(L-lysine) after 15 sec (lane 1), 30 sec (lane 2), 45 sec (lane 3), 1 min (lane 4), 2 min (lane 5), and 3 min (lane 6) of reaction.

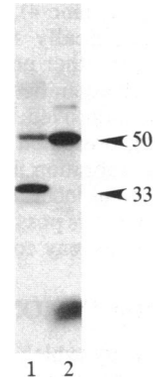

FIG. 4. Autoradiograph comparing coated vesicle proteins incubated in the presence of 20 μg of poly(L-lysine) with ATP at the standard specific activity (1000 cpm/pmol) (lane 1) with vesicles incubated with ATP at a specific activity of 10,000 cpm/pmol (lane 2).

of iodinated vesicle proteins (ref. 12 and unpublished data). However, to investigate the possibility that the phosphate was incorporated into another protein comigrating with CAP_2, we performed two different experiments. Fig. 5*A* shows the Coomassie blue-staining pattern of untreated coated vesicles and of the supernatant obtained after heating (100°C, 3 min) of labeled coated vesicles and subsequent ultracentrifugation to remove denatured protein and membranes. Lane 1 shows that, as expected (13), basically only two polypeptides remained in the supernatant, CAP_1 (M_r 36,000) and CAP_2 (M_r 33,000). The label is associated only with CAP_2 (Fig. 5*B*). Additional evidence that CAP_2 was the principal substrate of the polylysine-stimulated kinase is shown in Fig. 5*C*. When CAPs purified by heat treatment and ultracentrifugation as above were added to the reaction mixture containing polylysine, the incorporated label in the M_r 33,000 region of the gel nearly doubled (14,400 cpm vs. 9300 cpm), whereas there was essentially no change in pp50 labeling (5500 cpm vs. 5800 cpm) (see Fig. 5*C*, lanes 5 and 6).

In the same experiment, 13,600 cpm was incorporated into pp50 in the absence of polylysine (Fig. 5*C*, lane 4). A decrease in phosphate incorporation in the M_r 50,000 region of the gel was consistently observed when polylysine was present in the assay. Addition of polylysine to the incubation mixture was accompanied by an increase in turbidity and the occasional appearance of an additional labeled polypeptide mi-

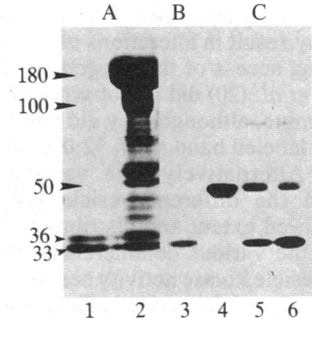

FIG. 5. Evidence that the labeled protein migrating at M_r 33,000 is CAP_2. (*A*) Coomassie blue-stained gel showing the supernatant obtained after heat-treatment of coated vesicles (lane 1) and the untreated coated vesicles (lane 2). (*B*) Autoradiograph showing the region of the gel corresponding to lane 1 in *A*. (*C*) Autoradiograph showing the results of adding CAPs to a coated vesicle incubation mixture prior to addition of ATP. Lane 4: coated vesicles plus 17 μg of CAPs but no polylysine. Lane 5: coated vesicles plus 20 μg polylysine. Lane 6: coated vesicles, 20 μg of polylysine, and 17 μg of CAPs.

grating at M_r 53,000 (Fig. 2, lane 4). Detection of this band seems to correlate specifically with the presence of polylysine and not with the other polymers that can induce CAP_2 phosphorylation. The significance of this observation awaits further study and improved resolution of these two polypeptides. In the work presented here, all tabulated data comparing phosphate incorporation into pp50 under different conditions represent the total label incorporated into the M_r 50,000 region of the gel; thus, it is possible that phosphorylation of two (or more) polypeptides may contribute to these values.

DISCUSSION

An important mechanism by which neuronal cells regulate specific processes in response to changes in their environment is protein phosphorylation (14, 15). The putative role of ATP in coated vesicle formation and/or function is far from being thoroughly understood. Several laboratories have reported the presence of a protein kinase activity in coated vesicles that appears to be specific to these organelles (1–4). Recently, additional roles for ATP in vesicle function have been postulated, including the generation of a pH gradient via a vesicle-associated proton pump (10, 16), the disassembly of the clathrin lattice (17, 18), and possibly in the recruitment of clathrin to form the coated vesicle (19).

Coated vesicles prepared in our laboratory without addition of protease inhibitors and using a Sephacryl S-1000 column buffer containing 20 mM Mes (pH 6.5), 0.15 M NaCl, 1 mM EGTA, and 7 mM 2-mercaptoethanol exhibited a casein kinase activity that did not require the presence of either histone or polylysine for its expression, in contrast to the kinase activity of the vesicles prepared by the protocol used in this study. This point is worth emphasizing because several laboratories have utilized exogenous substrates to investigate the properties of coated vesicle-associated kinases and have obtained results differing from ours (20, 21). Both groups have observed phosphorylation of phosvitin by their vesicle preparations, whereas we do not observe phosphorylation of this protein unless histone type II-AS or polylysine is added concurrently (Fig. 1). This difference may be explained by the differing protocols used by these laboratories to prepare and assay their vesicle preparations, since it is known that the coat structure is sensitive to high sucrose concentrations during centrifugation (22) and that the change in specificity of the vesicle kinase described in this report is correlated with an increase in turbidity suggestive of aggregation. Polyethylene glycol (M_r 3700) at 10% (wt/vol) final concentration did not induce CAP_2 phosphorylation (data not shown). The different preparation protocols and the presence of polylysine may result in alterations of coat and/or vesicle structure allowing access of the exogenous substrate to the enzyme. Usami *et al.* (20) did not observe labeling of added histone or protamine, although they did mention the appearance of a faintly labeled band at M_r 32,000 and a decrease in pp50 labeling. Alternatively, the variable presence of phosphatases in the different vesicle preparations may change the observed extent and distribution of label.

The effect of the various commercially obtained histone fractions upon vesicle kinase activity seems to correlate with the presence of lysine-rich histone in these preparations. The histone type VIII-S, which is arginine-rich, behaved much like polyarginine, in that it produced an overall decrease in total incorporation of label, with the appearance of some label in the CAP_2 region of the gel. The observed effects of polylysine and polyarginine suggest that charge effects alone cannot be causing the observed shift in phosphorylation, since polylysine and polyarginine should both be positively charged under the assay conditions (pH 7.5). Addition of either polymer results in increased turbidity, which suggests that aggregation *per se* is not alone responsible for the change in phosphorylation pattern. More recent results from our laboratory have shown that polylysine must be of $M_r >$ 14,000 to be effective and that a similar kinase activity is present in coated vesicles prepared from heart (unpublished observations).

The very obvious labeling of pp50 under the standard assay conditions, its presence in all species of coated vesicles studied to date (1–5), and the demonstration of its association in a complex that can stimulate assembly of the clathrin triskelion under physiological conditions of pH and ionic strength (6) all suggest an important regulatory role for this protein. Nevertheless, to date there has been no demonstration that phosphorylation plays a role in modifying the interaction of this protein with either clathrin or M_r 100,000 proteins associated with pp50. An interaction between pp50, which appears to be its own kinase (5), and the CAPs apparently can occur, since increased phosphorylation of pp50 has been observed when purified CAPs were added to a partially purified assembly-polypeptide preparation (7). We were unable to demonstrate an effect of CAPs upon pp50 phosphorylation in coated vesicles under our assay conditions, which use ATP of ≈90% lower specific activity. This difference between our work and that reported by Pauloin and Jolle (7) could be ascribed to the difference in specific activities of the ATP used and the fact that our studies were done with intact coated vesicles and theirs with a partially purified enzyme preparation. Their observation suggested that pp50 phosphorylation may be influenced by the environment and that the CAPs may play a role in the interaction of the assembly-polypeptide complex with the triskelion. Yet there is no defined role for this phosphorylation system in coated vesicle formation and/or function. Our results expand the apparent complexity of the phosphorylation mechanisms operational in coated vesicles and amplify the potential importance of CAPs in coated vesicle formation and/or function.

The addition of polybasic compounds has an effect upon a number of protein kinases (23–25). Polyarginine inhibits the activity of protein kinase C, of myosin light chain kinase, and, though less effectively, of both cAMP- and cGMP-dependent kinases (25). Polyglutamate had no effect upon the activity of protein kinase C and myosin light chain kinase; it is an inhibitor of the cAMP- and cGMP-dependent kinases (25). Spermidine was shown to inhibit the Ca^{2+}-dependent kinases but not to affect the activity of the cyclic nucleotide-dependent enzymes (23). In contrast, casein kinase II, a protein kinase with no known regulatory cofactors, was shown to be stimulated by polybasic compounds, including spermidine (25).

Our data suggest that the CAP_2 kinase activity was not related to any of the above systems, since we observed no inhibition with polyaspartate, no effect of spermidine, and varying stimulation by polylysine, polyethylenimine, and polyarginine. Also, none of the modulators of these various kinases affected CAP_2 kinase activity (data not shown). Our data are consistent with the pp50 kinase activity playing a role in CAP_2 phosphorylation, if we assume that the pp50 labeling is autocatalytic and that CAP_2 is a substrate, since the overall phosphate incorporation increased somewhat with a concomitant decrease in pp50 labeling. Sequential addition of ATP followed by polylysine demonstrated that the pp50 is not an intermediary form capable of transferring its phosphate to CAP_2 (data not shown), but independent phosphorylation sites are not ruled out. Alternatively, our data also are consistent with a single kinase utilizing both pp50 and CAP_2 as substrates. However, preliminary experiments utilizing inhibitors that block either pp50 or CAP labeling suggest that these activities are the result of independent kinases (unpublished observations). The final resolution of this problem must await purification and characterization of the kinase(s).

The demonstration of CAP_2 phosphorylation offers exciting possibilities for the regulation of clathrin assembly/disassembly and coated vesicle-organellar interactions, since the CAPs are known to protrude from the surface of the coated vesicle (26) and may play a role in triskelion assembly/disassembly (27, 28). The requirement for a nonvesicle-associated effector suggests that cytosolic components may be found that can produce the same effect as polylysine or histones and regulate phosphorylation of CAP_2.

Note Added in Proof. Recently, Usami *et al.* (29) described the phosphorylation of CAP_2 in the presence of histones.

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