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Increased Phosphorylation of Tyrosine in Vinculin Does Not Occur upon Transformation by Some Avian Sarcoma Viruses

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The level of phosphotyrosine in vinculin was determined in chicken embryo fibroblasts transformed by various strains of avian sarcoma virus. As previously reported (Sefton et al., Cell 24:165–174, 1981), vinculin was phosphorylated at tyrosine residues in most cultures examined, but the level varied greatly and no detectable change was found in cultures infected with Fujinami sarcoma virus or UR2 sarcoma virus. Regardless of the level of vinculin phosphorylation, the number of organized microfilament bundles was found to be decreased in all transformed cells. These results strongly suggest that tyrosine phosphorylation of vinculin is not an obligatory step in cell transformation by this class of oncogenes, nor is it correlated with the associated cytoskeletal disarray.

The transforming protein of Rous sarcoma virus (RSV), pp60^{src}, is a phosphoprotein of M_r 60,000 with a protein kinase activity that phosphorylates tyrosine (12). A similar protein kinase activity associated with the oncogene products of other avian retroviruses has been found, and there is evidence that tyrosine phosphorylation plays an important role in the process of transformation by these viruses (3). Primary substrates of the transforming proteins have been sought on the basis of enhanced phosphotyrosine content. Vinculin is a 130,000-dalton cytoskeletal protein which has been shown to contain a significantly greater amount of phosphotyrosine in cells transformed by RSV, Y73 sarcoma virus, or Abelson leukemia virus (32). The increase in phosphotyrosine content in vinculin is thermolabile in chick cells infected with an RSV mutant which is temperature sensitive in transformation (33). Vinculin has therefore been considered a possible substrate of the transforming proteins of these viruses in vivo.

Vinculin is associated with focal adhesion plaques, which are regions of close contact and anchorage between cell and substratum and are the sites of actin-membrane interaction at the ends of microfilament bundles (5, 18). In RSV-transformed cells, focal contacts are fewer and smaller, the cytoskeleton becomes disordered, and cells round up and are generally less adhesive (7). pp60^{src} has been shown to colocalize with vinculin in transformed cells (27, 29). In addition, isolated adhesion plaques and junctions of RSV-infected NRK cells have a protein kinase activity attributable to pp60^{src} (34). Thus tyrosine phosphorylation of vinculin, mediated by pp60^{src} within adhesion plaques, is a plausible determinant of various manifestations of the transformed state.

In this study we determined the extent of tyrosine phosphorylation in vinculin upon transformation by a number of avian sarcoma viruses. In addition, we used indirect immunofluorescence microscopy to examine the cellular disposition of microfilament bundles in the transformed cells.

Chicken embryo fibroblasts were infected and fully transformed with the following viruses: Y73; UR1; UR2; 16L; Fujinami sarcoma virus (FSV); Schmidt Ruppin RSV, subgroup A (SR-A); Bryan high-titer strain of RSV (BH-RSV); and a fusiform morphological mutant of BH-RSV (BH-RSV^f) isolated in this laboratory. These viruses are described in Table 1, which lists their transforming genes, their transforming proteins, and the transformed morphology which they induce. Criteria for transformation included morphology, refractility, and loss of anchorage dependence. Each plate containing log-phase cultures was labeled with 1.5 mCi of carrier-free $^{32}P_i$ (Amersham Corp., Arlington Heights, Ill.) per ml. Incubation was continued for 18 h, after which cell extracts were made as previously described (13), except for the use of phosphate-buffered RIPA (13) as lysis buffer. In some experiments, duplicate cultures were lysed in 1 ml of boiling sodium dodecyl sulfate (SDS) buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 1% SDS, 25 mM EDTA) instead of RIPA buffer, to ensure the absence of enzyme activity during the extraction procedure. After addition of SDS buffer, cells were scraped from the plate and vortexed, and 4 ml of Nonidet P-40 buffer (20) was added. SDS buffer extracts were centrifuged at 10,000 × *g* for 7 min, and the supernatants were stored in liquid nitrogen. ^{32}P-labeled cell extracts were immunoprecipitated with antivinculin monoclonal antibody or antivinculin rabbit serum (M. E. Greenberg, Ph.D. thesis, The Rockefeller University, New York, 1983) and analyzed on 5 to 15% gradient gels. For immunoprecipitation of RIPA buffer extracts, 40-μl portions of protein A-Sepharose CL4B (Sigma Chemical Co., St. Louis, Mo.) (a 50% [vol/vol] slurry in 20% glycerol) mixed with 40 μl of RIPA buffer were incubated with 10 μl of antivinculin monoclonal antibody for 10 min on ice. ^{32}P-labeled cell extract (200 μl) was added, and the mixture was incubated on ice for 1.5 h. Immunoprecipitation of SDS buffer extracts differed from RIPA buffer extracts in that 160 μl of protein A-Sepharose CL4B plus 40 μl of rabbit antivinculin antibody was incubated for 10 min on ice and 1 ml of ^{32}P-labeled SDS buffer extract was added, followed by incubation on ice for 2.5 h. Further procedures were as previously described (13). The vinculin used as an antigen for monoclonal antibody production and as a standard for electrophoretic migration was purified by the procedure of Feramisco and Burridge (16).

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TABLE 1. Viruses used for transformation of chicken embryo fibroblasts

Virus	Transforming gene	Transforming protein	Transformed morphology[a]	Reference(s)
SR-A	*src*	p60	r, s	4
BH-RSV	*src*	p60	r	4
BH-RSV[f]	*src*	p60	f	
FSV	*fps*	P130	f	13, 21
16L	*fps*	P142	f, s	26
UR1	*fps*	P150	f, s	1, 36
UR2	*ros*	P68	f	1, 15
Y73	*yes*	P90	f, s	24

[a] Induced in CEF; r, round; s, spindle-shaped; f, fusiform.

Vinculin bands were visualized both by a general protein stain (Fig. 1, lanes A through D) and by autoradiography (13) (lanes E through H). The vinculin bands were cut out, washed in 10% methanol, lyophilized, and rerun on an 8.5% SDS-polyacrylamide gel to remove visible background contamination (lane I). Immunoprecipitation with a control monoclonal antibody resulted in a pattern of bands with migrations distinct from that of vinculin (lanes A and E). Phosphoamino acid analysis of labeled vinculin was carried out by two-dimensional electrophoresis after partial acid hydrolysis (13). Phosphoamino acid spots visualized by autoradiography were quantified by densitometric tracing, as previously described (9). The results, expressed as a percentage of total phosphoamino acids, are shown in Table 2. Calibration with ^{32}P-labeled standards revealed a linear response within the range of experimental values (data not shown). Autoradiograms for which phosphotyrosine was visible but below the level of detection of the densitometer are shown in Fig. 2. The results with uninfected and SR-A-transformed cells are shown for comparison (Fig. 2a and b). Except for the densitometric tracing, the entire experimental protocol, from the infection of cultures to the analysis of phosphoamino acids, was duplicated. Virtually identical ratios and intensities of phosphoamino acid spots were obtained, except for a slight reduction of phosphotyrosine in 16L and UR1 and a slight increase in Y73.

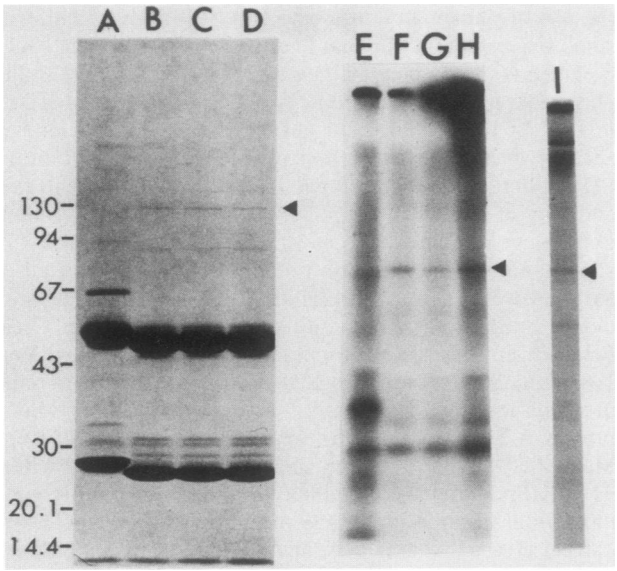

FIG. 1. Immunoprecipitation of vinculin. Lanes A through D represent Coomassie blue staining patterns; lanes E through I are autoradiograms. Lanes A through H are RIPA buffer extracts immunoprecipitated with either control monoclonal antibody (lanes A, E) or antivinculin monoclonal antibody (lanes B through D and F through H). Lane I is an SDS buffer extract immunoprecipitated with rabbit antivinculin antibody. A, B, E, F, I, Cells transformed by SR-A; C, G, cells transformed by FSV; D, H, cells transformed by UR2. Control monoclonal antibody is an uncharacterized monoclonal antibody which recognizes determinants in chicken embryo fibroblasts which are distinct from vinculin. ◀, Vinculin.

It is apparent that phosphorylation of tyrosine in vinculin occurred to various degrees upon transformation with the different sarcoma viruses (Table 2; Fig. 2). As previously described (32), transformation with both SR-A and Y73 caused a significant increase in the level of phosphotyrosine in vinculin, compared with that in uninfected cells. However, no phosphotyrosine was detected in vinculin extracted from cultures transformed with FSV and UR2 (Table 2; Fig. 2d and e). The extraction with SDS buffer permitted the detection of a faint spot of phosphotyrosine in uninfected cells (Fig. 2g), but a corresponding spot was even less intense in FSV-transformed cells (Fig. 2h). The results obtained with FSV or UR2 indicate that phosphorylation of tyrosine in vinculin is not an obligatory step in transformation by this class of viruses.

In contrast to FSV, both 16L and UR1 caused detectable increases in phosphotyrosine content, although at a lower level than that caused by SR-A or Y73 (Table 2; Fig. 2b, c, and f). All three viruses, FSV, 16L, and UR1, code for a *gag-fps* transforming protein (26). Previous work has shown that transformation by PRCII, another avian sarcoma virus containing *fps*, also causes no change in phosphotyrosine level in vinculin (32). The level of in vitro protein kinase activity in extracts from cultures transformed by these viruses was essentially the same when measured by autophosphorylation (data not shown). In addition, transformation by FSV and PRCII has been shown to elevate the level of phosphotyrosine in total cellular protein (2, 20, 28) and cause tyrosine phosphorylation of the 34-kilodalton protein (6, 11), another possible cellular substrate of the transforming proteins. The basis for the differences in phosphorylation in vinculin among these v-*fps*-containing viruses is

TABLE 2. Phosphoamino acid composition of vinculin

Cultures infected with:	Extraction and immunoprecipitation[a]	Relative abundance of phosphoamino acids (%):		
		Phosphoserine	Phosphothreonine	Phosphotyrosine
Nothing	A	96	4	U[b]
SR-A	A	58	8	33
UR2	A	86	14	U
FSV	A	92	8	U
16L	A	79	9	12
UR1	A	88	12	U
BH-RSV	A	80	6	14
BH-RSV[f]	A	56	7	38
Y73	A	62	3	35
Nothing	B	94	6	U
SR-A	B	58	9	32
FSV	B	95	5	U

[a] A, RIPA buffer extracts immunoprecipitated with antivinculin monoclonal antibody; B, SDS buffer extracts immunoprecipitated with antivinculin rabbit antibody.

[b] U, Undetectable.

currently unknown. Since both FSV and UR2 are highly tumorigenic (1, 21), the lack of detectable tyrosine phosphorylation in vinculin upon FSV and UR2 transformation indicates that malignant growth properties do not always correlate with the increased tyrosine phosphorylation of this cytoskeletal protein.

It is possible that the absence of tyrosine phosphorylation in vinculin seen upon expression of certain transforming proteins is related to a lack of association with the plasma membrane. Unlike RSV pp60^{src}, which associates tightly with the plasma membrane, FSV P130 appears to be a cytoplasmic, nonintegral membrane protein (14). In addition, localization studies by indirect immunofluorescence have revealed the absence of FSV *gag-fps* protein from adhesion plaques (R. L. Rohrschneider, personal communication).

In a previous study, a suggestion was made that tyrosine phosphorylation in vinculin may be less extensive in fusiform transformed cells (32). However, other workers observed no correlation between the morphology of transformed cells and phosphorylation in vinculin (23, 30). Our results support the latter view, particularly from the comparison of cultures transformed with BH-RSV and BH-RSV^f. The level of phosphotyrosine was higher in the thin and extremely elongated cells induced by BH-RSV^f transformation than in the round or slightly spindle-shaped cells transformed by BH-RSV (Table 2). Iwashita et al. (23) suggested that the amount of vinculin in cells may be related to morphology: an RSV mutant which induces fusiform morphological transformation causes a twofold higher elevation in the amounts of both vinculin and fibronectin than does the wild-type RSV. In this study, we found no direct correlation between vinculin content and cellular morphology (data not shown).

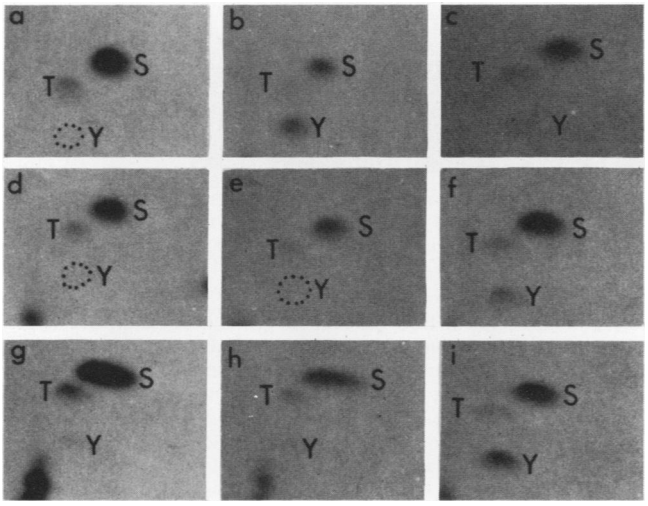

FIG. 2. Phosphoamino acid analysis of vinculin. Partial acid hydrolysates of ^{32}P-labeled vinculin were separated in two dimensions: electrophoresis at pH 1.9 is from left to right and electrophoresis at pH 3.5 is from bottom to top (13). Portions of autoradiograms are shown above. Positions of the internal phosphoamino acid markers, identified by ninhydrin staining, are indicated. Phosphoamino acid spots are circled only when no radioactivity in that spot is discernible on the film. Other spots appearing in the autoradiogram represent incompletely hydrolyzed phosphopeptides. Standard phosphoamino acids: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. a, g, Uninfected cells; b, i, cells transformed by SR-A; c, cells transformed by UR1; d, cells transformed by UR2; e, h, cells transformed by FSV; f, cells transformed by 16L. Samples a through f were lysed in RIPA buffer and immunoprecipitated with antivinculin monoclonal antibody; samples g through i were SDS extracts immunoprecipitated with antivinculin rabbit antibody.

Indirect immunofluorescence staining with the antivinculin monoclonal antibody was used to show that the monoclonal antibody used for immunoprecipitation recognized vinculin in all the cell types tested. Antibody preparations were made by mixing 120 μl of antivinculin monoclonal antibody with 12 ml of phosphate-buffered saline containing 10% calf serum, which was then filtered through a 0.45-μm (pore size) Millipore filter (Millipore Corp., Bedford, Mass.). The second antibody was fluorescein-conjugated rabbit antibody to mouse immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.), which was diluted 1:100 in phosphate-buffered saline containing 10% calf serum before use. Fixation of cells and further processing for immunofluorescence microscopy were done as previously described (19). The staining patterns shown in Fig. 3 resulted from the use of an extraction procedure that selectively removed the diffusely staining cytoplasmic vinculin so that the "organized" vinculin present at the membrane in focal adhesion plaques and less intense fibrillary patches could be better visualized (17). Both diffuse cytoplasmic and distinct organized vinculin were visible in the uninfected cells and in all the transformed cells studied (Fig. 3 and data not shown). Staining with an anti-(N-CAM) monoclonal antibody (22) showed the labeling patterns to be specific for vinculin immunofluorescence (data not shown).

To see whether cytoskeletal alterations occur even in cultures lacking observable tyrosine phosphorylation in vinculin, the disposition of microfilament bundles in transformed cells was examined by immunofluorescence staining with antiactin antibody. For actin visualization, 12 ml of rabbit antiactin antibody at an immunoglobulin concentration of 0.3 mg/ml with 10% calf serum was filtered for use. The second antibody was fluorescein-conjugated goat antibody to rabbit immunoglobulin G (Miles Laboratories). Further procedures were as described for vinculin immunofluorescence. The selective extraction procedure (17) removed much of the cytoplasmic soluble actin, which improved the visualization of any actin cables. Staining with preimmune sera showed the labeling patterns to be specific for actin immunofluorescence (data not shown).

The amount of organized actin decreased in all the transformed cells, compared with the amount in uninfected cells (Fig. 3). The decrease was apparent in the total number of actin cables, in the thickness of the cables, and in the crossing-over and network appearance of visible cables. Among transformed cells, there was no observable correlation between the decrease in actin organization and the degree of tyrosine phosphorylation in vinculin (Fig. 2 and 3 and other data not shown). For example, cells transformed by 16L or UR2 had a much lower phosphotyrosine content in vinculin than those transformed by SR-A, but the dissolution of actin cables was comparable.

The presence or absence of actin cables did not seem to correspond to a specific transformed morphology. This is clearly shown by the loss of cables in the rounded cells of SR-A-transformed cultures (Fig. 3e and f) as well as by the flat elongated cells induced by UR2 transformation (Fig. 3h and i).

The organization of the cytoskeleton has been implicated in growth control mechanisms, involving cell division, cell movement, and cell-cell interactions (8). It remains possible that cytoskeletal disarray seen in this study is related to

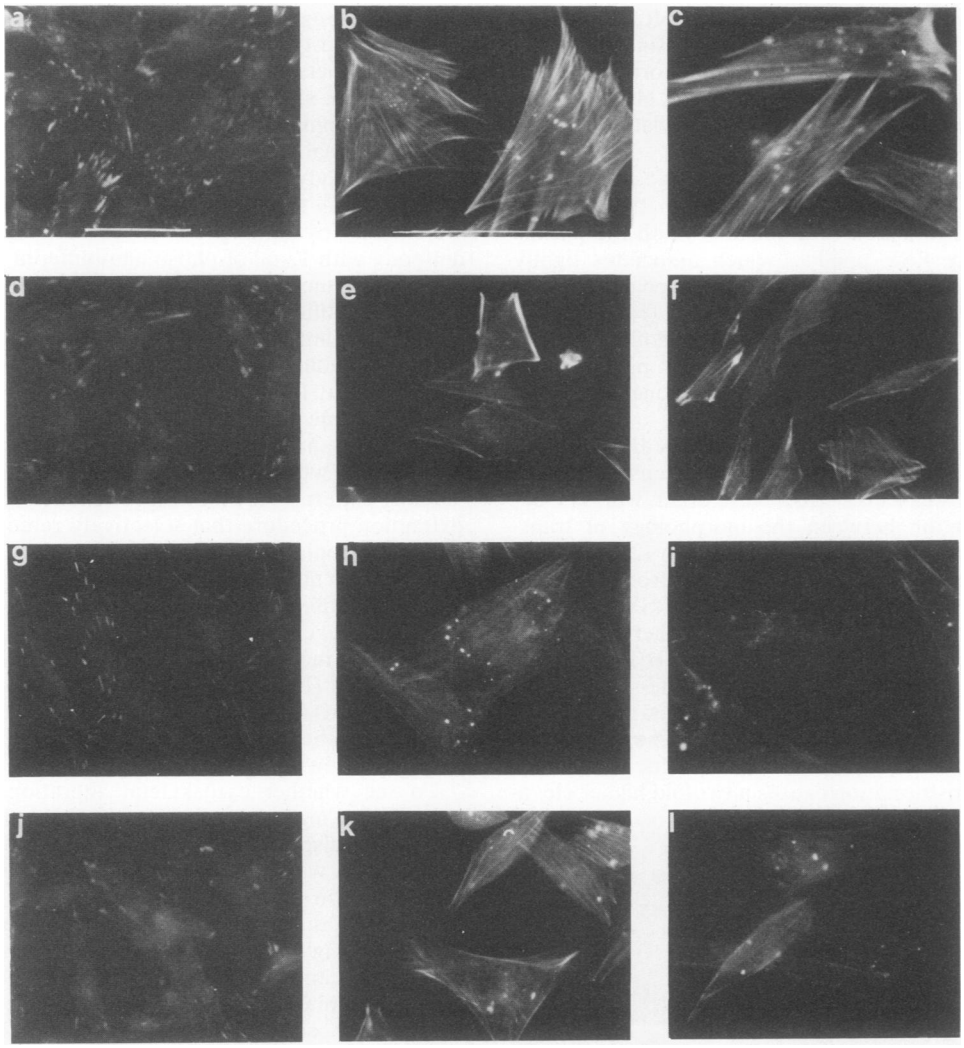

FIG. 3. Indirect immunofluorescence. Cells were stained with antivinculin monoclonal antibody (a, d, g, j) or antiactin antibody (b, c, e, f, h, i, k, l). a through c, Uninfected cells; d through f, cells transformed by SR-A; g through i, cells transformed by UR2; j through l, cells transformed by FSV. The bar shown in a and b represents 50 μm. Panels a, d, g, j, and the remaining panels were photographed at the same magnifications.

malignant growth properties resulting from viral transformation.

Studies with temperature-sensitive *src* mutants have revealed the loss of organized actin cables in fibroblasts to be dependent upon a functional transforming protein (10, 35). Vinculin is a likely target of the transforming proteins because of its proposed role in mediating the attachment of microfilament bundles to the cell membrane (18). However, Rosok and Rohrschneider (31) have shown that there is no detectable variation in the phosphotyrosine content of vinculin throughout the cell cycle, suggesting that normal cellular regulation of stress fiber dissolution before mitosis does not correspond to an increase in phosphorylation of tyrosine residues in vinculin. In addition, recent work with partial transformation mutants of RSV suggests that phosphorylation of tyrosine in vinculin is unrelated to stress fiber integrity (30). The reduction in thickness, amount, and organization of actin cables seen upon transformation by all the viruses of this study seems to indicate that tyrosine phosphorylation of vinculin is not obligatory to promote the disappearance of actin cables in the transformed state, nor is it a universal correlate of virus-induced cytoskeletal disarray.

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