Transformation by Rous Sarcoma Virus Induces Clathrin Heavy Chain Phosphorylation

Jorge Martin-Perez, Dudy Bar-Zvi, Daniel Branton, and R. L. Erikson

Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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 pp60csrc 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 pp60csrc 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 [35S]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.

The search for the underlying molecular mechanisms that explain transformation by Rous sarcoma virus (RSV) has resulted in the identification of many substrates phosphorylated by the tyrosine kinase, pp60csrc, 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; Bosdhi 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 pp60csrc (Decker, 1983; Cooper et al., 1983; Zetter et al., 1977).

Endocytosis often proceeds via coated pits and coated vesicles (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 phosphorylation both in vivo and in vitro (Keen and Black, 1986; Bar-Zvi et al., 1988b), 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 32P (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 [35S]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 alpha and beta light chains (Bar-Zvi et al., 1988b) 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 chain phosphorylated in chicken embryo fibroblasts transformed by 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 Myles, 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 Na3VO4, 2 mM EDTA, 2 mM EGTA, 0.002% NaN3, 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 homoogenized as before and centrifuged at 100,000 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 mM Na3VO4. The lysate was clarified by centrifugation at 12,000 g for 10 min at 4°C. Proteins were immunoprecipitated from the supernatant 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 supernatants were separated by SDS-PAGE (Laemmli, 1970). Where samples were labeled with [35S]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 [32P]Pi, the gels were exposed to -70°C to the same film with dried and exposed to Kodak X-Omat film at -70°C. Where samples were labeled with [32P]Pi, the gels were exposed to -70°C to the same film with dried and exposed to Kodak X-Omat film at -70°C. Where samples were labeled with [32P]Pi, the gels were exposed to -70°C to the same film with dried and exposed to Kodak X-Omat film at -70°C.

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 CEFs, cultures in DME containing 4% calf serum were labeled with [32P]Pi 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 j and 2). In addition, many of the phosphoprotein fragments detected on the corresponding autoradiograph comigrated with the major fragments of the 180-kD phosphoprotein stained by silver (Fig. 3, lanes j 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 aliquots. From one half, we determined the total amount of cellular clathrin heavy chain, proteins were precipitated with acetone. With the other half, we determined 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

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 V8 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., Burlington, 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 commigrates in SDS polyacrylamide gels with purified chicken clathrin heavy chain. The intensity of the Coomasie 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 [32P]Pi, 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 j and 2). In addition, many of the phosphoprotein fragments detected on the corresponding autoradiograph comigrated with the major fragments of the 180-kD phosphoprotein stained by silver (Fig. 3, lanes j 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 aliquots. From one half, we determined the total amount of cellular clathrin heavy chain, proteins were precipitated with acetone. With the other half, we determined 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, 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.

Martin-Perez et al. Clathrin Heavy Chain Phosphorylation by pp60\(^{src}\)

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\(^{src}\), 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\(^{src}\) 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\(^{src}\) were the only ki-
early as 15 min after the introduction of $^{32}$P, 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-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$^{v-c}c$, 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 V8, 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$^{v-c}c$ (Fig. 3, lanes 3 and 4).

Phosphorylation of clathrin heavy chain was detected as

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.

Figure 3. One-dimension V8 proteolytic maps of clathrin heavy chain. Immunoprecipitated clathrin heavy chain from $^{32}$P-labeled RSV-transformed cells (lanes 1 and 3) or clathrin heavy chain in vitro phosphorylated with pp60$^{v-c}c$ (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-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 Sefion, 1980).
Figure 5. In vitro phosphorylation of clathrin heavy chain by pp60<sup>ws</sup>. Triskelions were phosphorylated in vitro by pp60<sup>ws</sup> 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<sup>ws</sup>. The position of clathrin heavy chain (180 kD) and pp60<sup>ws</sup> (60 kD) are indicated.

The 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 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 <sup>32</sup>P-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...
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<sup>v-src</sup>, as the major phosphopeptide generated by the protease V8 from the in vivo immunoprecipitated clathrin comigrate with one of those obtained from in vitro phosphorylated clathrin heavy chain with pp60<sup>v-src</sup>. 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<sup>v-src</sup> 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 <sup>32</sup>P, 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 un assembled 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*^src. As described above, in RSV-transformed cells, at steady state, clathrin heavy chain contains \( \sim 0.06 \) 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 (Dossey 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.

The authors thank Drs. T. Martins, A. Bedard, A. Levin, and J. P. Garcia Ballesta for their critical review of the manuscript. In addition we are thankful to H.-P. Biemann for helping with the art work and to Dr. A. Levin for helping in the immunofluorescence experiments.

This work was supported by United States Public Health Service grant CA-42580 from the National Institute of Health (to R. L. Erikson), grant GM31579 from the National Institute of Health (to D. Branton), and by Financiacion Basal from the Consejo Superior de Investigaciones Científicas de Spain (J. Martin-Perez). R. L. Erikson is an American Cancer Society Professor of Cellular and Developmental Biology. J. Martin-Perez acknowledges the support of European Molecular Biology Organization Long Term Fellowship, a Leukemia Society of America Postdoctoral Fellowship, and a Juan March Short Term Fellowship. D. Bar-Zvi acknowledges the support of an American Heart Association Postdoctoral Fellowship.

Received for publication 28 December 1987 and in revised form 22 April 1988.

References

Ahle, S., and E. Ungewickell. 1986. Purification and properties of a new clathrin assembly protein. *EMBO J.* 5:3131–3149.

Ambros, V. R., L. B. Chen, and J. M. Buchanan. 1975. Surface ruffles as markers for studies of cell transformation by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 72:3144–3148.

Anderson, R. G. W., E. Vasile, R. J. Mello, M. S. Brown, and J. L. Goldstein. 1978. Immunocytochemical visualization of coated pits and coated vesicles in human fibroblasts: relation to low density lipoprotein receptor distribution. *Cell.* 15:919–933.

Balloo, M. L., M. Siegmund, and G. Thomas. 1988. S6 kinase in quiescent Swiss 3T3 cells is activated by phosphorylation in response to serum treatment. *Proc. Natl. Acad. Sci. USA.* 85:7154–7158.

Bar-Zvi, D., and D. Branton. 1986. Clathrin-coated vesicles contain two protein kinase activities. *J. Biol. Chem.* 261:9614–9621.

Bar-Zvi, D., A. E. Levin, and D. Branton. 1988a. Assembled clathrin in erythrocytes. *J. Biol. Chem.* 262:17717–17723.

Bar-Zvi, D., S. T. Mosley, and D. Branton. 1988b. In vivo phosphorylation of clathrin-coated vesicle proteins from rat reticulocytes. *J. Biol. Chem.* 263:4408–4415.

Blenis, J., and R. L. Erikson. 1985. Regulation of a ribosomal protein S6 kinase activity by the Rous sarcoma virus transforming protein, serum, or phorbol ester. *Proc. Natl. Acad. Sci. USA.* 82:7621–7625.

Boschek, C. B., B. M. Jockusch, R. Friis, R. Back, E. Grundmann, and H. Bauer. 1981. Early changes in the distribution and organization of microfilament protein during cell transformation. *Cell.* 24:175–184.

Brodsky, F. M. 1984. The role of monoclonal antibodies in cell transformation. *Cell.* 48:752–764.

Decker, S. 1981. Phosphorylation of ribosomal protein S6 in avian sarcoma virus-transformed chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA.* 78:4112–4115.

Decker, S. 1983. Reduced binding of epidermal growth factor by avian sarcoma virus-transformed rat cells. *Biochem. Biophys. Res. Commun.* 131:678–686.

Dowdy, S. F., J. M. Bodey, A. Blank, and A. Heleneus. 1987. Inhibition of endocytosis by anti-clathrin antibodies. *Cell.* 50:453–463.

Hirst, R., A. Horwitz, C. Back, and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 83:6470–6474.

Hunter, T., and J. A. Cooper. 1985. Protein-tyrosine kinases. *Annu. Rev. Biochem.* 54:897–930.

Hunter, T., and J. A. Cooper. 1986. Viral oncoproteins and tyrosine phosphorylation. In *The Enzymes*, Vol. 17, Control by Phosphorylation. Part A. P. D. Boyer and E. G. Krebs, editors. Academic Press, Inc., Orlando, FL. 192–237.

Jove, R., and H. Hanafusa. 1987. Cell transformation by the viral src oncogene. *Annu. Rev. Cell Biol.* 3:31–56.

Keen, J. H., and M. M. Black. 1986. The phosphorylation of clathrin membrane proteins in intact neurons. *J. Cell Biol.* 102:1325–1333.

Keen, J. H., M. H. Chestnut, and K. A. Beck. 1987. The clathrin coat assembly polypeptide complex. *J. Biol. Chem.* 262:3864–3871.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London).* 227:680–685.

Lisanti, M. P., L. S. Shapiro, N. Moskowitz, E. L. Hua, S. Puszkin, and W. Schook. 1982. Isolation and preliminary characterization of clathrin-associated proteins. *EuR. J. Biochem.* 125:463–470.

Oltmsid, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.

Pasquale, E. B., P. A. Maher, and S. J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 83:5507–5511.

Pastan, I., and M. C. Willingham. 1985. The pathway of endocytosis. *In* *Endocytosis*. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York/London. 1–44.

Pauloins, A., B. Bernier, and P. Jolles. 1982. Presence of cyclic nucleotide CA2+ independent protein kinase in bovine brain coated vesicles. *Nature (London).* 298:574–576.

Robinson, M. S., and B. M. F. Pearse. 1986. Immunofluorescence of 100K coated vesicle proteins. *J. Cell Biol.* 102:48–54.

Schook, W. J., and S. Puszkin. 1985. Brain clathrin light chain 2 can be phosphorylated by a coated vesicle kinase. *Proc. Natl. Acad. Sci. USA.* 82:8035–8043.

Sefon, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell.* 24:165–174.

Sugimoto, Y., E. Erikson, Y. Graziani, and R. L. Erikson. 1985. Inter-and intramolecular of highly purified Rous sarcoma virus-transforming protein, pp60^src. *J. Biol. Chem.* 260:13838–13843.

Ungewickell, E., and D. Branton. 1981. Assembly units of clathrin coats. *Nature (London).* 298:420–422.

Usami, M., A. Takahashi, T. Kadota, and K. Katoda. 1985. Phosphorylation of a clathrin light chain of coated vesicles in the presence of histones. *J. Biochem.* 97:1819–1822.

White, J. G., W. B. Amos, and M. Fordham. 1987. An evaluation of confocal versus conventional imaging of biological structures by immunofluorescence light microscopy. *J. Cell Biol.* 105:41–48.

Winkler, F. K., and K. K. Stanley. 1983. Clathrin heavy chain, light chain interactions. *EMBO (Eur. Mol. Biol. Organ.)J.* 2:1393–1400.

Zetter, B. R., L. O. Chen, and J. M. Buchanan. 1977. Binding and internalization of thrombin by normal and transformed chick cells. *Proc. Natl. Acad. Sci. USA.* 74:596–600.