Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6576–6580, October 1985
Cell Biology

Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells

(focal contacts/immunofluorescent staining)

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Contributed by S. J. Singer, June 11, 1985

ABSTRACT **We have used a high-affinity polyclonal antibody directed against phosphotyrosine (*P*-Tyr) to localize *P*-Tyr-containing proteins in normal and transformed cells in culture by immunofluorescence microscopy experiments. The distribution of the proteins with modified tyrosine was compared with that of F-actin in these cells. Cells infected with Abelson murine leukemia virus were found to contain elevated levels of *P*-Tyr, as expected. Various permanent lines of fibroblastic and epithelial cells exhibited lower, but easily detectable, levels of *P*-Tyr. The *P*-Tyr in fibroblasts was concentrated at the focal contacts at the termini of actin-containing microfilament bundles and, in the epithelial cells examined, at the intercellular junctions. Early passages of primary cultures of chicken embryo fibroblasts and chicken embryo heart cells also showed detectable levels of *P*-Tyr in focal contacts and cell–cell junctions. However, *P*-Tyr was not detectable in later passages of chicken embryo fibroblasts. The concentration of *P*-Tyr-containing proteins in intercellular junctions in normal cells suggests that these are sites of significant biochemical regulatory activities which may be important in the control of normal cell adhesivity, motility, and shape.**

Tyrosine protein kinase activity, which was originally found to be associated with the transforming proteins of a number of different tumor viruses (1–3), has been shown to be characteristic of normal cellular homologues of these transforming proteins (4), and of the membrane receptors for a variety of growth factors (5–8). Since the time that this enzyme activity was first recognized (2) an extensive search has been underway for the substrates of these protein kinases *in vivo*. In the traditional biochemical approach cells were labeled with [^{32}P]phosphate and were solubilized, then the phosphotyrosine (*P*-Tyr) content of the extract or of individual proteins isolated from it was determined by acid hydrolysis, followed by thin-layer separation and autoradiography (9). An alternative approach is to prepare antibodies to *P*-Tyr and use these to detect proteins containing *P*-Tyr residues. Both polyclonal (10–12) and monoclonal (13) antibodies directed to a *P*-Tyr analogue hapten have been described and applied to the analysis of *P*-Tyr-modified proteins in appropriately transformed and activated cells. In one case (11, 12), immunofluorescence studies have been carried out with cultured transformed and normal cells. Although *P*-Tyr was localized to several sites in the transformed cells, none was detected in the normal cells using these antibodies.

The production and isolation of high-affinity polyclonal antibodies to *P*-Tyr, elicited by immunization with the *P*-Tyr-modified transforming protein encoded by the v-*abl* sequence of Abelson murine leukemia virus (A-MuLV) has been achieved (unpublished work). With these antibodies some *P*-Tyr-modified proteins were detected in transformed cells that had not previously been recognized. Using these antibodies in immunofluorescence studies we report significant immunofluorescent labeling not only with appropriately transformed cells but also with normal cell lines and primary cultures of embryonic cells. The labeling for *P*-Tyr in the normal cells was concentrated at focal adhesions formed by the cells with the substratum and at cell–cell junctions. These findings suggest that the normal cell may in part regulate its adhesivity, motility, and shape by such *P*-Tyr enzymatic modification.

MATERIALS AND METHODS

Cell Culture. A-MuLV-transformed 3T3 cells (N54) were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Normal rat kidney (NRK) cells were cultured as described (14) from stocks originally provided by P. K. Vogt (University of Southern California, Los Angeles). Madin–Darby canine kidney (MDCK) and potoroo kidney (PtK2) cells were grown in Eagle's minimal essential medium with nonessential amino acids, 10% FCS and antibiotics. BALB/c 3T3 cells were grown in DME/Coon's F12 medium (14) (1:1) with 10% FCS. Chicken embryo fibroblasts (CEF) were prepared by trypsinization of decapitated, minced 8-day embryos. The cells were cultured in DME/Coon's F12 medium (1:1) containing 10% FCS and antibiotics and were either plated directly onto coverslips or cultured for two or three passages. Chicken embryo heart cells were prepared and cultured as described (15).

Immunofluorescence Microscopy. Cells were cultured for 24–72 hr on 18 mm^2 coverslips. All subsequent steps were carried out at room temperature. Cells were rinsed with phosphate-buffered saline (P_i/NaCl), pH 7.4, fixed for 5 min with 3% formaldehyde (vol/vol) in P_i/NaCl and permeabilized for 5 min with 0.5% Triton X-100 in 10 mM Hepes, 300 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, pH 7.4. Permeabilized cells were treated with rabbit anti-*P*-Tyr affinity-purified antibodies (≈10 μg/ml) in P_i/NaCl for 30 min, rinsed in P_i/NaCl, labeled with a mixture of affinity-purified rhodamine-conjugated goat anti-rabbit IgG (10 μg/ml) and nitrobenzoxadiazole (NBD)-phallacidin (20 units/ml; Molecular Probes, Junction City, OR) in P_i/NaCl for 10 min and mounted in 90% glycerol, 10 mM Tris·HCl, pH 8.0. Labeled cells were examined and photographed as described (14).

Antibodies and Other Reagents. The production and affinity purification of rabbit anti-*P*-Tyr antibodies used in this study will be described in detail elsewhere. Briefly, the v-*abl*-encoded transforming protein (the v-abl protein), which was expressed in bacteria, can phosphorylate itself on tyrosine residues (16). When this phosphorylated protein was isolated

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Abbreviations: *P*-Tyr, phosphotyrosine; CEF, chicken embryo fibroblasts; A-MuLV, Abelson murine leukemia virus; RSV, Rous sarcoma virus; NBD-phallacidin, nitrobenzoxadiazole-labeled phallacidin.

and used as an immunogen, anti-*P*-Tyr antibodies were generated in considerable titers, along with antibodies directed to the protein itself. The anti-*P*-Tyr antibodies were then isolated by affinity chromatography using *O*-phosphotyramine-coupled Sepharose as described (10), and shown to be specific for *P*-Tyr (unpublished studies; see also Figs. 1 and 2). The preparation and affinity purification of rhodamine-conjugated goat anti-rabbit IgG has been published previously (17). *O*-phospho-L-tyrosine and *O*-phospho-L-serine were obtained from Sigma. *O*-sulfo-L-tyrosine was a gift of J. Fessler (University of California, Los Angeles).

RESULTS

P-Tyr Distribution in Transformed and Normal Cell Lines in Culture. The presence and distribution of proteins phosphorylated on tyrosine has been examined in cultured cells by immunofluorescence microscopy (Fig. 1) and compared with the distribution of F-actin. The anti-*P*-Tyr antibody reacted very strongly with the proteins of a fibroblastic 3T3 cell line permanently transformed by A-MuLV (N54 cells, Fig. 1*B*). The *P*-Tyr labeling was prominent at the cell periphery, particularly at sites of cell–cell interaction (arrow in Fig. 1*B*). Small clumps of punctate staining were also observed in the extranuclear region of the cytoplasm. The distribution of *P*-Tyr at the cell periphery was generally correlated with that of F-actin (Fig. 1*A*). Much of the labeling with the anti-*P*-Tyr antibody in these cells is probably due to the 160-kDa protein encoded by v-*abl*, since immunoblotting (not shown) indicates that the oncogene product is the major *P*-Tyr-containing protein in these cells. Normal 3T3 cells, however, were also labeled with the anti-*P*-Tyr antibody (Fig. 1*E*). This label was restricted to discrete sites at the periphery of these cells corresponding to the termini of F-actin-containing stress fibers (arrowheads; Fig. 1 *D* and *E*). This pattern of *P*-Tyr labeling is identical to that obtained when the cells are immunolabeled with antibodies to vinculin (ref. 17; not shown here), indicating that the *P*-Tyr is localized to focal contacts. A closely similar distribution of *P*-Tyr-containing proteins, compared to F-actin, is observed in fibroblastic NRK cells (Fig. 1 *G* and *H*).

Two lines of epithelial cells have also been examined by immunofluorescence microscopy for the presence of *P*-Tyr-containing proteins. In both PtK2 cells (Fig. 1 *J* and *K*) and MDCK cells (Fig. 1 *M* and *N*), *P*-Tyr is localized to the termini of stress fibers in focal contacts (arrowheads) and at sites of cell–cell interaction (arrows). The latter localization is particularly evident in cultures of MDCK cells (Fig. 1 *M* and *N*) which show quite substantial labeling with the anti-*P*-Tyr antibody. These cells also exhibit strong nuclear staining.

It should be noted that to obtain these photographs of normal cells immunolabeled with the antibody to *P*-Tyr it was necessary to expose the film at least twice as long as with the transformed cells.

The specificity of the anti-*P*-Tyr antibody binding to the *P*-Tyr group in these immunofluorescence experiments is shown in Fig. 1 *C, F, I, L,* and *O* and Fig. 2 *C* and *H*. The presence of 10 mM *O*-phospho-L-tyrosine completely abolished the focal contact and intercellular labeling in all the normal cells as well as the membrane and cytoplasmic labeling in the transformed cells (Fig. 1*C*). The weak nuclear labeling observed in the NRK cells and more strongly in the 3T3 cells was not markedly affected by the presence of free *P*-Tyr suggesting it is nonspecific. However, the strong nuclear labeling of MDCK cells was partially reduced by *P*-Tyr (Fig. 1 *N* and *O*). The same concentrations of *O*-phospho-L-serine and *O*-sulfo-L-tyrosine had no effect on the intensity of labeling by the anti-*P*-Tyr antibodies with any of the cell lines (not shown).

P-Tyr Distribution in Primary Cells in Culture. To determine if the localization of *P*-Tyr-containing proteins to focal contacts and intercellular junctions was characteristic only of permanent cell lines or applied also to primary cultures, we examined primary CEF and chicken heart cells. CEF that were plated directly onto coverslips and labeled with anti-*P*-Tyr 24 hr later showed low but readily detectable labeling in focal contacts (arrowheads in Fig. 2*B*) that was specific for *P*-Tyr (Fig. 2*C*). The F-actin labeling (Fig. 2*A*) of the same cell labeled with anti-*P*-Tyr (Fig. 2*B*) showed actin was at the cell periphery, but intracellular stress fibers did not appear to be prominent. In the same cell population, in general cells with the most intense labeling for *P*-Tyr showed the fewest stress fibers; conversely, cells with the most developed stress fibers showed the least *P*-Tyr. When CEF were cultured for several passages and then plated onto coverslips for 24 hr and labeled, little or no *P*-Tyr labeling was observed (Fig. 2*E*), and actin stress fibers were prominent (Fig. 2*D*). Chick heart cells labeled with the anti-*P*-Tyr antibody 24 hr after their initial plating also showed a concentration of label at sites of cell–cell junctions (arrows in Fig. 2 *F* and *G*) and in focal contacts (not shown).

DISCUSSION

Elevated phosphorylation of tyrosine residues accompanies the transformation of cells by many RNA tumor viruses (1). The cellular oncogenes, from which the viral oncogenes encoding tyrosine kinases are derived, are present in the genome of normal cells and appear to be developmentally regulated (18–22). In normal cells, tyrosine kinase activity is also rapidly stimulated by the interaction of some polypeptide growth hormones with their receptors (5–8). In spite of the fact that tyrosine phosphorylation appears to play an important role in viral transformation, normal growth control, and cellular differentiation, the mechanism of the regulation or alteration of cellular activities consequent to increased tyrosine kinase activity is still not established. In fact, many of the minor *in vivo* substrates of tyrosine-specific protein kinases have probably not yet been recognized, because of the scarcity of *P*-Tyr in proteins and the insufficient sensitivity of the available methods of detection (23). Furthermore, even in the case of major substrates, the alterations induced by tyrosine phosphorylation on protein function or cellular location have not been clarified (1, 24).

The availability of an antibody preparation capable of recognizing *P*-Tyr residues specifically and with high affinity in immunofluorescence, immunoprecipitation, and immunoblotting experiments (unpublished results) has allowed us to localize *P*-Tyr-containing proteins not only in appropriate retrovirus-transformed cells but also in uninfected cells. In normal cells, *P*-Tyr represents only about 0.03% of the total phosphoamino acids in proteins (2) while in some transformed cells the *P*-Tyr level is increased about 10-fold. We have shown, utilizing conventional immunofluorescent labeling techniques, that in normal cells, *P*-Tyr appears to be concentrated at the focal contacts made with the substratum and at the intercellular junctions. The immunofluorescence localized to these sites is specific; excess phosphotyrosine, but not phosphoserine or sulfotyrosine, completely abolishes it. Antibodies capable of reacting with *P*-Tyr residues have been generated previously (10–13), and in one case have been used in immunofluorescence studies with both normal and Rous sarcoma virus (RSV)-transformed duck embryo fibroblasts (11), rat-1 fibroblasts (11) and 3T3 cells (11, 12). In the RSV-transformed cells, such antibodies immunolabeled the residual focal adhesions and the rosette structures described by David-Pfeuty and Singer (25). However, these antibodies did not detectably label the normal cells, and the results we have obtained using our anti-*P*-Tyr antibodies with normal

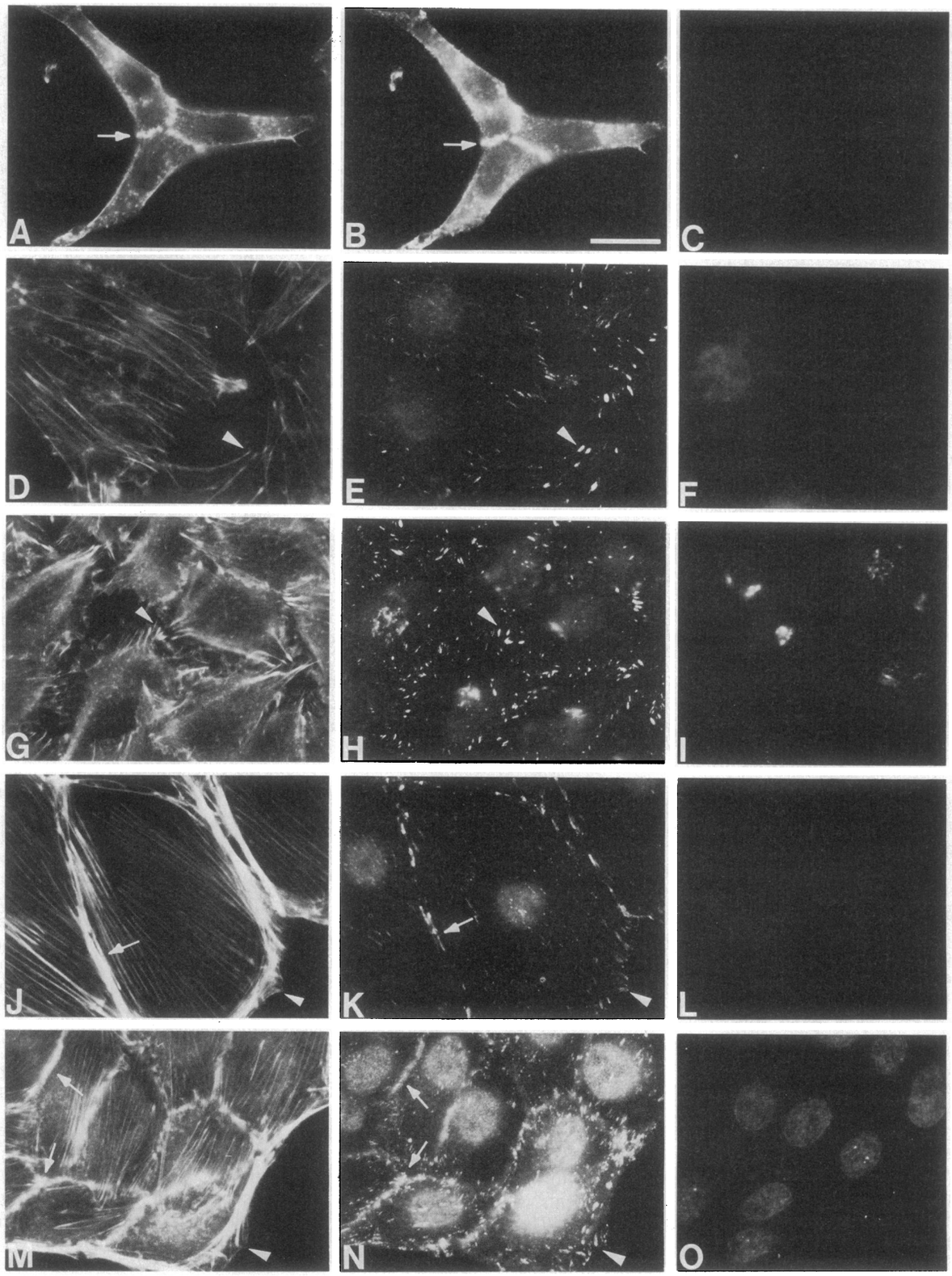

FIG. 1. Double fluorescent labeling of permanent cell lines with rabbit anti-*P*-Tyr antibodies followed by rhodamine-conjugated goat anti-rabbit IgG antibodies (*B*, *E*, *H*, *K*, and *N*) and NBD-phallacidin staining of F-actin (*A*, *D*, *G*, *J*, and *M*). (*C*, *F*, *I*, *L*, and *O*) Similar cells were indirectly immunolabeled for *P*-Tyr, but in the presence of excess free *P*-Tyr. (*A–C*) A-MuLV-transformed N54 cells. (*D–F*) 3T3 cells. (*G–I*) NRK cells. (*J–L*) PtK2 cells. (*M–O*) MDCK cells. Arrowheads indicate focal contacts and arrows point out cell–cell junctions. The fields in the controls (*C*, *F*, *I*, *L*, and *O*) are not the same as in the other two photographs of the same cell line. (Bar in *B* = 20 μm.)

cells therefore represent a novel finding. The differences in the properties of the two anti-*P*-Tyr antibody preparations may reflect differences in the way they were generated. Our anti-*P*-Tyr antibodies were made to a natural *P*-Tyr-containing protein, whereas the antibodies prepared by Comoglio *et al.* (11) were raised to a synthetic hapten-protein conjugate made by coupling the *p*-phosphonomethylene analogue of *O*-phosphotyramine to a protein. Subtle differences in the

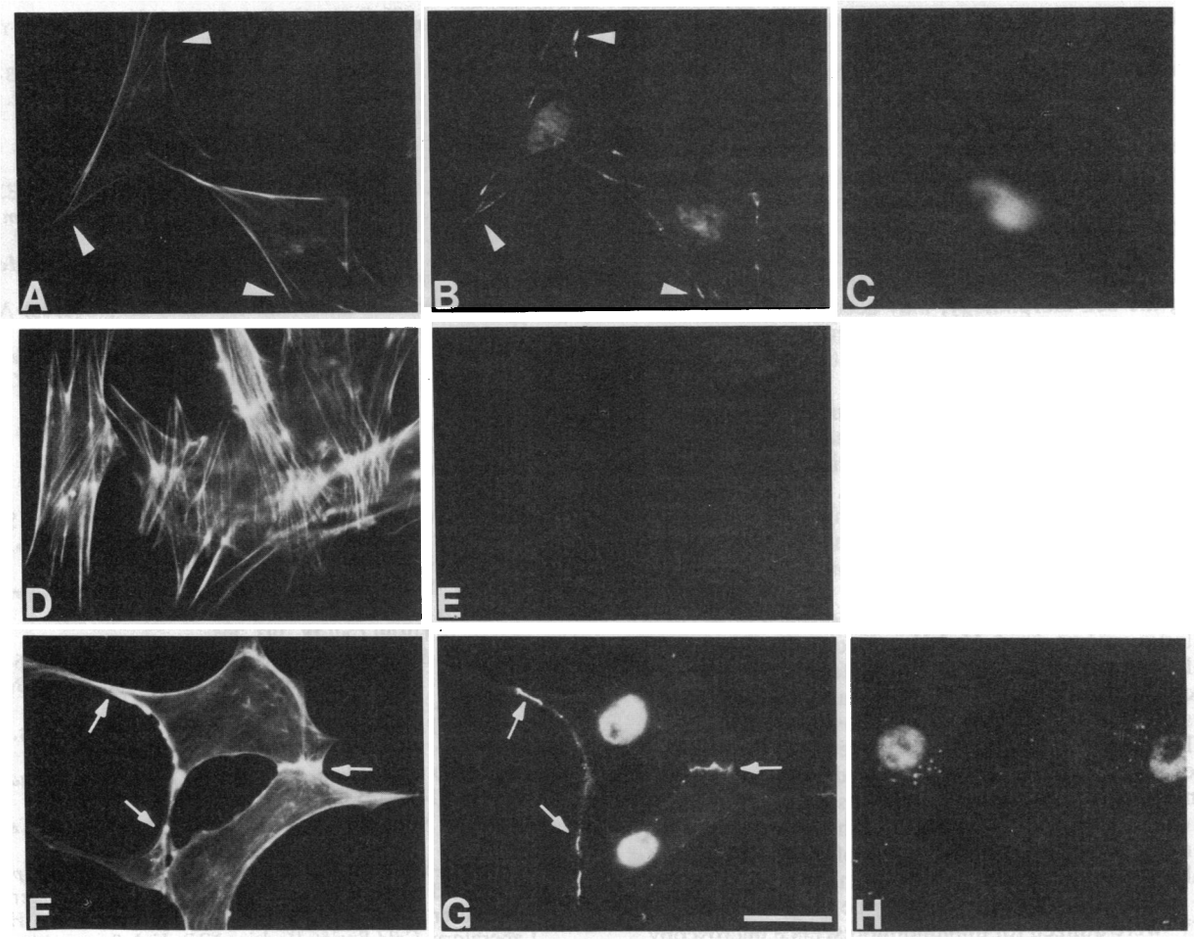

FIG. 2. Double fluorescent labeling of primary cell lines for *P*-Tyr as in Fig. 1 (*B*, *E*, and *G*) and NBD-phallacidin staining of F-actin (*A*, *D*, and *F*). (*C* and *H*) Similar cells were indirectly immunolabeled for *P*-Tyr, but in the presence of excess free *P*-Tyr. (*A–C*) First passage chick embryo fibroblasts. (*D* and *E*) Third passage chick embryo fibroblasts. (*F–H*) First passage chick embryo heart cells. Arrowheads indicate focal contacts and arrows point out cell–cell junctions. In *F* and *G* only small portions of two of the four junction forming cells are visible. The controls (*C* and *H*) are of different specimens from the other photographs of the same cell line. (Bar in *G* = 20 μm.)

specificities or affinities of the two antibodies for *P*-Tyr residues may therefore exist.

The concentration of *P*-Tyr in the focal contacts and intercellular junctions of normal cells is intriguing. The focal contacts are discrete, plaque-like regions at the periphery of the ventral cell surface where fibroblasts and other cells most closely approach the substratum (26, 27) and are the sites of strongest cell–substratum adhesion (28–30). Very similar focal contacts are present at sites of fibroblast cell–cell contact (31, 32). Inside the cell, focal contacts are where the actin-containing microfilament bundles terminate (33, 34) and where the protein vinculin is located (17, 32). Microfilament bundles also are associated with the adherent class of intercellular junctions (35). Focal contacts and intercellular junctions are not only involved in cell–cell adhesion, but in the inhibition of cell motility (31). Therefore, protein modification at the level of the focal contacts and intercellular junctions could modulate many critical properties of normal cells. Indeed, this appears to be an important feature of the infection and transformation of normal fibroblasts by RSV and certain related retroviruses. The tyrosine kinase, $p60^{src}$, encoded by the RSV oncogene, appears to be initially concentrated at fibroblast focal contacts (14, 36) along with vinculin but, when transformation is completed, the focal contacts are largely disrupted (25). Normally vinculin has small but detectable levels of *P*-Tyr but, in such transformed cells, vinculin has been shown to contain elevated levels of *P*-Tyr (37). Furthermore, in normal 3T3 fibroblasts stimulated with platelet-derived growth factor (PDGF), a transient disruption of focal contacts and redistribution of vinculin occurs (38) that closely resembles the more permanent effects produced by RSV transformation. PDGF binding to its specific receptor activates the tyrosine kinase activity of the receptor (6), but it is not known whether vinculin shows a transient elevation in its *P*-Tyr content. Thus, our finding that *P*-Tyr-containing proteins are concentrated at the focal contacts of normal fibroblasts accords with the indications from the RSV-transformed and the PDGF-stimulated cells that focal contacts are important sites of tyrosine kinase regulatory control. It is possible that in normal cells the tyrosine kinase receptors for some growth factors are concentrated in the plasma membrane in regions of focal contacts and may contribute to the *P*-Tyr immunolabeling at those contacts.

We have shown that CEF exhibited detectable but variable *P*-Tyr labeling at focal contacts at the first passage; the intensity of *P*-Tyr labeling at the focal contacts appeared to vary inversely with the density of actin-containing stress fibers from one cell to another. From the discussion in the previous paragraph, this observation is consistent with a correlation between the extent of tyrosine phosphorylation and the disruption of focal contacts, since the latter structures attach the stress fibers to the cell membrane. CEF that were passaged several times showed no detectable *P*-Tyr immunolabeling (Fig. 2*E*), in contrast to the cells at first passage. Correspondingly, the stress fibers (Fig. 2*D*) were

much more prominent. The basis for this depletion in *P*-Tyr labeling after multiple passages of the cells is not clear, but it may reflect some differentiative or aging process. From the limited observations we have so far made, the established normal cell lines exhibited more intense labeling for *P*-Tyr than the primary cell cultures (compare Fig. 1 with Fig. 2), although not as much as transformed cells. This observation is particularly interesting in relation to previous studies (39) showing that permanent cell lines are more easily transformed by tumor viruses than primary cells. Therefore, some of the alterations characteristic of malignancy, but not greatly affecting the cell morphology, may accompany the immortalization of cells adapted to grow indefinitely in culture and be reflected in an increased *P*-Tyr content of the components of the focal contacts.

Enhanced *P*-Tyr levels have been found previously in certain embryonic or differentiating normal cells and tissues. Some authors detected elevated tyrosine protein kinase activity during embryogenesis (18, 20, 40) and elevated expression of $p60^{csrc}$ in neural tissue during development (19, 22). The presence of tyrosyl protein kinases in normal rat liver (41) and normal lymphocyte membranes (42) has also been demonstrated.

As expected, we have found that the presence of *P*-Tyr is particularly evident in A-MuLV-transformed cells (N54). Not only were the focal contacts and intercellular junctions immunolabeled, but substantial cytoplasmic labeling was also observed (Fig. 1*B*). In N54 cells, most of the labeling by anti-*P*-Tyr antibody is probably associated with the 160-kDa tyrosine kinase (the v-abl protein) encoded by the tumor virus and containing the majority of *P*-Tyr residues in the transformed cells. The anti-*P*-Tyr labeling of the N54 cells that we obtained (presumably largely indicative of the distribution of the v-abl protein) is in general agreement with previous results (43) in which antibodies to the *gag* sequence of $p120^{gag\text{-}abl}$ were utilized for immunofluorescence microscopy of A-MuLV-transformed cells.

In summary, a high-affinity antibody that permits the use of immunofluorescence and immunoblotting techniques for the identification of many of the substrates of tyrosine-specific protein kinases, including very minor ones, has proven valuable in an initial screening of various cell lines for their *P*-Tyr content and distribution. Of particular interest is the discovery that, in cells with a normal phenotype, the proteins modulated by tyrosine phosphorylation are concentrated at sites of microfilament–membrane and membrane–substratum interaction, namely, the focal contacts and intercellular junctions. A more detailed characterization of the spectrum of proteins that can undergo phosphorylation of one, or more, of their tyrosine residues will be extremely helpful for understanding, at the molecular level, the role of this protein modification in the regulation of specific cellular functions.

We gratefully acknowledge the excellent technical assistance of Mrs. Margie Adams. We would like to thank Dr. Anne Dutton for the primary chicken heart cells. These studies were supported by National Cancer Institute Grant CA 38006-01 (P.A.M.), National Science Foundation Grant PCM 83-14300 (J.Y.J.W.), and National Institutes of Health Grant GM-15971 (S.J.S.). E.B.P. is a fellow of the Arthritis Foundation. S.J.S. is an American Cancer Society Research Professor. J.Y.J.W. is a Searle Scholar.

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