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The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions

Tova Volberg[1], Yehiel Zick[1], Ruth Dror[1], Ilana Sabanay[1], Chaim Gilon[2], Alexander Levitzki[3] and Benjamin Geiger[1,4]

[1]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, [2]Department of Organic Chemistry and [3]Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel

[4]Corresponding author

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Adherens-type junctions (AJs) are major subcellular targets for tyrosine specific protein phosphorylation [Volberg *et al.* (1991) *Cell Regul.*, 2, 105-120]. Here we report on the apparent effect of such phosphorylation events on the assembly and integrity of AJs. We show that incubation of MDCK cells with potent inhibitors of tyrosine-specific phosphatases (PTP), namely H_2O_2 and vanadate, leads to a dramatic increase in AJ-associated phosphotyrosine which was apparent already within 2-5 min of treatment and progressed upon further incubation. Examination of H_2O_2 vanadate treated cells at later time points indicated that intercellular AJs rapidly deteriorated, concomitantly with a marked increase in the number and size of vinculin and actin containing focal contacts. In parallel, major changes were observed in cell structure and topology, as revealed by electron microscopy. These were manifested by rapid rounding-up of the cells followed by reorganization of the cell monolayer. Other intercellular junctions, including desmosomes and tight junctions, visualized by staining with desmoplakin and ZO-I antibodies, were not significantly affected. To verify that modulation of AJs was indeed related to tyrosine phosphorylation, we have carred out reciprocal experiments in which Rovs Sarcoma virus (RSV) transformed chick lens cells, expressing high levels of $pp60^{src}$ kinase, were treated with inhibitors of tyrosine kinases, (tyrphostins). We show that following such treatment, intercellular AJs which were deteriorated in the transformed cells, were reformed. Based on these observations, we propose that specific tyrosine phosphorylation of AJ components is involved in the downregulation of these cellular contacts.

Key words: adherens junctions/cell adhesion/malignant transformation/tyrosine phosphorylation/tyrphostins

Introduction

Adherens junctions (AJs) are a family of microfilament associated cell contacts formed with other cells or with the extracellular matrix (Geiger *et al.*, 1987, 1989, 1990). Attempts to determine the molecular architecture of AJs revealed batteries of proteins which partake in the junctional interactions, including the local association of microfilaments with the membrane. Some of these 'linking' molecules (i.e. vinculin and α-actinin) are present in essentially all AJs while others are selectively associated with either intercellular- or cell-matrix adhesions (Burridge and Connell, 1983a,b; Damsky *et al.*, 1985; Geiger *et al.*, 1985a,b; Kapprell *et al.*, 1987; Burridge *et al.*, 1988; Takeichi, 1988). These molecules, concertedly form extensive multimolecular complexes containing proteins which directly participate in the transmembrane linkage as well as minor components and different enzymes (mostly proteases and kinases), which presumably play regulatory roles (Geiger and Ginsberg, 1991).

Studies on the formation and modulation of AJs pointed to the highly dynamic nature of these cellular structures. In migrating cells for example, new focal contacts are formed under the leading lamella, which later change their size and eventually fade and disappear (Abercrombie, 1980; Burridge *et al.*, 1988; Rinnerthaler *et al.*, 1988). Intercellular junctions are also periodically modulated during processes such as cell migration and mitosis. The physiological mechanisms involved in the modulation of AJs in cells are still poorly understood. As mentioned above, some of the molecular residents of AJs are believed to play a regulatory role, yet information on their nature and precise mode of action is still scarce.

One of the mechanisms implicated in the modulation of AJ mediated interactions involves protein phosphorylation on tyrosyl residues (Comoglio *et al.*, 1984; Maher *et al.*, 1985; Takata and Singer, 1988; Volberg *et al.*, 1991). This notion is supported by several lines of evidence: (i) over-expression of oncogenic protein tyrosine kinases (PTK) in cells is accompanied by reduction in adhesion and deterioration of AJ; (ii) some of the prominent AJ constituents including vinculin (Sefton *et al.*, 1981), talin (Pasquale *et al.*, 1986), paxilin (Glenney and Zokas, 1989) and possibly integrin (Hirst *et al.*, 1986) are primary substrates for these PTK oncogenes (see also Kellie *et al.*, 1991); (iii) there is evidence suggesting that tyrosine phosphorylation affects intermolecular interactions (i.e. talin-integrin) in AJs (Horwitz *et al.*, 1990); (iv) AJs, even in normal cells, are major sites of cellular PTK activity. The latter notion is supported both by immunolabeling of embryonic tissues with anti-phosphotyrosine (Takata and Singer, 1980) and by the dramatic increase in junctional phosphotyrosine levels following inhibition of phosphotyrosine phosphatases (PTP) by vanadate in conjuction with H_2O_2 (Volberg *et al.*, 1991). Such inhibition indicated that cell-cell AJ, cell-matrix AJ or both contain high levels of P-Tyr (up to 60 to 50-fold over background) following 10-30 min treatment with H_2O_2/vanadate). This also indicated that the levels of P-Tyr in cells are controlled by a highly dynamic balance PTK and PTP.

In the present study we have perturbed the PTK/PTP balance by specifically inhibiting tyrosine phosphorylation

or dephosphorylation and examined the effect of this treatment on the integrity of AJs and the organization of the associated cytoskeleton. We show here that addition of H_2O_2/vanadate to cultured epithelial cells results in the induction of a rapid increase in P-Tyr levels along intercellular AJs. Upon prolonged incubation (>30 min) these junctions and the associated actin bundles deteriorated while vinculin containing focal contacts and stress fibres became abundant. Unlike AJs, desmosomes and ZO-1 containing tight junctions were not significantly affected by the H_2O_2/vanadate treatment.

In a reciprocal set of experiments chick epithelial cells, infected with Rous Sarcoma Virus (RSV), were treated with PTK inhibitors (tyrophostins). Notably following several hours of treatment, intercellular AJs were reformed in the transformed cells and epithelioid morphology restored. These observations support the notion that tyrosine phosphorylation is involved in the specific modulation of AJ mediated interactions.

Results

Tyrosine phosphorylation in H_2O_2/vanadate treated MDCK cells

Addition of H_2O_2/vanadate to cultured MDCK cells resulted in a rapid and dramatic increase in the cytoplasmic levels of P-Tyr as observed by immunoblotting assay (Figure 1). The presence of Tyrosine phosphorylated bands was already apparent 2 min after addition of H_2O_2/vanadate and its levels increased upon further incubation up to 1−2 h. Since prolonged exposure to vanadate and H_2O_2 often resulted in rounding up and detachment of cells, we have restricted the incubation to 15 min and replaced the H_2O_2/vanadate containing medium with normal medium for the rest of the incubation period. The major bands undergoing phosphorylation were distinct from those previously detected in RSV transformed chicken lens cells (Volberg *et al.*, 1991) and included major bands with apparent molecular masses of 180, 160, 130, 114, 100, 80−90, 68, 50−55 and 47 kDa. The reactivity of the anti P-Tyr antibodies was apparently specific since it could be completely abolished by 100 μM P-Tyr but not by P-Ser or P-Thr, even when applied at 10-fold higher concentrations (Heffetz and Zick, 1989).

Immunofluorescent microscopic localization of the P-Tyr containing components in H_2O_2/vanadate treated MDCK cells indicated that the labeling was specifically associated with areas of intercellular contact (Figure 2). Following 2 min of treatment only scattered groups of cells were positively labeled, displaying somewhat variable intensities while other cells in the same cultures were still negative (Figure 2b). Longer exposure (5 min) to H_2O_2/vanadate resulted in an intensely labeled band at the periphery of essentially all cells (Figure 2c), which became somewhat more restricted in width apparently associated with intercellular junctions following incubation for additional 10 min (Figure 2d). Interestingly, longer incubation of the cells (beyond 15 min) following H_2O_2 treatment, resulted in weaker and apparently discontinuous labeling at the cell periphery. Incubation of cells for 2 h after the H_2O_2/vanadate treatment resulted in largely diffuse staining and nearly complete loss of junctional P-Tyr labeling. Double immunolabelling of H_2O_2/vanadate treated MDCK cells for both vinculin and P-Tyr indicated that only cell−cell AJs were labeled for the latter while vinculin-rich focal contacts did not contain detectable P-Tyr (Figure 3).

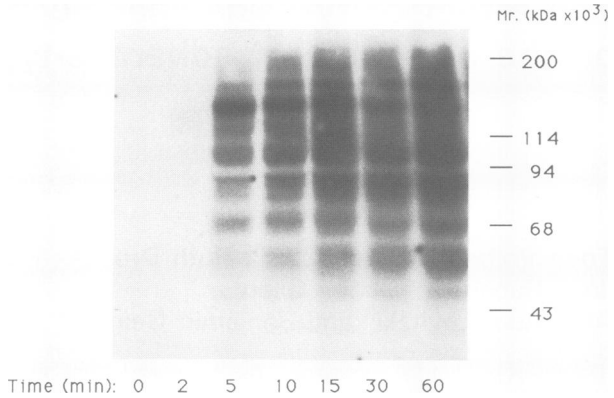


Fig. 1. Effect of H_2O_2/vanadate on the presence of P-Tyr in MDCK cells. Cells were incubated with H_2O_2/vanadate mixture for up to 15 min. For longer incubations, normal medium was restored after 15 min pulse with H_2O_2/vanadate and incubation continued as indicated. Cells were harvested in buffer B and subjected to Western blot analysis using P-Tyr specific, affinity purified rabbit antibodies followed by ^{125}I-labeled goat anti-rabbit antibodies. All lanes contained equal amounts of protein.

Effect of H_2O_2/vanadate treatment on AJ in MDCK cells

Double immunofluorescence labeling of MDCK cells for actin and vinculin, before and after H_2O_2/vanadate treatment revealed dramatic reorganization of AJ and the associated microfilament system. In confluent non-treated cells most of the actin and vinculin labeling (Figure 4a and b) was associated with the sub-apical circumference of the cells. In less confluent regions some stress fibres could also be detected close to the ventral focal plane, associated with vinculin-rich focal contacts (not shown, see for example Geiger, 1979).

Short exposure (15 min) to H_2O_2/vanadate already had a significant effect on the distribution of both cytoskeletal proteins; the circumferential actin ring became only partly associated with the intercellular junction while the rest of it contracted centripetally (Figure 4d). Vinculin was not associated with the contracting belt and still remained largely associated with cell−cell contact areas (Figure 4d). The peripheral vinculin staining became, however, quite discontinuous, often displaying laterally aligned radial striations.

Following longer incubation (30 min), well defined junction associated actin bundles deteriorated and stress fibres, associated with vinculin-rich focal contacts, progressively developed within the cells (Figure 4e and f). After 2 h of incubation vinculin and actin containing intercellular junctions were no longer detectable and the cells developed an elaborate ventral network of vinculin associated actin stress fibres (Figure 4h and g). Staining of cells for uvomorulin confirmed that AJs indeed deteriorated following long H_2O_2/vanadate treatment though the loss of junctional staining was partly masked since significant amounts of uvomorulin are present in these cells throughout the basolateral membrane (not shown, see also Gumbiner and Simons, 1986).

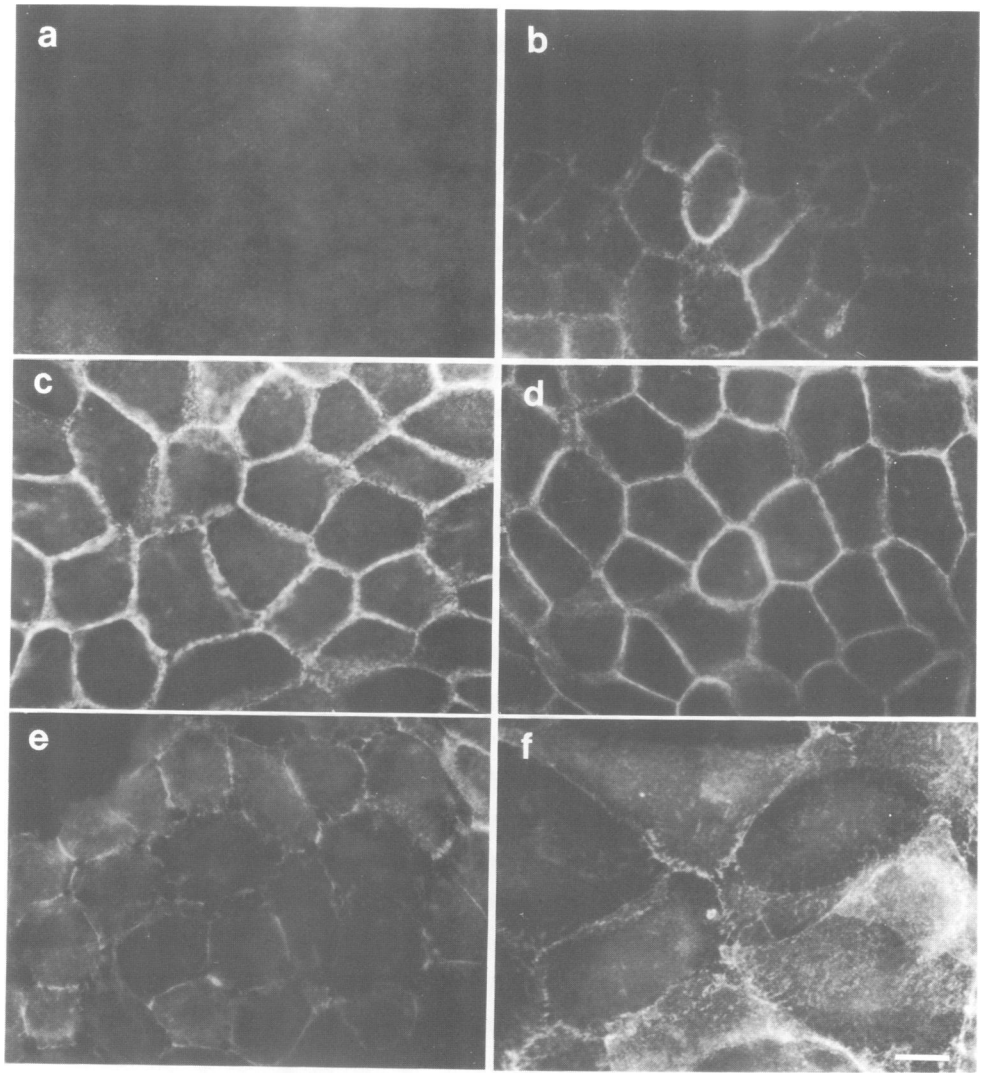


Fig. 2. Effect of H_2O_2/vanadate on the distribution of P-Tyr in cultured MDCK cells. Cells were exposed to the vanadate and H_2O_2 for different intervals [0 min (**a**), 2 min (**b**), 5 min (**c**), 15 min (**d**), 30 min (**e**) and 120 min (**f**)], then fixed–permeabilized and indirectly immunofluorescently labeled with P-Tyr-specific antibodies. The bar indicates 10 μm.

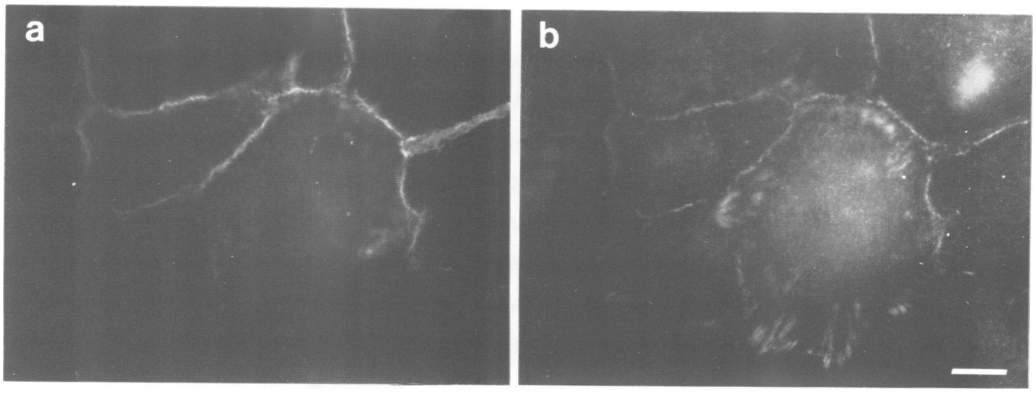


Fig. 3. Double immunofluorescence labeling of MDCK cells for P-Tyr (**a**) and vinculin (**b**), following 15 min of incubation with H_2O_2/vanadate. Note that the vinculin-containing cell–cell junction is positively labeled, while the vinculin-rich focal contacts contain no detectable P-Tyr. The bar indicates 10 μm.

These changes in AJ structure and cytoskeletal organization were accompanied by conspicuous changes in cell morphology. Transmission electron microscopic analysis indicated that untreated MDCK cells form a coherent, well organized monolayer with typical junctional complexes at the subapical region (Figure 5a and b). The apical aspects of the cells are rather flat, running nearly parallel to the substrate with sparsely distributed microvilli. As early as

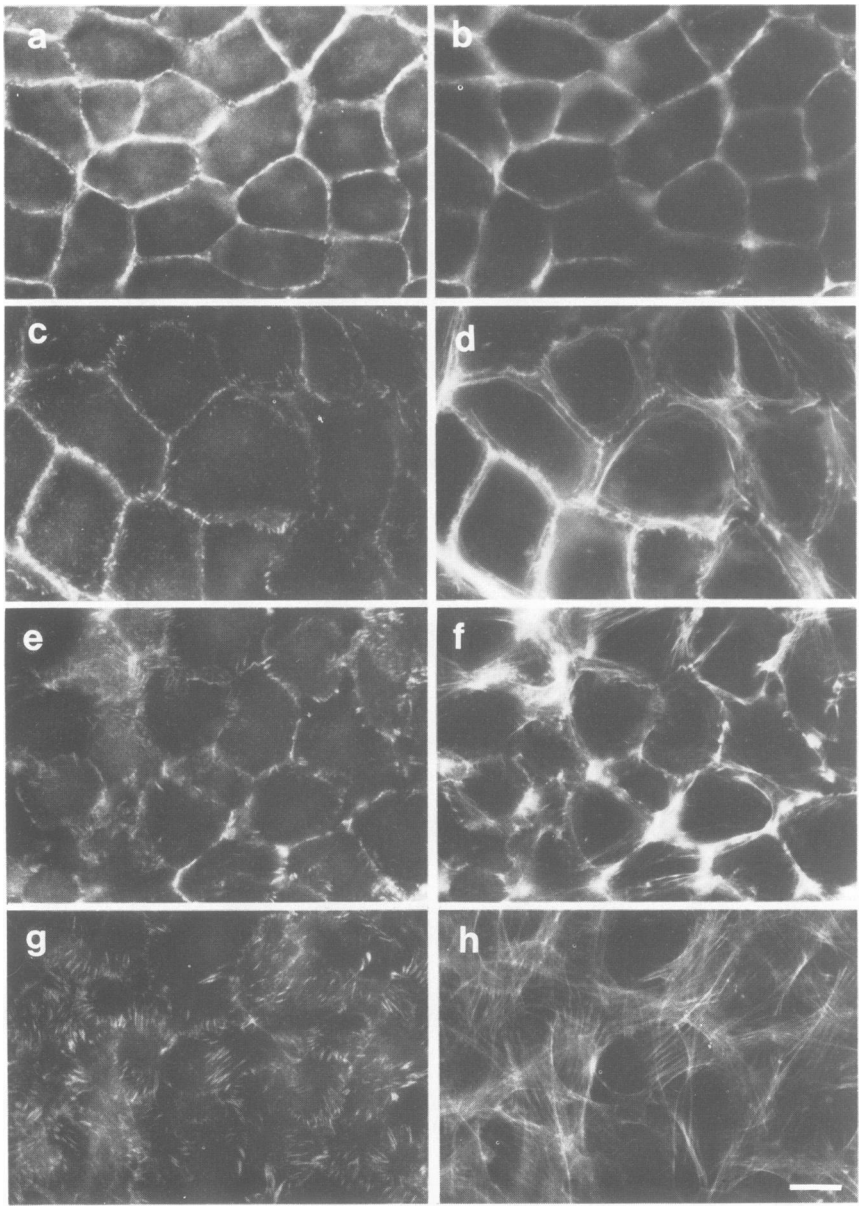


Fig. 4. Effect of H_2O_2/vanadate treatment on the distribution of vinculin (**a, c, e** and **g**) and actin (**b, d, f** and **h**) in cultured MDCK cells. The incubation times were 0 min (a and b), 15 min (c and d), 30 min (e and f) and 2 h (g and h). Note the disappearance of the circumferential microfilament belt and formation of an elaborate network of stress fibres. Bar indicates 10 μm.

15 min after addition of H_2O_2/vanadate to the medium, rounding up of the apical cell surfaces was noted (Figure 5c). This process was accompanied by the formation of large protrusions and blebs over the apical cell surface. Within several hours of treatment the overall organization of the culture was perturbed and multilayers of cells were frequently noted.

Notably, unlike AJs which were apparently affected by H_2O_2/vanadata treatment, desmosomal junctions were retained even after prolonged incubation (see insert in Figure 5f). This notion was further confirmed by immunolabeling of H_2O_2/vanadata treated cells with antibodies to desmoplakin (Figure 6). As shown, the pattern of desmosome distribution was somewhat altered by the treatment, yet elaborate arrays of desmosomes were still detected at intercellular contacts.

The third member of the junctional complex, namely the tight jucntion was also affected to only limited extent by H_2O_2/vanadate treatment. Since these junctions in MDCK cells cannot always be definitively identified by regular transmission electron microscopy, we have examined the distribution of a tight junction proteins, ZO-1 and cingulin by immunoflourescence microscopy. As shown in Figure 7, the continuous junctional belt present in non-treated cells became somewhat fragmented, yet it was prominently retained in areas of contact at the cell periphery even after relatively long H_2O_2/vanadate treatment.

Restoration of AJ in RSV-transformed cells treated with tyrphostins (PTK inhibitors)

To substantiate further the relationships between tyrosine phosphorylation or dephosphorylation and AJ formation we have added PTK inhibitors to RSV transformed cells which overexpress the PTK $pp60^{v\text{-}src}$. It was previously

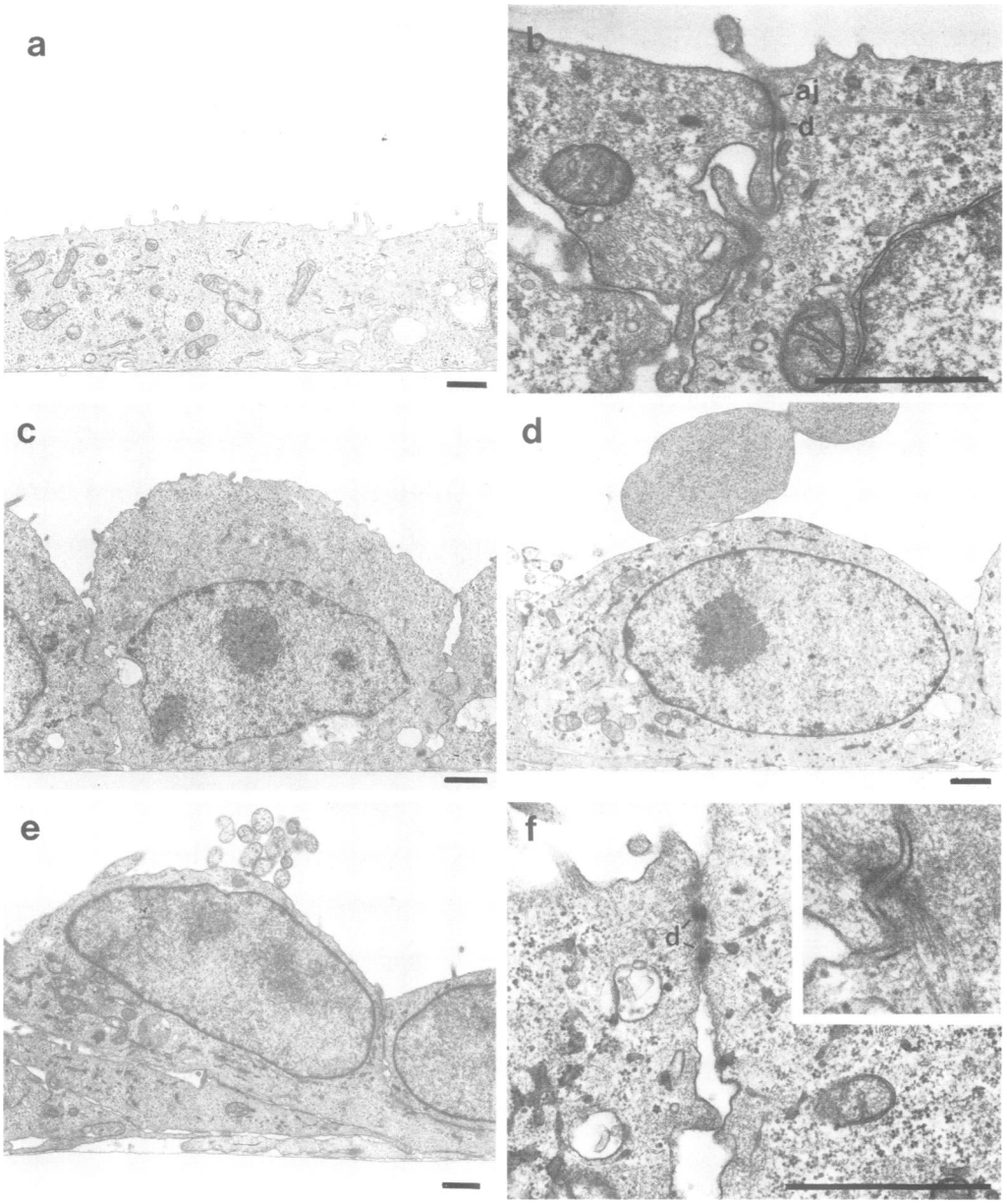


Fig. 5. Transmission electron micrographs showing the effect of H_2O_2/vanadate on the morphology of MDCK cells. Untreated cells (**a** and **b**) formed a coherent monolayer, displaying flat apical surface and intact junctional complexes containing tight junctions, adherens junctions (aj) and desmosomes (d). H_2O_2/vanadate-treated cells were fixed following different incubation times including: 15 min (**c**), 30 min (**d**) and 4 h (**e** and **f**). Notice the perturbation of the monolayer morphology and the rounding-up of the cells following the treatment. Desmosomes are, nevertheless, retained at intercellular interfaces even after prolonged treatment (see insert in f). The bar indicates 0.2 μm.

demonstrated (Volberg *et al.*, 1991) that transformation of cultured chicken lens cells with RSV leads to an essentially complete deterioration of intercellular AJ. We have added different tyrphostins to such cells and examined their effect on P-Tyr and cadherin distribution. As shown in Figure 8a, P-Tyr labeling in non-transformed lens cells was essentially negative, while cells transformed with a temperature sensitive mutant of RSV were brightly stained at both temperatures. In cells maintained at the restrictive temperature, P-Tyr labeled intercellular junctions became most prominent (Figure 8c), while at the permissive temperature junctional staining was essentially absent and only focal contacts were labeled (Figure 8b). Addition of certain tyrphostins to the transformed cells at the permissive temperature resulted in the appearance of prominent P-Tyr containing intercellular junctions (Figure 8d and Table I).

To confirm that the added tyrphostins indeed induced the *de novo* assembly of AJs in the RSV transformed cells we have also labeled the same cultures for *N*-cadherin (Volberg *et al.*, 1991). As shown in Figure 9, non-transformed lens cells had prominent AJs which disappear upon RSV transformation (Figure 9b). Shifting of the transformed cells from the permissive to the non-permissive temperature resulted in the reacquisiton of a more epithelioid morphology, concomitantly with the establishment, within 2 to 4 h, of *N*-cadherin containing AJs (Figure 9c). The restoration of junctions was also confirmed by electron microscopy, essentially as previously described (Volberg *et al.*, 1991). Addition of AG-18 to transformed cells maintained at the permissive temperature induced the formation of cell–cell AJs, indistinguishable from those of non-transformed lens cells (Figure 9d, compare to Figure 9a). It is noteworthy

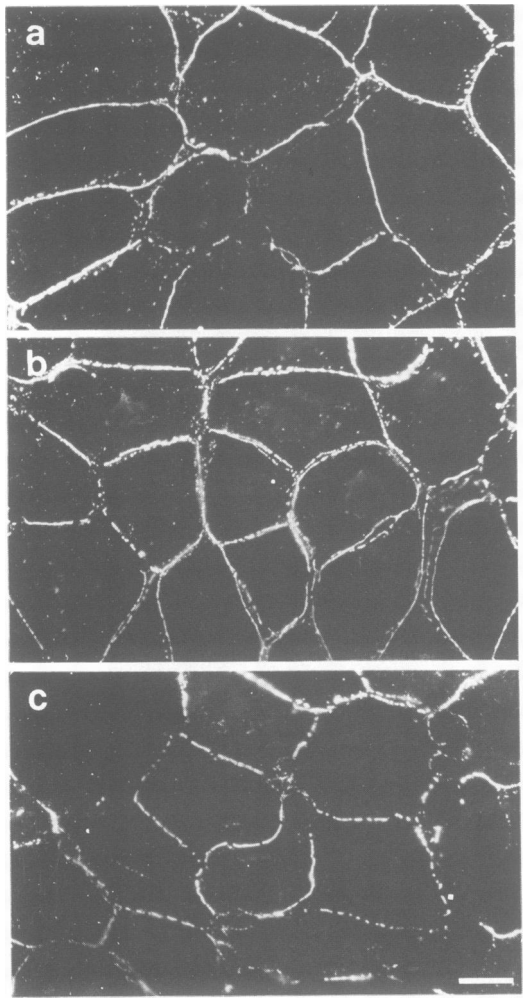


Fig. 6. Distribution of desmosomes in cultured MDCK cells following treatment with H_2O_2/vanadate. Desmosomes were visualized here by immunofluorescence labeling using anti desmoplakin antibodies. Untreated cells are shown in (**a**), cells 15 min and 2 h following H_2O_2/vanadate treatment are shown in (**b**) and (**c**), respectively. Bar indicates 10 μm.

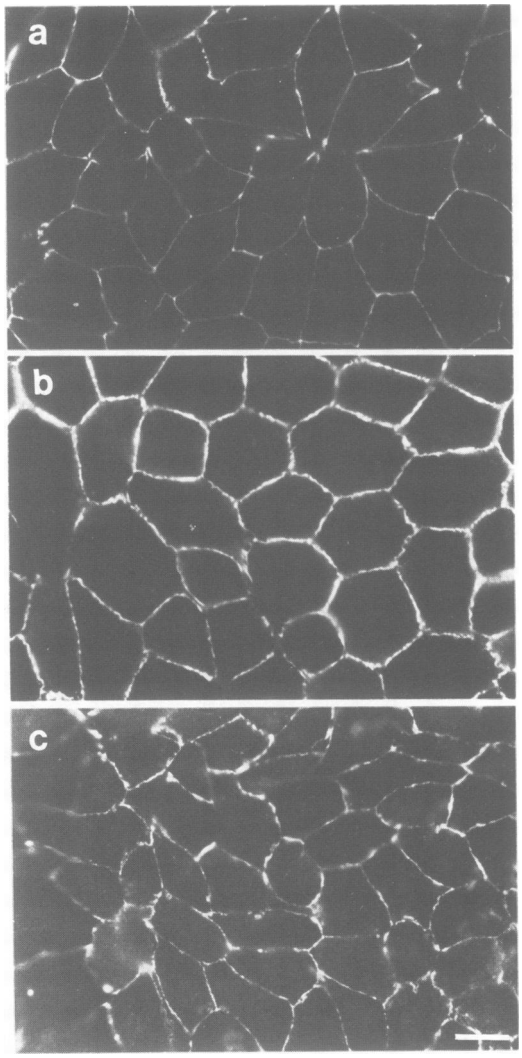


Fig. 7. Distribution of tight junctions, visualized with anti ZO-1 antibodies in H_2O_2/vanadate-treated MDCK cells, control cells are shown in (**a**), cells following 15 min and 2 h of H_2O_2/vanadate treatment are shown in (**b**) and (**c**) respectively. Bar indicates 10 μM.

that the P-Tyr levels in cells maintained at 42°C were lower than those found at 37°C, yet were significantly higher than those of non-transformed cells (see Volberg *et al.*, 1991).

Notably, different tyrphostins had variable effects on cell morphology. The potency of eight different tyrphostins added to RSV transformed lens cells in order to restore cell junctions is summarized in Table I. It is also shown that the concentrations of different tyrphostins needed to exert a significant effect on the cells were variable, ranging from 75 to 200 μM. Most effective in restoring P-Tyr and *N*-cadherin containing intercellular AJs were AG-18 and AG-82, which caused prominent changes at relatively low concentrations. Tyrphostins AG-34, AG-30 and AG-537 induced AJ formation only at high concentrations (>200 μM) while AG-213, AG-17 and AG-370 were apparently without effect. The effect was usually maximal following 8 h of incubation with the relevant inhibitors.

Discussion

Cell–cell adhesion and in particular the formation of AJs, are generally believed to be nucleated by cadherin mediated intracellular interactions (Takeichi, 1988; Geiger *et al.*, 1990). This surface event is followed by the consecutive recruitment of peripheral membrane proteins and cytoskeletal elements leading, eventually to the assembly of an elaborate multi-molecular structure. This scheme, however, does not take into account the intrinsic regulatory mechanisms involved in the spatial and temporal control of junction formation and structure. The very existence of such mechanisms is evident from many observations which indicate that the size and topology of AJs is dynamically modulated in cells during cell locomotion, mitosis, etc. (Abercrombie, 1980; Vasiliev and Gelfand, 1981).

In an attempt to gain insight into the cellular processes which are involved in the regulation of AJ organization, we have specifically investigated here one potential mechanism, namely tyrosine phosphorylation. Previous studies have established that AJs are primary subcellular target sites for PTK, either during embryonic development (Maher and Pasquale, 1988; Takata and Singer, 1988) or following transformation with PTK oncogenes (Rohrschneider, 1980; Nigg *et al.*, 1982; Tsukita *et al.*, 1991). We have shown further that also in non-transformed cells, AJs are primary sites of tyrosine phosphorylation (Volberg *et al.*, 1991).

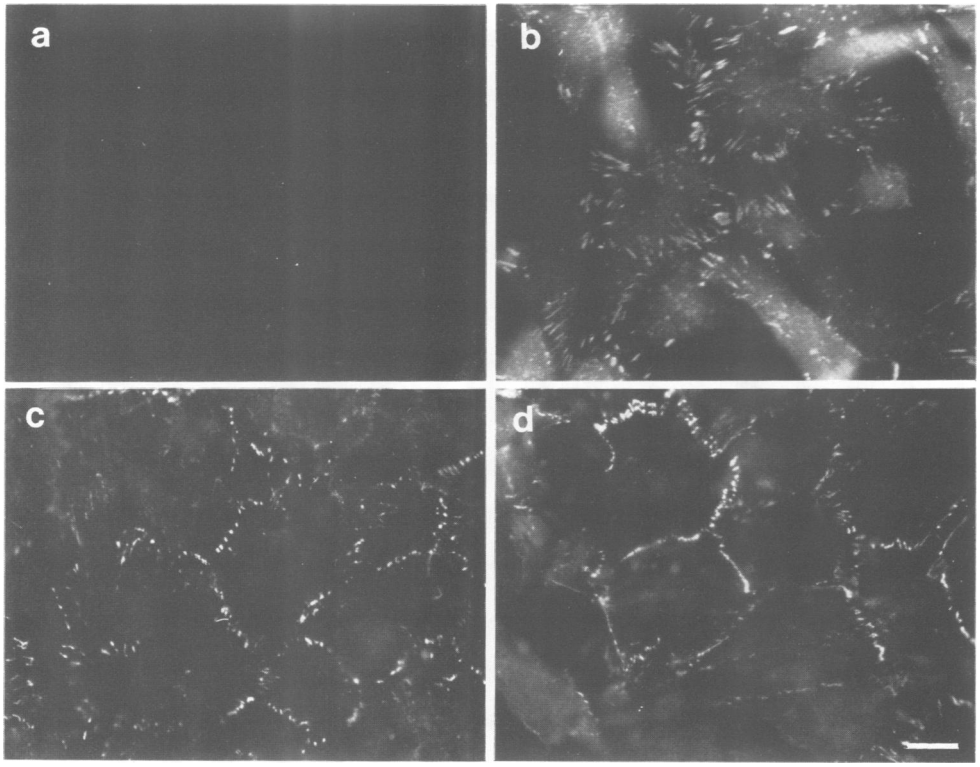


Fig. 8. Effect of tyrphostin AG-18 on P-Tyr distribution in ts-RSV transformed chicken lens cells maintained at the permissive or restrictive temperatures. (**a**) Normal chicken lens cells (**b**) cells transformed with ts-RSV, cultured at the permissive temperature and maintained in normal medium, (**c**) cells transformed with ts-RSV, cultured at the restrictive temperature and maintained in normal medium, (**d**) ts-RSV transformed cells as in b, but cultured in the presence of the tyrphostin AG-18 for 8 h. Bar indicates 10 μm.

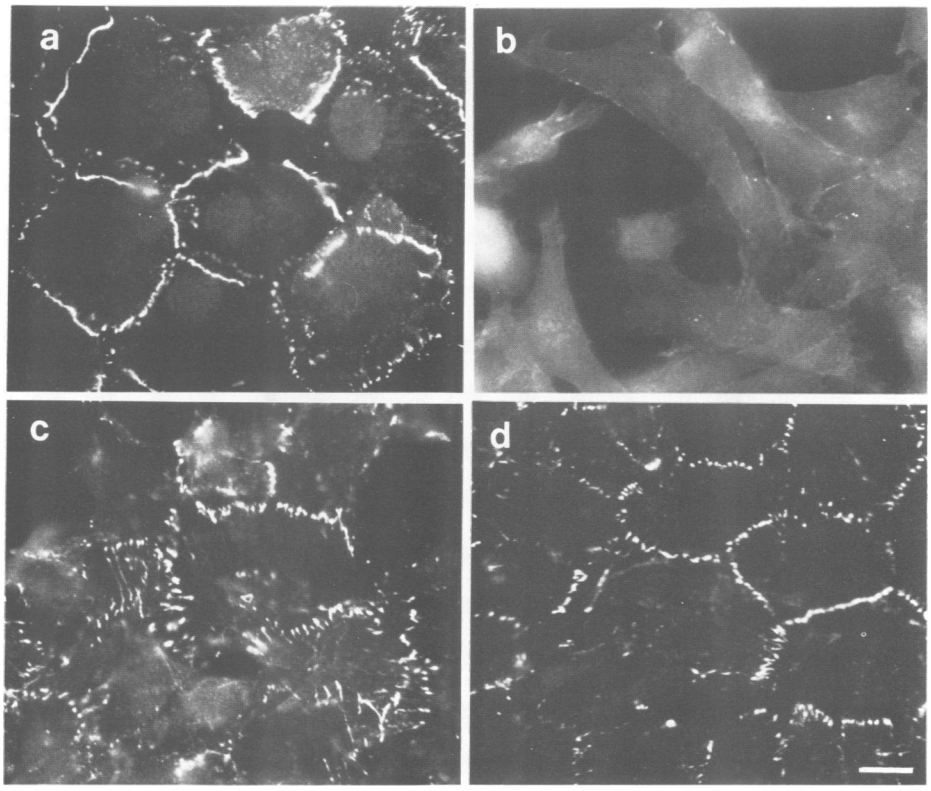
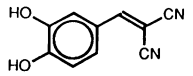
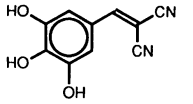
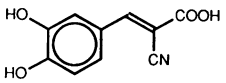
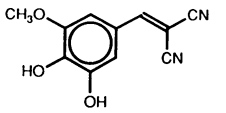
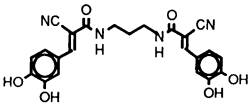
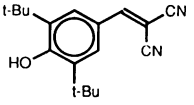
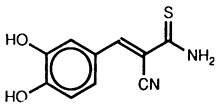
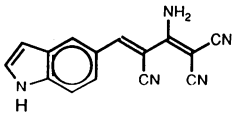


Fig. 9. Effect of tyrphostin AG-18 on *N*-cadherin (A-CAM) distribution in RSV transformed chicken lens cells: (**a**) normal chicken lens cells fixed, permeabilized and immunolabeled with monoclonal anti A-CAM (ID 7.2.3), (**b**) chicken lens cells, transformed with ts-RSV and maintained at the permissive temperature. Note the apparent loss of *N*-cadherin-containing intercellular junctions, (**c**) chicken lens cells transformed with ts-RSV and incubated at the restrictive temperature. *N*-cadherin containing junctions are retained under these conditions, (**d**) chicken lens cells cultured at the permissive temperature but treated for 8 h with the tyrphostin AG-18 (100 μM). Note that junctional structures with organized *N*-cadherin are restored in these cells (compare to the untreated transformed controls shown in (b). Bar indicates 10 μm.

Table I. Restoration of cell junction in RSV-transformed lens cells by tyrphostins

AG No.	Structure	Effective concentration μM
18		10–100
82		10–100
30		≥200
34		≥200
537		≥200
17		ineffective
213		ineffective
370		ineffective

The results presented here address the physiological significance of these processes and point to the close correlation between the modulation of P-Tyr levels and the state of AJ assembly and integrity. Based on the observations described here, we would like to propose that local increase in P-Tyr levels in AJs affects the intermolecular interactions in these junctions. This notion is supported by the reciprocal effects of the PTK and PTP inhibitors on junction structure. Increased intracellular levels of protein tyrosine phosphorylation were obtained by treating cells with a combination of H_2O_2 and vanadate. Such treatment has been previously shown to inhibit intracellular PTP activities in a rapid, specific and reversible manner and thus indirectly activate certain PTK, which maintain their highest tyrosine kinase activity when fully autophosphorylated (Heffetz and Zick, 1989; Heffetz *et al.*, 1990; Zick and Sagi-Eisenberg, 1990). Treatment with H_2O_2 and vanadate did not have any adverse effects on overall cellular metabolism as judged by the fact that the cells remained fully viable and their intracellular ATP content was not perturbed (not shown). Vanadate has been shown to act in synergy with H_2O_2 both in the inhibition of PTP activities and the consequent stimulation of protein tyrosine phosphorylation (Heffetz *et al.*, 1990; Bushkin *et al.*, 1991; Volberg *et al.*, 1991) yet the exact molecular targets for the action of H_2O_2 and vanadate are not fully elucidated. If vanadate alone is added to intact cells for these short time periods it only has limited effects on either PTP activity or on protein tyrosine phosphorylation, in sharp contrast with the known inhibitory effects of vanadate on PTP activity *in vitro* (Swarup *et al.*, 1982). The reason for this discrepancy is currently unknown, but may be attributed to the poor accumulation of vanadate within the cells during our relatively brief treatments. In the presence of H_2O_2, orthovanadate is presumably oxidized to pervanadate (Howarth *et al.*, 1979) which readily enters the cells. After entry into cells, most of the vanadate is reduced into vanadyl ions which could inhibit PTP activities similar to its effects on alkaline phosphatase (Cantley and Aisen, 1979). It is noteworthy that, in addition to its effect on vanadate, H_2O_2 on its own is a potent inhibitor of PTP both in intact cells (Heffetz *et al.*, 1990) and cell-free systems (D.Hecht and Y.Zick, unpublished). Overall, by severely perturbing the equilibrium between tyrosine phosphorylation and dephosphorylation reactions, H_2O_2 and vanadate are proven to be as powerful tools in the amplification of protein tyrosine phosphorylations as others described in this paper, but which are otherwise difficult to observe.

The effect of H_2O_2/vanadate on P-Tyr in cells was clearly apparent within 2–5 min of incubation and the junctional reorganization noticed within 15–30 min. The effect was manifested by a rapid collapse of the junctional belt of actin and its contraction, rounding up of the cells and subsequently, development of elaborate focal contact bound arrays of stress fibres. This effect is reminiscent of the apparent epithelial-to-mesenchymal transition observed in MBT-II cells following treatment with acidic FGF, which also increases, P-Tyr levels (Boyer *et al.*, 1900). It is noteworthy that the earliest detectable effect in the MDCK cells was the dissociation of the junctional actin bundle from the membrane without apparent splitting of the junctions themselves, suggesting that the primary target for the vanadate-induced effects is the junction bound cytoskeleton and not the junctional receptors. This notion is also supported by the fact that other elements of the junctional complex in the MDCK cells (desmosomes and ZO-1-containing tight junctions) showed only limited sensitivity to the vanadate treatment and by previous results indicating that *N*-cadherin is not Tyrosine phosphorylated in cultured lens cells under conditions similar to those employed in the present study (Volberg *et al.*, 1991).

The reciprocal effect was obtained in the present study by the addition of PTK inhibitors, namely tyrphostins, to cells which overexpress PTK and hence have deteriorated junctions. The inhibitory activity of the various tyrphostins on several potent tyrosine kinases and their specificity were previously described and discussed (Levitzki, 1990). It should nevertheless be indicated that both Tyrphostins AG 18 and AG-82 (Yaish *et al.*, 1988; Lyall *et al.*, 1989) which proved to be most effective in the restoration of junctional strucutres, are potent broad spectrum inhibitors of PTK including those of the *src* family (Anafi *et al.*, 1992). Examination of the specificity of inhibition of the various tyrphostins indicated that both AG-537 and 213 are potent blockers of EGF receptor kinase and that AG 17 and 370 effectively inhibit the PDGF receptor kinase. All the tyrphostins are very weak inhibitors of Serine/Threonine kinases (concentrations needed for effective inhibitions

in vitro are 10^3-10^4 higher than those needed for PTK inhibition). For example, addition of 100 μM AG 18 to NIH 3T3 expressing human EGF receptors (not shown) or human keratinocytes (Dvir *et al.*, 1991) had no apparent effect on the incorporation of ^{32}P into serine or threonine residues. In addition, for the concentration range used here, the various tyrphostins were not toxic to cells.

An intriguing observation made here is in the presence of tyrosine phosphorylated proteins in intercellular junctions of tyrphostin-treated RSV infected cells as well as in transformed cells maintained at the non-permissive temperature. If local tyrosine phosphorylation indeed leads to dissociation of AJs how can such modified components be associated with apparently intact junctions? While we have no definitive answer to this question, several possibilities may be considered. These include qualitative differences in the phosphorylated components (probably minor components which are not readily detectable by immunoblotting) or in the sites of phosphorylation on specific proteins. Alternatively, there might be a threshold level of Tyrosine phosphorylation above which the cells appear transformed and below which they appear normal. Attempts to distinguish between these possibilities are currently in progress.

The effect of tyrphostins on the formation of intercellular AJs in chicken lens cells, overexpressing an oncogenic PTK was somewhat slower than that of H_2O_2/vanadate and required for a full effect 8 h. This time, however, is similar to that required for junction formation following plating of epithelial cells in culture, or after recovery from EGTA induced disassembly of cell contacts.

It is noteworthy that while the results presented here suggest the involvement of tyrosine phosphorylation in the modulation of AJs, they do not imply that this mechanism is solely or directly responsible for this activity. In previous studies, for example, it was shown that adhesion molecules may be targets for proteolytic cleavage (Volk *et al.*, 1990) and that AJs may contain urokinase (Pöllänen *et al.*, 1987) and in transformed cells, exert a conspicuous proteolytic activity (Chen, 1990). It appears conceivable that all these mechanisms are concertedly partaking in the regulation of adhesive interactions in living cells.

An interesting phenomenon detected both in the H_2O_2/vanadate and tyrphostin treated cells is the reciprocal relationship between intercellular and cell matrix adhesions. In the H_2O_2/vanadate treated MDCK cells P-Tyr labeling was initially associated with cell−cell AJs and apparently absent from focal contacts (Figure 3). This was true both for sparsely plated cells in which focal contacts were quite abundant and for dense cultures in which cell−cell AJs predominated. However, even in the confluent cultures long incubations (>30 min) with vanadate and H_2O_2 resulted not only in the dissociation of intercellular adhesions but also in a progressive assembly of focal contacts and development of stress fibres. Such reciprocal relationships between cell−cell and cell−matrix junctions were noted a long time ago, (i.e. Weston and Roth, 1969) yet the molecular basis for these interrelationships is still not clear. An intuitive explanation might be that since some of the cytoplasmic components are shared by both junctions, selective dissociation of cell−cell junctions may make these constituents available in larger amounts and thus promote the assembly of cell−matrix adhesions and stress fibres. This mechanism is also compatible with the observation that disassembly of cell−cell junctions preceded the formation of stress fibres.

The molecular target(s) for PTK activity, which are directly involved in AJ modulation were not identified. The immunoblotting analyses presented here (Figure 1) and in the previous study (Volberg *et al.*, 1991), revealed a multitude of phosphorylated bands whose cellular localization and relation to AJs is still unknown. Attempts are now in progress to identify some of the relevant molecular targets for junctional PTK, mostly by the preparation of monoclonal antibodies to purified Tyrosine phosphorylated proteins.

Materials and methods

Cells

Madin-Darby canine kidney (MDCK cells; ATCC-CCL) were cultured on coverslips in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Cultured lens cells were prepared from 7−8 day old chick embryos as described (Volk and Geiger, 1986b). The cells were infected with a temperature sensitive (ts-68) mutant of Schmitt-Ruppin strain of RSV (denoted ts-RSV, Kawai and Hanafusa, 1971) in the presence of 8 mg/ml Polybrene (Sigma, USA). Cells were initially cultured at the permissive temperatrue (37°C) in DMEM containing 10% fetal calf serum (FCS; Biolab, Israel) as described (Volk and Geiger, 1984, 1986a,b) and shifted when indicated to the non-permissive temperature (42°C) in the same medium.

H_2O_2/vanadate treatment

Cultured cells on glass coverslips were washed with serum-free DMEM. H_2O_2 (2 mM) and sodium orthovanadate (1 mM) in serum-free medium were added to the cells for a period of 15 min or as indicated at 37°C in a humidified incubator. For the examination of long-term effect cells were 'pulsed' with H_2O_2/vanadate for 15 min as indicated, then washed once with 10% FCS-containing medium and further incubated in serum-containing medium for different periods of time. The treatment was terminated by washing the cells with PBS, followed by fixation.

Tyrphostin

The different tyrphostins were synthesized as described earlier (Gazit *et al.*, 1989) stock solutions were prepared in DMSO and were stable for long periods of time at −10°C. Aliquots of the various tyrphostins were directly added to complete culture medium, yielding final concentrations ranging from 25−200 μM.

Immunofluorescence labeling

For immunofluorescence labeling, cells were permeabilized by a 2 min exposure to 0.5% Triton X-100 in 50 mM morpholinoethane sulfonate (MES) buffer pH 6.0, and then fixed for 30 min with 3% paraformaldehyde. Double immunofluorescence labeling was carried out, largely as previously described using mouse and rabbit antibodies in conjunction (Geiger, 1979). It was routinely verified that the secondary goat antibodies were exclusively reactive with the respective primary antibodies.

Antibodies

Mouse monoclonal antibodies reactive with P-Tyr were prepared by immunization of BABL/c mice with KLH-P-Tyr as described (Heffetz *et al.*, 1989). Affinity purufied rabbit anti-P-Tyr antibodies were generated as previously decribed (Heffetz and Zick, 1989). Rhodamine and fluorescein labeled secondary antibodies were purchased from Jackson Immuno-Research Labs, Inc. (West-grove, PA, USA). Rhodamine labeled phalloidine was purchased from Sigma (St. Louis, USA), Anti-A-CAM (*N*-cadherin) was a monoclonal antibody ID-7.2.3 (Volk and Geiger, 1984, 1986a,b), now available from Sigma Immunochemicals. Anti-human vinculin was kindly provided by Dr V.Koteliansky (CNRS-Ecole Normale Superieure, Paris). Anti-desmoplakin was provided by Prof. W.W.Franke (German Cancer Research Center, Heidelberg, FRG, see Cowin *et al.*, 1985). ZO-1 antibodies were provided by Prof. M.Mooseker (Yale University, New Haven, USA, see Stevenson *et al.*, 1986).

Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide slab gels to nitrocellulose papers was performed essentially as described (Laemmli, 1970; Burnette, 1981). The transfer was carried out for 3 h at 200 mA in 50 mM

glycine, 50 mM Tris–HCl buffer, 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% BSA. The blots were incubated for 16 h at 4°C with affinity purified (1 μg/ml) anti-P-Tyr antibodies, intensely washed in buffer A, containing 0.1% BSA and further incubated with ^{125}I-goat anti-rabbit antibodies (3×10^5 c.p.m./ml) for 2 h at 22°C. The papers were then washed, air dried and autoradiographed.

Electron microscopy

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

Cell extracts

Cells were cultured on 60 mm tissue culture plates (Falcon, USA). One hour before treatment, the medium was replaced and H_2O_2 and/or sodium orthovanadate were added to the medium as indicated. After incubation, cells were washed three times with ice-cold PBS and immediately frozen in liquid nitrogen. Cells were extracted with buffer B (150 mM sucrose, 80 mM β-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 μg 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

Abercombie,M. (1980) *Proc. R. Soc. Lond. B*, **207**, 614–628.
Anafi,M., Gazit,A., Gilon,C., Ben Neria,Y. and Levitzki,A. (1992) *J. Biol. Chem.*, in press.
Boyer,B., Valles,A.M., Tucker,G.C., Jouanneau,J. and Thiery,J.-P. (1990) *J. Cell Biol.*, **111**, 420a.
Burnette,W.W. (1981) *Anal. Biochem.*, **112**, 195–203.
Burridge,K. and Connell,L. (1983a) *Cell Motil.*, **3**, 405–417.
Burridge,K. and Connell,L. (1983b) *J. Cell Biol.*, **94**, 359–367.
Burridge,K., Fath,K., Kelly,T., Nuckolls,G. and Turner,C. (1988) *Annu. Rev. Cell Biol.*,**4**, 487–525.
Bushkin,I., Roth,J., Heffetz,D. and Zick,Y. (1991) *J. Biol. Chem.*, **266**, 11890.
Cantley,L.C. and Aisen,P. (1979) *J. Biol. Chem.*, **254**, 1781–1784.
Chen,W.-T. (1990) *Cell Differ. Develop.*, **32**., 329–336.
Comoglio,P.M., DiRenzo,M.F., Tarone,G., Giancotti,F.G., Naldini,L. and Marchisio,P.C. (1984) *EMBO J.*, **3**, 483–489.
Cowin,P., Capprell,H.-P. and Franke,W.W. (1985) *J. Cell Biol.*, **101**, 1442–1454.
Damsky,C.H., Knudsen,K.A., Bradley,D., Buck,C.A. and Horwitz,A.F. (1985) *J. Cell Biol.*, **100**, 1528–1539.
Dvir,A., Millner,Y., Chomsky,O., Gilon,C., Gazit,A. and Levitzki,A. (1991) *J. Cell Biol.*, **113**, 857–865.
Gazit,A., Yaish,P., Gilon,C. and Levitzki,A. (1989) *J. Med. Chem.*, **32**, 2344–2352.
Geiger,B. (1979) *Cell*, **18**, 193–205.
Geiger,B. and Ginsberg,D. (1991) *Cell Motil. Cytoskel.*, **20**, 1–6.
Geiger,B., Avnur,Z., Volberg,T. and Volk,T. (1985a) In Edelman,G.M. and Thiery,J.-P. (eds), *Cell In Contact: Adhesions and Junctions as Morphogenetic Determinants*, Wiley and Sons, New York, pp. 461–489.
Geiger,B., Volk,T. and Volberg,T. (1985b) *J. Cell Biol.*, **101**, 1523–1531.
Geiger,B., Volk,T., Volberg,T. and Bendori,R. (1987) *J. Cell Sci.*, Suppl. **8**, 251–272.
Geiger,B. (1989) *Curr. Opin. Cell Biol.*, **1**, 103–109.
Geiger,B., Ginsberg,D., Salomon,D. and Volberg,T. (1990) *Cell Differen. Develop.*, **32**, 343–354.
Glenney,J.R. and Zokas,L. (1989) *J. Cell Biol.*, **108**, 2401–2408.
Gumbiner,B. and Simons,K. (1986) *J. Cell Biol.*, **102**, 457–468.
Heffetz,D. and Zick,Y. (1989) *J. Biol. Chem.*, **264**, 10126–10132.
Heffetz,D., Bushkin,I., Dror,R. and Zick,Y. (1990) *J. Biol. Chem.*, **265**, 2896–2902.
Hirst,R., Horwitz,A., Buck,C. and Rohrschneider,L. (1986) *Proc. Natl. Acad. Sci, USA*, **83**, 6470–6474.
Horwitz,A., Bozyczko,D. and Buck,C.A. (1990) In Edelman,G.M., Cunningham,B.A. and Thiery,J.-P. (eds), *Morphoregulatory Molecules*. Wiley and Sons, New York, pp. 217–230.
Howarth,O.W. and Hunt,J.R. (1979) *Chem. Soc. Dalton. Trans.*, **9**, 1388–1391.
Kapprell,H.P., Cowin,P. and Franke,W.W. (1987) *Eur. J. Biochem.*, **166**, 505–517.
Kawai,S. and Hanafusa,H. (1971) *Virology*,**46**, 470–479.
Kellie,S., Horvath,A.R. and Elmore,M.A. (1991) *J. Cell Sci.*, **99**, 207–211.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Levitzki,A. (1990) *Biochem. Pharmacol.*, **40**, 913–918.
Lyall,R.M., Zilberstein,A., Gazit,A., Gilon,C., Levitzki,A. and Schlessinger,J. (1989) *J. Biol. Chem.*, **264**, 14503–14509.
Maher,P.A. and Pasquale,E.B. (1988) *J. Cell Biol.*, **106**, 1747–1755.
Maher,P.A., Pasquale,E.B., Wang,J.Y.J. and Singer,S.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6576–6580.
Nigg,E.A., Sefton,B.M., Hunter,T., Walter,G. and Singer,S.J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5322–5326.
Pasquale,E.B., Maher,P.A. and Singer,S.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5507–5511.
Pöllänen,J., Saksela,O., Salonen,E.-M., Andersen,P., Nielsen,L., Danno,K. and Vaheri,A. (1987) *J. Cell Biol.*, **104**, 1085–1096.
Rinnerthaler,G., Geiger,B. and Small,J.V. (1988) *J. Cell Biol.*, **106**, 747–760.
Rohrschneider,L.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3514–3518.
Sefton,B., Hunter,T., Ball,E.H. and Singer,S.J. (1981) *Cell*, **24**, 165–174.
Stevenson,B.R., Siliciano,J.D., Mooseker,M.S. and Goodenough,D.A. (1986) *J. Cell Biol.*, **103**, 755–766.
Swarup,G., Cohen,S. and Garbers,D.L. (1982) *Biochem. Biophys. Res. Commun.*, **107**, 1104–1109.
Takata,K. and Singer,S.J. (1988) *J. Cell Biol.*, **106**, 1757–1764.
Takeichi,M. (1988) *Development*, **102**, 639–655.
Tsukita,S., Oichi,K., Akiyama,T., Yamanashi,Y., Yamamoto,T. and Tsukita,S. (1991) *J. Cell Biol.*, **113**, 867–879.
Vasiliev,J.M. and Gelfand,I.M. (1981) *Neoplastic and Normal Cells in Culture*, Cambridge University Press, Cambridge, U.K.
Volberg,T., Geiger,B,, Dror,R. and Zick,Y. (1991) *Cell Regul.*, **2**, 105–120.
Volk,T. and Geiger,B. (1984) *EMBO J.*, **3**, 2249–2260.
Volk,T. and Geiger,B. (1986a) *J. Cell Biol.*, **103**, 1441–1450.
Volk,T. and Geiger,B. (1986b) *J. Cell Biol.*, **103**, 1451–1464.
Volk,T., Volberg,T., Sabanay,I. and Geiger,B. (1990) *Dev. Biol.*, **139**, 314–326.
Weston,J.A. and Roth,S.A. (1969) In Smith,R.T. and Good,R.A. (eds), *Cellular Recognition*. Appelton-Century-Crofts, NY, pp. 29–37.
Yaish,P., Gazit,A., Gilon,C. and Levitzki,A. (1988) *Science*, **242**, 933–935.
Zick,Y. and Sagi-Eisenberg,R. (1990) *Biochemistry*, **29**, 10240–10245.

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