Transformation by Rous Sarcoma Virus Induces Clathrin Heavy Chain Phosphorylation

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Abstract. We have shown that the heavy chain of clathrin is phosphorylated in chicken embryo fibroblast cells transformed by Rous sarcoma virus, but not in normal cells. Approximately 1 mol of phosphate is bound for every 5 mol of heavy chain in the maximally phosphorylated transformed cells. Two-thirds of the phosphate is on serine and one-third on tyrosine residues. Clathrin heavy chain is a substrate for pp60^{v-src} in vitro. Cleveland analysis of the in vivo and in vitro clathrin heavy chain phosphopeptides, generated by protease V8 digestion, show labeled proteolytic fragments of similar molecular weight, suggesting that pp60^{v-src} could be directly responsible for the in vivo phosphorylation of clathrin. Phosphate is equally incorporated into clathrin in both the unassembled and the assembled clathrin pools, whereas [^{35}S]methionine is preferentially incorporated into the assembled pool. In normal cells, clathrin visualized by immunofluorescent staining appears in a punctate pattern along the membrane surface and concentrated around the nucleus; in transformed cells the perinuclear staining is completely absent. The phosphorylation of clathrin heavy chain in transformed cells may be linked to previously observed transformation-dependent alterations in receptor-mediated endocytosis of ligands such as EGF and thrombin.

T HE search for the underlying molecular mechanisms that explain transformation by Rous sarcoma virus (RSV)[1] has resulted in the identification of many substrates phosphorylated by the tyrosine kinase, pp60^{v-src}, as well as numerous substrates phosphorylated by other kinases whose activities are indirectly regulated by RSV (for reviews see Hunter and Cooper, 1985, 1986; Jove and Hanafusa, 1987). Morphological alterations of the cell surface and cytoskeleton are among the earliest manifestations of the src gene product (Ambros et al., 1975; Boschek et al., 1981). Among the cell surface changes, it has been observed that a number of receptors, including those for epidermal growth factor and thrombin, are cleared from the surface by endocytic events that may be altered by pp60^{v-src} (Decker, 1983; Cooper et al., 1983; Zetter et al., 1977).

Endocytosis often proceeds via coated pits and coated vesicles (for review see Pastan and Willingham, 1985), structures whose coats are known to include several phosphoproteins, among them the assembly factor polypeptides (Pauloin et al., 1982; Campbell et al., 1984; Keen et al., 1987) and the clathrin light chains (Usami et al., 1985; Schook and Puszkin, 1985; Bar-Zvi and Branton, 1986). Although previous studies have examined coated vesicle protein phos-

phorylation both in vivo and in vitro (Keen and Black, 1986; Bar-Zvi et al., 1988*b*), phosphorylation of clathrin heavy chain, the major component of coated vesicles, has not been observed in normal cells. Here we show that transformation of secondary chick embryo fibroblasts (CEF) by RSV induces the phosphorylation of clathrin heavy chains on tyrosine and serine residues. The effect is paralleled by a partial cellular redistribution of clathrin.

Materials and Methods

Cell Culture and Labeling

CEF were cultured in DME supplemented with 5% calf serum and antibiotics at 41.5°C. They were transformed with the RSV Schmidt-Rupin subgroup, strain A (SR-A) and were used for experiments several passages after infection.

Cultures in 100-mm dishes were labeled with 0.4 mCi/ml ^{32}P$_i$ (carrier free; ICN Biochemicals Inc., Irvine, CA) in 5 ml of phosphate-free medium or preincubated for 30 min in the same volume of methionine-free medium and then radiolabeled with 50 μCi/ml [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear, Boston, MA) for the time and at the temperature indicated in each experiment.

Antisera

The rabbit anti-bovine clathrin light chain (clathrin LC) IgG was raised against native clathrin triskelions and affinity-purified against a mixture of bovine brain alfa and beta light chains (Bar-Zvi et al., 1988*b*) and was used for immunoprecipitations and immunofluorescence. For this last technique we have also used another rabbit anti-bovine clathrin LC IgG obtained from Daniel Louvard (Pasteur Institute). The rabbit anti-bovine clathrin heavy

1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; RSV, Rous sarcoma virus.

chain (clathrin HC) IgG was raised against clathrin heavy chain excised from SDS gels of highly purified bovine brain-coated vesicles, and affinity-purified against bovine clathrin heavy chain (a gift from Dr. Stephen Mosley, Harvard University). This IgG specifically recognized clathrin HC on immunoblots, but failed to immunoprecipitate the protein from cell lysates.

Coated Vesicle Preparation

Coated vesicles and clathrin triskelions were prepared from chicken livers obtained from a local slaughterhouse and processed within 1 h of slaughter. All of the steps were carried out at 4°C according to procedures described previously (Bar-Zvi and Branton, 1986).

Subcellular Fractionation

The medium was aspirated and cells were scraped from the culture dish into 1 ml of ice-cold isolation buffer (10 mM MES-NaOH, 100 mM NaF, 1 mM Na_3VO_4, 2 mM EGTA, 2 mM EDTA, 0.002% NaN_3, and 50 mg/ml PMSF, pH 6.5, at room temperature) supplemented with 0.5% Triton X-100. Cells were homogenized on ice with 30 strokes of a Dounce homogenizer with a tight-fitting pestle. All subsequent steps were carried out at 4°C. The cell homogenate was centrifuged at 10,000 g for 10 min, and the resulting supernatant was centrifuged at 100,000 g for 30 min. The high speed supernatant was saved, while the high speed pellet was resuspended in 1 ml isolation buffer, homogenized as before and centrifuged at 10,000 g for 10 min to remove aggregated material. High speed supernatants and pellets provided source material for the immunoprecipitation of soluble and assembled clathrin, respectively.

Immunoprecipitation and Western Blotting Analysis

The high speed supernatant and pellet were mixed with RIPA buffer (final concentration 150 mM NaCl, 10 mM Tris/HCl, pH 7.2, 1% Na Deoxycholate, 1% Triton X-100, 0.1% SDS). In cases where cells were not fractionated, they were directly lysed in 1 ml ice-cold RIPA buffer containing 50 mM NaF and 100 μM Na_3VO_4. The lysate was clarified by centrifugation at 12,000 g for 10 min at 4°C. Proteins were immunoprecipitated from the supernatants by incubation with excess antibody for 1 h on ice, followed by incubation with fixed *Staphylococcus aureus* cells for 30 min on ice. The *Staphylococcus aureus* cells were washed several times by centrifugation at 10,000 g for 1 min, with resuspension of the pellet in RIPA buffer after each centrifugation, and one time with 1 M NaCl. Finally, pellets were transferred to fresh tubes and boiled in 2× Laemmli sample buffer (Laemmli, 1970), centrifuged as above, and supernatant proteins were separated by SDS-PAGE (Laemmli, 1970). Where samples were labeled with [^{35}S]methionine, the gels were washed in water, incubated in 1 M sodium salicylate, dried and exposed to Kodak X-Omat film at −70°C. Where samples were labeled with $^{32}P_i$, the gels were exposed to −70°C to the same film with the addition of a Cronex Lightning Plus (DuPont Co., Wilmington, DE) intensifying screen.

Western blotting was carried out as described by Olmsted (1981).

Determination of the stoichiometry of clathrin heavy chain phosphorylation: To estimate the stoichiometry of clathrin heavy chain phosphorylation in RSV-transformed CEF, cultures in DME containing 4% calf serum were labeled with 1.2 mCi/ml of $^{32}P_i$ to equilibrium as previously described (Sefton et al., 1981). Clathrin heavy chain was immunoprecipitated from cell lysates and analyzed by a 10% SDS polyacrylamide gel, as above. The amount of clathrin on the gel band was determined by scanning the Coomassie blue-stained gel with a Hoefer GS300 scanning densitometer using known amounts of BSA as a standard. The amount of radioactivity in clathrin was determined by excising the gel band and counting in a scintillation counter using Liquiscint (National Diagnostics Inc., Somerville, NJ) as scintillation fluid.

In vitro phosphorylation of clathrin heavy chain by $pp60^{v-src}$: Clathrin triskelions were prepared from coated vesicles purified from chicken liver (Bar-Zvi and Branton, 1986) and dialyzed against 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). Purified $pp60^{v-src}$ was kindly prepared by Sugimoto (Sugimoto et al., 1985). The reaction was carried out in a final volume of 30 μl of buffer A containing 10 mM $MgCl_2$, 20 μM gamma-^{32}P-ATP (1 μCi/μmol), 1.2 μg of triskelions and 0.1 μg of $pp60^{v-src}$. After 15 min at 30°C, the reaction was stopped by addition of 6 μl of 5× SDS sample buffer, the reaction tube was boiled for 2 min, and proteins were analyzed on a 7.5% SDS polyacrylamide gel (Laemmli, 1970). The gel was stained with Coomassie blue, dried, autoradiographed, and the clathrin heavy chain band was then excised and counted as described above.

Phosphopeptide Analysis

For one-dimensional peptide mapping of clathrin heavy chain, the band was excised from the wet unfixed gel and treated with 100 ng of *Staphylococcus aureus* V_8 protease (Miles Laboratories, Inc., Naperville, IL). The proteolytic fragments generated were analyzed in a 11-20% SDS polyacrylamide gradient gel as previously described (Cleveland et al., 1977). The peptides were then visualized by silver stain of the gel and the phosphopeptides detected by autoradiography.

Indirect Immunofluorescence

Normal and RSV-transformed CEFs were cultured on glass coverslips. Transformed cells were plated at a higher density to assure their survival. Cells were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature, washed three times for 5 min in PBS, then extracted for 5 min with 0.1% Triton X-100 in PBS. Clathrin was detected by incubation of the cells on the glass coverslips with an affinity-purified rabbit anti-bovine clathrin light chain IgG and then with a goat anti-rabbit IgG conjugated with fluoresceine (Vector Laboratories, Inc., Burlingame, CA) as previously described (Bar-Zvi et al., 1988a). Immunofluorescence was analyzed with both conventional and scanning confocal microscopes (White et al., 1987). For Golgi staining, the fixed and permeabilized cells on glass coverslips were incubated with rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, Inc.), and the fluorescence pattern was analyzed by conventional microscopy.

Results

An anti–clathrin light chain antibody was used to immunoprecipitate clathrin light and heavy chains from chicken fibroblast cell homogenates. The antibody immunoprecipitated at 180-kD protein that commigrate in SDS polyacrylamide gels with purified chicken clathrin heavy chain. The intensity of the Coomassie blue stain of the 180-kD immunoprecipitated protein was similar in both normal and RSV transformed cells (Fig. 1 *A*). When cultures of normal or transformed cells were labeled with $^{32}P_i$ for 3 h at 41.5°C, autoradiographs of the immunoprecipitates revealed that the 180-kD protein was phosphorylated only in transformed cells (Fig. 1 *B*). An antibody to the clathrin heavy chain stained the 180-kD immunoprecipitated protein on immunoblots (Fig. 2), suggesting that the immunoprecipitated 180-kD polypeptide was the clathrin heavy chain. This identity was confirmed by partial V8 digests of the immunoprecipitated 180-kD phosphoprotein. The immunoprecipitated phosphoprotein-generated proteolytic fragments were identical to authentic clathrin heavy chain from chicken liver, as detected by silver stain (Fig. 3, lanes *1* and *2*). In addition, many of the phosphoprotein fragments detected on the corresponding autoradiograph commigrate with the major fragments of the 180-kD phosphoprotein stained by silver (Fig. 3, lanes *1* and *3*). That the clathrin heavy chain was immunoprecipitated by an antibody to clathrin light chain was expected, given the strong association of the two polypeptides in native triskelions (Ungewickell, 1983; Winkler and Stanley, 1983). Nevertheless, we determined the portion of clathrin heavy chain immunoprecipitated by the anti–clathrin light chain IgG from homogenates of both normal and RSV-transformed cells. Cells were lysed in RIPA buffer (see Materials and Methods) and the lysates were divided in two equal alliquots. From one half, to determine the total amount of cellular clathrin heavy chain, proteins were precipitated with acetone. With the other half, we performed quantitative immunoprecipitations with the anti–clathrin light chain to estimate the portion of the cellular clathrin heavy chain pulled down by the antibody (see Materials and Methods). The
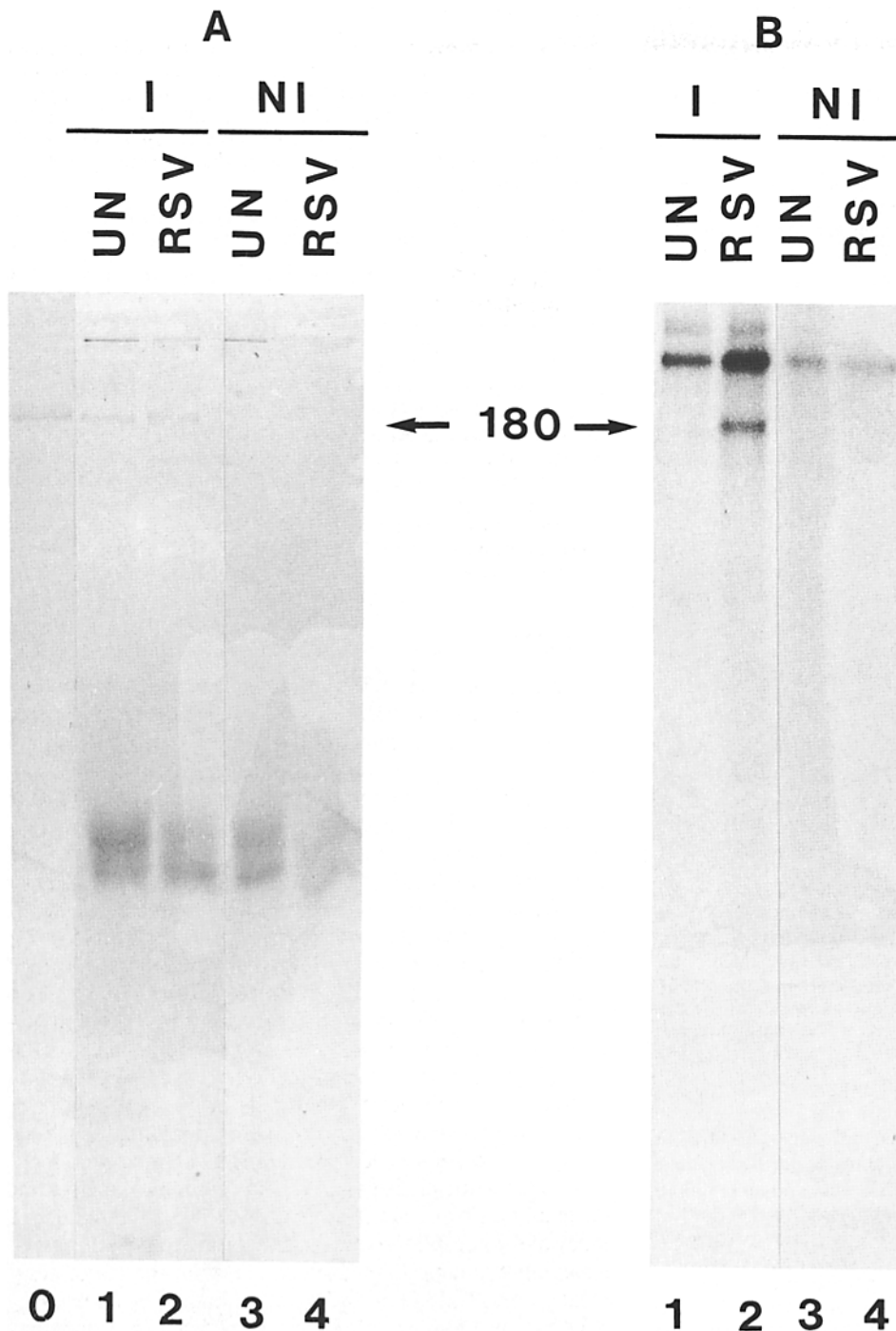
Figure 1. Immunoprecipitation of clathrin heavy chain from uninfected and RSV-transformed cells. Uninfected (*UN*, lanes *1* and *3*) and RSV-transformed cells (*RSV*, lanes *2* and *4*) were labeled with $^{32}P_i$ and the lysates were immunoprecipitated with an anti-clathrin light chain IgG (*I*, lanes *1* and *2*) or nonimmune IgG (*NI*, lanes *3* and *4*) as described in Materials and Methods. The immunoprecipitates were analyzed by electrophoresis in a 10% SDS polyacrylamide gel. (*A*) Coomassie blue pattern containing authentic clathrin from chick triskelions (lane *0*); (*B*) corresponding autoradiogram. The position of clathrin heavy chain (180 kD) is indicated.

amounts of clathrin heavy chain contained in each of these fractions were analyzed by a 10% SDS-PAGE, the gel was subsequently blotted and immunodecorated with the anti-clathrin heavy chain antibody. The immunoprecipitates of both normal and RSV-transformed cells contained 25% of the amount of clathrin heavy chain found in acetone precipitates of cell homogenates (data not shown).

The stoichiometry of phosphorylation of clathrin heavy chain was determined by immunoprecipitation of this protein from RSV-transformed cells labeled to equilibrium with ^{32}Pi (see Materials and Methods; Sefton et al., 1981). Approximately 1 mol of phosphate was bound for every 5 mol of immunoprecipitated clathrin heavy chain. About one-third of the phosphate was bound on tyrosine residues, the remainder on serine residues, with minor traces on threonine residues (Fig. 4). We therefore inferred that pp60^{v-src}, in addition to directly phosphorylating clathrin heavy chain, may stimulate a separate serine kinase or inhibit a serine phosphatase. We examined the capacity of pp60^{v-src} and a variety of serine protein kinases (including cAMP-dependent protein kinase catalytic subunit, S6 kinase from *Xenopus laevis* oocytes, casein kinase type II, and protein kinase C) to phosphorylate clathrin heavy chain in purified chicken liver triskelions. Protein kinase C and pp60^{v-src} were the only ki-
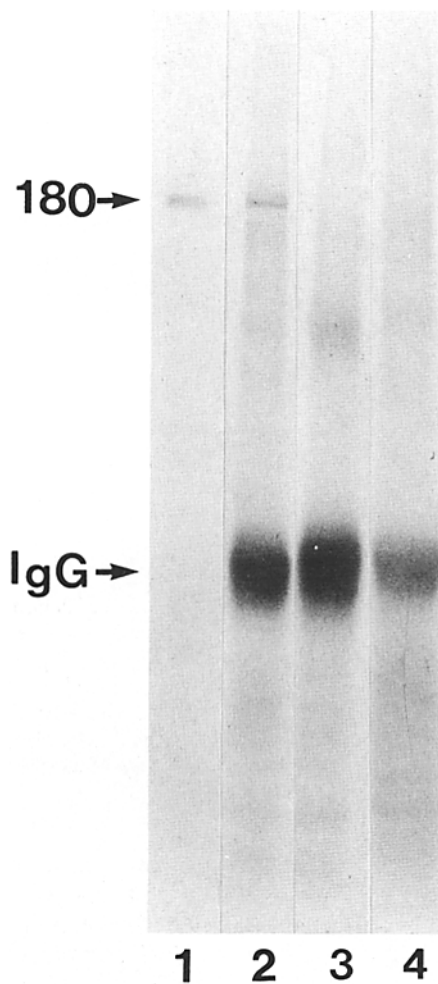
Figure 2. Immunoblot identification of clathrin heavy chain in the transformed cell immunoprecipitate. Proteins were immunoprecipitated from the total cell lysate of an RSV-transformed culture with an anti–clathrin light chain IgG (lanes 2 and 4) or nonimmune IgG (lane 3) as described in Materials and Methods. The immunoprecipitates were resolved on a 10% SDS polyacrylamide gel and proteins were transferred to nitrocellulose and probed with an affinity-purified anti–clathrin heavy chain IgG (lanes 1 and 2) or nonimmune IgG (lanes 3 and 4). Lane 1 contains purified clathrin from chicken liver. The position of clathrin heavy chain (180 kD) is indicated.

early as 15 min after the introduction of $^{32}P_i$ into the culture medium, and increased progressively over a 6-h period (Fig. 6). When the radioactive phosphate was chased with non-radioactive DME >50% of the initial phosphate was turned over in the first 90 min of the chase (Fig. 6). In contrast, the quantity of [^{35}S]methionine labeled clathrin unchanged after a 2-h chase (data not shown).

Clathrin heavy chain is found in both membrane-bound (assembled) and soluble (unassembled) forms. To determine the distribution of newly synthesized and phosphorylated clathrin heavy chain in membrane-bound and soluble forms, homogenates of [^{35}S]methionine or $^{32}P_i$-labeled RSV-transformed cells were fractionated, and clathrin was immunoprecipitated from both fractions with an anti–clathrin light chain as described (see Materials and Methods). The total amount of clathrin heavy chain in both assembled and unassembled fractions was the same, as determined by Coomassie blue staining of the gels after immunoprecipitation. However, a greater quantity of newly synthesized protein was found in the membrane fraction, as determined by fluorogra-
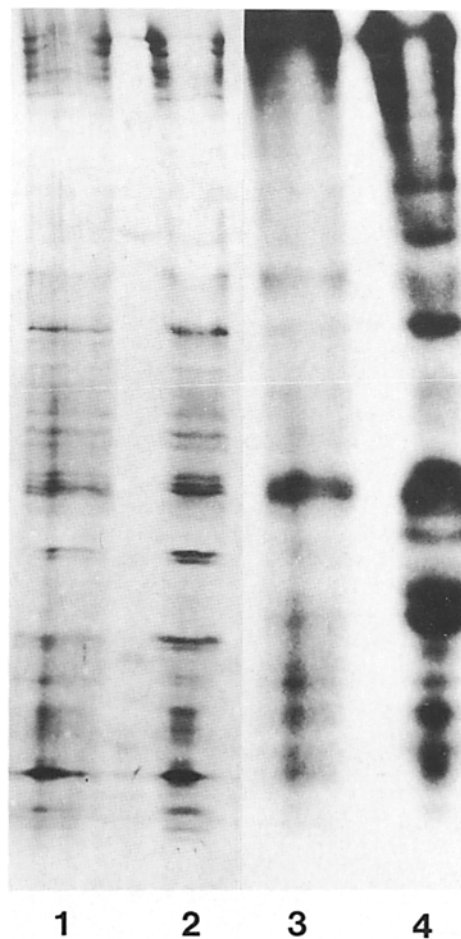
nases able to phosphorylate the clathrin heavy chain. In vitro phosphorylation by protein kinase C was dependent on the addition of phospholipids; however, it was not stimulated by addition of phorbol esters (PMA) to CEF cultures (data not shown). The stoichiometry of clathrin heavy chain phosphorylation by purified pp60^{v-src}, under the experimental conditions used (see Materials and Methods), was 0.1 mol of phosphate bound per every 100 mol of clathrin (Fig. 5). Cleveland analysis of the in vivo immunoprecipitated clathrin heavy chain, digested by V$_8$, showed that the major phosphopeptide and few minors, of low molecular weight, commigrate with those obtained from the in vitro phosphorylated clathrin heavy chain with pp60^{v-src} (Fig. 3, lanes 3 and 4).

Phosphorylation of clathrin heavy chain was detected as

Figure 3. One-dimension V8 proteolytic maps of clathrin heavy chain. Immunoprecipitated clathrin heavy chain from $^{32}P_i$-labeled RSV-transformed cells (lanes 1 and 3) or clathrin heavy chain in vitro phosphorylated with pp60^{v-src} (lanes 2 and 4) were isolated by SDS-PAGE. The gel slices corresponding to clathrin heavy chain were reelectrophoresed in the presence of 100 ng of S. aureus V8 protease (see Materials and Methods). The gel was then silver stained (lanes 1 and 2) and autoradiographed (lanes 3 and 4).
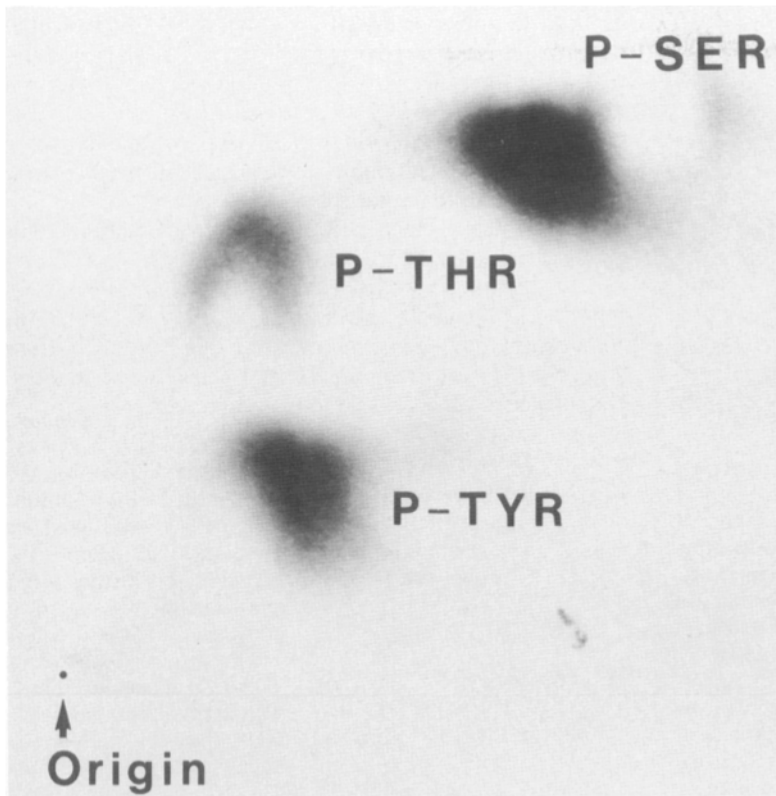
Figure 4. Phosphoamino acid analysis of clathrin heavy chain. Clathrin was immunoprecipitated from 18-h $^{32}P_i$-labeled RSV-transformed cells as in Fig. 1. The clathrin band was excised from the gel, and it was eluted and hydrolyzed with 6 N HCl for 2 h at 110°C. The phosphoamino acids analysis by thin layer electrophoresis was performed as previously described (Hunter and Sefton, 1980).

phy of the gel (Fig. 7). The distribution of phosphorylated clathrin heavy chain in the assembled and unassembled fractions was determined by excising and counting the immunoprecipitated clathrin bands and subtracting the background radioactivity in the neighboring gel regions lacking specific bands. This method of $^{32}P_i$ qualification was necessary because immunoprecipitates from membrane fraction had consistently higher nonspecific background radioactivity, in both immune and nonimmune lanes, than the immunoprecipitates from soluble fractions. This condition made photographic reproduction of side by side immunoprecipitates from both fractions difficult. Using these means of protein and $^{32}P_i$ measurement, we did not find significant differences in the extent of phosphorylation of clathrin heavy chain between these two fractions (data not shown). It should be noted that initially, these experiments were conducted in the absence of the tyrosine phosphatase inhibitor sodium vanadate in the isolation buffer. While the [^{35}S]methionine-labeled clathrin heavy chain distribution had not been modified, in the absence of the tyrosine phosphatase inhibitor, we found more $^{32}P_i$-labeled clathrin heavy chain associated with the soluble fraction than with the membrane-bound fraction (data not shown). These results indicate the presence of clathrin heavy chain phosphatase activity in the membrane-bound fraction.

Transformation by RSV is known to cause phenotypic changes, in part through alterations in cytoskeletal organization (Ambros et al., 1975). We compared the distribution of clathrin in normal and transformed cells by immunofluorescent staining using either of two anti-clathrin light chain antibodies. While normal cells yielded a classical punctate distribution of clathrin, with a higher concentration of the

antigen in a polar perinuclear region (Fig. 8 *A*) thought to be the Golgi apparatus (Anderson et al., 1978; Lisanti et al., 1982; Robinson and Pearse, 1986), in RSV-transformed cells, clathrin appeared in large dots, primarily at peripheral regions of the cell, just inside of the plasma membrane and was practically undetected in the perinuclear region (Fig. 8 *B*). Detection of the Golgi apparatus with rhodamine-conjugated wheat germ agglutinin did not show any alteration after RSV transformation of chicken cells (Fig. 8, *C* and *D*). This redistribution of clathrin may be related to alterations of the receptor-mediated endocytosis mechanisms previously observed in RSV-transformed cells (Decker, 1983; Cooper et al., 1983; Zetter et al., 1977).

Discussion

Using anti-clathrin light chain IgG to immunoprecipitate cell homogenates from normal and RSV-transformed CEF, we have identified a new protein substrate for phosphorylation in RSV-transformed cells: the heavy chain of clathrin. Five lines of evidence support this identification: (*a*) the molecular mass of the immunoprecipitated and phosphorylated protein was 180 kD; (*b*) the protein was tightly bound to the light chains of clathrin, since it was immunoprecipitated under high salt conditions (see Materials and Methods) by anti-clathrin light chain antibodies; (*c*) the immunoprecipitated protein was labeled by affinity-purified anti-clathrin heavy chain antibodies on immunoblots; (*d*) the partial V_8 digest of the phosphoprotein results in a silver stain pattern identical to that of authentic chicken clathrin heavy chain; and (*e*) the protein was not resolved on electrofocusing gels, but appeared as a broad smear (data not shown). This broad
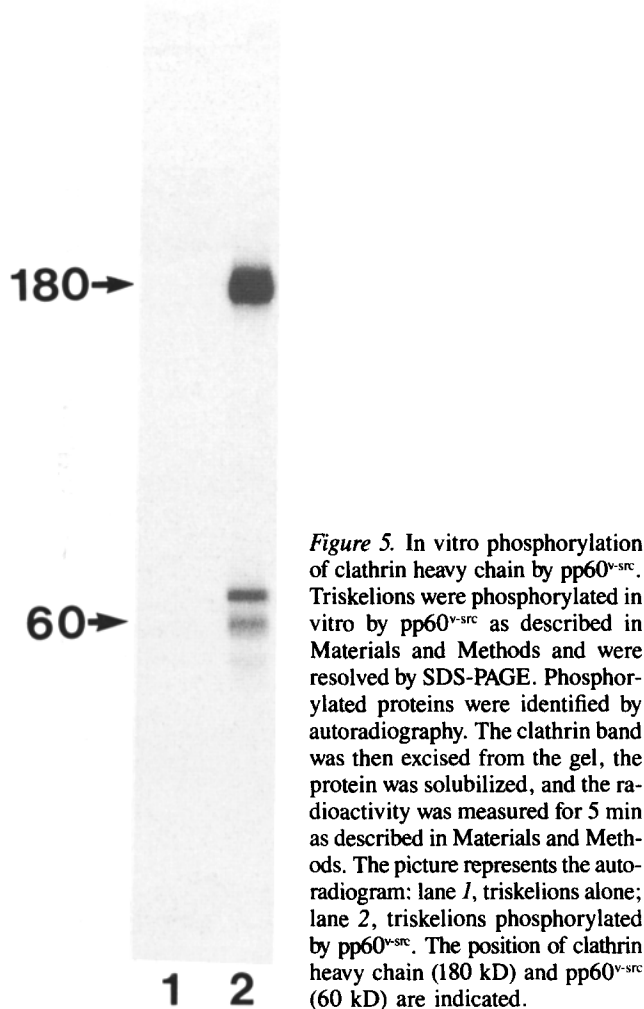
Figure 5. In vitro phosphorylation of clathrin heavy chain by pp60[v-src]. Triskelions were phosphorylated in vitro by pp60[v-src] as described in Materials and Methods and were resolved by SDS-PAGE. Phosphorylated proteins were identified by autoradiography. The clathrin band was then excised from the gel, the protein was solubilized, and the radioactivity was measured for 5 min as described in Materials and Methods. The picture represents the autoradiogram: lane *1*, triskelions alone; lane *2*, triskelions phosphorylated by pp60[v-src]. The position of clathrin heavy chain (180 kD) and pp60[v-src] (60 kD) are indicated.

Figure 7. Immunoprecipitation of assembled and soluble clathrin. RSV-transformed cells were labeled with [35S]methionine. Cells were lysed and fractionated as described in Materials and Methods. Proteins from the high speed supernatants (*S*, lanes *1* and *3*) and the pellets (*P*, lanes *2* and *4*) were immunoprecipitated with anti–clathrin light chain IgG (*I*, lanes *1* and *2*) or non-immune IgG (*NI*, lanes *3* and *4*). The immunoprecipitates were resolved by PAGE on a 10% SDS gel that was subsequently fluorographed. The position of clathrin heavy chain (180 kD) is indicated.

smear is typical of purified clathrin and distinctly different from the pattern for the high molecular mass assembly protein recently described (Ahle and Ungewickell, 1986).
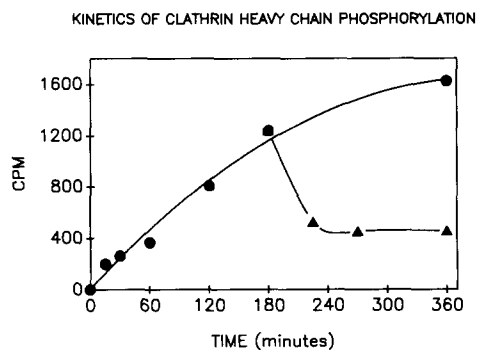
The absence of phosphorylation of the clathrin heavy

Figure 6. Kinetics of clathrin heavy chain phosphorylation in RSV-transformed cells. Cells cultures were labeled with $^{32}P_i$ (●) for the indicated length of time. In addition, in some of them, the $^{32}P_i$ was chased with regular DME after the first 3 h of labeling (▲). Clathrin heavy chain was then immunoprecipitated with the affinity-purified anti–clathrin light chain IgG. The immunoprecipitates were resolved on a 10% SDS polyacrylamide gel and autoradiographed. The clathrin heavy chain bands were excised from the gel, the protein was solubilized in Liquiscint (National Diagnostics Inc.) and radioactivity was measured for 5 min (vertical axis).

chain in normal cells is in agreement with previous in vivo studies of coated vesicle phosphorylation in rat neurons and rat reticulocytes (Keen and Black, 1986; Bar-Zvi et al., 1988b).

The stoichiometry of clathrin heavy chain phosphorylation in the immunoprecipitate was found to be 1 mol of phosphate per every 5 mol of protein. Only 25% of the cellular clathrin heavy chain from both normal and RSV-transformed cells was immunoprecipitated by the anti–clathrin light chain antibody. This could be due to the existence of free cellular pools of clathrin heavy chain, as it has been previously observed for clathrin light chain (Brodsky, 1985). Therefore, we cannot assess the phosphorylation state of the total clathrin heavy chain by this indirect method of immunoprecipitation. Unfortunately, the anti–heavy chain antibodies from bovine, rat, and chicken available to us, were ineffective for immunoprecipitation. The extent of phosphorylation of individual heavy chains within the triskelion population also remains to be determined.

About two-thirds of the label was found in serine, while one third was in tyrosine residues. When RSV-transformed cells were $^{32}P_i$-labeled for a period of 3 h instead of 18 h, we obtained the same phosphoamino acid composition of the immunoprecipitated clathrin heavy chain (data not shown). As is the case for ribosomal protein S6 (Decker, 1981; Blenis and Erikson, 1985; Ballou et al., 1988), phosphorylation of clathrin in serine residues may result from a cascade initiated
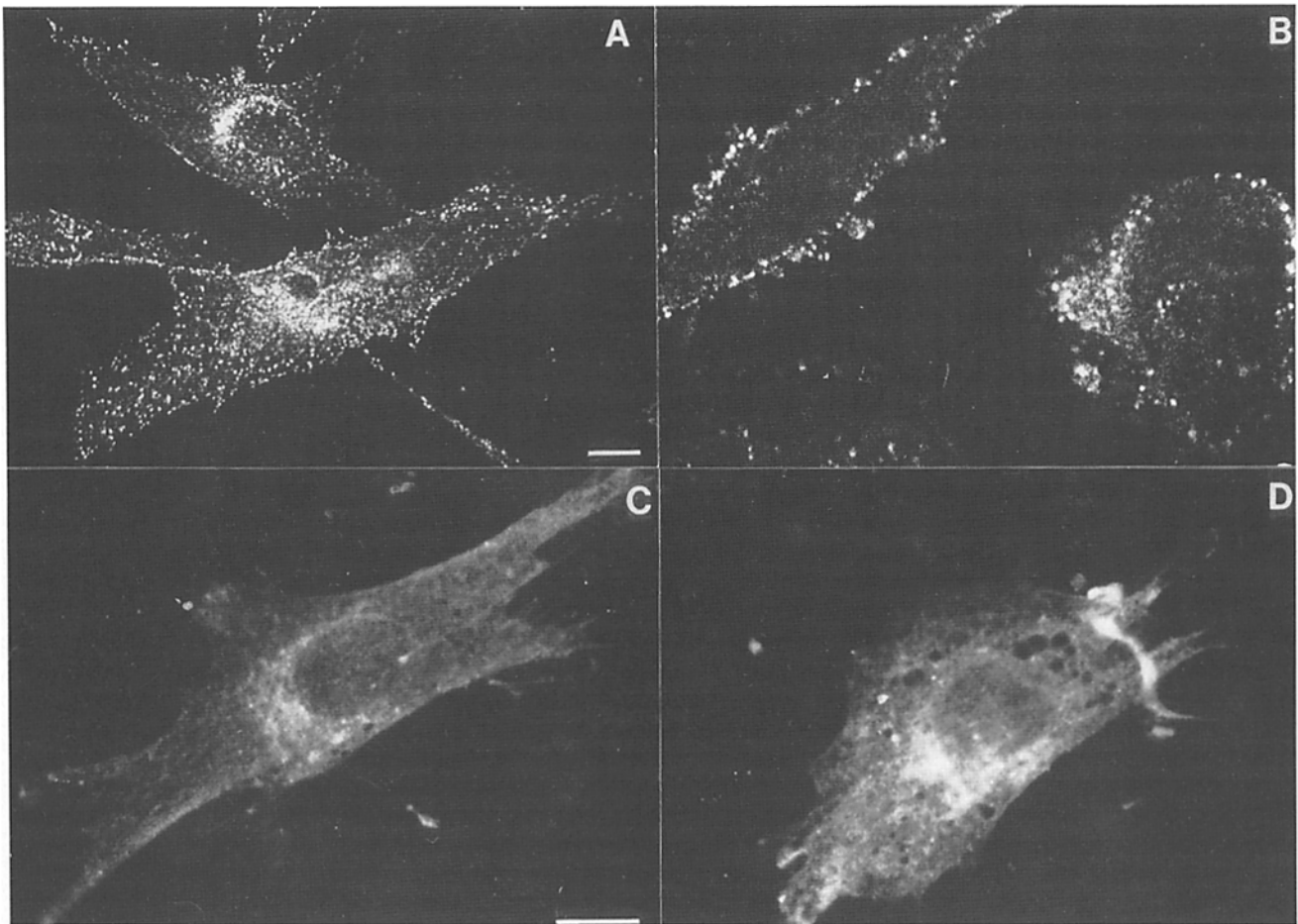
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Figure 8. Immunofluorescent staining of clathrin and detection of the Golgi apparatus in normal and RSV-transformed cells. Actively growing normal and RSV-transformed CEF on glass coverslips were fixed and permeabilized. For staining of clathrin, cells were incubated with an affinity-purified anti–clathrin light chain IgG and then with a goat anti–rabbit IgG conjugated with fluoresceine as described in Materials and Methods. The preparations were examined with a scanning confocal light microscope: (*A*) normal CEF cells; (*B*) RSV-transformed CEF cells. For staining of the Golgi apparatus, the fixed and permeabilized cells were stained with rhodamine-conjugated wheat germ agglutinin and analyzed by conventional light fluorescence microscope: (*C*) normal CEF cells; (*D*) RSV-transformed CEF cells. Bar, 10 μm.

by the tyrosine kinases; alternately, serine phosphorylation in transformed cells could be the consequence of the inhibition of a serine protein phosphatase. The tyrosine phosphorylation of clathrin heavy chain could be directly catalyzed by pp60^{v-src}, as the major phosphopeptide generated by the protease V8 from the in vivo immunoprecipitated clathrin commigrate with one of those obtained from in vitro phosphorylated clathrin heavy chain with pp60^{v-src}. In addition, when CEF cultures infected with a temperature-sensitive mutant of the RSV (NY 72-4) were transferred to permissive temperature, we found a temporal correlation between activation of pp60^{v-src} tyrosine kinase activity and clathrin heavy chain phosphorylation (data not shown).

The phosphorylation did not appear to be dependent on the synthesis of clathrin, since the $^{32}P_i$ label was turned over in the absence of protein degradation (data not shown), suggesting a phosphorylation–dephosphorylation equilibrium. The analysis of the distribution of clathrin heavy chain phosphorylation between the assembled and the unassembled pools was initially carried out in the absence of the tyrosine phosphatase inhibitor, sodium orthovanadate, in the isolation

buffer. Under those conditions, the most highly phosphorylated clathrin heavy chain was found in the unassembled fraction. However, when the experiments were repeated with orthovanadate in the isolation buffer, the extent of clathrin heavy chain phosphorylation was the same in both pools. As the newly synthesized clathrin heavy chain accumulates preferentially in the assembled pool, we conclude that the turnover of phosphorylation was faster in the membrane-bound pool.

Other target proteins for oncogenes encoding for tyrosine kinases have been found at the cortical cytoskeleton, among them: talin (Pasquale et al., 1986), the fibronectin receptor complex (Hirst et al., 1986), vinculin (Sefton et al., 1981), erzin, and calpactin I (for reviews see Hunter and Cooper, 1985, 1986). The range of their content of phosphotyrosine varies from ∼0.25 mol/mol of calpactin I down to 0.01 mol/mol of vinculin (Hunter and Cooper, 1985, 1986). Because of their localization and function, these phosphoproteins could be relevant in the mechanisms of oncogenesis. However, at the present time, there is no clear demonstration whether their phosphorylation is biologically significant

or is simply due to the partial cellular colocalization with pp60^v-src. As described above, in RSV-transformed cells, at steady state, clathrin heavy chain contains ~0.06 mol of phosphotyrosine per mole of protein.

Clathrin heavy chain appears to play an important role in endocytosis; CV-1 cells, loaded with anti-clathrin heavy chain antibodies, showed a substantial reduction of the number of coated pits and the receptor-mediated endocytosis uptake of the Semliki Forest virus (Doxsey et al., 1987). Cellular transformation by RSV causes a loss of dependence on the presence of ligand for receptor-mediated endocytosis of both EGF and thrombin (Decker, 1983; Cooper et al., 1983; Zetter et al., 1977). This effect, that correlates with the results described here, suggests that the phosphorylation and redistribution of clathrin heavy chain could be implicated in the internalization of growth factor receptors in the absence of their ligand, making the transformed cell more independent of external stimuli.

Demonstrating the biological significance of the heavy chain phosphorylation and its possible role in the transformation-induced redistribution of clathrin will require both in vitro and in vivo experiments. In vitro, it should be possible to establish whether heavy chain phosphorylation alters clathrin's assembly properties or binding affinities. In vivo experiments are needed to examine the effects of clathrin heavy chain phosphorylation on receptor-mediated endocytosis. If heavy chain phosphorylation alters coated pit–coated vesicle function, it should be possible to demonstrate a quantitative relationship between phosphorylation and ligand-receptor internalization.

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