

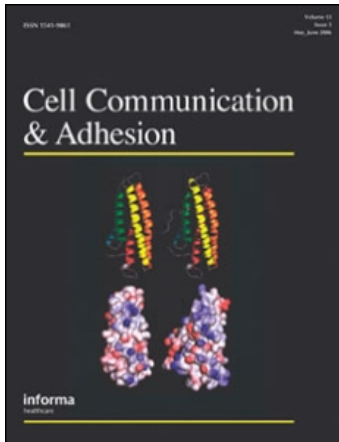
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Augmentation of Adherens Junction Formation in Mesenchymal Cells by Co-expression of N-CAM or Short-term Stimulation of Tyrosine-phosphorylation

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Adherens-type junctions (AJ) are specialized intercellular contacts, mediated by cadherins and characterized by the association with actin filaments through a vinculin- and catenin-rich submembrane plaque. We describe here two mechanisms which potentiate AJ formation in mesenchymal cells. These include the augmentation of AJ by the co-expression of another adhesion molecule, namely NCAM, and the stimulation of tyrosine phosphorylation. These effects were obtained in NIH-3T3 cells, which, under normal conditions, have poor cadherin- and vinculin-containing intercellular junctions. The transfection of these cells with cDNA encoding the 140kD NCAM resulted in the extensive formation of cadherin- and vinculin-rich AJ, demonstrating a cooperativity between the two junctional systems. AJ could also be induced in 3T3, and in CEF and COS cells, upon a brief exposure to H₂O₂/vanadate, which elevates cellular levels of phosphotyrosine due to inhibition of tyrosine-specific phosphatases. This induction was, however, transient since prolonged exposure to H₂O₂/vanadate resulted in an overall destruction of AJ and detachment of cells from each other and from the extracellular matrix. AJ formation appears, therefore, to be modulated by a variety of factors including the level of expression of its intrinsic components, the cooperative effect of other adhesion molecules, and by tyrosine-phosphorylation.

KEYWORDS: adherens junction, cadherins, NCAM, tyrosine phosphorylation

INTRODUCTION

Intercellular adhesion or the attachment of cells to the extracellular matrix (ECM) play a major role in cellular and tissue morphogenesis (Geiger 1989). Moreover, such interactions exert long term effects on cell growth, motility and differentiation (Hynes and Lander 1992) and are often disturbed in tumors (Ruoslahti and Giancotti 1989; Juliano and Varner 1993). Four major superfamilies of cell surface receptors are known to participate in adhesive interactions: cadherins, members of the immunoglobu-

lin superfamily, selectins, and integrins. These molecules are differentially distributed in cells and exhibit distinct binding specificities (Albelda and Buck 1990). Cells may, often, express simultaneously several types of adhesion molecules, yet the cooperativity between them is still poorly characterized. Similarly, the transmembrane interactions of these receptors as well as the mechanisms responsible for adhesion mediated signal transduction has not been elucidated yet. It is, however, becoming evident that the submembrane plaque present in AJ plays an important role in junction formation and stability. Similarly, there is compelling evidence that transmembrane signaling in adhesion sites involves phosphorylation of either the cell surface receptor molecules themselves or of molecules associated

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with them inside the cells including components of the submembrane junctional plaque, such as vinculin, α -actinin (Geiger, 1991), talin (Pasquale *et al.* 1986), paxillin (Glenny and Zokas 1989), zyxin (Sadler *et al.* 1992), tensin (Bockholt and Burridge 1993) and catenins (Ozawa *et al.* 1989; Reynolds *et al.* 1992; Geiger and Ginsberg 1991). Moreover, it has been recently demonstrated that adhesive interactions can trigger tyrosine phosphorylation (Guan and Shalloway 1992; Schaller *et al.* 1993). Antibody mediated clustering of integrins (Kornberg *et al.* 1991) or activation by ligands (Hanks *et al.*, 1992) causes an increase in tyrosine phosphorylation in focal contacts, possibly by p125FAK or Src-kinase (Zachary and Rozengurt 1992).

Multiple mechanisms are implicated in the regulation of AJ formation, primarily the level of expression of the relevant adhesion molecules. It has been shown that reduced levels of these molecules are often associated with reduced adhesive properties and with transformed phenotype (Takeichi 1993; Vlemminckx *et al.* 1991; Birchmeier *et al.* 1993). Furthermore, introduction of adhesion molecules such as cadherins (Nose *et al.* 1990) or integrins (Guan *et al.* 1990) into cells leads to a remarkable augmentation of intercellular or cell-matrix adhesion, respectively. Elucidation of the molecular basis for the regulation of cell adhesion is further complicated by the fact that most cells express on their surface not a single but rather a combination of adhesion molecules which might cooperate or compete with each other. Recent studies have shown that modulation (over-expression or down regulation) of plaque and cytoskeletal proteins such as vinculin (Rodrigues Fernandes *et al.* 1992), α -actinin (Gluck *et al.* 1993) and gelsolin (Vasconcellos and Lind 1993) affects cell adhesiveness and suppresses the tumorigenicity of tumor cells.

Post-translational modification, and in particular tyrosine phosphorylation, was also shown to affect cell adhesion (Matuyoshi *et al.* 1992; Hanagushi *et al.* 1993b). For example, invasiveness of transformed cells was correlated with tyrosine-phosphorylation of the E-cadherin- β -catenin complex (Behrens *et al.* 1993), suggesting that β -catenin is another target for modulation of adherens junctions. Tyrosine phosphorylation of integrins by the transforming or cellular src also leads to a concomitant alteration in cell-matrix interactions and in a cytoskeletal reorganization (Volberg *et al.* 1991; Kellie *et al.* 1991). In general, tyrosine hyper-phosphorylation of AJ molecules, usually leads to a reversible de-stabilization of cellular junctions (Volberg *et al.* 1991, 1992).

In this study we have focused on two alternative mechanisms for the regulation of AJ formation. These include an examination of the possible cooperativity between distinct adhesion systems and the effect of tyrosine phosphorylation of junctional molecules. We show that expression of one adhesion molecule (NCAM) may greatly potentiate the organization of another junctional system (cadherins). Furthermore, we report here on the rapid and transient induction of AJ formation following short stimulation of tyrosine phosphorylation.

MATERIALS AND METHODS

Cells: Mouse 3T3 fibroblasts, African Green monkey kidney (COS) cells and chicken embryo fibroblasts (CEF) were cultured on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 3T3-NCAM cells were transfected with the cDNA encoding the 140kD isoform of the neural cell adhesion molecule, NCAM, in the pH-*Apr-1-neo* vector (Doherty *et al.* 1991), kindly provided by Dr. F. Walsh (Guy's Hospital, London). The transfections were performed by the standard calcium phosphate procedure (Graham and van der Eb 1973). Positive transfectants were analyzed by FACS-scan analysis. The 3T3 cells used in these experiments did not express endogenous NCAM, since they did not stain with broad reactive rabbit antiserum against NCAM that cross reacts with NCAM of other species.

H₂O₂/vanadate treatment: Cultured cells on glass coverslips were washed with serum-free DMEM. H₂O₂(2mM) and sodium orthovanadate (2mM), in serum-free medium, were added to the cells for 10 minutes at 37°C. The cells were then washed with 10% FCS-containing medium and incubated in normal medium for various time periods. The treatment was stopped by washing the cells three times with PBS, followed by fixation.

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

were exclusively reactive with the respective primary antibodies. Mouse monoclonal antibodies reactive with phosphotyrosine (P-tyr), was obtained from Prof. Y. Zick (Weizmann Institute, Rehovot, (Heffetz and Zick 1989) or purchased from Sigma Immunochemicals (St. Louis, USA). Rabbit anti-pan-cadherin, R-156 (Geiger et al. 1990), and mouse monoclonal antibody 123C3 (Moolenaar et al. 1990) were used as described. Anti-human vinculin, cross reactive with vinculin of other species, was kindly provided by Dr. V. Kotliansky, (CNRS-Ecole Normale Supérieure, Paris), rabbit anti-talin by Dr. K. Burridge (University of North Carolina, Chapel Hill). Rhodamine labeled phalloidine was purchased from Sigma (St. Louis, USA).

Cell extracts and Immunoblotting: Cells, cultured on 60 mm tissue culture disks were treated with H₂O₂ as described above, washed three times with ice-cold PBS and immediately frozen in liquid nitrogen. Cells were extracted with lysis buffer (150mM sucrose, 80mM β -glycerophosphate, 2mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 10 mM pyrophosphate, 50 mM NaF, 1% Triton X-100, 0.1% SDS, 1mM PMSF, 10 mg/ml aprotinin, pH 7.6). The cells were scraped off the plates, homogenized and centrifuged for 15 minutes at 4°C at 12000g. Supernatants were collected and aliquots were mixed with concentrated (5x) sample buffer (Laemmli, 1970), and electrophoresed on a 10% reducing SDS-PAGE. Electrophoretic transfer of the proteins from the gel was performed as described by Burnette (1981), and carried out for 3 h at 200mA in 50mM glycine, 50mM Tris-HCl buffer, pH 8.8. The nitrocellulose filters were presoaked in 150mM NaCl, 10mM Tris-HCl, 0.05% Tween-20, pH 7.6, containing 0.1% BSA in case of anti P-tyr, or in 10% skim milk powder (Oxoid, England) in PBS for the other antibodies. The blots were incubated for 16h at 4°C with 1mg/ml antibody and further incubated with ¹²⁵I-labeled goat anti mouse or anti rabbit antibody (5x10⁵ cpm/ml) for 2 hour at 22°C. The filters were then washed, air dried and exposed on Kodak-X-ray films.

RESULTS

Induction of AJ Formation by NCAM

Intercellular adherens junctions are characterized by the association of cadherin molecules with actin filaments, through a submembrane plaque which con-

tains vinculin, catenins, plakoglobin and several actin associated proteins (Geiger 1989; Geiger and Ginsberg 1991). These junctions are most common in epithelial and endothelial cells and only scarcely distributed in mesenchymal cells. Immunolocalization of cadherin molecules in NIH 3T3 cells with anti-pan-cadherin revealed occasional patches in cell-cell contact areas, as shown in Figure 1A. These AJ-like structures did not exhibit, however, extensive association with vinculin and actin (Figure 1 C, D), as is seen in cells with an overt expression of AJs (Volberg et al., 1991) or in 3T3-NCAM cells (Figure 1). Due to the low level of expression of cadherins or vinculin in the cells used in these experiments,

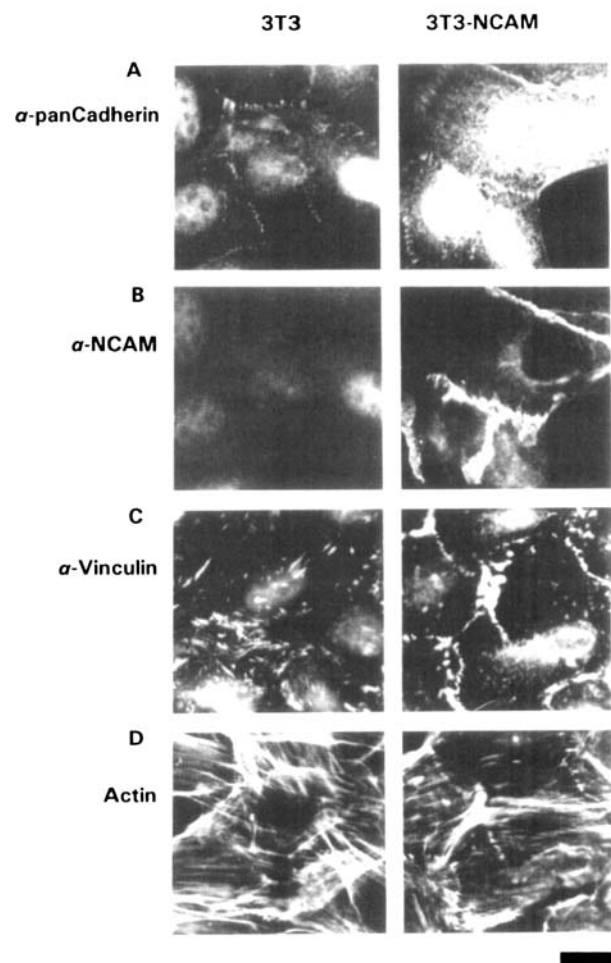


FIGURE 1. Distribution of Cadherins (A), NCAM (B), Vinculin (C), and Actin (D) in 3T3 cells and 3T3-NCAM cells. The cells were double stained for cadherin and NCAM, (A and B), and for vinculin and actin (C and D). Note that only in 3T3-NCAM cells vinculin and actin are apparently associated with intercellular junctions. The vinculin staining in 3T3 cells is mostly associated with cell-substrate focal contacts.

long exposure times had to be used to detect these molecules. As a result of this, nuclear background staining apparently increased (see Figure 1, but also Figures 3, 4 and 6). Vinculin and actin staining in 3T3 cells were mostly associated with focal contacts and stress fibers, respectively.

To assess the possible effect of a second type of adhesion molecule on AJ formation we have transfected 3T3 cells with NCAM-140 cDNA and examined the consequent distributions of cadherin, vinculin and actin. NCAM, as can be seen in Figure 1B, was present in these cells as a ribbon in between adjacent cells, as previously reported by Bloch (1992), and does cover, but not co-localize with AJs. The presence of NCAM in 3T3 cells had a dramatic effect on the development of AJ in the cells, as revealed in 3 independent transfected cell lines and suggested that expression of another adhesion molecule, NCAM, suffices to markedly augment the assembly of cadherins and associated molecules into prominent AJ. Now, vinculin and cadherin structures are apparent in intercellular AJ (Figure 1, and data not shown). To determine whether this effect is attributable to cooperativity between the two

junctional systems or to induced changes in the expression of AJ components, we compared the levels of expression of talin, cadherins, vinculin and NCAM between 3T3 and 3T3-NCAM cells by quantitative immunoblotting. As shown in Figure 2, no major alterations were detected in the level of expression of cadherins (2B) and vinculin (2C); NCAM was present only in the transfected cells, as expected. We therefore propose that the expression of NCAM potentiates the assembly of cadherins, vinculin and actin into typical AJ. A difference in the level of expression of talin was observed between 3T3 and 3T3-NCAM cells (Figure 2A), which might have resulted from clonal variation or from transfection of NCAM. We hold it unlikely that the difference in talin expression affects the stability of the intercellular AJs.

AJ Induction by Tyrosine Phosphorylation

Inhibition of tyrosine-phosphatases by a brief treatment of cells with H₂O₂/vanadate resulted in a rapid tyrosine phosphorylation which is most prominent in AJ such treatment leads to dissocia-

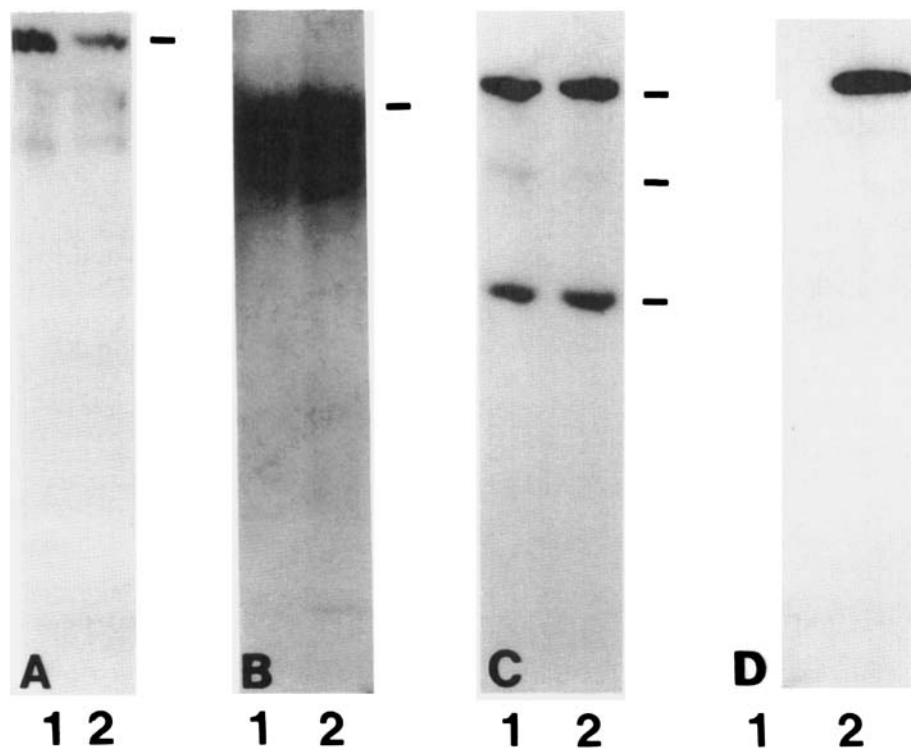


FIGURE 2. Presence of Talin (A), Cadherins (B), Vinculin (C), and NCAM (D), in 3T3 (1) or 3T3-NCAM (2) cells. Identical number of cells were lysed in sample buffer and subjected to quantitative immunoblotting analysis using the antisera described in Materials and Methods, and ¹²⁵I-labeled goat anti-rabbit IgG (A and B), or goat anti-mouse IgG (C and D), to identify the reactive bands. Notice that there are no major difference in the amounts of talin, cadherin or vinculin between 3T3 and 3T3-NCAM cells. The apparent molecular mass of talin is 215 kD, cadherins are represented by multiple bands ranging from 90-120 kD, vinculin is present as a major 116 kD band (plus two proteolytic breakdown products), and NCAM appears as a single band of 140kD.

tion of AJ after 30 minutes of treatment or more. (Volberg et al. 1991; 1992, Hadari et al. 1993). This phenomenon is reversible, and normal adhesions are formed upon further incubation of the cells in normal medium. While some of the molecular targets and specific kinases present in AJ are known (see Discussion, below), the relevance of specific phosphorylation events for the deterioration of AJ remains to be determined.

In 3T3 cells, intercellular AJs are very scarce, as indicated above. However, brief exposure of the cells to H₂O₂/vanadate (10 minutes, Figure 3) resulted in a marked augmentation in AJ, as seen by staining for cadherins, actin and, particularly, vinculin. This effect was transient since longer incuba-

tion (30 minutes after H₂O₂/vanadate stimulation), resulted in a re-distribution and deterioration of AJs. Vinculin and actin staining became mostly associated with focal contacts and with distorted AJ structures between the cells (Figure 3A). The P-tyr staining followed a similar pattern, namely was first (after 10 minutes of treatment) associated with intercellular AJ and later (after 30 minutes) switched to focal contacts (Figure 3B). Interestingly, talin exhibited a reciprocal distribution, namely, was associated with focal contacts in untreated cells and rapidly dissociated from them upon treatment with H₂O₂/vanadate (concomitantly with formation of intercellular AJ- which are devoid of talin). At the 30 minutes time point, when the cell-cell contacts

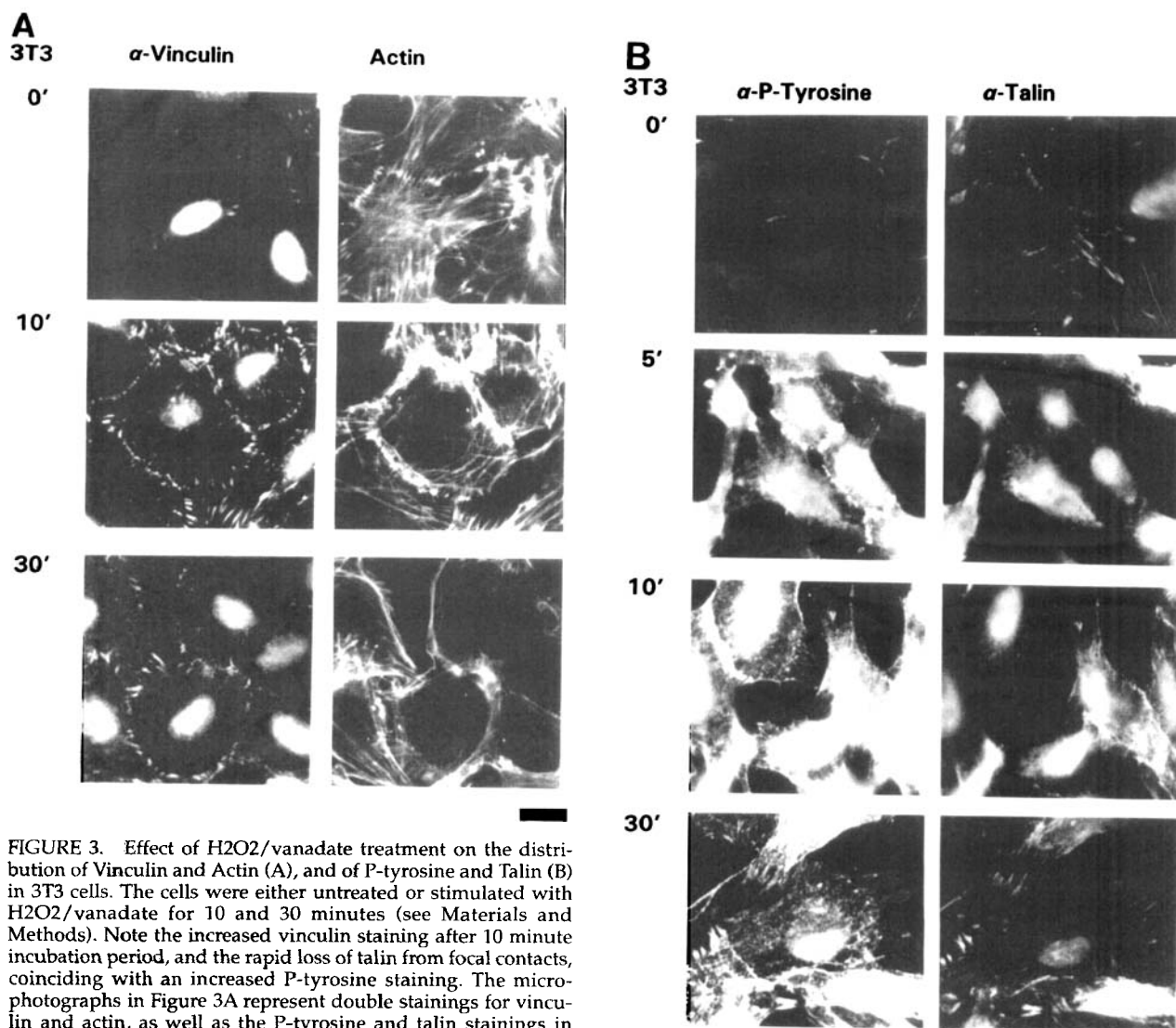


FIGURE 3. Effect of H₂O₂/vanadate treatment on the distribution of Vinculin and Actin (A), and of P-tyrosine and Talin (B) in 3T3 cells. The cells were either untreated or stimulated with H₂O₂/vanadate for 10 and 30 minutes (see Materials and Methods). Note the increased vinculin staining after 10 minute incubation period, and the rapid loss of talin from focal contacts, coinciding with an increased P-tyrosine staining. The microphotographs in Figure 3A represent double stainings for vinculin and actin, as well as the P-tyrosine and talin stainings in Figure 3B.

deteriorated, talin-rich focal contacts reappeared. It should be emphasized that such two-step process was only noticeable in cells without an overt presence of AJ, since a similar effect was not noted in epithelial cells (Volberg *et al.* 1991) nor in 3T3-NCAM cells, (Figure 4), where AJ are prominent irrespective of vanadate-induced phosphorylation. In cells with an overt expression of AJs, the early, H₂O₂/vanadate induced assembly of AJs as detectable by vinculin staining, is not observed. The early assembly of AJs upon H₂O₂/vanadate treatment in 3T3 cells was concluded from the vinculin patterns that differed between the control (0') and 10 minutes after treatment, whereas the cadherin patterns were not different. These were similar to those seen in 3T3NCAM cells (Figure 4A).

Treatment of 3T3 cells with H₂O₂/vanadate potentiated the tyrosine phosphorylation of multiple proteins (Figure 5, lane 2) in line with the immunocytological examination. The anti P-tyrosine antibody used in these experiments only recognized

epitopes of phosphorylated tyrosine (Volberg *et al.* 1992), therefore, phosphorylation of serine- and of threonine residues on proteins are unlikely to be detected in this experiment. In 3T3-NCAM cells, the same array of tyrosine-phosphorylated proteins was observed (Figure 5, lane 4), with a detectable over-phosphorylation of proteins in the molecular weight range of 140-180kD. NCAM itself was not phosphorylated in this experiment (data not shown).

To study the generality of the apparent augmentation of AJ formation by tyrosine phosphorylation we have examined the effect of vanadate on other cell types, such as chicken embryo fibroblasts (CEF), and monkey kidney cells, COS. As shown in Figure 6, both cell types showed a poor organization of AJ, when cultured in regular medium, yet, after 3 minutes of H₂O₂/vanadate treatment, AJ became conspicuous as demonstrated by the staining for vinculin (Figure 6). Again, this effect was transient since upon longer incubation (7 minutes), vinculin staining became faint and the AJ started to deteriorate.

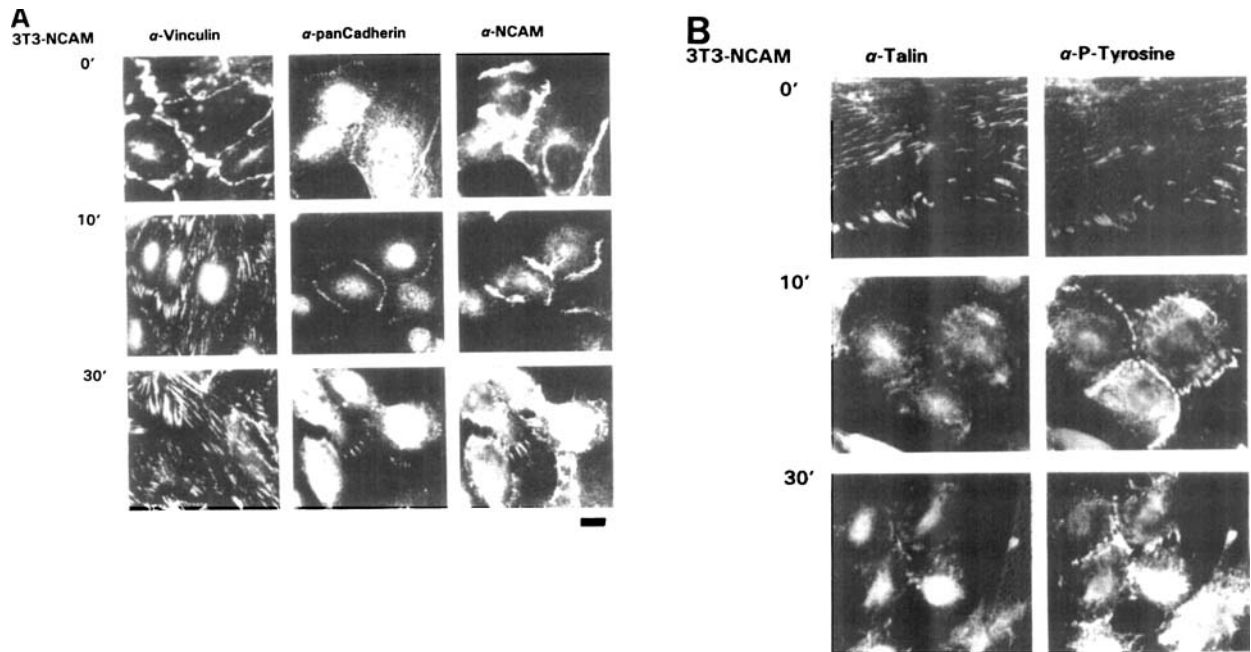


FIGURE 4. Effect of H₂O₂/vanadate treatment on the distribution of Vinculin, Cadherin, and NCAM, (panel A) and of Talin and P-tyrosine (Panel B) in 3T3-NCAM cells. The cells were either untreated or treated with H₂O₂/vanadate for 10 or 30 minutes. Note the presence of vinculin in intercellular sites already at t=0, and the extensive AJ patterns stained with vinculin and pan-cadherin at t=0. Cells were double stained for cadherin and NCAM, and for talin and P-tyrosine. Bar indicates 10 μ m.

FIGURE 5. Effect of H₂O₂/vanadate treatment on tyrosine phosphorylation in 3T3 cells (lanes 1 and 2) and in 3T3-NCAM cells (lanes 3 and 4). Cells were treated with H₂O₂/vanadate for 10 minutes, rapidly washed with serum-free medium, and either directly extracted with lysis buffer (lanes 1 and 3) or further incubated with serum containing medium for 20 minutes and then lysed (lanes 2 and 4). Cell extracts were examined by 10% SDS-PAGE, transferred to nitrocellulose paper and reacted with anti-P-tyrosine antibody. Multiple proteins become tyrosine-phosphorylated in the treated cells with major bands of 140-180 kD. The pattern in 3T3-NCAM cells, as compared with 3T3 cells was indistinguishable.

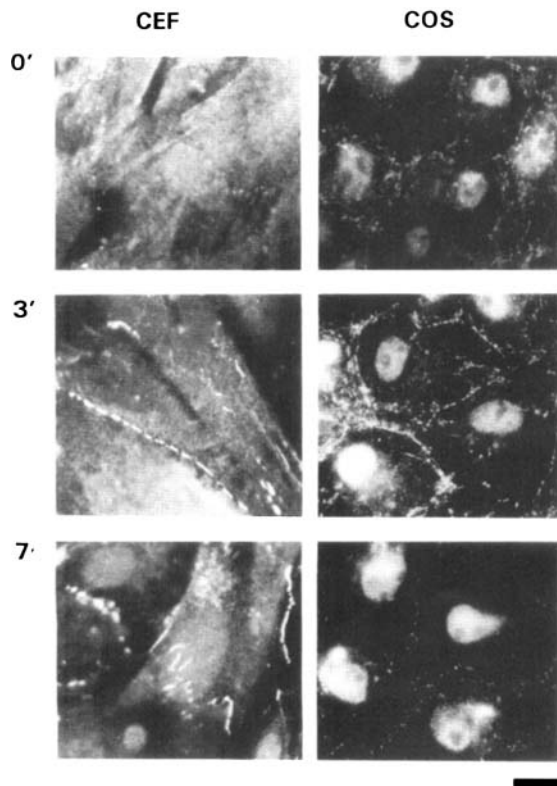
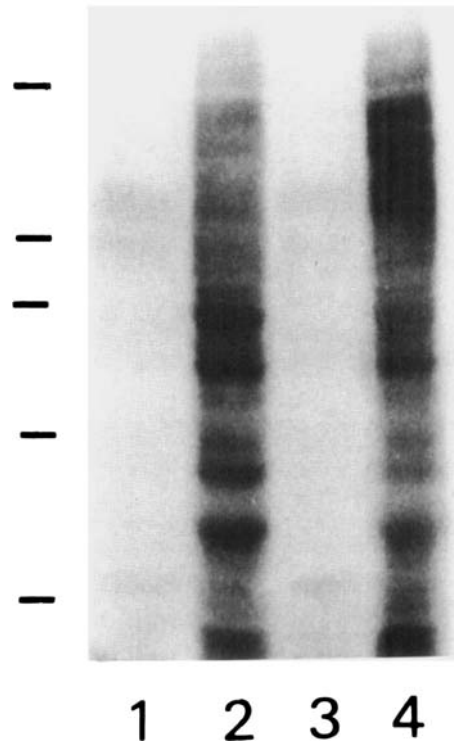


FIGURE 6. Distribution of Vinculin in chicken embryo fibroblast (CEF) and in COS cells following H₂O₂/vanadate treatment. CEF and COS cells were either untreated or treated with H₂O₂/vanadate for 3 or 7 minutes. The cover slips were then washed with PBS and the cells were fixed and stained for vinculin. Note the increased vinculin staining in cell-cell adhesions following short (3 minute) treatment. Bar indicates 10 μ m.

DISCUSSION

The assembly of cellular junctions can be regulated at multiple levels, including the modulation of expression and transmembrane interactions with the cytoskeleton of the relevant adhesion molecules (Takeichi 1990; Geiger and Ayalon, 1992). The results presented in this study point to two additional mechanisms which might be responsible for the regulated adhesion, namely the synergism between different adhesion systems, and the effect of post-translational modification (tyrosine phosphorylation) on AJ formation. It might not be surprising that an adhesion molecule such as NCAM does directly contribute to the morphogenesis of cells, in view of its localization along adhesions between adjacent cells and its capacity to interact in an apparently homophilic manner (Figure 1; Bloch, 1992; Edelman, 1985). In this study we show an additional, synergistic effect, whereby the presence of NCAM markedly augments the assembly of a different class of adhesion molecules to assemble into conspicuous junctions. NCAM expression did not alter the level of expression of cadherin or vinculin, but had a stabilizing effect on the assembly of vinculin into AJs. The exact mechanism of this synergism is not elucidated here, yet it is likely that NCAM could either nucleate AJ formation or markedly stabilize them. It is interesting to note, however, that the two molecules did not precisely co-localize in the transfected cells and that down regulation of AJ by H₂O₂/vanadate treatment had, initially, little or no apparent effect on NCAM distribution. Only after prolonged incubation with H₂O₂/vanadate did NCAM adhesions deteriorate too. This may either involve a late direct effect of vanadate on NCAM or, more likely, a secondary, passive, effect induced by AJ dissociation without affecting the homophilic binding affinity of NCAM itself. It should be mentioned that there is no evidence for tyrosine phosphorylation of NCAM itself, nor for co-localization of NCAM and phosphotyrosine.

Is the mechanism whereby the assembly of specific junctions may be affected not only by the availability of the constituents of that particular junction, but also by the presence of adhesion molecules of a different class, physiologically significant? The experiments described here do not directly address this question, yet cellular systems in which multiple adhesion molecules are present are very common. For example, nerve and muscle cells co-express NCAM and N-cadherin (Fredette *et al.* 1993;

Seilheimer *et al.* 1989), endothelial cells contain both PECAM-1 and several types of cadherins (Geiger and Ayalon 1992).

Another mechanism shown here to induce or augment AJ formation (at least transiently) is tyrosine phosphorylation. This effect, shown here with 3T3 cells was initially surprising in view of our previous results with epithelial cells where elevation of tyr-phosphorylation resulted in deterioration, rather than potentiation of intercellular AJ (Volberg *et al.*, 1991;1992). Careful examination of the results show, however, that there is no conflict between the two observations. It appears that in mesenchymal cells, as well as other cell types which primarily form cell-matrix, rather than cell-cell adhesions, a short-term stimulation of tyr-phosphorylation leads to a rapid (<10 min) assembly of the latter and, concomitantly, deterioration of the former. It still remains to be determined which is the primary target for the vanadate effect, namely- do focal contacts deteriorate first, releasing their constituents (for example, vinculin or a-actinin) which, in turn are used to build cell-cell AJ, does the phosphorylation directly and transiently stimulate junction formation or is it a combination of the two.

However, regardless of the precise mechanism, it is evident from the results described here that tyrosine phosphorylation is an effective modulator of cell adhesion, affecting mainly AJ structure. The generality of these observations is demonstrated here by the stimulation of several cell types with vanadate (Figure 6) as well as by the preferential effect of this treatment on cadherin distribution and the relative stability of NCAM (Figure 4). In a different line of studies we have recently demonstrated that cytoskeletal and junctional changes similar to those of H₂O₂/vanadate can be induced in starved endothelial cells, following stimulation with serum or specific growth factors (Ayalon, Yarden and Geiger, unpublished results). This biphasic (constructive-destructive) effect of tyrosine-phosphorylation on AJ structures is in agreement with apparently conflicting current views on the role of tyrosine-phosphorylation in junction formation: the early adhesive response, shown in this study is reminiscent of the early stimulatory effects of tyrosine phosphorylation on focal contact formation and platelet adhesion (BurrIDGE *et al.* 1988; Hynes 1992; Shattil and Brugge 1992). The late effects, on the other hand, are similar to those observed in v-src transformed cells (Behrens *et al.* 1993), or in other tumors (Frixen *et al.* 1991; Ottenhoff-Kalff *et al.* 1992). Based

on previous results in which AJ were modulated by transfection with vinculin sense and anti sense cDNA constructs (Rodrigues et al., 1992) as well as on the results reported here, we would like to propose that de-stabilization of AJ may potentiate invasive growth of tumor cells. The finding of reduced levels of cadherin expression in invasive tumors (Oka et al. 1993; Umbas et al. 1992; Kadowaki et al. 1994) is in line with this hypothesis.

The molecular basis for the effects described here is still unclear. Multiple proteins become tyrosine phosphorylated upon H₂O₂/vanadate treatment (Figure 5), but no particular AJ associated targets were identified in this study. Recent studies have established the association of tyrosine kinases such as FAK125 (Schaller et al. 1992), pp60src (Tsukita et al. 1991; Hamaguchi et al., 1993b, Clark and Brugge, 1993) with cell adhesions, and identified some possible substrates which might be phosphorylated by them. These include vinculin (Sefton and Hunter 1986, talin (Pasquale et al. 1986), integrin (Hirst et al. 1986), paxillin (Glenney and Zokas 1989), tensin (Bockholt and Burridge 1993) and catenins (Behrens et al. 1993). A major challenge for future studies will be the identification of the specific kinases involved in this process, as well as of the respective junctional substrates whose phosphorylation leads to the modulation of AJ and focal contact formation.

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