In Vivo Phosphorylation of Adaptors Regulates Their Interaction with Clathrin

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Abstract. The coat proteins of clathrin-coated vesicles (CCV) spontaneously self-assemble in vitro, but, in vivo, their self-assembly must be regulated. To determine whether phosphorylation might influence coat formation in the cell, the in vivo phosphorylation state of CCV coat proteins was analyzed. Individual components of the CCV coat were isolated by immunoprecipitation from Madin-Darby bovine kidney cells, labeled with [^{32}P]orthophosphate under normal culture conditions. The predominant phosphoproteins identified were subunits of the AP1 and AP2 adaptors. These included three of the four 100-kD adaptor subunits, α and β2 of AP2 and β1 of AP1, but not the γ subunit of AP1. In addition, the μ1 and μ2 subunits of AP1 and AP2 were phosphorylated under these conditions. Lower levels of in vivo phosphorylation were detected for the clathrin heavy and light chains. Analysis of phosphorylation sites of the 100-kD adaptor subunits indicated they were phosphorylated on serines in their hinge regions, domains that have been implicated in clathrin binding. In vitro clathrin-binding assays revealed that, upon phosphorylation, adaptors no longer bind to clathrin. In vivo analysis further revealed that adaptors with phosphorylated 100-kD subunits predominated in the cytosol, in comparison with adaptors associated with cellular membranes, and that phosphorylated β2 subunits of AP2 were exclusively cytosolic. Kinase activity, which converts adaptors to a phosphorylated state in which they no longer bind clathrin, was found associated with the CCV coat. These results suggest that adaptor phosphorylation influences adaptor–clathrin interactions in vivo and could have a role in controlling coat disassembly and reassembly.

CLATHRIN-MEDIATED vesicular transport occurs at two sites within the cell. At the plasma membrane, clathrin-coated vesicles (CCV)[1] facilitate receptor-mediated endocytosis (Pearse and Robinson, 1990) to internalize soluble extracellular molecules such as nutrients, antigens, and growth factors and to down-regulate their receptors. CCV are also formed at the TGN where they sort newly synthesized lysosomal enzymes to the endocytic pathway and play a role in secretory granule biogenesis (Pearse and Robinson, 1990). The CCV coat is formed by polymerization of triskelion-shaped clathrin molecules into a lattice, catalyzed by adaptor molecules. The AP2 and AP1 adaptors nucleate clathrin assembly at the plasma membrane and the TGN, respectively, and play a role in selecting integral membrane proteins for concentration in CCVs. Purified clathrin and adaptor molecules, when sufficiently concentrated, will coassemble under physiological conditions in vitro (Keen, 1990).

The cellular assembly of coat proteins needs to be tightly regulated to ensure that the coat forms only at the membrane to initiate vesiculation, and that coat disassembly occurs only after the CCV has formed. Thus, there must be regulatory factors in the cell to ensure that clathrin and adaptors interact at the appropriate cellular location but avoid coassembly in the cytosol. Results from several studies have suggested CCV coat protein phosphorylation may play a role in controlling assembly state in vivo (Georgieva-Hanson et al., 1988; Pypaert et al., 1991). Endocytosis is inhibited during mitosis by cytosolic factors that block CCV invagination in an in vitro assay, an effect that could be partially reproduced by addition of purified cdc2 kinase to the assay (Pypaert et al., 1991). The mitotic inhibition of CCV invagination in vitro was reversed by diluting cytosol, and this reversal was sensitive to okadaic acid, a protein phosphatase inhibitor. Furthermore, phosphorylation of coat proteins by CCV-associated kinases destabilizes the clathrin coat of isolated brain CCV (Georgieva-Hanson et al., 1988). Together, these data indicate phosphorylation may influence CCV assembly or disas-

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1. *Abbreviations used in this paper*: CCV, clathrin-coated vesicle; CIAP, calf intestinal alkaline phosphatase; MDBK, Madin-Darby bovine kidney; PVDF, polyvinylidene difluoride.

sembly, but the target molecules for the implicated kinases and phosphatases have not yet been identified.

Conversely, in vitro studies have established that some CCV components can be phosphorylated by CCV-associated kinases, but the functional significance of the phosphorylation and whether it occurs in vivo has yet to be established (Morris et al., 1990). Each heterotetrameric adaptor has two 100-kD subunits (designated α and β2 [β] in AP2, and β1 [β′] and γ in AP1) and one medium and one small subunit (designated μ1 [AP47] and σ1 [AP19] in AP1, and μ2 [AP50] and σ2 [AP17] in AP2). When the kinase activity of purified CCV is stimulated, it is possible to phosphorylate all four 100-kD adaptor subunits. The medium subunits of AP1 and AP2 have both been reported to become phosphorylated in vitro (Merrese et al., 1990; Pauloin and Thurieau, 1993). Indeed, μ1 was reported to have an intrinsic casein kinase II–like activity (Merrese et al., 1990). σ1 and σ2 have not been found to be phosphorylated in any conditions. In vitro phosphorylation of clathrin, which comprises a 180-kD heavy chain and two homologous light chains, LCa and LCb, has revealed a casein kinase II activity associated with CCV that phosphorylates LCb but not LCa. The target site in LCb was mapped to serines 11 and 13, in the amino-terminal region, which are absent in LCa (Hill et al., 1988). In vitro, the clathrin heavy chain can be phosphorylated by $pp60^{v\text{-}src}$ (Mooibroek et al., 1992).

A limited number of studies on the in vivo phosphorylation of CCV coat proteins have been carried out in specialized cell types such as neurons and reticulocytes (Bar-Zvi et al., 1988; Corvera and Capocasale, 1990; Keen and Black, 1986). One study found that in cultured neurons, the 100-kD CCV coat proteins are phosphorylated, as well as the μ2 but not μ1 (Keen and Black, 1986). It was not established which of the 100-kD proteins became phosphorylated, as antibodies distinguishing the different 100-kD proteins were not available at the time these studies were carried out. In reticulocytes, it was found that the distribution of phosphorylated 100 and 50-kD CCV proteins apparently correlated with their assembly state, further supporting the idea that phosphorylation of adaptors may influence CCV formation (Bar-Zvi et al., 1988). Again, due to lack of appropriate reagents, the phosphorylated adaptor subunits were not identified. A third study reported that, in chick embryo fibroblast cells transformed with Rous sarcoma virus, phosphorylation of the clathrin heavy chain occurred on tyrosine and serine residues (Martin-Perez et al., 1989). Tyrosine phosphorylation of the clathrin light chains was also shown to occur upon the stimulation of cells with EGF (Mooibroek et al., 1992). Each of these studies of clathrin phosphorylation implicated $pp60^{v\text{-}src}$ as the tyrosine kinase responsible.

The data summarized above demonstrate the potential for many CCV coat proteins to be phosphorylated both in vivo and in vitro, and hint at a possible role for phosphorylation in cellular regulation of CCV coat assembly or disassembly. It was therefore of interest to establish more accurately which CCV coat protein subunits are modified by phosphorylation in vivo, and to correlate their modification with coat protein interactions. Here we identify the CCV coat protein subunits that are phosphorylated in vivo and show that phosphorylation of adaptors is a negative regulator of their interaction with clathrin. This phosphorylation effect was reproduced in vitro by stimulation of CCV-associated kinase activity. In addition, the β2 subunits of AP2 adaptors in the cytosol were all phosphorylated, while the β2 subunits of membrane-associated AP2 were predominantly dephosphorylated, further supporting a role for phosphorylation in regulating the assembly state of CCV coat proteins in the cell.

Materials and Methods

Materials

Clathrin and adaptors were purified using a method based on that of Manfredi and Bazari (1987) with the modifications described by Chang et al. (1993). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated in the text. The antibodies X22, LCB.1, TD.1, X16, and AP.6 were generated in this laboratory (see Table I). Antibodies 100/1, 100/2, and 100/3, and antibodies GD/1 and RY/1 were obtained from E. Ungewickell and L. Traub (Washington University, St. Louis, MO), respectively. Antibodies AC1-M11 and AP50-2 were obtained from M. Robinson (University of Cambridge, UK).

Cell Culture, Phosphate Labeling, and Immunoprecipitation

Madin-Darby bovine kidney (MDBK) cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in DME (Chang et al., 1993) to 80–90% confluency. Cells were washed in phosphate-free DME (GIBCO BRL, Gaithersburg, MD), and then labeled for 3 h in a medium containing 250 mCi/ml [^{32}P]orthophosphate (Dupont–New England Nuclear, Wilmington, DE). Cells were then solubilized in 0.5 ml of IP buffer (0.5M Tris/HCl, pH 7.2, 1% Triton X-100, 20 mM EDTA, 10 mM NaF, 30 mM Na_4PPi, 2 mM benzamidine, 1 mM Na_3VO_4, 1mg/ml pepstatin A, aprotinin, and leupeptin, and 0.1 mM PMSF) and centrifuged (13,000 *g*, 10 min, 4°C); the supernatant was removed and used for immunoprecipitation by antibodies prebound to protein A–sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970) and by Western blotting and subsequent autoradiography using x-ray film (X-OMAT; Eastman-Kodak Co., Rochester, NY).

Phosphoamino Acid Analysis

MDBK cells were labeled as described previously, and the immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting of the protein onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA). Phosphorylated proteins were excised from the membrane and heated at 110°C for 1.5 h in 6 N HCl (Pierce Chemical Co., Rockford, IL). The resultant solution of hydrolyzed protein was analyzed by one-dimensional thin-layer electrophoresis on 0.1 mm plates (EM Separations, Gibbstown, NJ) using 10% acetic acid and 1% pyridine running buffer to separate the different phosphorylated amino acids. The migration of standards of the phosphoamino acids serine, threonine, and tyrosine was visualized with ninhydrin, and the migration of ^{32}P-containing phosphoamino acids was determined by autoradiography.

Fractionation of Labeled Cells

MDBK cells were labeled with [^{32}P]orthophosphate as above. Cells were harvested in 100 mM MES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.2 mM DTT and lysed by repeated freeze thaw in liquid nitrogen. Cell lysate was centrifuged (1,000 *g*, 10 min), to remove intact cells and nuclei, and the supernatant was removed and centrifuged (100,000 *g*, 30 min) to separate membranes (pellet) from the cytosol (supernatant). To the cytosolic fraction, Tris/HCl, pH 7.2, and Triton X-100 were added to final concentrations of 0.5 M and 1%, respectively, before being used for immunoprecipitation as described above. The membrane fraction was solubilized in IP buffer and centrifuged (13,000 *g*, 10 min), and the supernatant was used for immunoprecipitation as described before. Immunoprecipitations were analyzed by SDS-PAGE, Western blotting, and autoradiography. The amount of protein found in the different fractions was determined by immunoblotting using the ECL detection system (Amersham Corp., Ar-

lington Heights. IL) and by quantitation using the Molecular Dynamics Personal Densitometer (Sunnyvale, CA). The relative levels of ^{32}P-labeled proteins in each of the fractions were determined using a phosphorimager (Molecular Dynamics). To determine the ratio of phosphorylated proteins in the membrane as compared with those in the cytosol, the phosphorimager values were normalized according to the protein levels determined from the immunoblot experiments.

Two-dimensional Gel Electrophoresis

Proteins were resolved in the first dimension by isoelectric focusing on a 3.3% acrylamide gel containing 9 M urea and 2% ampholytes (Pharmacia). The ampholyte mixture was a 3:1 mix of the pH 5.0–7.0 ampholytes and the pH 3.5–10 ampholytes, respectively. Proteins were then separated on the basis of size on a standard 8% acrylamide SDS-PAGE gel (O'Farrell, 1975) and transferred onto nitrocellulose before detection by immunoblotting.

Protease Digestion of Immunoprecipitated Adaptors

MDBK cells were labeled with [^{32}P]orthophosphate, as above, and AP1 and AP2 were immunoprecipitated with the 100/3 and AP.6 antibodies. Immunoprecipitated coat proteins were resuspended in 50 μl of 50 mM Tris/HCl, pH 8.0, and digested at 37°C for either 1, 3, or 5 min by the addition of either 0.025 mg of elastase or 0.1 mg of trypsin. The resultant digest was analyzed by SDS-PAGE, Western blotting, and autoradiography.

Clathrin Cage-binding Assay

Purified clathrin was concentrated to >1 mg/ml, and then dialyzed overnight against 100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.2 mM DTT at 4°C to allow clathrin cages to assemble. The cages were harvested by centrifugation (100,000 *g*, 15 min), and the pelleted cages were resuspended in the dialysis buffer. Clathrin cages were added to cytosol made from [^{32}P]orthophosphate-labeled MDBK cells harvested in 100 mM MES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.2 mM DTT, and incubated for 3 h at 4°C. Cages were then pelleted and solubilized; adaptors were then immunoprecipitated from both the pellet and the supernatant; and immunoprecipitates were analyzed by SDS-PAGE, Western blotting, and autoradiography. In experiments where cytosol from nonradiolabeled cells was tested for cage binding, the presence of adaptors in pellets or supernatants was analyzed directly by SDS-PAGE and immunoblotting.

Dephosphorylation of Cytosolic Adaptors

Cytosol was prepared from MDBK cells labeled with [^{32}P]orthophosphate as above in the absence of phosphatase inhibitors. Adaptors were dephosphorylated by the addition of calf intestinal alkaline phosphatase (CIAP) (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 U/ml, and incubated at 37°C for 30 min. The cytosol was then used in the cage-binding assay described above. Mock treatment was by incubation at 37°C for 30 min without adding CIAP.

Stimulation of Clathrin-coated Vesicle-associated Kinase Activity

CCVs were purified from bovine brain using the method described by Bauxbaum and Woodman (1995). Proteins were stripped from purified CCVs using 0.5 M Tris, pH 7.4. Kinase activity was stimulated as described by Morris et al. (1990), by the addition of KCl, $MgCl_2$, polylysine, and ATP to final concentrations of 0.15 M, 5 mM 100 μg/ml and 100 μM, respectively, and by diluting the stripped protein solution such that the final Tris concentration was 100 mM. This solution was then incubated at room temperature for 30 min. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM.

Results

Adaptor Subunits Are Major CCV Phosphoproteins

The purpose of this study was to identify components of the CCV coat that are phosphorylated in vivo and to investigate the role of these modifications in regulating coat assembly. In recent years, our laboratory and others have developed and characterized antibodies specific for each of the subunits of clathrin and the AP1 and AP2 adaptors (Table I). These antibodies were used to immunoprecipitate components of the CCV coat from solubilized MDBK cells, biosynthetically labeled with [^{32}P]orthophosphate. Analysis of the immunoprecipitated proteins after SDS-PAGE and autoradiography revealed that, under these steady state culture conditions, components of both adaptors and clathrin are constitutively phosphorylated, and that several adaptor subunits appear to be the dominant targets for phosphorylation among the CCV components analyzed (Fig. 1).

To verify which subunits of CCV proteins were phosphorylated, immunoprecipitates were further analyzed by immunoblotting with specific antibodies, as well as by autoradiography, and the resulting films were overlaid (Fig. 2). Immunoprecipitated 100-kD adaptor subunits were resolved on SDS-PAGE gels containing 6 M urea before this analysis (Ahle and Ungewickell, 1986). Once phosphorylated polypeptides were identified from both adaptors and clathrin, gel bands were excised and hydrolyzed, and the phosphoamino acid composition was determined by thin-layer electrophoresis (Fig. 3).

In AP1, the β1 subunit was phosphorylated (Fig. 2 *A*), and its phosphorylation was shown to be exclusive to serine residues (Fig. 3 *A*). In AP2, both the α and β2 subunits were found to be phosphorylated (Fig. 2 *B*) on serine residues (Fig. 3 *A*). Quantitation of the amount of [^{32}P]phosphate incorporated in the α and β2 subunits showed that the β2 subunit contained twice as much phosphate as α (data not shown). For both AP1 and AP2, the medium subunits were also phosphorylated (Fig. 2, *C* and *D*). The μ1 subunit of AP1 was phosphorylated equally on serine and threonine residues. In contrast, the μ2 subunit of AP2 was phosphorylated predominantly on serine residues with only a minor amount of threonine phosphorylation (Fig. 3 *B*).

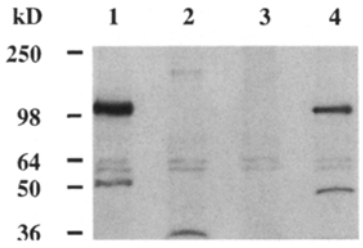

Figure 1. In vivo–phosphorylated CCV coat proteins. Autoradiograph of the immunoprecipitated coat protein components of CCV from MDBK cells labeled in vivo with [^{32}P]orthophosphate. AP2 immunoprecipitated with antibody AP.6 (lane *1*). Clathrin immunoprecipitated with antibody X22 (lane *2*). Immunoprecipitation using a control antibody DA6 that recognizes the α chain of class II histocompatibility molecules (not expressed in MDBK cells) (lane *3*). AP1 immunoprecipitated with antibody 100/3 (lane *4*). Immunoprecipitation was carried out in the presence of 0.5 M Tris, pH 7.0, to prevent coat protein assembly. Immunoprecipitates were analyzed on a 10% acrylamide–SDS gel. The migration of molecular weight standards in kilodaltons (kD) is indicated on the lefthand side of this figure and all subsequent figures.

Table I. Antibodies Used in This Study

Antibody	Antigen	Reference
100/1	β1 subunit of AP1 and β2 subunit of AP2	(Ahle et al., 1988)
100/2	α subunit of AP2	(Ahle et al., 1988)
100/3	γ subunit of AP1	(Ahle et al., 1988)
AC1-M11	α subunit of AP2	(Robinson, 1987)
AP.6	α subunit of AP2	(Chin et al., 1989)
G/D1	β1 subunit of AP1 and β2 subunit of AP2	(Traub et al., 1995)
RY/1	μ1 of AP1	gift from L. Traub
AP50-2	μ2 of AP2	(Page and Robinson, 1995)
X16	Clathrin light chain a(LCa)	(Brodsky, 1985)
X22	Clathrin heavy chain	(Brodsky, 1985)
LCB.1	Clathrin light chain b (LCb)	(Brodsky et al., 1987)
TD.1	Clathrin heavy chain	(Näthke et al., 1992)

Under the same steady state culture conditions, clathrin heavy chain was weakly phosphorylated (Fig. 2 *E*), predominantly on tyrosine residues but with some serine phosphorylation (Fig. 3 *C*). Clathrin light chains, LCa and LCb, were both phosphorylated (Fig. 2 *F*) on serine residues (Fig. 3 *C*). LCb was the predominant target for phosphorylation in clathrin, correlating with in vitro findings that LCb has a target site for CCV-associated casein kinase II activity that is absent in the LCa sequence (Hill et al., 1988).

Identification of the Phosphorylated Domains of the 100-kD Adaptor Subunits

The 100-kD subunits of adaptors have proline/glycine-rich hinge regions within which are proteolysis sites that allow the adaptors to be fragmented into their constituent domains. Limited cleavage releases the globular "ear" domains, from the "head" of the adaptors, which comprises the NH_2-terminal 60–70-kD portion of each 100-kD subunit plus the medium and small subunits. A number of studies have assigned specific functions to the proteolytically defined domains of adaptors (Goodman and Keen, 1995; Keen and Beck, 1989; Shih et al., 1995; Traub et al., 1995). The head domains of both AP1 and AP2 have localization specificity for the intracellular membrane where each type of adaptor is found (Page and Robinson, 1995; Peeler et al., 1993). The hinge regions of both β1 and β2, as well as sequences in the α chain of AP2, can interact with clathrin (Goodman and Keen, 1995; Shih et al., 1995). The domains in which adaptors are phosphorylated were identified to establish a potential functional consequence for these modifications.

AP1 or AP2 adaptors were separately immunoprecipitated from ^{32}P-labeled MDBK cells and cleaved with either trypsin or elastase, and the resulting fragments were analyzed by SDS-PAGE and immunoblotting (Fig. 4). The trypsin cleavage site lies in the hinge domain of the 100-kD adaptor subunits, closest to the head domain, and proteolysis with trypsin produces a low molecular weight fragment, consisting of the hinge and ear domains, and a high molecular weight fragment, consisting of the head fragment alone (Schröder and Ungewickell, 1991). The elastase cleavage site lies at the opposite end of the hinge domain closest to the ear domain. Cleavage with elastase produces a low molecular weight fragment, consisting of the ear domain alone, and a high molecular weight fragment, consisting of the head and hinge domains (Shih et al., 1995). Analysis of phosphorylated AP1 digested with trypsin revealed a ^{32}P-labeled fragment corresponding to the ear plus hinge domains (Fig. 4 *A*), while elastase diges-

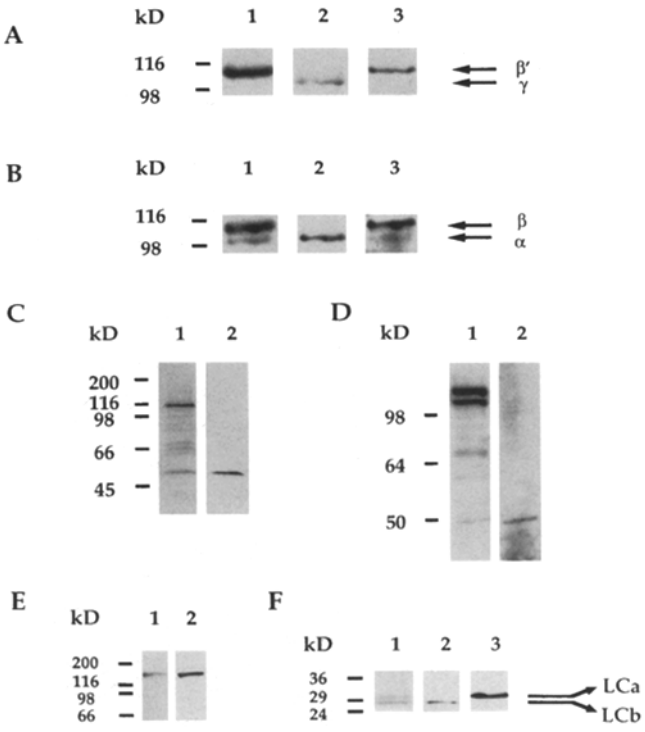

Figure 2. Identification of the phosphorylated subunits within each of the CCV coat protein complexes. MDBK cells were labeled in vivo with [^{32}P]orthophosphate, and components of the CCV coat were immunoprecipitated. The individual subunits of each precipitated complex were separated by SDS-PAGE and analyzed both by Western blotting and autoradiography. (*A*) AP1 immunoprecipitated with antibody 100/3 and analyzed on an 8% acrylamide–SDS gel, containing 6 M urea, followed by autoradiography (lane *1*), immunoblotting with antibody 100/3 to detect the γ subunit (lane *2*), and immunoblotting with the antibody 100/1 to detect the β1 (*β′*) subunit (lane *3*). (*B*) AP2 immunoprecipitated with antibody AP.6 and analyzed on an 8% acrylamide–SDS gel, containing 6 M urea, followed by autoradiography (lane *1*), immunoblotting with antibody AC1-M11 to detect the α subunit (lane *2*), and immunoblotting with the antibody 100/1 to detect the β2 (*β*) subunit (lane *3*). (*C*) AP1 immunoprecipitated with antibody 100/3 and analyzed on a 10% acrylamide–SDS gel, followed by autoradiography (lane *1*) and immunoblotting with antibody RY/1 against μ1 (lane *2*). (*D*) AP2 immunoprecipitated using antibody AP.6 and analyzed on a 10% acrylamide–SDS gel, followed by autoradiography (lane *1*) and immunoblotting with antibody AP50-2 against μ2 (lane *2*). (*E*) Clathrin immunoprecipitated using the antibody X22 and analyzed on an 8% acrylamide–SDS gel, followed by autoradiography (lane *1*) and by immunoblotting with the clathrin heavy chain–specific antibody TD.1 (lane *2*). (*F*) Clathrin immunoprecipitated with the antibody X22 and analyzed on a 12% acrylamide–SDS gel, followed by autoradiography (lane *1*), immunoblotting with the antibody LCB.1 that recognizes LCb (lane *2*), and immunoblotting with the antibody X16 that recognizes LCa (lane *3*).

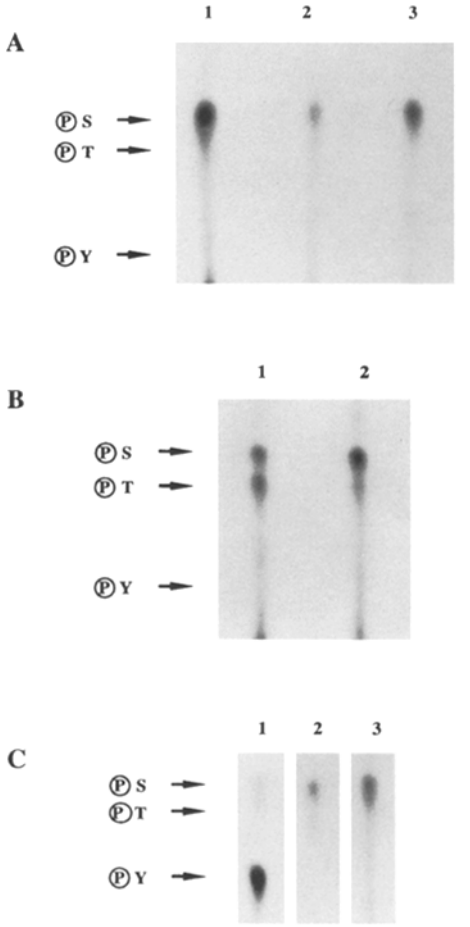

Figure 3. Phosphoamino acid analysis of the phosphoproteins of the CCV coat. Protein complexes of the CCV coat were immunoprecipitated from MDBK cells labeled in vivo with [^{32}P]orthophosphate and separated by SDS-PAGE, followed by blotting onto PVDF membranes. Phosphorylated proteins were visualized by autoradiography, the corresponding region of the PVDF membrane was excised and hydrolyzed in 6 N HCl, and phosphoamino acids were analyzed by thin-layer electrophoresis. Migration of phosphorylated amino acids was detected by autoradiography and compared with the migration of standard, nonradioactive phosphoamino acids, phospho-serine (P-S), phospho-threonine (P-T), and phospho-tyrosine (P-Y). (*A*) Analysis of the 100-kD subunits of AP1 and AP2: (lane *1*) α subunit of AP2; (lane *2*) β2 subunit of AP2; (lane *3*) β1 subunit of AP1. (*B*) μ1 of AP1 (lane *1*) and μ2 of AP2 (lane *2*). (*C*) Clathrin heavy chain (lane *1*), LCa (lane *2*), and LCb (lane *3*).

tion produced a labeled peptide of higher molecular weight corresponding to a fragment containing the head and hinge domains. These results indicate that the hinge region of the β1 subunit becomes phosphorylated in AP1, as each of the labeled fragments produced only contains the hinge domain in common, which was confirmed by immunoblotting with a hinge-specific antibody (data not shown). Labeled AP2 yielded similar results upon proteolysis, demonstrating that the phosphorylated regions of the α and β2 subunits segregated with the ear or head domains, depending on whether trypsin or elastase was used, and thereby mapping phosphorylation of AP2 to the hinge region of both subunits. Immunoblotting of the digested

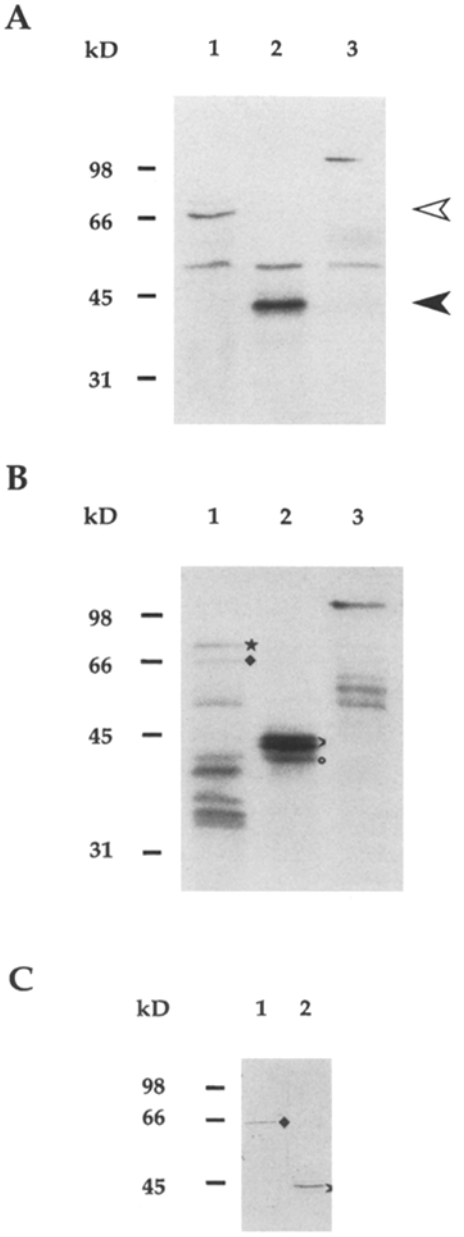

Figure 4. Phosphorylated domains within the 100-kD subunits of AP1 and AP2. AP1 and AP2 were immunoprecipitated from [^{32}P]orthophosphate-labeled MDBK cells and digested with trypsin or elastase. The digested products were separated by SDS-PAGE and transferred to nitrocellulose. Labeled peptide fragments were visualized by autoradiography. (*A*) ^{32}P-labeled AP1 digested with elastase (lane *1*), trypsin (lane *2*), and undigested (lane *3*). (*Open arrow*) Position of the head plus hinge fragment of β1 in lane *1*; (*closed arrow head*) position of the ear plus hinge fragment of β1 in lane *2*. (*B*) ^{32}P-labeled AP2 digested with elastase (lane *1*), trypsin (lane *2*), and undigested (lane *3*). Peptides corresponding to the different domains of each of the phosphorylated 100-kD adaptor subunits were identified by immunoblotting using domain-specific antibodies as indicated. The star and diamond indicate the positions of the α and β2 head plus hinge fragments, respectively, in lane *1*. The bracket and open circle indicate the position of the ear plus hinge fragments of β2 and α fragments, respectively, in lane *2*. (*C*) Immunoblot of the digest shown in *B* (lanes *1* and *2*), using the antibody GD/1 against the β-subunit hinge domains. The blot is aligned with the molecular weight markers of *B*, from which it was produced, and the fragments are designated by the same symbols as in *B*.

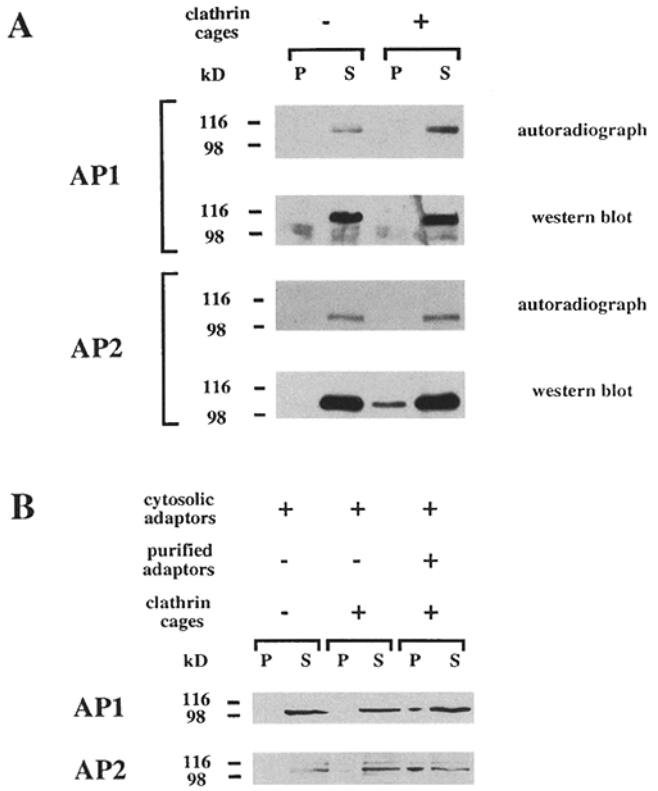

Figure 5. Adaptors phosphorylated in vivo are impaired in their ability to bind to clathrin cages. (*A*) Cytosol was prepared from [^{32}P]orthophosphate-labeled MDBK cells and incubated in the presence or absence of preformed clathrin cages produced by polymerization of purified bovine brain clathrin. Cages were harvested by centrifugation, and adaptors were first immunoprecipitated (using antibody 100/3 for AP1 and antibody AP.6 for AP2) from both the pellet (*P*) and the soluble fraction (*S*); then the immunoprecipitates were analyzed by SDS-PAGE, immunoblotting (*lower panel*), and autoradiography (*upper panel*). AP1 was detected using the antibody 100/3, and AP2 was detected using the antibody 100/2. (*B*) Cytosol was prepared from MDBK cells and incubated in the presence or absence of preformed clathrin cages with or without purified adaptors. The solution was centrifuged, and both the pelletable (*P*) and soluble (*S*) material was analyzed directly by SDS-PAGE and immunoblotting. AP1 was detected by immunoblotting with the 100/3 antibody. AP2 was detected by immunoblotting with the 100/2 antibody.

AP2 products with an antibody recognizing the hinge domain of the β2 subunits (Traub et al., 1995) showed the hinge region segregated along with the ^{32}P-labeled peptides into the low molecular weight trypsin fragment and the high molecular weight elastase fragment of the β2 subunit (Fig. 4 *C*). The same digestion patterns were seen for phosphorylated adaptors immunoprecipitated from labeled normal rat kidney cells, establishing that hinge phosphorylation was not cell specific (data not shown). Furthermore, in vivo–phosphorylated AP1 and AP2 from MDBK and normal rat kidney cells, fragmented using CNBr, produced phosphopeptide maps consistent with the phosphorylation site being in the hinge region (data not shown). The hinge regions of the α, β1, and β2 subunits of adaptors extend approximately between amino acid residues 600 and 750 in each of these 100-kD subunits (Kirchhausen et al., 1989). As serine residues within these domains do not lie within classical kinase consensus sequences, their phosphorylation must be mediated by a novel kinase or a known kinase using a nonclassical consensus site.

Phosphorylated Adaptors Have Impaired Binding to Clathrin Cages

Phosphorylation of the β1 and β2 subunits within their clathrin-binding hinge region suggested that one role of adaptor phosphorylation might be to regulate the interaction between adaptors and clathrin within cells. To investigate this possibility, an in vitro clathrin-binding assay was used. Purified bovine brain clathrin was polymerized into polyhedral cages, which were added to cytosol from ^{32}P-labeled MDBK cells. This cage-binding assay can detect adaptor–clathrin interactions by cosedimentation of adaptors with preformed clathrin cages (Ahle and Ungewickell, 1989). The presence of bound (pelletable) or unbound (nonpelletable) adaptors was assayed by immunoprecipitation followed by immunoblotting and autoradiography (Fig. 5 *A*). Phosphorylated adaptors, derived from MDBK cytosol, were only associated with the supernatant of these reactions and absent from pellets of clathrin cages. Blotting for adaptor subunits indicated that no adaptors from the cytosol bound to the preformed cages in this experiment, suggesting that cytosolic adaptors were impaired in their ability to associate with clathrin cages. This was confirmed using cytosol from unlabeled MDBK cells (Fig. 5 *B*). No adaptors of cytosolic origin bound to clathrin cages, although when purified adaptors were added to cytosol in the same reaction, adaptor binding to clathrin cages was detected. This demonstrated that cytosol does not contain inhibitory factors that prevent binding of adaptors to clathrin cages and further suggested that cytosolic, but not purified adaptors, are impaired for clathrin binding, possibly as a result of modification.

Purified and cytosolic adaptors would be expected to differ in their states of phosphorylation. Adaptors purified from bovine brain CCV have been previously shown to be dephosphorylated by a potent phosphatase activity that copurifies with adaptors and efficiently dephosphorylates adaptors within the time course of purification (Morris et al., 1990). Furthermore, the purified adaptors tested for clathrin binding in the above experiment were isolated in the absence of phosphatase inhibitors, while the adaptors present in the cytosol were clearly phosphorylated to some extent (Fig. 5 *A*). If phosphorylation prevents cytosolic adaptors from binding to clathrin, then dephosphorylation should induce clathrin binding. To test this theory, conditions for dephosphorylation of cytosolic adaptors were established, using CIAP. Dephosphorylation of cytosolic adaptors, detected by loss of ^{32}P-label without comparable loss of protein, was achieved after incubation with 5 U/ml for 30 min at 37°C (Fig. 6 *A*). Unlabeled cytosol was then prepared from MDBK cells, incubated with CIAP under the same conditions, and the cytosolic adaptors were tested for binding to clathrin cages (Fig. 6 *B*). The CIAP-treated cytosolic adaptors were rendered competent to bind cages, while mock-treated cytosolic adaptors did not. These experiments indicate that phosphorylated adaptors are impaired in binding to clathrin and suggest that phos-

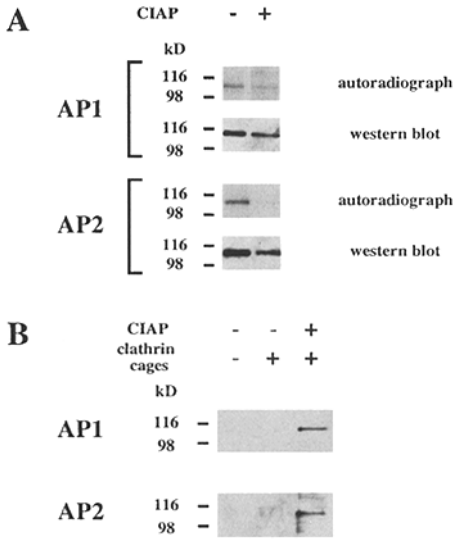

Figure 6. CIAP will dephosphorylate cytosolic adaptor complexes, allowing them to bind to clathrin cages. (*A*) Cytosol prepared from [^{32}P]orthophosphate-labeled MDBK cells was treated with CIAP to establish whether the CIAP was effective at dephosphorylation. AP1 or AP2 was first immunoprecipitated, using the antibodies 100/3 and AP.6, respectively, from cytosol, and then analyzed by SDS-PAGE, immunoblotting, and autoradiography. AP1 was detected using the antibody 100/3, and AP2 was detected using the antibody 100/2. (*B*) Cytosol was prepared from MDBK cells, treated or mock treated with calf intestinal phosphatase, and then incubated in the presence or absence of preformed clathrin cages. Material was pelleted by centrifugation and analyzed directly by SDS-PAGE and immunoblotting for AP1 using the antibody 100/3 or for AP2 using the antibody 100/2.

phorylation of adaptors could influence the assembly state of CCV coat proteins within the cell.

Cellular Localization of Phosphorylated Adaptors

Further evidence for a cellular role of adaptor phosphorylation in regulating adaptor–clathrin interactions was sought in vivo. First, the intracellular distribution of phosphorylated adaptors was investigated to determine whether phosphorylated adaptors predominated in the cytosol. MDBK cells were labeled with [^{32}P]orthophosphate and, after freeze-thaw lysis, separated by differential centrifugation into membrane and cytosol fractions. AP1 or AP2 adaptors were immunoprecipitated from each fraction and analyzed by SDS-PAGE and autoradiography (Fig. 7). In addition, the degree of phosphorylation of each of the adaptor subunits in each fraction was measured using a Molecular Dynamics phosphorimager. This value was then normalized for protein concentration as determined by estimating the relative amount of protein in each fraction by quantitative immunoblotting. Phosphorylated adaptor subunits of both AP1 (β1) and AP2 (α and β2) predominated in the cytosol fraction relative to their presence in the membrane fraction, in ratios of >3:1. This distribution correlated with a tendency of dephosphorylated adaptors to be coassembled with clathrin on cellular membranes.

The predominance of phosphorylated β1 and β2 adaptor subunits in the cytosol (Fig. 7), in conjunction with the

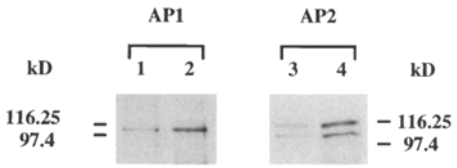

Figure 7. Cellular localization of phosphorylated adaptors. MDBK cells were labeled with [^{32}P]orthophosphate, and then membrane and cytosol fractions were isolated. AP1 and AP2 were immunoprecipitated from the fractions using the antibodies 100/3 and AP.6, respectively, and analyzed by SDS acrylamide gel containing 6 M urea, followed by Western blotting onto nitrocellulose and autoradiography. (Lanes *1* and *3*) Membrane fractions. (Lanes *2* and *4*) Cytosolic fractions.

impaired ability of cytosolic adaptors to bind clathrin (Fig. 5), suggested that most cytosolic adaptors may be phosphorylated in the β1 or β2 hinge region that influences clathrin binding. Therefore, the degree of phosphorylation of the β2 subunit of AP2 was investigated in membrane and cytosolic fractions of MDBK cells, using two-dimensional electrophoresis (Fig. 8). Isoelectric focusing, followed by SDS-PAGE and autoradiography, revealed that the β2 subunit of AP2, immunoprecipitated from ^{32}P-labeled MDBK cytosol, was present in two phosphorylated forms and

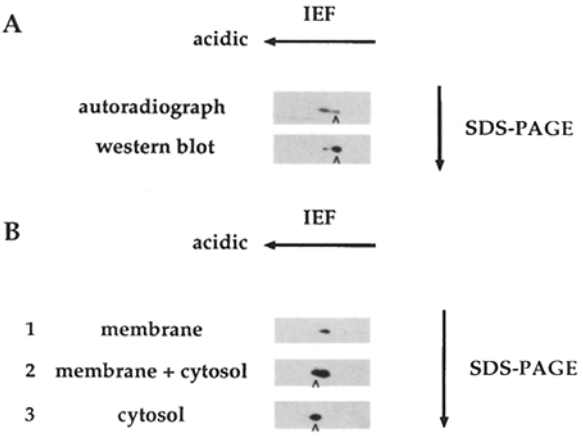

Figure 8. Two-dimensional gel electrophoresis of the β2 subunit of AP2 from membrane and cytosolic fractions. ^{32}P-labeled (*A*) or unlabeled (*B*) MDBK cells were lysed by freeze thawing, and membrane and cytosol fractions were prepared. In each of the fractions, AP2 was analyzed by isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. (*A*) AP2 was immunoprecipitated from the cytosolic fraction of [^{32}P]orthophosphate-labeled MDBK cells with the antibody AP.6 and analyzed by two-dimensional gel electrophoresis, followed by autoradiography and immunoblotting with the 100/1 antibody to detect the β2 subunit of AP2. (*B*) AP2 was immunoprecipitated with AP.6, and the β2 subunit was detected by immunoblotting with the antibody 100/1. (Lane *1*) β2 subunit immunoprecipitated from MDBK membranes. (Lane *2*) β2 subunit immunoprecipitated separately from membrane and cytosolic fractions of MDBK cells. These immunoprecipitates were then mixed and analyzed on the same two-dimensional gel. (Lane *3*) β2 subunit immunoprecipitated from MDBK cytosol. (*Arrows*) Equivalent spots in the two experiments; i.e., the major form of β2 in the cytosol, which is phosphorylated, but to a lesser extent than the minor form visible in *A*, which is more acidic and not detected by blotting in *B*.

therefore must have at least two phosphorylation sites (Fig. 8 *A*). Immunoblotting indicated that no dephosphorylated β2 subunit was present in the cytosol, and that the only dectectable β2 subunit protein in the cytosol corresponded to the two phosphorylated forms detected by autoradiography. Blotting also indicated that the hyperphosphorylated form of β2 subunit constituted a minor subpopulation. In contrast, when the β2 subunit of AP2 isolated from the membrane fraction was analyzed, it migrated to a less acidic position than either phosphorylated form. When the two samples were mixed (AP2 from membrane and AP2 from cytosol) and analyzed together on the same gel, it was confirmed that a distinctly charged form of β2 subunit predominated in each of the cellular subfractions. The more acidic form was exclusive to the cytosol, comigrating with the major phosphorylated form (not the hyperphosphorylated form). A less acidic form, which is consistent with migration of dephosphorylated β2, was exclusive to membrane-associated AP2 (Fig. 8 *B*). These results indicate that all the β2 subunits in the cytosol are phosphorylated, and the membrane-associated β2 is dephosphorylated.

Location of the Adaptor Kinase

Previous studies have shown that many different kinase activities are associated with the CCV coat (Bar-Zvi and Branton, 1986; Campbell et al., 1984; Merrese et al., 1990; Pauloin et al., 1982). Coat proteins were extracted from purified CCV, and their kinase activity was stimulated to determine whether associated kinase activities could phosphorylate the extracted adaptors and affect their ability to bind clathrin. Kinase activity was low unless polylysine was added to the reaction, an observation consistent with previous studies (Morris et al., 1990). Addition of polylysine resulted in phosphorylation of 100-kD adaptor subunits present in the extracted coat proteins (data not shown). Adaptors phosphorylated in this manner did not bind to preformed clathrin cages, in contrast with adaptors that were extracted from CCV without stimulation of kinase activity (Fig. 9). This indicates that, within the clathrin coat, there is a kinase capable of modifying adaptors in a manner analogous to that achieved by in vivo phosphorylation.

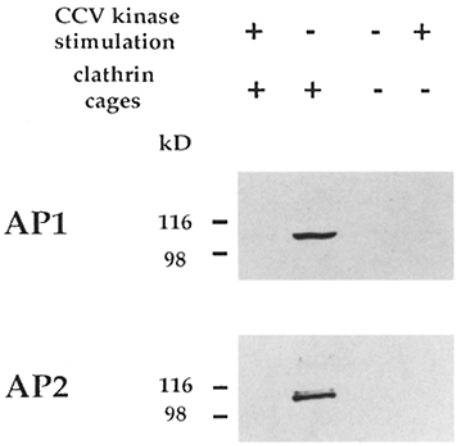

Figure 9. In vitro, a CCV-associated kinase phosphorylates adaptors and impairs their binding to preformed clathrin cages. Coat proteins were extracted from CCV with 0.5 M Tris, pH 7.0, and diluted 10-fold. CCV-associated kinase activity was then stimulated (+) or not (−), by the addition of poly-L-lysine and ATP. Phosphorylated (+) or unphosphorylated (−) CCV coat proteins were incubated in the presence or absence of preformed clathrin cages. The clathrin cages were harvested by centrifugation and analyzed directly by SDS-PAGE and immunoblotting to detect the presence of bound adaptors with the anti-AP1 antibody 100/3 or the anti-AP2 antibody 100/2.

Discussion

The results reported in this study identify the individual CCV coat proteins that become phosphorylated in vivo (Table II). Phosphorylation of adaptors prevents their binding to clathrin. Thus, one physiological function of adaptor subunit phosphorylation is apparently to regulate the assembly state of CCV coat proteins. The α, β1, β2, μ1, and μ2 subunits of the adaptors were all identified as phosphoproteins. However, the most likely targets for the phosphorylation that regulates adaptor–clathrin binding in vivo are the 100-kD adaptor subunits. All three, α, β1, and β2 subunits, have been implicated in clathrin binding (Goodman and Keen, 1995; Shih et al., 1995; Traub et al., 1995), and all three are phosphorylated in their hinge regions (Fig. 4). The hinge region of the β1 and β2 subunits have been previously identified as clathrin-binding domains (Shih et al., 1995), and they are more homologous to each other than to α, whose counterpart γ subunit in AP1 is not phosphorylated. Therefore, we suggest that the site at which phosphorylation regulates interaction between the adaptors and clathrin is most likely the hinge region of both the β1 and β2 subunits.

The hinge regions of the β1 and β2 subunits (approximately residues 600–750) are rich in proline and glycine. Hinge phosphorylation might influence the orientation of the adaptor head and ear domains, which are separated by the hinge region and thereby conceal the clathrin-binding site. The differential phosphorylation of the α and γ subunits could have a role in one of several distinct cellular functions attributed to the α and γ subunits. The α subunit, but not γ, has been shown to interact with dynamin (Wang et al., 1995), as well as clathrin. Differential phosphorylation of α and γ is also consistent with the known differential regulation of membrane interactions of AP1 and AP2 adaptors (Robinson and Kreis, 1992; Wong and Brodsky, 1992).

Kinase activity that can phosphorylate adaptors and

Table II. CCV Phosphoprotein Properties

Coat protein	Phosphoamino acid	Domain
α subunit, AP2	S	hinge
β1 subunit, AP1	S	hinge
β2 subunit, AP2	S	hinge
μl of AP1	S T	nd
μ2 of AP2	S (T)	nd
LCa	S	nd
LCb	S	nd
Clathrin heavy chain	Y S	nd

S, serine; *T*, threonine; *Y*, tyrosine; *nd*, not done.
Parentheses indicate a low level of phosphorylation.

prevent their binding to clathrin was found to be associated with the CCV coat (Fig. 9). Taking this observation into account, two possible models can be proposed for how adaptor phosphorylation could regulate the assembly state of the CCV coat in vivo (Fig. 10). CCV uncoating is believed to be mediated by the cytoplasmic heat shock family protein, hsc 70 (Schlossman et al., 1984). This protein catalyzes ATP-dependent depolymerization of the clathrin lattice, leaving adaptors behind on the membrane, which are then removed by an additional uncoating activity (Hinshaw, J.E., and S.L. Schmid, unpublished observations). Thus, uncoating of the clathrin lattice by hsc 70 might activate the CCV-associated adaptor kinase or reveal the target site for the kinase (Model I). Alternatively, the kinase involved in adaptor phosphorylation could be activated before CCV uncoating (Model II) to contribute to the destabilization of clathrin–adaptor interactions and thereby initiate depolymerization of the clathrin lattice by hsc 70. Model II is consistent with the observation that stimulation of CCV-associated kinase activity destabilizes the CCV coat (Georgieva-Hanson et al., 1988). In both models, phosphorylation of adaptors on the uncoated CCV would prevent clathrin rebinding. Subsequent release of phosphorylated adaptors to the cytosol would further prevent their nonproductive interaction with cytosolic clathrin. These models are consistent with our finding a low level of adaptor phosphorylation associated with membranes (Fig. 7), and with the fact that all AP2 adaptors in the cytosol had phosphorylated β2 subunits (Fig. 8). In both pathways, adaptor recruitment to membranes during coated pit formation would have to be accompanied by dephosphorylation by a yet unidentified phosphatase. This suggests a further step at which clathrin polymerization can potentially be regulated.

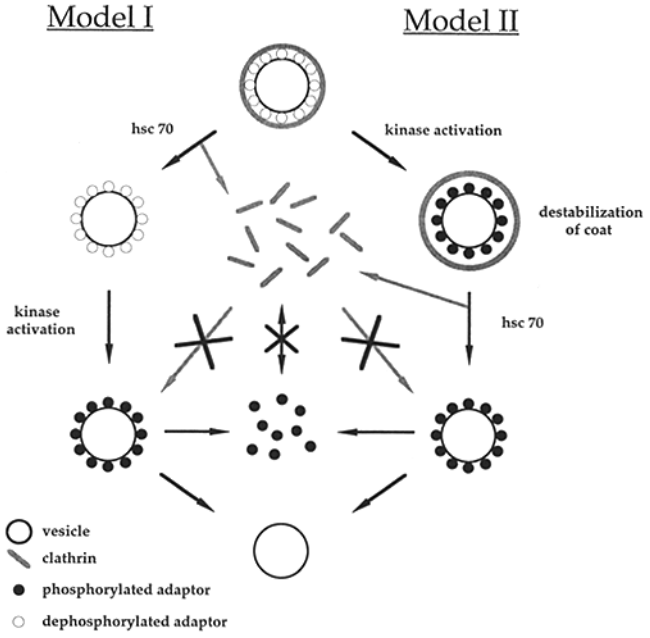

Figure 10. Models for how adaptor phosphorylation could regulate the assembly and disassembly of CCV coat proteins. The "X" indicates nonproductive adaptor–clathrin interactions that would be inhibited by adaptor phosphorylation. See text for further details.

In previous in vitro studies, each of the 100-kD adaptor subunits had been shown to be a potential substrate for CCV-associated kinase activities (Morris et al., 1990). In vivo, it had not been possible to identify the actual subunits of the CCV coat that were phosphorylated, as subunit-specific antibodies were not available (Bar-Zvi et al., 1988; Keen and Black, 1986). The two previous in vivo studies of CCV coat phosphorylation had some findings that appear to differ from those reported here. Bar-Zvi et al. (1988), studying reticulocytes, found that adaptors with phosphorylated 100-kD subunits predominated in fractions containing assembled clathrin. A second study of phosphoproteins in cultured neurons (Keen and Black, 1986) showed that in vivo–phosphorylated 100-kD proteins would coassemble with purified clathrin and adaptors in an in vitro assay. These discrepancies with our findings might be explained both on the basis of cell type and methodology. Neurons and reticulocytes have high endocytic rates. Consequently, different patterns of phosphorylation might be observed when compared with cell types with lower endocytic rates. In addition, reticulocytes develop into erythrocytes, which are endocytically dormant and contain empty clathrin cages (Bar-Zvi and Branton, 1987). Thus, regulation of CCV assembly in erythrocytes is likely to be highly specialized. In studying neuronal phosphoproteins, Keen and Black (1986) used a low pH buffer to drive clathrin cage formation in the presence of coat proteins. The low pH conditions favoring clathrin cage assembly may have overidden any regulatory effects that phosphorylation has on adaptor–clathrin interactions. Our cage-binding experiments, which demonstrate that phosphorylation has a role in regulating adaptor–clathrin interactions, were carried out at a higher pH, allowing us to identify this role for adaptor subunit phosphorylation.

The phosphorylation of other CCV coat proteins has yet to be correlated with a function for their modification, but demonstration of their in vivo phosphorylation suggests some interesting possibilities. For example, the medium subunits of both AP1 and AP2 have recently been implicated in receptor binding (Ohno et al., 1995), in which phosphorylation could have a regulatory role. This is the first report of in vivo phosphorylation of μ1, and it was found to be phosphorylated equally on serine and threonine residues. μ1 has previously been attributed with an in vitro casein kinase II activity that copurifies with AP1, influences AP1 recruitment to TGN membranes, and modifies TGN-associated mannose-6-phosphate receptors (Merrese et al., 1990). However, it is not yet established whether μ1 actually has endogenous kinase activity and whether the observed activity functions in vivo. Phosphorylation of μ2 in vivo has been observed previously in rat reticulocytes (Bar-Zvi et al., 1988) and found to occur predominantly on threonine residues (66%), but with some occurring on serine residues (33%). In MDBK cells, we also find that μ2 is phosphorylated, but primarily on serines, with a low level of threonine phosphorylation. The differences in the relative amounts of phosphorylation of the different residues of μ2 may again be a function of the specialized endocytic processes in reticulocytes and, consequently, which kinases are most active in those cells. Previous in vitro studies have demonstrated that the phosphorylation pattern of μ2 differs according to the assembly state

of AP2. When in the CCV, μ2 was observed to be phosphorylated on threonines, but in the disassembled coat, μ2 was phosphorylated predominantly on serines with only a minor amount occurring on threonine residues (Campbell et al., 1984). This suggests that different kinases may be able to act on μ2 depending on its cellular location.

It is also worth considering the role that in vivo phosphorylation might have in influencing clathrin function. A previous study reported that clathrin heavy chain was phosphorylated in Rous sarcoma virus–transformed cells but not in untransformed cells, suggesting it was a substrate for $pp60^{v\text{-}src}$ (Martin-Perez et al., 1989). In MDBK cells, we observed tyrosine phosphorylation of clathrin heavy chain, implicating cellular tyrosine kinases. Due to the low level of clathrin heavy chain phosphorylation, it was not possible to determine where the phosphorylated form predominated. However, as only a minor population of the clathrin heavy chain was phosphorylated, phosphorylation may affect only a specialized function of a subset of cellular clathrin. Several minor pools of clathrin have been described: one being found on endosomes (Stoorvogel et al., 1996), another found at sites of cell adhesion (Nicol and Nermut, 1987), and a third contributing to the formation of pentagons rather than hexagons in the polymerized clathrin lattice (Crowther et al., 1976; Heuser, 1989). Interestingly, $pp60^{c\text{-}src}$ and other *SRC* family protein tyrosine kinases are localized to endosomes and to adhesion plaques, as well as to the plasma membrane, where they would be available to modify such clathrin subpopulations. In addition, each of the clathrin light chains, LCa and LCb, are phosphorylated in vivo, on serine residues. LCb was more heavily phosphorylated than LCa, as observed in reticulocytes (Bar-Zvi et al., 1988), and consistent with the unique casein kinase II consensus site, in the NH_2 terminus of LCb (Hill et al., 1988). The level of clathrin light chain phosphorylation varied between experiments, so it was not possible to establish where phosphorylated forms were localized within the cell. The clathrin light chains are thought to modulate clathrin assembly, and their phosphorylation might regulate aspects of this modulating role or again influence the function of specialized subsets of clathrin.

The data presented here indicate that adaptor phosphorylation and dephosphorylation could influence the assembly/disassembly cycle of the CCV coat within the cell. The potential for phosphorylation to control additional steps in CCV formation is also demonstrated. Identification of the kinases and phosphatases involved in the cycling of CCV coat proteins on and off the membrane will provide further insight into how this process is regulated.

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