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Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells

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Transformation of cultured chick lens epithelial cells with a temperature-sensitive mutant of Rous sarcoma virus (tsRSV) leads to radical changes in cell shape and interactions. When cultured at the restrictive temperature (42°C), the transformed cells largely retained epithelial morphology and intercellular adherens junctions (AJ), whereas on switch to the permissive temperature (37°C) they rapidly became fibroblastoid, their AJ deteriorated, and cell adhesion molecules (A-CAM) (N-cadherin) largely disappeared from intercellular contact sites. The microfilament system that was primarily associated with these junctions was markedly rearranged on shift to 37°C and remained associated mainly with cell-substrate focal contacts. These apparent changes in intercellular AJ were not accompanied by significant alterations in the cellular content of several junction-associated molecules, including A-CAM, vinculin, and talin. Immunolabeling with phosphotyrosine-specific antibodies indicated that both cell-substrate and intercellular AJ were the major cellular targets for the pp60$^{v\text{-}src}$ tyrosine-specific protein kinase. It was further shown that intercellular AJ components serve as substrates to tyrosine kinases also in nontransformed lens cells, because the addition of a combination of vanadate and H_2O_2—which are potent inhibitors of protein tyrosine phosphatases—leads to a remarkable accumulation of immunoreactive phosphotyrosine-containing proteins in these junctions. This finding suggests that intercellular junctions are major sites of action of protein tyrosine kinases and that protein tyrosine phosphatases play a major role in the regulation of phosphotyrosine levels in AJ of both normal and RSV-transformed cells.

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Introduction

Intercellular adhesion or attachment to the extracellular matrix are widely believed to play a central role in the regulation of cell and tissue morphogenesis (Ben-Ze'ev, 1986, 1989; Geiger *et al.,* 1987; Geiger, 1989). In recent years major progress has been made in the identification of a multitude of adhesion-related molecules and the elucidation of their roles in the establishment of cell contacts. Such studies include the identification of extracellular adhesion-promoting and -modulating proteins and their respective membrane-bound receptors, the study of specific cell-adhesion molecules (CAMs) that mediate long-term intercellular interactions, and the characterization of cytoskeletal assemblies involved in junction formation and the determination of their mechanical properties and stability (for references see Edelman *et al.,* 1990).

Particularly prominent among the cytoskeleton-bound junctions are adherens-type junctions (AJ), which are specifically associated with the actin-containing microfilament system (Geiger *et al.,* 1985a, 1987). There are two major subfamilies of AJ with distinct structure, molecular architecture, and (presumably) function (Geiger *et al.,* 1985b): cell-matrix AJ such as focal contacts (Burridge *et al.,* 1988) or analogous structures are involved in the anchorage to specific adhesive proteins of the extracellular matrix (ECM), whereas intercellular AJ are apparently involved in a direct interaction between integral membrane CAMs of neighboring cells (Geiger *et al.,* 1985b, 1987; Takeichi, 1988). At the molecular level, it has been shown that, although the two subfamilies share some molecular constituents (i.e., vinculin, α-actinin, and actin), there are some components that are specific for each of them. The cytoplasmic plaque molecule talin (Burridge 1983a,b) and the integrin ECM receptors (Damsky *et al.,* 1985) were shown to be specific for cell-matrix AJ, whereas the plaque molecule plakoglobin (Kapprell *et al.,* 1987) and the transmembrane receptors of the cadherin family (Takeichi, 1988) are uniquely associated with cell–cell AJ.

Still poorly understood are the regulatory mechanisms that control AJ formation and stability. Several lines of evidence have suggested that the expression of different junctional molecules is closely correlated to the actual formation of AJ and may be modulated by specific adhesive stimuli (Ungar *et al.,* 1985; Ben Ze'ev *et al.,* 1986, 1989; Bendori *et al.,* 1987). In other cases it was argued that controlled and localized proteolysis might also be involved in the down regulation of AJ (Volk *et al.,* 1990).

Another possibility that has been considered was the involvement of posttranslational modifications and, particularly, phosphorylation in AJ assembly. This possibility was especially attractive in view of the apparent deterioration of cell adhesions in transformed cells. It had been shown that, on transformation of fibroblastic cells with Rous sarcoma virus (RSV; McNutt *et al.,* 1973; Ash *et al.,* 1976; Wang and Goldberg, 1976; David-Pfeuty and Singer, 1980; Boschek *et al.,* 1981), focal contacts and the associated actin bundles deteriorate. The residual contacts were found to be associated with the RSV-specific oncogene product, $pp60^{v\text{-}src}$ (Rohrschneider, 1980; Nigg *et al.,* 1982) and to contain tyrosine-phosphorylated components (Comoglio *et al.,* 1984; Maher *et al.,* 1985; Takata and Singer, 1988). At the biochemical level, it had been shown that several focal contact proteins are prominent substrates of $pp60^{src}$, including vinculin (Sefton *et al.,* 1981), talin (Pasquale *et al.,* 1986), and integrin (Hirst *et al.,* 1986). The exact physiological effect of phosphorylation on the molecular interactions in AJ is not clear, yet it had been proposed that specific tyrosine phosphorylation of the cytoplasmic tail of the b_1 chain of integrin may be involved in the detachment of the latter from the ECM and/or the cytoskeleton (Horwitz *et al.,* 1990).

Results

Effect of transformation on the morphology of cultured lens cells

Nontransformed chicken lens cells form, in culture, flat epithelioid sheets (Volk and Geiger, 1984, 1986a,b). After transformation with a temperature-sensitive (ts) RSV and incubation at the permissive temperature (37°C), the cells formed dense multiple layers assuming an elongated fibroblastoid morphology (Figure 1, a and b). After elevation of the temperature to the restrictive level (42°C), cells gradually flattened down and regained an epithelioid appearance. These changes were apparent already within 2–4 h (Figure 1, c and d) and were essentially complete within 12–24 h after the temperature shift (Figure 1e). This cellular reorganization was readily reversible, and, after a temperature shift back to 37°C, the cells lost their epithelial morphology and reacquired the transformed phenotype (Figure 1, f–j). Notably, the latter process was somewhat more rapid, and the loss of epithelial morphological characteristics was apparent already within 1–2 h after the temperature shift.

Electron microscopic examination of the transformed lens cells cultured at 37°C disclosed multiple cell layers with numerous areas of intercellular contacts, yet membrane-bound actin filaments were not detected in these contact areas (Figure 2a). Focal contacts were apparent although their abundance was relatively low. In contrast, transformed lens cells, cultured at 42°C (Figure 2b), formed flat monolayers with many focal contacts and conspicuous microfilament-associated AJs, essentially indistinguishable from those of normal cells (see Volk and Geiger, 1986b).

Distribution of AJ components in RSV-transformed chick lens cells

The distribution of A-CAM (N-cadherin) in RSV-transformed cells, maintained at the restrictive temperature, was similar to that found in nontransformed cells (Volk and Geiger, 1986a,b). The labeling was primarily associated with peripheral membrane–associated bands and streaks corresponding to the intercellular AJ belt (Figure 3a). After the shift to 37°C, A-CAM staining gradually disappeared from these contact sites and became more uniformly distributed over the cell surface (Figure 3, b–d). As for the reciprocal process (see Figure 3, e–h), it was noticed that A-CAM became associated with intercellular contact sites within 4 h after the shift to 42°C and reformed apparently normal junctional belts within 24–48 h of incubation at the restrictive temperature. Concomitantly with these changes in A-CAM distribution (Figure 4, a, c, e, and g), several other AJ components underwent major reorganization. As shown in Figure 4 (b) actin, in transformed cells cultured at 37°C, was organized in numerous thin bundles which apparently terminated in cell-substrate focal contacts and were rarely associated with residual A-CAM containing patches or with intercellular contacts. Similarly, a-actinin (Figure 4d) showed a delicate striated distribution along stress fibers. Vinculin was primarily associated with focal contacts, displaying only minor apparent association with residual surface-bound A-CAM (Figure 4f). Talin, a plaque component present only in cell-matrix attachments, re-

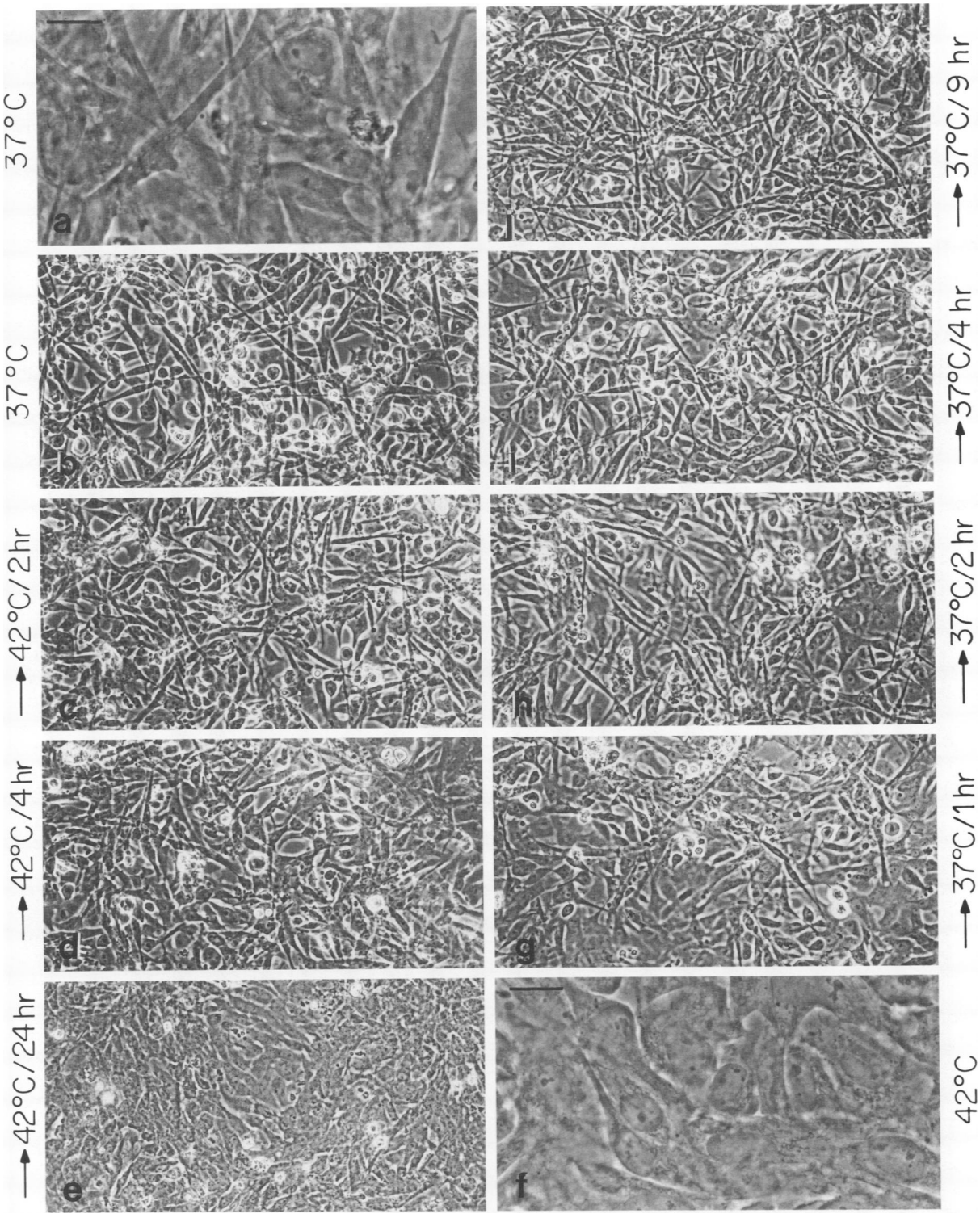
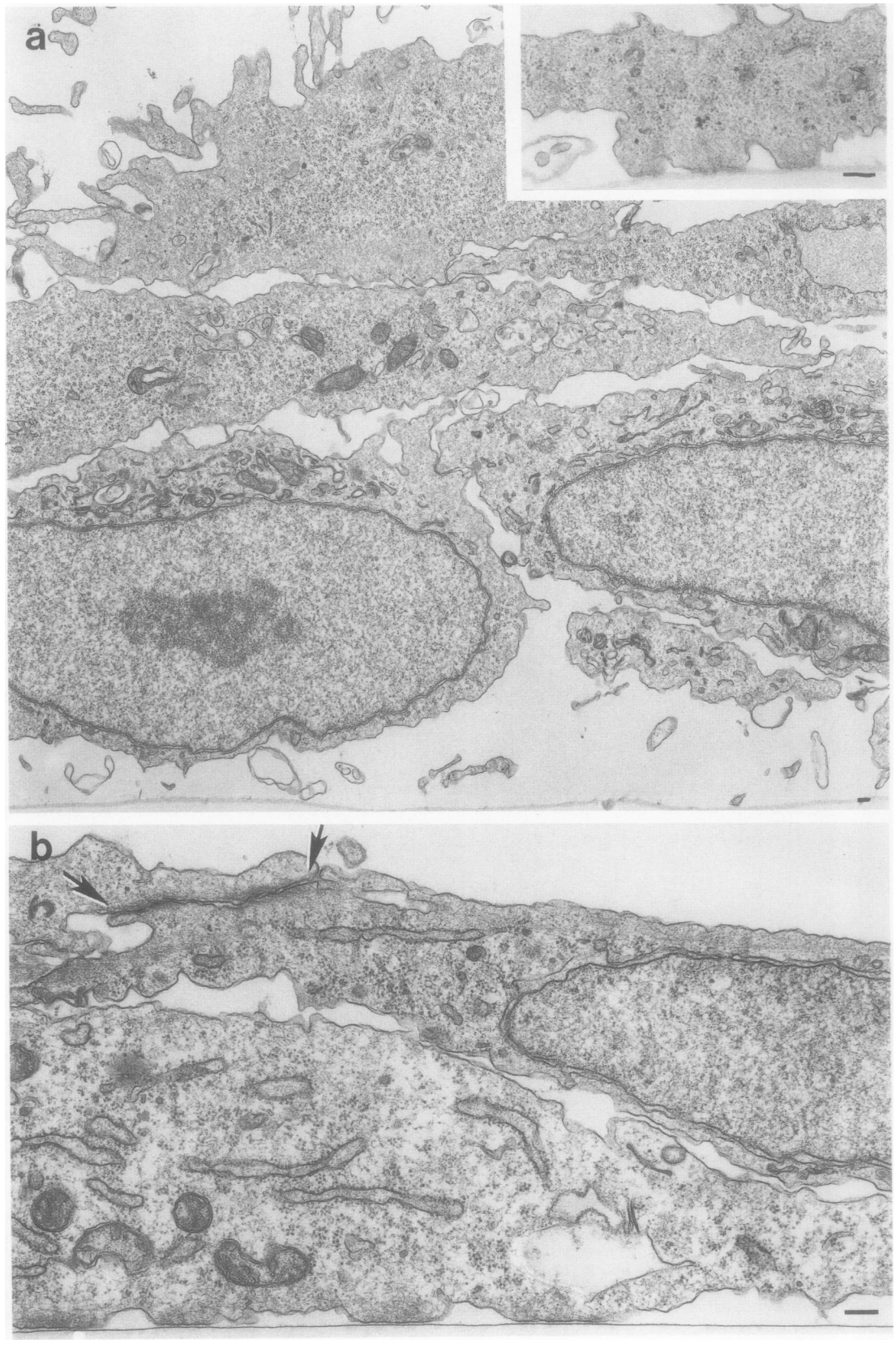


Figure 1. **Effect of transformation by tsRSV on the morphology of cultured chicken lens cells as visualized by phase contrast microscopy.** Cells cultured at the permissive temperature (37°C, a and b) were incubated at the restrictive temperature (42°C) for 2 h (c), 4 h (d), and 24 h (e and f). Notice the gradual transition from fibroblastoid to a "normal" epithelioid morphology. Changes in cell morphology after transition from the restrictive to the permissive temperature (42°C to 37°C) are shown in h–j. Time incubations at 37°C after the temperature shifts are 1 h (g), 2 h (h), 4 h (i), and 9 h (j). Bar indicates 20 mm.


a
b

mained associated with focal contacts (Figure 4h) and exhibited no apparent relationship to A-CAM (Figure 4h).

At 42°C the entire microfilament system of the cultured lens cells underwent reorganization and became primarily associated with the A-CAM rich intercellular AJ (Figure 5, a, c, e, and g). Actin filaments were most prominently present in the junctional belt with only occasional ventral stress fibers (Figure 5b). α-actinin and vinculin exhibited a similar association with the A-CAM-containing junctions (Figure 5, d and f), and their association with focal contacts was apparent mainly at the periphery of epithelial islands. Talin, when present, was exclusively associated with focal contacts at the ventral cell level and showed no relationship to A-CAM (Figure 5h).

Expression of AJ proteins in RSV-transformed chick lens cells

The dramatic decrease in intercellular AJ, associated with the transformed phenotype, raised the possibility that the expression of A-CAM or another junctional molecule was reduced to levels that are not compatible with maintenance of junctional structure, leading to deterioration of AJ. To examine this possibility, we shifted RSV-transformed lens cells from 42°C to 37°C for 24 h and compared, by quantitative immunoblotting, the amounts of talin, A-CAM, and vinculin to those expressed in cultures maintained at 42°C. As shown in Figure 6, there was no apparent difference in the amounts of the three proteins between cells growing under the restrictive and permissive conditions. Moreover, trypsinization of these cultures in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (as in Volk *et al.*, 1990) indicated that A-CAM was fully susceptible to the proteolytic enzyme at both temperatures, suggesting that it was exposed on the cell surface (not shown).

AJ as major cellular targets for tyrosine phosphorylation

To reveal the basis for the modulation of AJ in RSV-infected cells, we have localized tyrosine phosphorylated proteins in normal and transformed lens cells by the use of phosphotyrosine (P-Tyr)-specific antibodies. The results show that tsRSV transformants were strongly labeled both at 37°C (Figure 7b) or 42°C (Figure 7d). Cells at 37°C showed intense and spotty labeling corresponding mainly to small focal contacts (arrow in Figure 7b), whereas cells maintained at 42°C displayed, in addition, an intense labeling of intercellular junctions (Figure 7, b and d, arrowheads). Comparison between P-Tyr and A-CAM labeling indicated that at 42°C both essentially colocalized to the same intercellular junctions, whereas at 37°C only residual cell–cell contacts were detected and P-Tyr was primarily associated with focal contacts.

To localize the major sites of tyrosine phosphorylation in nontransformed cells, primary lens cell cultures were also directly double-labeled for A-CAM and P-Tyr. The results (Figure 8, a and b) indicate that P-Tyr labeling was essentially undetectable in these cells, whereas A-CAM was associated with the intercellular AJ.

In view of the fact that tyrosine-phosphorylated proteins usually constitute <0.1% of the total phosphoproteins (Hunter, 1987) because of the action of potent protein tyrosine phosphatases (PTPases, Chan *et al.*, 1988; Tonks *et al.*, 1988; Lau *et al.*, 1989), we suppressed their activity with specific PTPase inhibitors. We have previously shown (Heffetz and Zick, 1989; Heffetz *et al.*, 1990) that H_2O_2 effectively inhibits intracellular PTPase activity in living cells and that this effect is markedly potentiated in the presence of vanadate. Indeed, as shown in Figure 8d, treatment of normal lens cells for 20 min with a combination of 3 mM H_2O_2 and 1 mM vanadate markedly potentiated protein tyrosine phosphorylation, which localized to A-CAM positive AJ (arrows in Figure 8, c and d).

The profile of protein tyrosine phosphorylation was studied in tsRSV-transformed chicken lens cells cultured at either the permissive or at the restrictive temperature. Total cell homogenates were prepared in a buffer containing phosphatase and protease inhibitors, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti P-Tyr antibodies (Figure 9). As seen in Figure 9, lane g, small amounts of P-Tyr-containing proteins could be detected in nontransformed chicken lens cells. Transformation with the tsRSV resulted in a marked enhancement of

Figure 2. **Electron microscopic examination of tsRSV-transformed lens cells cultured at 37°C (a) and 42°C (b).** Notice that at the permissive temperature the cultures become multilayered and form loose attachments to the substrate, with relatively small, scarce focal contacts (see inset in a). Intercellular contacts are small and apparently devoid of membrane-bound electron dense microfilaments. In the restrictive temperature (42°C) (b) the cells form a flat monolayer with conspicuous focal contact and microfilament containing AJ (marked with arrows). Bar indicates 0.2 mm.

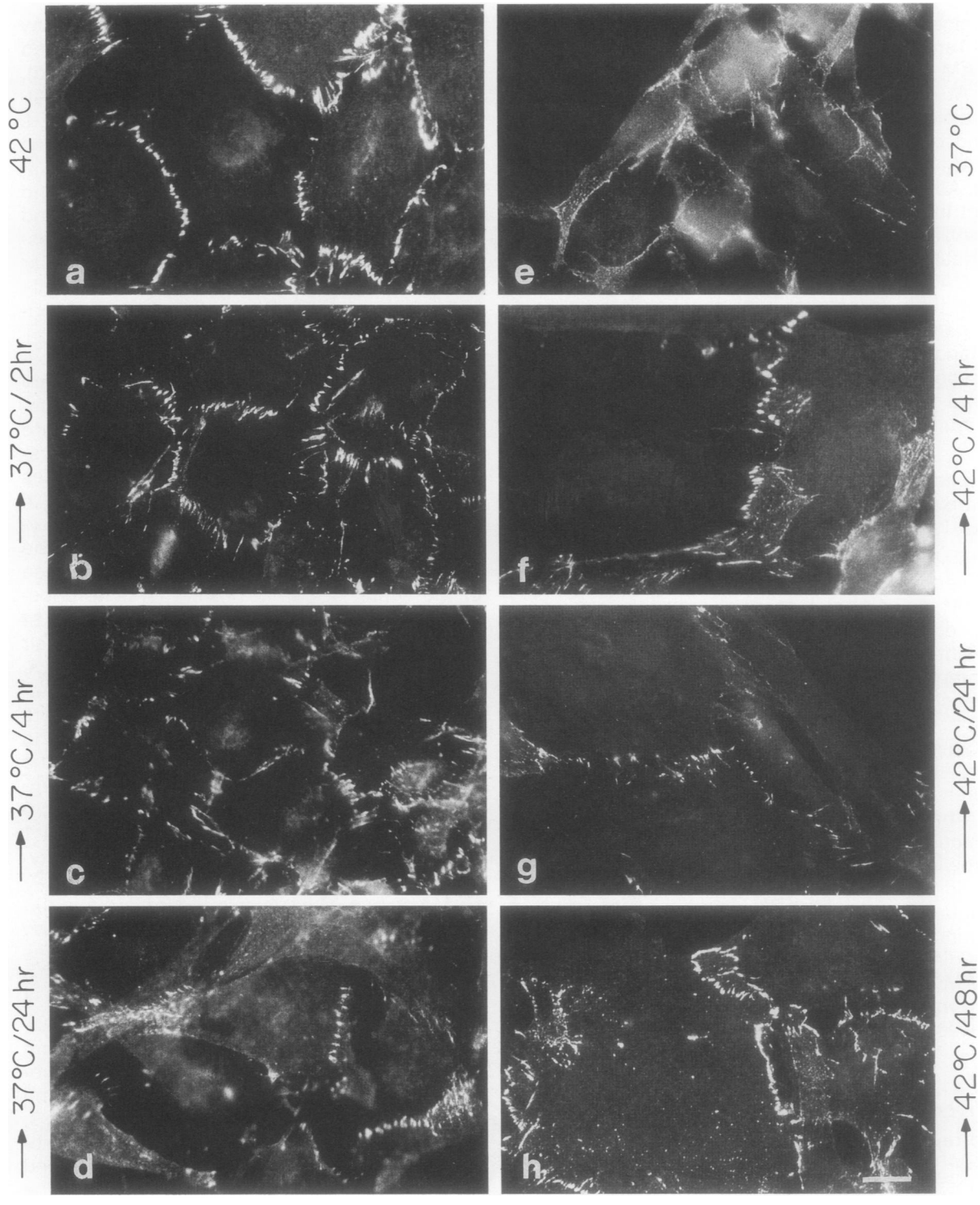


Figure 3. **Effect of transformation on A-CAM distribution in cultured lens cells.** At 42°C the cells display large A-CAM containing AJ (a) that diminish in size after switch to 37°C for 2 h (b), 4 h (c), and 24 h (d). (e–h): the reciprocal shift from 37°C (e) to 42°C (f–h). Incubation periods at 42°C were 4 h (f), 24 h (g), and 48 h (h). Notice that the acquisition of the transformed phenotype is accompanied by progressive loss of A-CAM from cell–cell contact sites and its appearance either in small patches or in diffuse membrane labeling. The switch back to 42°C resulted in a rapid (~4 h) re-reformation of new intercellular junctions. Bar indicates 10 mm.

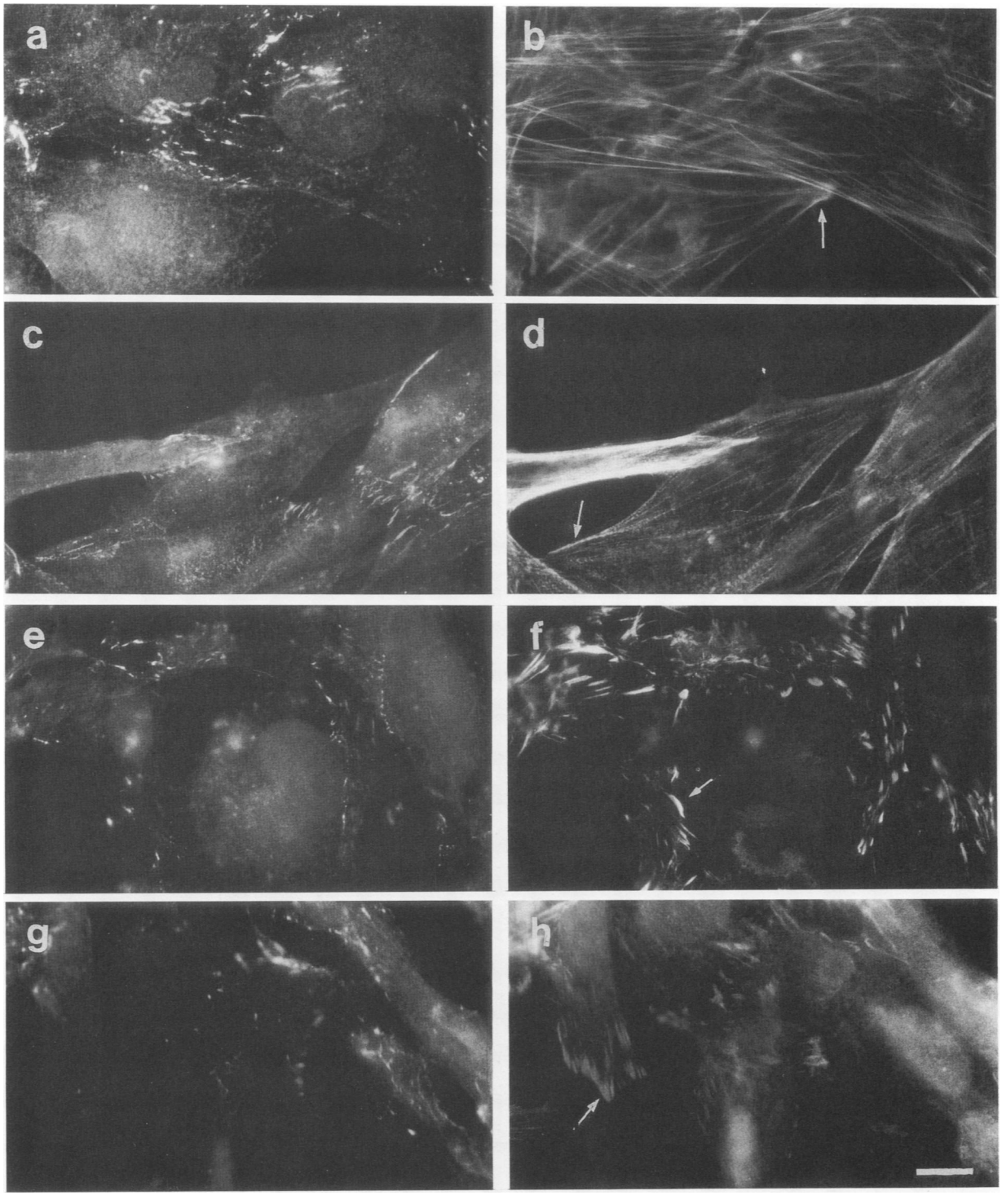


Figure 4. **Distribution of AJ-associated cytoskeletal molecules in tsRSV-transformed chicken lens cells cultured at 37°C.** The cells were double labeled for A-CAM (a, c, e, and g) and actin (b), α-actinin (d), vinculin (f), and talin (h). Notice that A-CAM is nearly absent from cell–cell contacts and that all elements of the microfilament system are apparently associated with cell-substrate focal contacts (arrows) and the associated microfilament bundles. Bar indicates 10 mm.

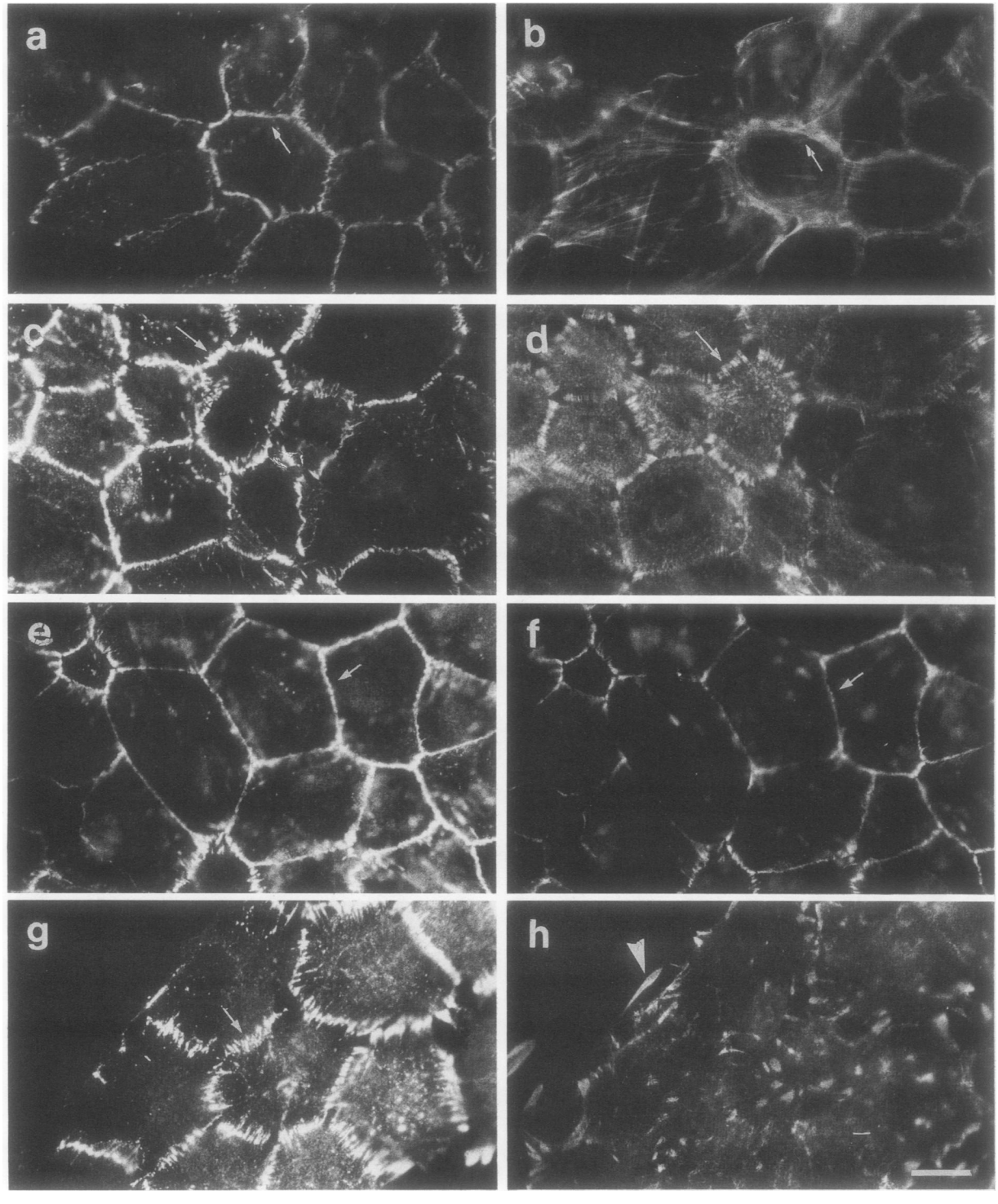


Figure 5. **Distribution of AJ-associated cytoskeletal molecules in tsRSV-transformed chicken lens cells cultured at the restrictive temperature (42°C) for 48 h.** The cells were double labeled for A-CAM (a, c, e, and g) and actin (b), α-actinin (d), vinculin (f), and talin (h). Notice that A-CAM is associated with intercellular AJs in an apparent association with actin, α-actinin, and vinculin (see matching arrows). Talin is exclusively associated with focal contacts (arrowhead in h) and does not show significant relationship to A-CAM. Generally, these patterns are similar to those found in nontransformed lens cells. Bar indicates 10 mm.

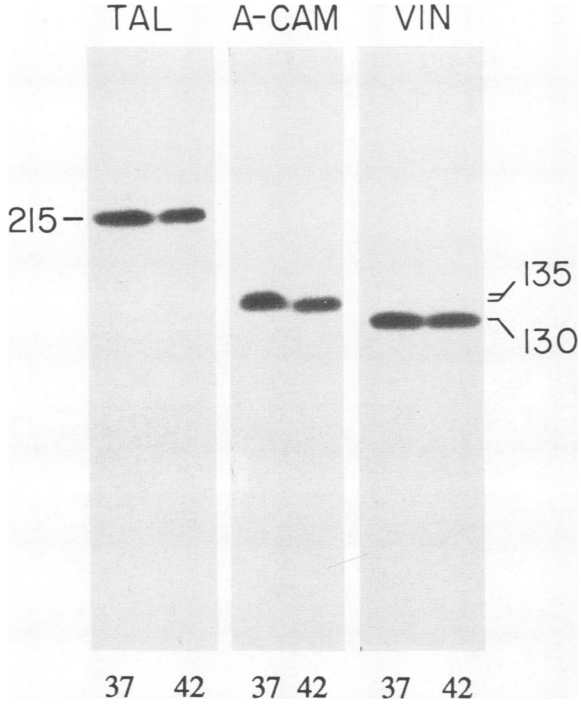


Figure 6. **Quantitative determination of talin, A-CAM, and vinculin in tsRSV-transformed lens cells cultured at 37°C or 42°C.** Identical number of cells were lysed in sample buffer and subjected to quantitative immunoblotting analysis. As shown, no major difference is noted between the amount of the three AJ proteins in cells cultured at the two temperatures. It is notable that A-CAM in the transformed cells cultured at 37°C displays a slightly lower mobility.

protein tyrosine phosphorylation in cells maintained at 37°C (Figures 9, lane i, and 10). Tyr-phosphorylated proteins having molecular masses of 160, 135, 110, 92, 76, 62, 60, and 30 kDa were readily detected. The protein migrating at ~60 kDa presumably corresponds to the $pp60^{v\text{-}src}$. RSV-transformed cells maintained at 42°C (Figure 9, lane h) expressed a similar set of phosphoproteins, although the extent of their phosphorylation was reduced by ~50%. All phosphorylations occurred exclusively on tyrosine residues, as immunoblotting with anti-P-Tyr antibodies could be fully inhibited with 1 mM P-Tyr but not with 1 mM phosphoserine (P-Ser) or phosphothreonine (P-Thr) (not shown).

To avoid PTPase activity, we treated tsRSV-transformed chicken lens cells as well as nontransformed cells for 20 min either with 3 mM H_2O_2 or with a combination of H_2O_2 and sodium orthovanadate as above. As seen in Figure 9, lane b, incubation of nontransformed cells with H_2O_2 enabled detection of two tyrosine-phosphorylated proteins having molecular mass of 140 and 110 kDa. Their phosphorylation was further enhanced 2- to 3-fold when the cells were preincubated with a combination of H_2O_2 and vanadate (Figure 9, lane a). These proteins were most likely localized in intercellular junctions as these areas were prominently labeled with P-Tyr-specific antibodies (Figure 8d). Preincubation for 20 min at 37°C of tsRSV-transformed cells with H_2O_2 potentiated 6- to 10-fold protein tyrosine phosphorylation (compare Figure 9, lane i with f) and an additional 2- to 3-fold increase could be detected on inclusion of vanadate (lane e vs. f). Treatment with H_2O_2-vanadate potentiated phosphorylation of several proteins already detected in ts-RSV-transfected cells maintained at 37°C (e.g., pp160, 110, 76, 60, and 30; see Figure 9 lane e vs. i) and enhanced the phosphorylation of proteins that were undetectable otherwise (e.g., pp120 and pp94). Treatment of tsRSV-transformed cells, maintained at 42°C with H_2O_2 alone (Figure 9, lane d) or in combination with vanadate (Figure 9, lane c), yielded proteins that were tyrosine-phosphorylated to the extent of 20 and 56%, respectively, compared with their counterparts maintained at 37°C (Figure 10). However, the phosphoprotein repertoire was not qualitatively different from that observed in cells maintained at 37°C.

To determine whether A-CAM is specifically phosphorylated on tyrosyl residues, we carried out the following experiment. Normal or RSV-transformed chicken lens cells were treated with H_2O_2-vanadate and lysed, and the extract was chromatographed through a Sepharose-bound anti-P-Tyr column. The original extract and the unbound fraction, as well as fractions specifically eluted with P-Tyr, were examined by immunoblotting with either anti-P-Tyr or anti-A-CAM antibodies. The results indicated that the antibody column effectively adsorbed all (>95%) the P-Tyr-containing proteins and that the latter could be completely and specifically recovered from the column. A-CAM, on the other hand, did not bind to the anti-P-Tyr column (<5%, not shown). It was thus concluded that A-CAM is not one of the substrates for either the $pp60^{v\text{-}src}$ or the endogenous tyrosine-specific protein kinase.

Discussion

The transformation of cells by oncogenic viruses or related oncogenes triggers a cascade of cellular responses leading to major cytomorphological changes and to the modulation of cell growth characteristics (Wang and Goldberg, 1976; Maher *et al.*, 1985; Hirst *et al.*, 1986; Glenney and Zokas, 1989; Tapley *et al.*, 1989). Although the correlation between oncogene

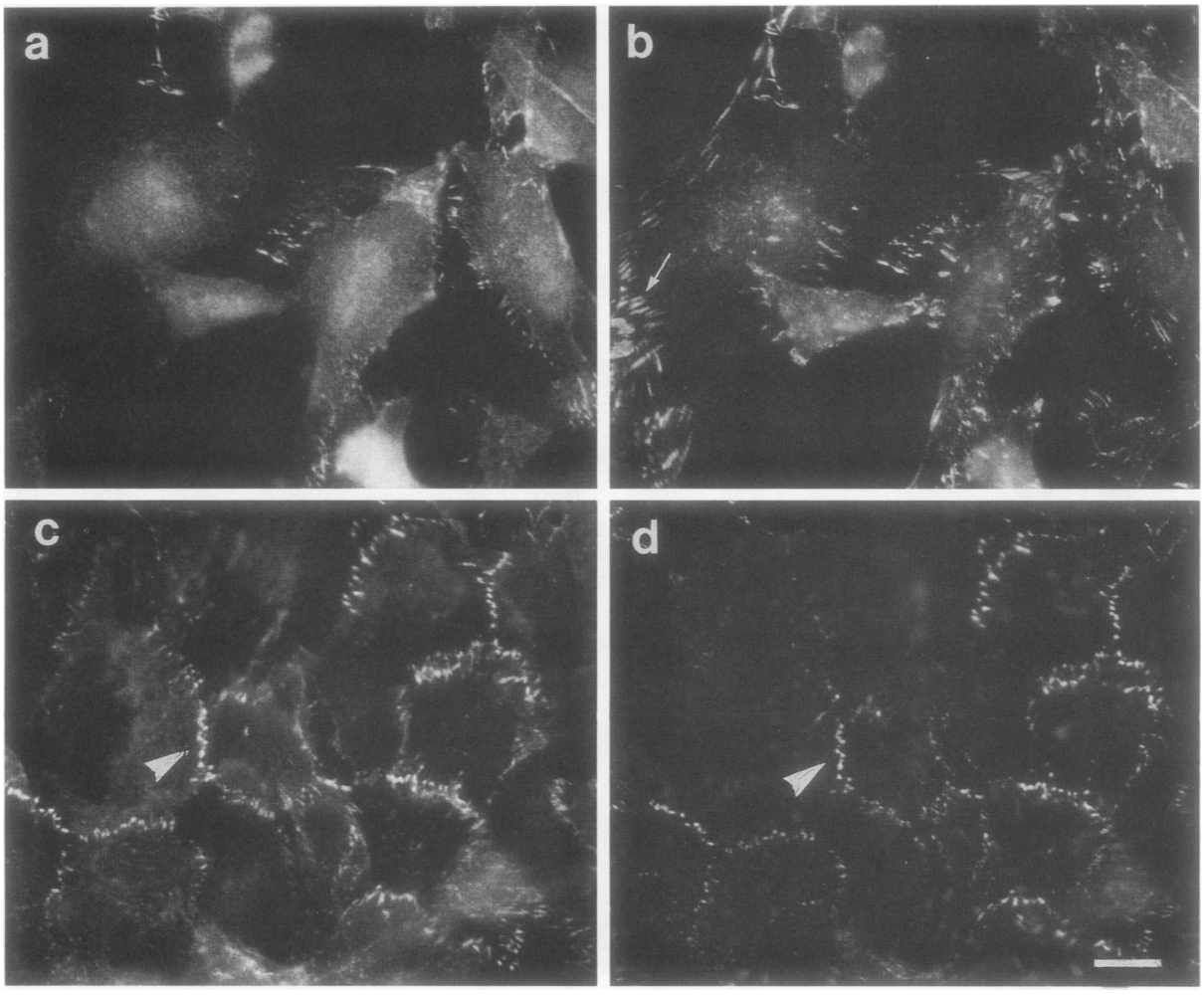


Figure 7. **Double immunofluorescence labeling of tsRSV-transformed lens cells for A-CAM (a and c) and P-Tyr (b and d).** The transformed cells were cultured either at the permissive (37°C) temperature (a and b) or the restrictive (42°C) temperature (c and d). Notice that at 37°C P-Tyr is predominantly associated with focal contacts (arrow in b), whereas at 42°C the A-CAM-containing intercellular junctions are stains (matching arrowheads in c and d). Bar indicates 10 mm.

expression and the acquisition of the transformed phenotype has been extensively documented in recent years, the precise molecular responses to specific oncogenic stimuli is, in most cases, still poorly understood (Hunter and Cooper, 1986; Bishop, 1987; Jove and Hanafusa, 1987). In RSV-infected cells, the oncogenic transformation is known to be triggered by protein tyrosine phosphorylation, catalyzed by the oncogene product $pp60^{v\text{-}src}$ (Comoglio *et al.*, 1984; Maher *et al.*, 1985; Glenney and Zokas, 1989; Hamaguchi *et al.*, 1990; Kozma *et al.*, 1990). It was further shown that in fibroblastic cells $pp60^{v\text{-}src}$ is associated with focal contacts (Rohrschneider, 1980; Nigg *et al.*, 1982) and that several focal contact proteins may be partly phosphorylated by it (Sefton *et al.*, 1981; Maher *et al.*, 1985; Hirst *et al.*, 1986; Pasquale *et al.*, 1986; Tapley *et al.*, 1989; Glenney and Zokas, 1989). Moreover, immunolocalization studies using P-Tyr-specific antibodies have indicated that, in transformed epithelial cells (Maher *et al.*, 1985), including chicken lens cells (Maher and Pasquale, 1988; Takata and Singer, 1988), significant labeling is also detectable along intercellular contact sites. As shown in Figure 8 above, we have detected essentially no labeling with P-Tyr antibodies in nontransformed lens cells, in an apparent contrast with the results of Maher and Pasquale (1988). This could be attributed primarily to the presence of growth factors in the latter cultures or to differences in the sensitivity of the antibodies used for labeling.

In the cellular system studied here, we were able to show that the major contact site affected

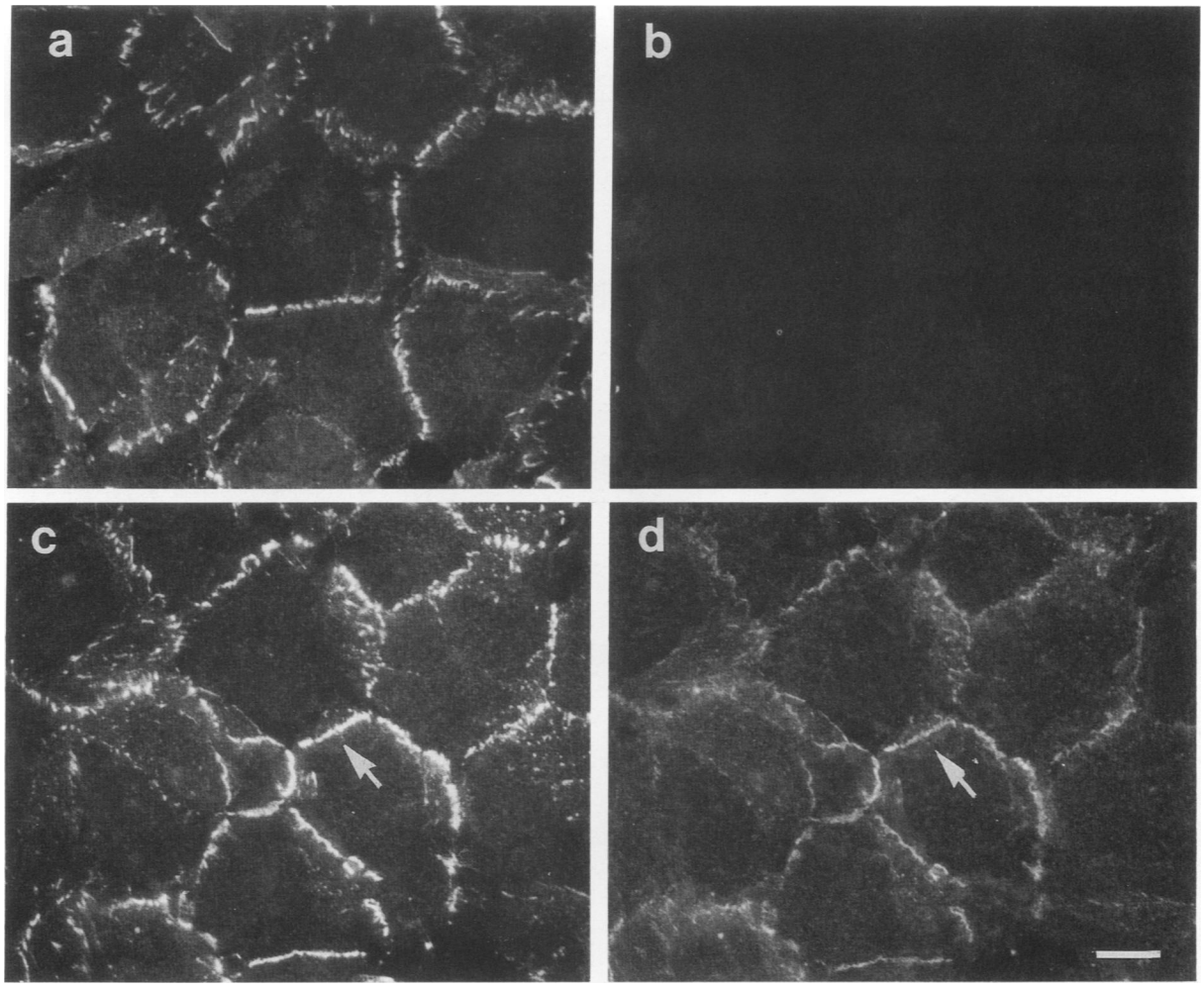


Figure 8. **Double immunofluorescence labeling of nontransformed chicken lens cells for A-CAM (a and c) and P-Tyr (b and d).** The cells were cultured in serum-free medium in the absence (a and b) or presence (c and d) of H_2O_2-vanadate (see text). Notice that the nontreated cells disclose essentially no labeling for P-Tyr, whereas the treated cells display intense labeling along the A-CAM-positive AJ (matching arrows in c and d). Bar indicates 10 μm.

by transformation with tsRSV is the intercellular AJ. Moreover, we show that modulation of AJ in cells, shifted from the permissive to the restrictive temperature or vice versa, leads to major reorganization of the transmembrane junctional "receptor" A-CAM and of the AJ-bound microfilament system. It is noteworthy that, in contrast to fibroblasts (Hirst *et al.,* 1986; Glenney and Zokas, 1989), the effect of transformation in the lens epithelial cells was most prominent on intercellular contacts, whereas focal contacts were affected to a more limited extent and apparently did not develop into podosomes (Tarone *et al.,* 1985). Concomitantly with the changes in intercellular AJ, major effects were noted on cell shape and culture organization: the monolayer of flat epithelioid cells that predominated at 42°C was transformed into multilayered fibroblastoid culture after shift to 37°C.

These changes were reversible on repeated temperature transitions, although the changes occurred at slightly different rates: the acquisition of a transformed phenotype was already apparent 1–4 h after transition to 37°C, whereas the reacquisition of the normal epithelioid appearance required 12–24 h. The basis for these differences is presently not clear.

It is amply documented and widely accepted that transformation with a variety of oncogenic agents may have a profound effect on cell adhesion (McNutt *et al.,* 1973; Ash *et al.,* 1976; Wang and Goldberg, 1976; Boschek *et al.,* 1981); yet adhesive processes may occur via a variety of molecularly distinct junctional systems, and the identity of the affected junction

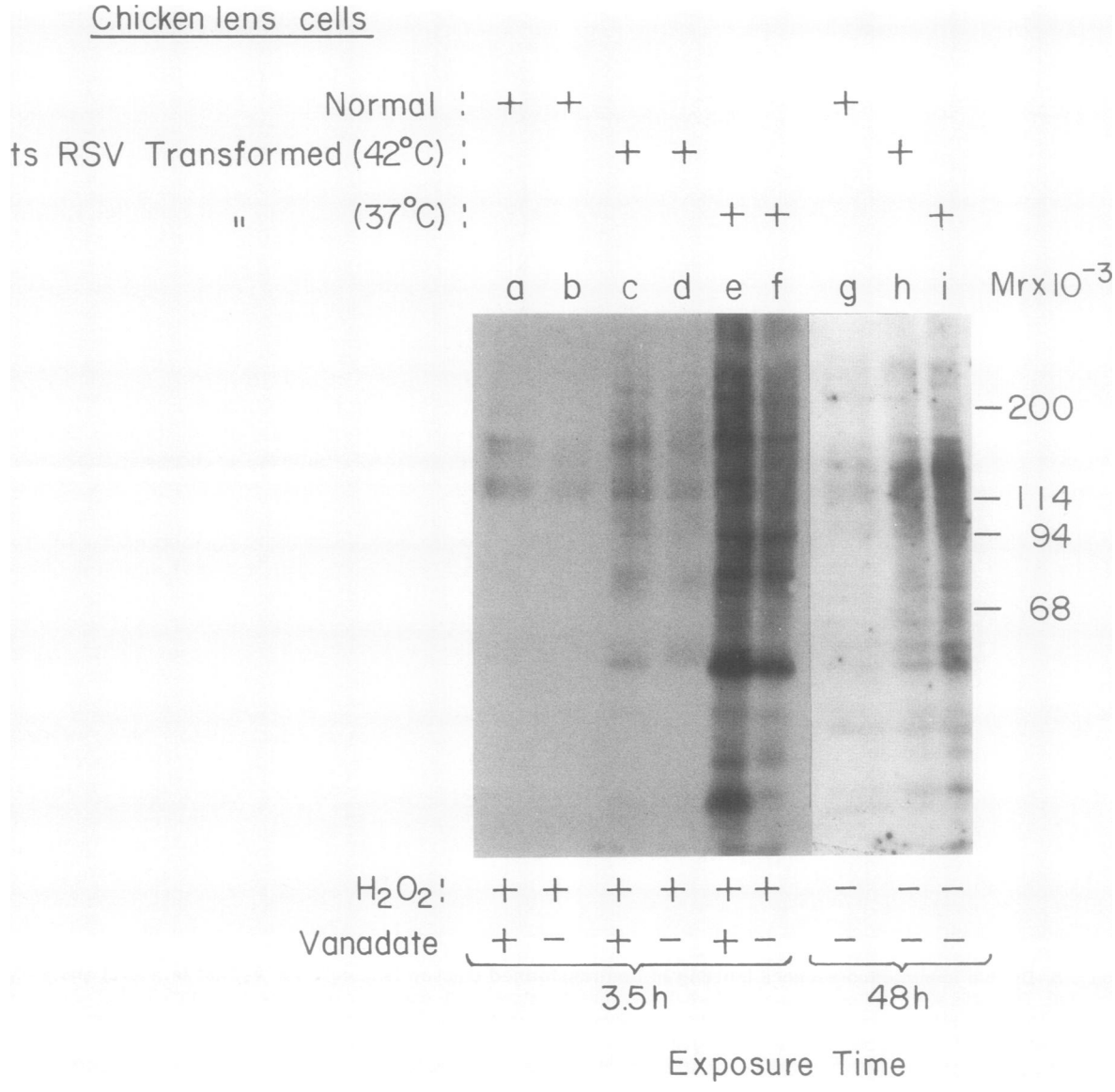


Figure 9. **Effects of H_2O_2 or H_2O_2-vanadate on protein tyrosine phosphorylation in tsRSV-transformed and nontransformed lens cells.** The transformed cells were cultured either at the permissive (37°C) temperature (lanes e, f, and i) or the restrictive (42°C) temperature (lanes c, d, and h). Nontransformed cells were cultured at 37°C (lanes a, b, and g). All three cell lines were deprived of serum for 10 h before the experiment. One hour before treatment, the medium was replaced. Phosphate-buffered saline (lanes g–i), H_2O_2 (lanes a–f), and vanadate (lanes a, c, and e) were added to the medium to yield the final concentrations of 3 mM and 1 mM, respectively. After 20 min incubation, the cells were washed three times with ice-cold PBS and frozen in liquid nitrogen. Cell extracts (50 mg; prepared as described under Methods) were resolved by 10% SDS–PAGE, transferred to nitrocellulose papers and blotted with anti P-Tyr antibodies.

and junctional constituent has not been unequivocally determined. In the case of cultured chick lens cell monolayers, the predominant stable cell–cell junctions present are AJ. Electron microscopic analysis of lens cells, cultured under the conditions described here, indicated that they contain little or no gap junctions and no desmosomes (not shown). Moreover, the ubiquitous association of the microfilament system with the plasma membrane in AJ renders them likely candidates for affecting cell morphology (see Geiger *et al.*, 1987) and thus potential targets for the oncogene action. It should nevertheless be mentioned that Menko and Boettiger (1988) indicated that cultured lens cells maintained under conditions that favor formation of lentoid bodies and gap junctions lose these structures on oncogenic transformation.

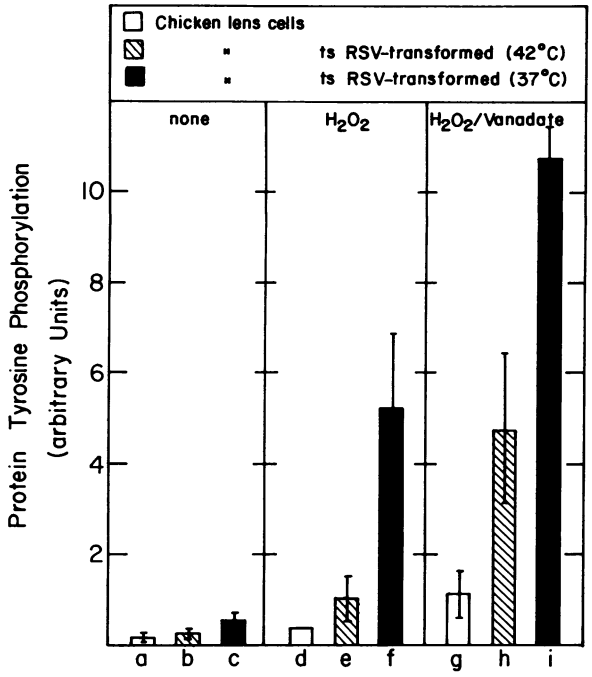


Figure 10. **Quantitative analysis of protein tyrosine phosphorylation detected in tsRSV-transformed and nontransformed lens cells.** The experiment was carried out exactly as described in the legend to Figure 9. The radioactive lanes were excised from the blotted nitrocellulose paper and counted in a g-counter. Results represent means ± SD of two experiments.

In principle, deterioration of AJ could be the result of down regulation of junctional molecules or of post-translational events that disrupt the molecular interactions between AJ components. Several lines of evidence suggest that the latter mechanism is more relevant. These include the rapid reacquisition of AJ on shift to the restrictive temperature and the insensitivity of this process to cycloheximide treatment (Volberg and Geiger, not shown). Moreover, the total amounts of A-CAM, vinculin, and talin were not significantly altered on transition from the permissive to the restrictive temperature or vice versa. It has also been found that A-CAM, under both conditions, was fully exposed on the cell surface.

For RSV-transformed cells protein tyrosine phosphorylation seems to be the posttranslational modification that is most likely involved in the modulation of AJ. This is supported by the fact that the RSV oncogene product $pp60^{v\text{-}src}$ is a potent tyrosine kinase capable of phosphorylating several proteins, including those of cell–substrate and cell–cell AJ (Sefton *et al.*, 1981; Hirst *et al.*, 1986; Pasquale *et al.*, 1986; Tapley *et al.*, 1989). In addition, direct localization of the $pp60^{v\text{-}src}$ pointed to its association with focal contacts (Rohrschneider, 1980; Nigg *et al.*, 1982). Several junctional molecules, including vinculin (Sefton *et al.*, 1981), talin (Pasquale *et al.*, 1986), and integrin (Hirst *et al.*, 1986), have been implicated as targets for $pp60^{v\text{-}src}$, although the nature of the specific intermolecular interactions that are perturbed by their phosphorylation has not been definitively determined. It was recently suggested that phosphorylated integrin might exhibit a reduced affinity for talin (Tapley *et al.*, 1989), yet both talin and integrin are absent from cell–cell AJ, which are the primarily affected site in cultured chick lens cells. It is noteworthy that A-CAM/N-cadherin, which mediates intercellular interactions in the lens cells, does contain several tyrosyl residues, flanking acidic amino acids in its cytoplasmic domain, that might, potentially, be phosphorylated. Yet, direct examination of P-Tyr-containing proteins, purified on anti-P-Tyr column, suggested that A-CAM was not phosphorylated either in the normal or in the RSV-transformed lens cells. Attempts to use antibodies to a battery of junctional molecules to identify relevant targets for the tyrosine-specific protein kinase are presently in progress.

The results presented here further indicate that both normal and transformed intercellular AJ do contain tyrosine kinase activity. Immunofluorescent labeling of tsRSV-transformed cells cultured at 42°C indicated that both cell substrate and intercellular AJ contain P-Tyr, whereas small focal contacts are most heavily labeled at 37°C. Consistent with previous studies (Kozma *et al.*, 1990), the level of tyrosine phosphorylation at the restrictive temperature was significantly lower than that obtained at 37°C, which presumably reflects the leakiness of the lesions—yet the pattern of phosphorylated proteins at the two temperatures was indistinguishable (although much higher and different) from that found in nontransformed cells. This finding is quite intriguing because there was a major difference with respect to the fate of the junction and the localization of A-CAM, vinculin, and the P-Tyr-modified components between the tsRSV-infected cells maintained at the two temperatures. Thus, the limited yet significant phosphorylation of tsRSV-transformed lens cells at 42°C seems to be insufficient for the induction of gross junction disassembly, whereas this process readily occurs at 37°C. It still remains to be determined whether this disassembly reflects some qualitative difference in the phosphorylation of a specific junction proteins or whether the difference just reflects the need for some threshold level of tyrosine phosphorylation of several junctional components.

The application of H_2O_2-vanadate was the key for the localization of proteins phosphorylated on tyrosine residues in nontransformed cells. As shown here as well as in previous reports (Heffetz and Zick, 1989; Heffetz *et al.*, 1990), the addition of H_2O_2 either alone or together with vanadate to cells dramatically augmented the apparent levels of tyrosine phosphorylation, mostly because of the rapid and reversible inhibitory effect of both substances on PTPase activity (Swarup *et al.*, 1982; Heffetz *et al.*, 1990). It is noteworthy that treatment of various cell types with H_2O_2-vanadate, even for $\geq$1 h, had no adverse effect on cell viability, intracellular ATP content, membrane permeability, and Ser/Thr phosphorylation (Heffetz and Zick, 1989; Heffetz *et al.*, 1990; Zick and Sagi-Eisenberg, 1990). Such treatment of normal chick lens cells elevated the overall levels of phosphotyrosine-containing proteins to those observed in RSV-transformed cells. Similar findings were observed when baby hamster kidney fibroblasts were treated with 0.1 mM orthovanadate, although overnight incubations with vanadate were necessary to detect its effects (Marchisio *et al.*, 1988). Yet, within the time frame of our experiments (20 min), AJs in H_2O_2-vanadate-treated cells were apparently unaffected. It is, however, notable that there were significant differences between the phosphotyrosine distribution in tsRSV-transformed (at 42°C) and normal (H_2O_2-vanadate treated) cells: in the former, both focal contacts and intercellular junctions were labeled, whereas in the nontransformed lens cells the labeling was confined to the intercellular junctions. Moreover, major differences were also noted in the P-Tyr-containing proteins between the transformed and nontransformed cells as described above. Whereas in normal (H_2O_2-vanadate treated) cells only two major tyrosine-phosphorylated proteins (pp140 and pp190) were detected, several additional proteins appeared phosphorylated in tsRSV-transfected cells maintained at 37°C. We can therefore conclude that intercellular junctions in normal lens cells are subjected to tyrosine phosphorylation by endogenous, as yet undefined, tyrosine kinases. Their state of phosphorylation is maintained at extremely low levels because of the action of potent PTPases. This conclusion leads us to hypothesize that PTPase may play a key role in regulating cell adhesion. Attempts are currently in progress to identify the various phosphopeptides and determine their functions and cellular localization. It is anticipated that such information will shed light on the regulation and maintenance of AJ and on the involvement of specific tyrosine phosphorylation events in the modulation of cell contacts.

Materials and methods

Cells

Cultured lens cells were prepared from 7- to 8-d-old chick embryos as described. The cells were infected with a ts mutant of Schmitt-Ruppin strain of RSV denoted ts-68 (Kawai and Hanafusa, 1971) in the presence of 8 mg/ml Polybrene (Sigma, St. Louis, MO). Cells were initially cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Biolab, Israel) as described (Volk and Geiger, 1984, 1986a,b). Cells were shifted as indicated to the restrictive temperature in the same medium.

Antibodies

Affinity-purified rabbit anti-P-Tyr antibodies were generated as previously described (Heffetz and Zick, 1989). Rhodamine- and fluorescein-labeled secondary antibodies were prepared and applied as previously described (Geiger *et al.*, 1979). Rhodamine-labeled phalloidine was purchased from Sigma, Anti-A-CAM was a monoclonal antibody ID-7.2.3 (Volk and Geiger, 1984, 1986a,b), now available from Sigma Immunochemicals (St. Louis, MO). Anti-α-actinin (Geiger *et al.*, 1979) and anti-vinculin (Geiger, 1979) were both raised in rabbits; anti-talin was kindly provided by Dr. Keith Burridge, University of North Carolina, Chapel Hill.

Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide slab gels to nitrocellulose papers was performed essentially as described (Burnette, 1981). The transfer was carried out for 3 h at 200 mA in 50 mM glycine, 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 8.8. The nitrocellulose papers were then soaked in buffer A (150 mM NaCl, 10 mM Tris HCl, 0.05% Tween 20, pH 7.6) containing 1% bovine serum albumin (BSA). The blots were incubated for 16 h at 4°C with affinity-purified (1 mg/ml) anti-P-Tyr antibodies. After intensive washing in buffer A, containing 0.1% BSA, the presence of anti-P-Tyr antibodies was determined by adding 3×10^5 cpm/ml of ^{125}I-goat anti-rabbit antibodies for 2 h at 22°C. This was followed by intensive washing in buffer A. The papers were then air dried and autoradiographed.

Electron microscopy

Cultured cells in 35-mm dishes (Falcon, Lincoln Park, NJ) were rinsed and rapidly fixed with 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated, and embedded in Epon (Polybed, 812, Polysciences, Warrington, PA). Sections stained with uranyl acetate and lead nitrate were examined in Phillips EM 410 at 80 kV.

Cell extracts

Cells, cultured on 60-mm tissue culture plates (Falcon), were deprived of serum for 16 h before each experiment to avoid stimulating effects of growth factors present in serum. One hour before treatment, the medium was replaced. H_2O_2 and/or sodium orthovanadate were added to the medium as indicated. After incubation, cells were washed three times with ice-cold phosphate-buffered saline and frozen in liquid nitrogen. Cells were extracted with buffer B (150 mM sucrose, 80 mM b-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 0.1% SDS, 1 mM

phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 5 mg/ml leupeptin, pH 7.6). The cells were scraped off the plates, homogenized, and centrifuged for 15 min at 4°C at 12 000 × *g*. Supernatants were collected and 50-mg aliquots were mixed with concentrated (5-fold) sample buffer (Laemmli, 1970). Samples were examined by 10% SDS-PAGE under reducing conditions.

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References

Ash, J.F., Vogt, P.K., and Singer, S.J. (1976). Reversion from transformed to normal phenotype by inhibition of protein synthesis in rat kidney cells infected with a temperature sensitive mutant of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *73,* 3603–3607.

Bendori, R., Salomon, D., and Geiger, B. (1987). Contact-dependent regulation of vinculin expression in cultured fibroblasts: a study with vinculin-specific cDNA clones. EMBO J. *6,* 2897–2905.

Ben-Ze'ev, A. (1986). The relationship between cytoplasmic organization, gene expression and morphogenesis. Trends Biochem. Sci. *11,* 478–481.

Ben-Ze'ev, A. (1987). The role of changes in cell shape and contacts in the regulation of cytoskeleton expression during differentiation. J. Cell. Sci. (Suppl.) *8,* 293–312.

Ben-Ze'ev, A. (1989). Cell shape and cell contacts: molecular approaches to cytoskeleton expression. In: Cell Shape: Determinants Regulation and Regulatory Role, ed. D. Stein and F. Bronner, San Diego: Academic Press, 95–119.

Bishop, J.M. (1987). The molecular genetics of cancer. Science *235,* 305–311.

Boschek, C.B., Jockusch, B.M., Friis, R.R., Back, R., Grundmann, E., and Bauer, H. (1981). Early changes in the distribution and organization of microfilament proteins during cell transformation. Cell *27,* 175–184.

Burnette, W.W. (1981). "Western blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. *112,* 195–203.

Burridge, K., and Connell, L. (1983a). Talin: a cytoskeleton component concentrated in adhesion plaques and other sites of actin-membrane interaction. Cell Motil. *3,* 405–417.

Burridge, K., and Connell, L. (1983b). A few protein of adhesion plaques and ruffling membranes: J. Cell Biol. *94,* 359–367.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. *4,* 487–525.

Chan, C.P., McNall, S.J., Krebs, E.G., and Fischer, E.H. (1988). Stimulation of protein phosphatase activity by insulin and growth factors in 3T3 cells. Proc. Natl. Acad. Sci. USA *85,* 6257–6261.

Comoglio, P.M., DiRenzo, M.F., Tarone, G., Giancotti, F.G., Naldini, L., and Marchisio, P.C. (1984). Detection of phosphotyrosine-containing proteins in the detergent-insoluble fraction of RSV-transformed fibroblasts by azobenzene phosphonate antibodies. EMBO J. *3,* 483–489.

Damsky, C.H., Knudsen, K.A., Bradley, D., Buck, C.A., and Horwitz, A.F. (1985). Distribution of the cell-substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. *100,* 1528–1539.

David-Pfeuty, T., and Singer, S.J. (1980). Altered distributions of the cytoskeletal proteins vinculin and a-actinin in cultured fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *77,* 6687–6691.

Edelman, G.M., Cunningham, B.A., and Thiery, J.-P. (eds.). (1990). Morphoregulatory Molecules. New York: John Wiley & Sons.

Geiger, B. (1979). A 130 K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. Cell *18,* 193–205.

Geiger, B. (1989). Cytoskeleton-associated cell contacts. Curr. Opinion Cell Biol. *1,* 103–109.

Geiger, B., Avnur, Z., Volberg, T., and Volk, T. (1985a). Molecular domains of adherens junctions. In: Cell in Contact: Adhesions and Junctions as Morphogenetic Determinants, ed. G.M. Edelman and J.-P. Thiery, New York: John Wiley & Sons, 461–489.

Geiger, B., Tokuyasu, K.T., and Singer, S.J. (1979). Immunocytochemical localization of a-actinin in intestinal epithelial cells. Proc. Natl. Acad. Sci. USA *76,* 2833–2837.

Geiger, B., Volk, T., and Volberg, T. (1985b). Molecular heterogeneity of adherens junctions. J. Cell Biol. *101,* 1523–1531.

Geiger, B., Volk, T., Volberg, T., and Bendori, R. (1987). Molecular interactions in adherens-type junctions. J. Cell Sci. Suppl. *8,* 251–272.

Glenney, J.R., and Zokas, L. (1989). Novel tyrosine kinase substrates from Rous sarcoma virus–transformed cells are present in membrane skeleton. J. Cell Biol. *108,* 2401–2408.

Hamaguchi, M., Matsuda, M., and Hanafusa, H. (1990). A glycoprotein in the plasma membrane matrix as a major potential substrate of $p60^{v\text{-}src}$. Mol. Cell. Biol. *10,* 830–836.

Heffetz, D., Bushkin, I., Dror, R., and Zick, Y. (1990). The insulinomimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. J. Biol. Chem. *265,* 2896–2902.

Heffetz, D., and Zick, Y. (1989). H_2O_2 potentiates phosphorylation of novel putative substrates for the insulin receptor kinase in intact Fao cells. J. Biol. Chem. *264,* 10126–10132.

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

Horwitz, A., Bozyczko, D., and Buck, C.A. (1990). The integrin family and neighbors. In: Morphoregulatory Molecules, ed. G.M. Edelman, B.A. Cunningham, and J.-P. Thiery, New York: John Wiley & Sons, 217–230.

Hunter, T. (1987). A thousand and one protein kinases. Cell *50,* 823–829.

Hunter, T., and Cooper, J.A. (1986). Viral oncogenes and tyrosine phosphorylation. Enzymes *17,* 191–243.

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

Kapprell, H.P., Cowin, P., and Franke, W.W. (1987). Biochemical characterization of the soluble form in the junctional plaque protein, plakoglobin, from different cell types. Eur. J. Biochem. *166,* 505–517.

Kartenbeck, J., Schmid, E., Franke, W.W., and Geiger, B. (1982). Different modes of internalization of proteins associated with adhaerens junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material. EMBO J. *1,* 725–732.

Kawai, S., and Hanafusa, H. (1971). The effect of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. Virology *46,* 470–479.

Kozma, L.M., Reynolds, A.B., and Weber, M.J. (1990). Glycoprotein tyrosine phosphorylation in Rous sarcoma virus–transformed chicken embryo fibroblasts. Mol. Cell. Biol. *10,* 837–841.

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

Lau, K.H.W., Farley, J.R., and Baylink, D.J. (1989). Phosphotyrosyl protein phosphatases. Biochem. J. *257,* 23–36.

Maher, P.A., and Pasquale, E.B. (1988). Tyrosine phosphorylated proteins in different tissues during chick embryo development. J. Cell Biol. *106,* 1747–1755.

Maher, P.A., Pasquale, E.B., Wang, J.Y.J., and Singer, S.J. (1985). Phospahotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. Proc. Natl. Acad. Sci. USA *82,* 6576–6580.

Marchisio, P.C., D'Urso, N., Comoglio, P.M., Giancotti, F.G., and Tarone, G. (1988). Vanadate-treated baby hamster kidney fibroblasts show cytoskeleton and adhesion patterns similar to their Rous sarcoma virus–transformed counterparts. J. Cell. Biochem. *37,* 151–159.

McNutt, N.S., Culp, L.A., and Black, D.H. (1973). Contact-inhibited revertant cell lines isolated from SV-40 transformed cells. IV. Microfilament distribution and cell shape in untransformed, transformed, and revertant BALB/c3T3 cells. J. Cell Biol. *56,* 412–428.

Menko, S.A., and Boettiger, D. (1988). Inhibition of chicken embryo lens differentiation and lens junction formation in culture by $pp60^{v\text{-}src}$. Mol. Cell. Biol. *8,* 1414–1420.

Nigg, E.A., Sefton, B.M., Hunter, T., Walter, G., and Singer, S.J. (1982). Immunofluorescent localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic *src* peptide. Proc. Natl. Acad. Sci. USA *79,* 5322–5326.

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

Rohrschneider, L.R. (1980). Adhesion plaques of Rous sarcoma virus transformed cells contain the *src* gene product. Proc. Natl. Acad. Sci. USA *77,* 3514–3518.

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

Swarup, G., Speeg, K.V., Cohen, S., and Garbers, D.C. (1982). Phosphotyrosyl-protein phosphatase of TCRC-2 cells. J. Biol. Chem. *257,* 7298–7301.

Takata, K., and Singer, S.J. (1988). Phosphotyrosine-modified proteins are concentrated at the membranes of epithelial and endothelial cells during tissue development in chick embryos. J. Cell Biol. *106,* 1757–1764.

Takeichi, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development *102,* 639–655.

Tapley, P., Horwitz, A., Buck, C., Duggan, K., and Rohrschneider, L. (1989). Integrins isolated from Rous sarcoma virus–transformed chicken embryo fibroblasts. Oncogene *4,* 325–333.

Tarone, G., Girillo, D., Giancotti, F.G., Comoglio, P.M., and Marchisio, P.C. (1985). Rous sarcoma virus transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. Exp. Cell. Res. *159,* 141–157.

Tonks, N.K., Diltz, C.D., and Fischer, E.H. (1988). Characterization of the major protein-tyrosine-phosphatases of human placenta. J. Biol. Chem. *263,* 6731–6737.

Ungar, F., Geiger, B., and Ben-Ze'ev, A. (1985). Cell contact and cell shape–dependent regulation of vinculin synthesis in cultured fibroblasts. Nature *319,* 787–791.

Volk, T., and Geiger, B. (1984). A 135 Kd membrane protein of intercellular adherens junctions. EMBO J. *3,* 2249–2260.

Volk, T., and Geiger, B. (1986a). A-CAM: a 135 Kd receptor of intercellular adherens junctions. A. Immunoelectron microscopic and biochemical studies. J. Cell Biol. *103,* 1441–1450.

Volk, T., and Geiger, B. (1986b). A-CAM: a 135 Kd receptor of intercellular adherens junctions. B. Antibody-mediated modulation of junction formation. J. Cell Biol. *103,* 1451–1464.

Volk, T., Volberg, T., Sabanay, I., and Geiger, B. (1990). Cleavage of A-CAM by endogenous proteinases in culture lens cells and in developing chick embryos. Dev. Biol. *139 (in press).*

Wang, E., and Goldberg, A.R. (1976). Changes in microfilament organization and surface topography upon transformation of chick embryo fibroblasts with Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *73,* 4065–4069.

Zick, Y., and Sagi-Eisenberg, R. (1990). A combination of H_2O_2 and Vanadate concomitantly stimulates protein tyrosine phosphorylation and phosphoinositide breakdown in different cell lines. Biochemistry *(in press).*