Involvement of microtubules in the control of adhesion-dependent signal transduction

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Background: The adhesion of cells to the extracellular matrix (ECM) generates transmembrane signals that affect cell proliferation, differentiation and survival. These signals are triggered by interactions between integrin and the ECM and involve tyrosine phosphorylation of specific proteins, including focal adhesion kinase (FAK) and paxillin, and the assembly of focal adhesions and actin bundles. In matrix-adherent, serum-starved Swiss 3T3 cells, the system of focal adhesions and actin bundles is poorly developed, and the level of tyrosine phosphorylation of FAK and paxillin is low. A number of growth factors rapidly stimulate tyrosine phosphorylation of these proteins and the assembly of focal adhesions and actin bundles. Growth factors and adhesion to the ECM are both necessary for the subsequent transition of cells to the S-phase of the cell cycle.

Results: In serum-starved Swiss 3T3 cells, the disruption of microtubules by nocodazole or vinblastine, without the addition of external growth factors, induces the rapid assembly of focal adhesions and microfilament bundles, tyrosine phosphorylation of FAK and paxillin, and subsequent enhancement of DNA synthesis. All these effects require cell adhesion to the ECM and do not occur when cells are plated on substrates coated with poly-L-lysine or concanavalin A. Inhibitors of tyrosine phosphorylation and cell contractility also eliminate the effects of microtubule disruption on adhesion-dependent signal transduction.

Conclusions: In ECM-attached cells, microtubule disruption activates the integrin-dependent signaling cascade, which leads to the assembly of matrix adhesions and the induction of DNA synthesis. The increase in cell contractility is an indispensable intermediate step in this signaling process.

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Background

The adhesion of cells to the extracellular matrix (ECM) produces a signal that, together with signals induced by soluble ligands, controls major events in the cell's life. Thus, cell proliferation, differentiation, and even survival, strongly depend on specific contacts with appropriate ECMs [1,2]. Contacts between cell-surface receptors known as integrins and their counterpart ECM molecules, attached to a solid surface, induce a rapid sequence of protein assembly and modification events in the cytoplasm which culminates in formation of focal adhesions. These structures are associated with bundles of actin filaments (known as stress fibers) and contain complexes of signal transduction proteins [3,4]. The formation of focal adhesions and stress fibers is accompanied by the tyrosine phosphorylation of several specific proteins, including focal adhesion kinase (FAK) and paxillin [4–6]; inhibitors of tyrosine phosphorylation interfere with the assembly of focal adhesions [6–8]. These early changes in protein phosphorylation and assembly are also thought to be required for stimulating the transition through G1–S phase after cell attachment, through the activation of the Ras/MAP kinase (mitogen-activated protein kinase) cascade [9–11].

Besides attachment to the specific ECM, assembly of mature focal adhesions and stress fibers usually requires co-stimulation by certain soluble ligands — including lysophosphatidic acid, bombesin and PDGF — which induce the rapid assembly of large focal adhesions and stress fibers in serum-starved cells [12,13]. The effect of such growth factors was shown to be mediated through the activation of the small GTP-binding protein Rho [12,13]. The addition of growth factors to the medium, or the introduction of activated Rho into cells, induces tyrosine phosphorylation of FAK and paxillin [14–17].

It has been extensively documented that an intact actin cytoskeleton is also essential for integrin-dependent focal adhesion assembly and signal transduction. Cytochalasins were shown to inhibit the early events of this pathway, including growth factor- and ECM-induced protein tyrosine phosphorylation [14–16,18] and MAP kinase activation

[9,11]. Consequently, the later events, such as hyperphosphorylation of the retinoblastoma protein (Rb), cyclin D1 induction [19] and, finally, the G1–S transition [19,20], also depend on the integrity of actin cytoskeleton.

In the majority of cell types, the actin cytoskeleton functions in concert with other cytoskeletal components, particularly the microtubular system. The interactions between the actin cytoskeleton and microtubules may apparently be either cooperative or antagonistic, depending on the specific cellular system tested [21,22]. Although there is no direct information on the involvement of microtubules in adhesion-dependent signaling, the paradoxical finding that microtubule disruption induces the stimulation of DNA synthesis in a variety of quiescent cell types is well documented [23–26].

Here, we show that the microtubular network, together with the actin system, participates in the control of integrin-dependent signal transduction. We demonstrate that microtubule disruption induces signal transduction events in quiescent cells similar to those triggered by a variety of growth factors, including tyrosine phosphorylation of FAK and paxillin, and the assembly of focal adhesions and stress fibers. Subsequently, the cells emerge from quiescence and enter the S-phase of the cell cycle. We have characterized this phenomenon and present evidence suggesting that the involvement of microtubules in adhesion-dependent signaling is related to their interaction with, or restraining effect on, the contractile actomyosin system.

Results

Microtubule disruption in quiescent cells induces focal adhesions and stress fiber formation and phosphorylation of FAK and paxillin

The microtubular system of serum-starved cells, as detected by immunofluorescence staining, is well developed and has a typical radial organization (Fig. 1a). On the other hand, these cells have poorly organized actin bundles (Fig. 1b), and small and sparse vinculin- and paxillin-containing focal contacts (Fig. 1f,h) compared with non-starved cells. The addition of 10 μM nocodazole to these cells resulted in the rapid destruction of microtubules (Fig. 1c) and induced a dramatic increase in the number and size of microfilament bundles. Development of actin bundles, mainly at the cell periphery, was already apparent 3 minutes after the addition of nocodazole (Fig. 1d); upon longer incubation, numerous prominent bundles were detected throughout the entire cytoplasm (Fig. 1e). In parallel, both the number and the size of vinculin- and paxillin-rich focal contacts dramatically increased (Fig. 1g,i). Direct microscopic observation of live cells suggested that shortly after the addition of nocodazole these cells displayed some peripheral contraction (data not shown), as described by Danowski [27].

Fluorescence anti-phosphotyrosine labeling of serum-starved cells revealed only weak staining associated with the dot-like structures. Nocodazole-treated cells, on the other hand, had large prominent and brightly labeled focal adhesions (Fig. 2a). The effect of microtubule disruption on tyrosine phosphorylation of cellular proteins was also studied using immunoblotting and immunoprecipitation techniques. Immunoblot analysis of total cell lysates revealed that the level of tyrosine phosphorylation of several proteins — including major species with apparent molecular weights of 125, 90 and 68 kDa (Fig. 2b) — rapidly increased after the addition of nocodazole. Phosphorylation of several other proteins (for example, the ~60 kDa species) was not affected by nocodazole treatment. As shown in Figure 2c, the 125 kDa protein immunoprecipitated by the anti-phosphotyrosine antibodies reacted with antibodies directed against FAK, and the 68 kDa species reacted with anti-paxillin antibodies. Phosphorylation of both proteins reached maximal levels after 20 minutes of nocodazole treatment and declined after longer incubation. The identity of the 90 kDa band is still unclear.

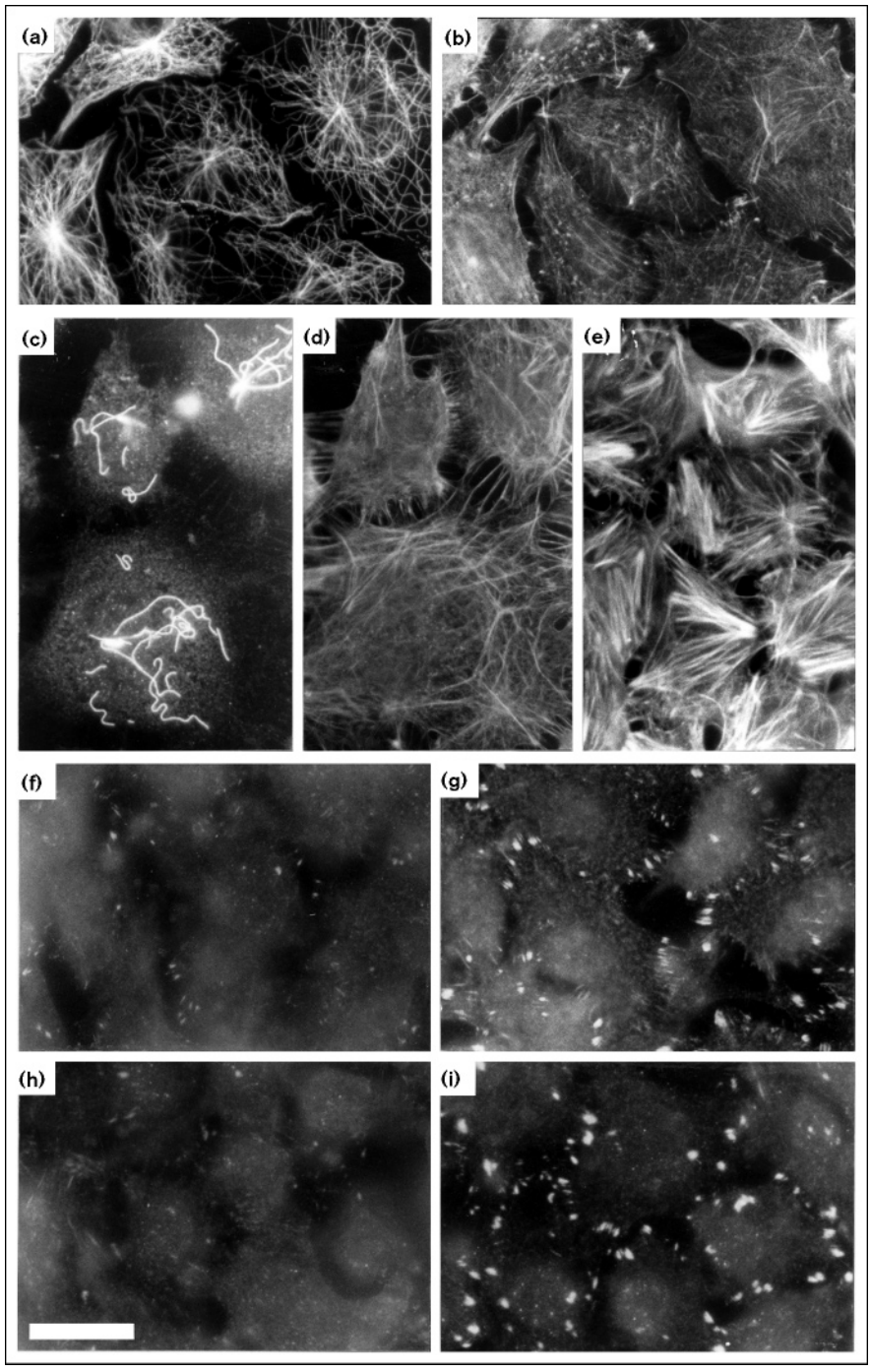
All the effects described above were attributable to microtubule disruption, because they could also be induced using microtubule-disrupting drugs other than nocodazole, including vinblastine (Fig. 3a,b,e), which disrupts microtubules using a different mechanism to that of nocodazole [28] and produces characteristic tubulin 'paracrystals' (Fig. 3a). On the other hand, taxol, a microtubule-stabilizing agent [28], did not stimulate the assembly of stress fibers or the tyrosine phosphorylation of FAK and paxillin (Fig. 3c–e). Moreover, pretreatment with taxol efficiently prevented all the effects exerted by nocodazole on quiescent fibroblasts. When the cells were preincubated for 2 hours with 20 μM taxol, and 10 μM nocodazole was added to the same medium for an additional 30 minutes, neither microtubule disruption, nor tyrosine phosphorylation and stress fiber formation were observed (data not shown).

Inhibitors of tyrosine phosphorylation and cell contractility prevent formation of focal adhesions induced by microtubule disruption

Genistein, a broad spectrum tyrosine kinase inhibitor [29], and H-7, a serine/threonine kinase inhibitor [30], both completely abolished the nocodazole-induced effects on focal contact formation and protein tyrosine phosphorylation (Fig. 4a–d,f). Genistein treatment strongly inhibited the phosphorylation of most of the labeled bands, whereas H-7 more specifically inhibited the nocodazole-induced increase in the phosphorylation of FAK, paxillin and the 90 kDa protein, without significantly affecting the phosphorylation of several other major species (Fig. 4f). H-7 is a broad specificity serine/threonine kinase inhibitor with a high inhibitory activity against protein kinase C (PKC) [30]. However, a specific PKC inhibitor, known as bisindolylmaleimide (GF 109203X) [31], did not interfere with

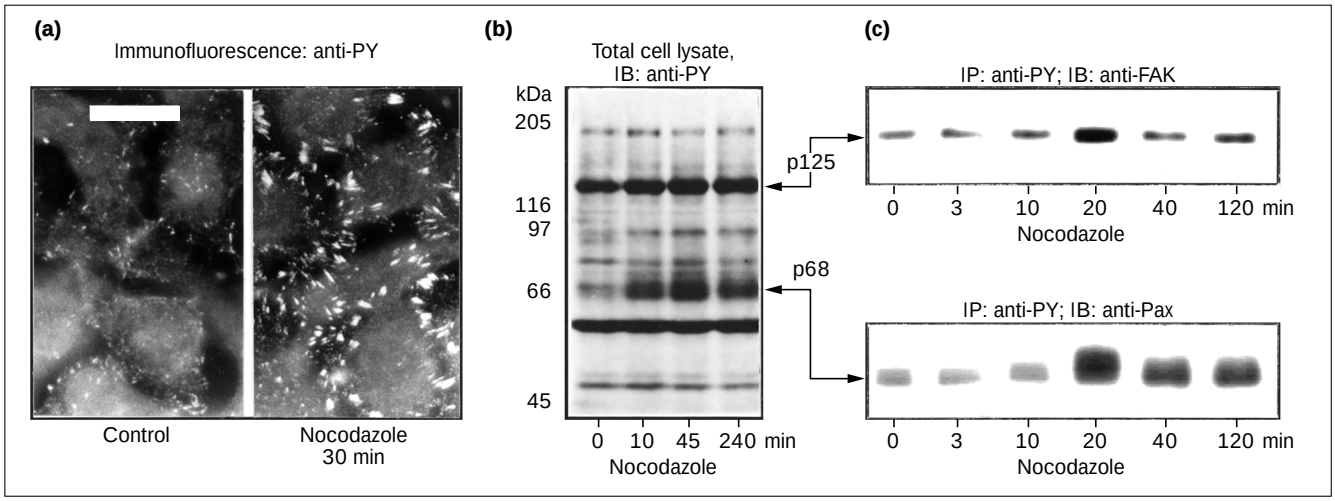
the effects of nocodazole (data not shown). On the other hand, H-7 has previously been shown to inhibit actomyosin-based contractility, most probably *via* inhibition of myosin light chain kinase (MLCK) [32]. We therefore examined the effect of a specific MLCK inhibitor, KT5926 [33], on the nocodazole-induced events, and found that this drug significantly, but not completely, inhibited the formation of focal adhesions (Fig. 4e) and strongly diminished the nocodazole-induced augmentation of tyrosine phosphorylation of the 68 kDa (paxillin) and 90 kDa proteins (Fig. 4f). The effect of this inhibitor on the tyrosine phosphorylation of FAK was limited. None of the kinase inhibitors used affected microtubule depolymerization by nocodazole.

Figure 1



Formation of actin cables (stress fibers) and focal adhesions in serum-starved Swiss 3T3 cells after microtubule disruption. (a,b,f,h) Untreated cells 24 h after serum starvation; the radial system of microtubules is well preserved (a), while actin cables are poorly organized and not prominent (b). Note the dot-like sparsely distributed vinculin-positive (f) and paxillin-positive (h) focal adhesion-like structures. Treatment of similar cultures with nocodazole for 3 min (c,d) induces almost complete disruption of microtubules (c), and the formation of prominent actin cables at the cell periphery (d). 30 min after the addition of nocodazole, numerous thick stress fibers were formed in all the cells (e). At that time both vinculin (g) and paxillin (i) were concentrated in numerous large focal adhesions. Immunofluorescence staining was carried out using antibodies directed against tubulin (a,c), vinculin (f,g) and paxillin (h,i). (b,d,e) Visualization of filamentous actin with phalloidin; (b) and (d) are photographs of the same fields as (a) and (c), respectively. Nocodazole was added to serum-free medium at a final concentration of 10 μM. Scale bar in (h) = 20 μm.

Figure 2



Tyrosine phosphorylation of cellular proteins. (a) Immunofluorescence staining of serum-starved untreated (left) and nocodazole-treated (right) Swiss 3T3 cells using anti-phosphotyrosine (anti-PY) antibodies. Scale bar = 20 μm. (b) Immunoblot (IB) analysis of total lysates of Swiss 3T3 cells after serum starvation and nocodazole treatment for different times. Blots were stained with anti-PY antibodies. (c) Identification of the two major tyrosine-phosphorylated proteins in nocodazole-treated cells after immunoprecipitation (IP) of total cell lysates with anti-PY antibody and immunoblotting (IB) of the precipitate with anti-FAK and anti-paxillin (anti-Pax) antibodies.

Cell attachment to the ECM is essential for the formation of focal adhesions and tyrosine phosphorylation events induced by microtubule disruption

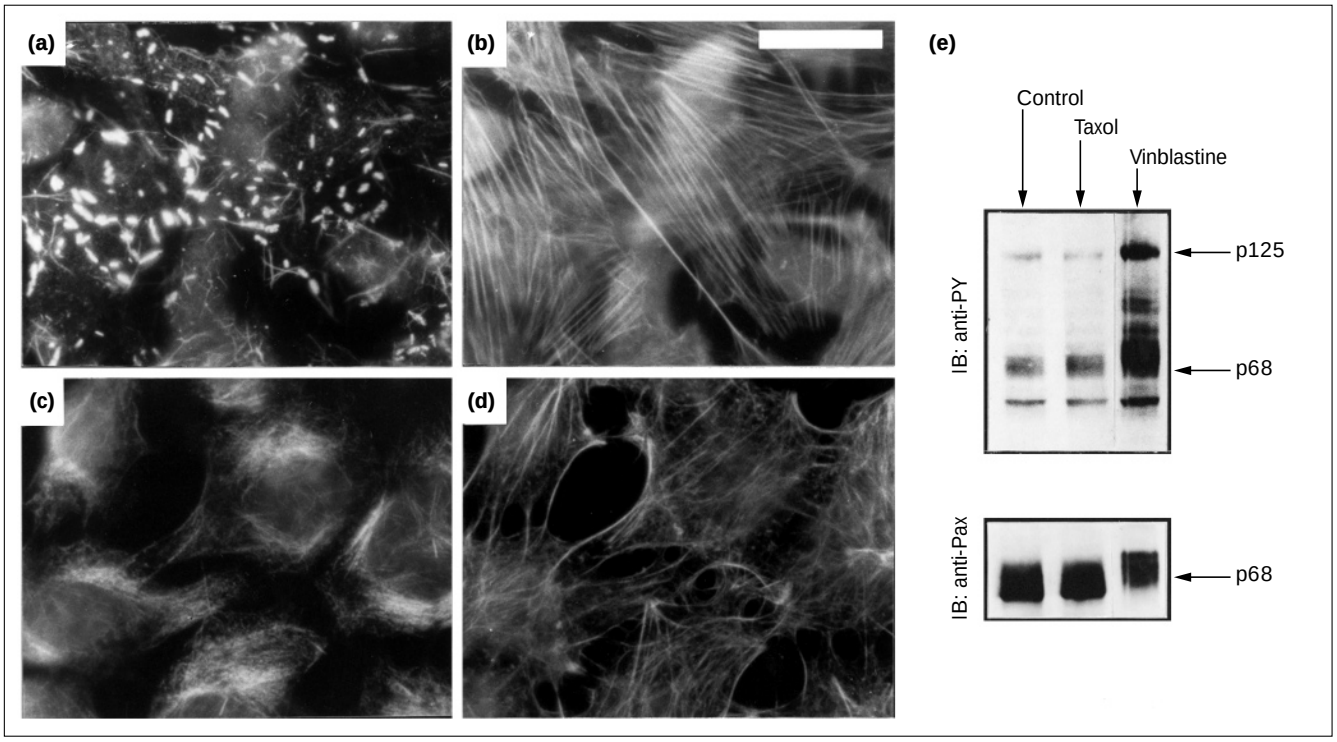
Further experiments indicated that the nocodazole-induced tyrosine phosphorylation and enhancement of focal adhesion and actin bundle organization required the specific adhesion of cells to the ECM. Thus, cells plated in the serum free-medium on a fibronectin-coated substrate produced a strong response to nocodazole treatment (Fig. 5a,c,e). The effect of fibronectin depended on its concentration: at low concentrations (10 $\mu g\ ml^{-1}$) the cells contracted and rounded after nocodazole treatment (data not shown); if, however, the concentration of fibronectin was sufficiently high (100 $\mu g\ ml^{-1}$), the nocodazole-induced contraction was accompanied by the formation, at the cell periphery, of numerous radially oriented and tyrosine phosphorylated focal adhesions (Fig. 5c). The system of stress fibers was assembled in association with the focal adhesions (data not shown). The increase in the level of protein tyrosine phosphorylation was mainly attributable to paxillin and FAK (Fig. 5e).

In contrast, cells plated in serum-free medium on substrates coated with poly-L-lysine or concanavalin A (ConA) did not show a significant increase in tyrosine phosphorylated FAK and paxillin, and did not form the conspicuous, phosphotyrosine-rich focal adhesions after microtubule disruption (Figs. 5b,d,e). The inability of cells to form focal adhesions on poly-L-lysine was not related to any toxic effect of this compound, because the fibronectin-coated coverslips were precoated with poly-L-lysine.

Stimulation of DNA synthesis by microtubule disruption requires cell–ECM adhesion and can be blocked by inhibitors of tyrosine phosphorylation and cell contractility

One of the extensively documented yet poorly understood effects of microtubule disruption is the stimulation of DNA synthesis in quiescent cells [23–26]. We have confirmed these results by demonstrating that the fraction of cells incorporating bromodeoxyuridine (BrdU) into nascent DNA after pulse labeling increased 2–3 fold following overnight incubation of serum-starved cells with nocodazole. This increase was not as strong as the 15–20-fold elevation (compared to basal levels) seen after serum stimulation (Fig. 6a–f), but was highly significant and reproducible. In accordance with the published data [24] we were able to detect the increase in BrdU incorporation even after short-term treatment with nocodazole, followed by overnight incubation in serum-free medium (Fig. 6g). These kinetics of the nocodazole stimulation made it possible to study whether inhibitors that interfere with the early signaling events induced by microtubule depolymerization would also block the later event, namely the G1–S transition. In particular, we studied the effect of H-7 and genistein on the nocodazole-induced stimulation of DNA synthesis. In these experiments the cells were incubated with nocodazole for 3 hours and BrdU incorporation was measured 16 hours later. As seen in Figure 6, H-7 completely prevented the stimulatory effect of nocodazole, while it had a minimal effect on the background level of BrdU incorporation in serum-starved cells. Genistein abolished the effect of nocodazole and reduced the incorporation of BrdU to almost zero in nocodazole-treated and

Figure 3



The effects of vinblastine and taxol on the organization of actin cables, and tyrosine protein phosphorylation. (a–d) Effects on the actin cytoskeleton; Swiss 3T3 cells were incubated in serum-free medium for 24 h and treated with 50 μM vinblastine for 90 min (a,b), or with 20 μM taxol for 240 min (c,d). (a,c) Anti-tubulin staining; (b,d) phalloidin staining of the same fields. In vinblastine-treated cells microtubules are disrupted, tubulin is concentrated in numerous paracrystals (a), and all cells contain prominent straight actin cables (b). In taxol-treated cells, large bundles of microtubules are formed (c), while the actin cytoskeleton remains poorly organized (d). Scale bar in (b) = 20 μm. (e) Effects of vinblastine and taxol on protein tyrosine phosphorylation. Lysates of untreated (control) serum-starved Swiss 3T3 cells, and of the same cells treated with 20 μM taxol or 50 μM vinblastine for 60 min, were analyzed by immunoblotting using anti-phosphotyrosine antibodies. Treatment with taxol did not induce any apparent change in tyrosine phosphorylation compared to the serum starved control, while vinblastine induced tyrosine phosphorylation of the same set of proteins as nocodazole (namely, FAK, paxillin and the ~90 kDa protein). Upper panel: immunoblotting of total cell lysates with anti-phosphotyrosine antibodies; lower panel: the same blot, stripped and restained with anti-paxillin antibodies to illustrate equal protein loading.

untreated cells (Fig. 6g). This inhibition, however, was reversible, as the addition of nocodazole after the removal of genistein increased BrdU incorporation more than 3 fold over the basal level (data not shown).

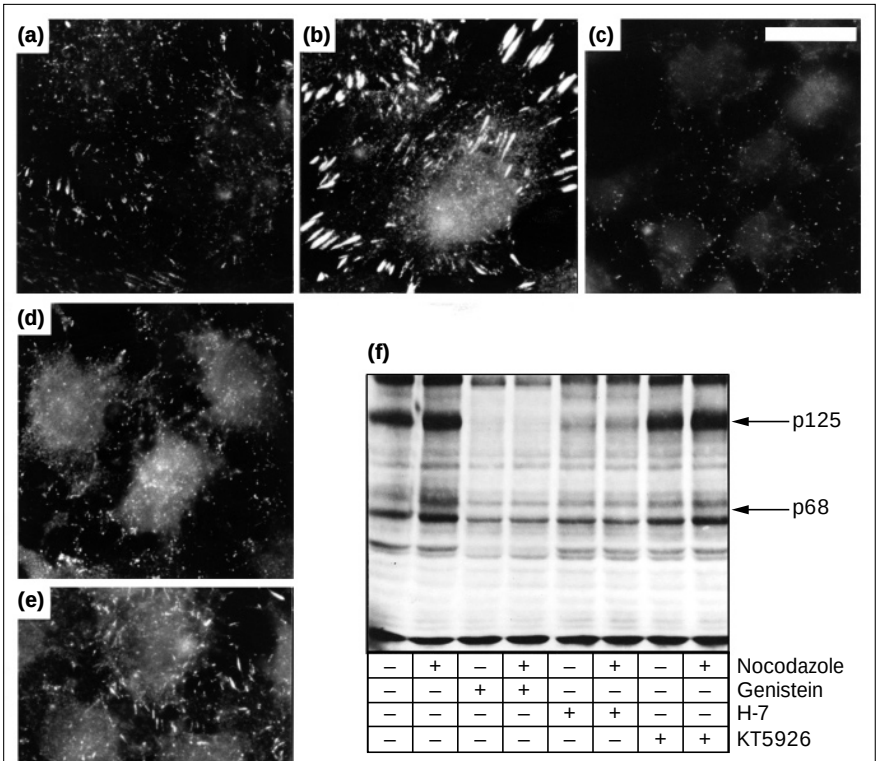
Furthermore, we showed that the stimulatory effect of microtubule disruption on DNA synthesis — as well as its effects on protein tyrosine phosphorylation and focal adhesion formation — depends on integrin-mediated cell adhesion. Cells plated on fibronectin-coated coverslips showed the same background level of BrdU incorporation as the cells in stationary culture after an identical period of serum starvation. Incubation with nocodazole after plating on fibronectin increased the percentage of BrdU-positive nuclei (Fig. 6g). At the same time, the cells plated on poly-L-lysine in serum-free medium showed lower levels of BrdU incorporation than serum-starved cells in stationary culture, and did not show any increase in BrdU incorporation after microtubule disruption (Fig. 6g).

Discussion

The primary objective of these studies was to elucidate the molecular basis for the diverse pleiotropic effects of microtubule disruption in quiescent cells. These effects include the assembly of prominent actin cables, an increase in the number and size of vinculin-containing focal adhesions, local tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin, and the stimulation of DNA synthesis. We observed these effects in serum-starved cells; however, similar although less dramatic effects could also be seen in the cells growing in serum-containing medium. Some of the structural effects, namely the assembly of actin bundles and focal contacts, are in line with previous reports [34,35]. In our system the effects are more prominent, presumably because of the use of serum-starved 3T3 cells.

One clear observation is that microtubule disruption alone is not sufficient for the induction of tyrosine phosphorylation, the assembly of focal adhesions and stress fibers, or

Figure 4



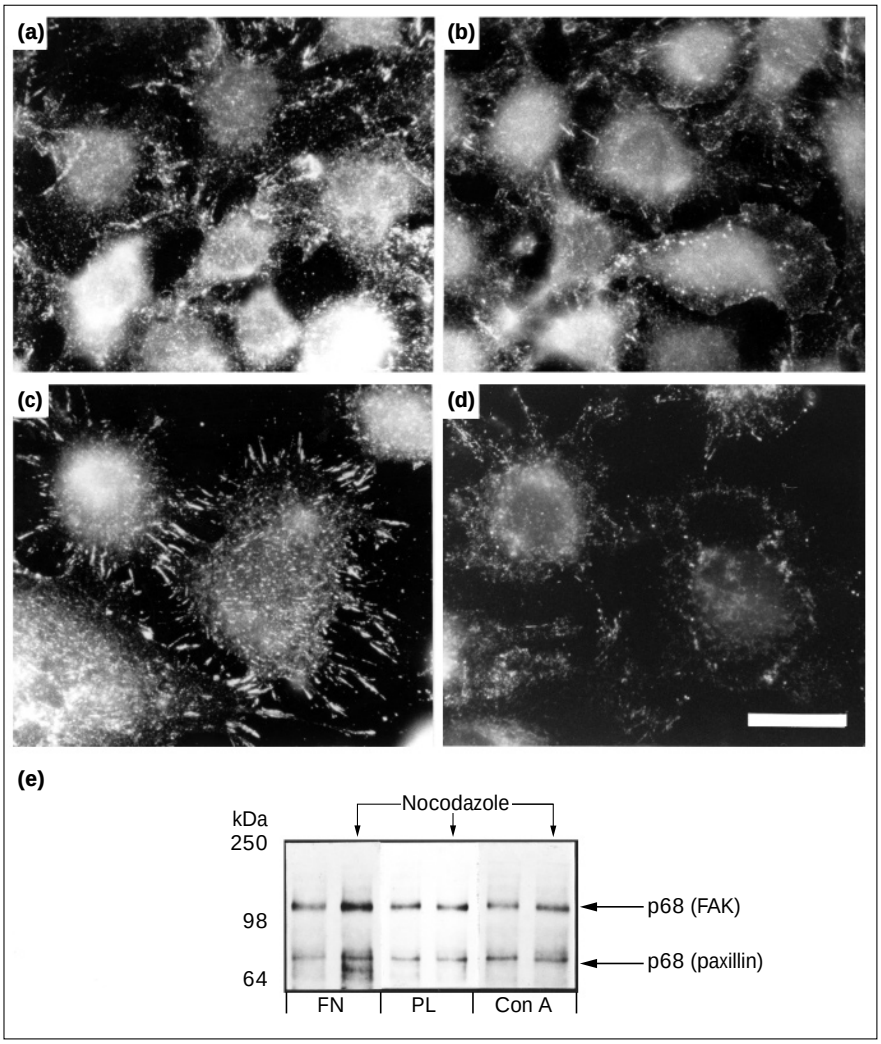
Effects of different kinase inhibitors on nocodazole-induced protein tyrosine phosphorylation and the formation of focal adhesions. Immunofluorescence staining with anti-phosphotyrosine antibody of control, serum-starved cells (**a**), the same cells treated with nocodazole alone (**b**), and with nocodazole in the presence of genistein (**c**), H-7 (**d**), or KT5926 (**e**). Scale bar in (c) = 20 μm. (**f**) Immunoblot analysis of cell lysates with the anti-phosphotyrosine antibody PT66. Final concentrations of the drugs were 10 μM for nocodazole, 100 μg ml^{-1} for genistein, 300 μM for H-7, and 20 μM for KT5926. Cells exposed to the combined action of kinase inhibitor and nocodazole were preincubated first with kinase inhibitor for 1 h, and then the nocodazole was added to the same medium for additional 30 min.

for the stimulation of DNA synthesis: for a complete effect the cells have to be attached to specific ECM proteins, such as fibronectin, and to be contraction-competent. Control cells attached to 'non-specific' substrates coated with poly-L-lysine- or concanavalin A, did not exhibit such responses, suggesting that there is an essential cross-talk between microtubule organization and the ECM-activated integrin-dependent signal transduction pathway. Moreover, interference with cell contractility abolished the 'nocodazole stimulation', suggesting that activation of actomyosin contractility following microtubule disruption is essential for the manifestation of the pleiotropic effect.

Experiments with genistein, a non-selective tyrosine phosphorylation inhibitor, show that the downstream events following microtubule disruption in ECM-attached cells apparently depend on the triggering of tyrosine phosphorylation signals. In order to elucidate the role of specific kinases we have now begun to examine the effects of different tyrosine kinase inhibitors (tyrphostins) on the nocodazole-induced formation of focal adhesions and stress fibers. In particular, we have found that tyrphostin AG213, which inhibits FAK tyrosine kinase activity *in vitro* and prevents FAK phosphorylation *in vivo* [7], does not interfere with nocodazole-induced formation of focal adhesions and stress fibers in quiescent cells; in contrast, tyrphostin AG879, an inhibitor of the Neu/HER-2 tyrosine kinase [36] efficiently prevents these effects of nocodazole (T. Volberg, A.B., A. Levitzki and B.G., unpublished observations). Elucidation of the role of specific tyrosine kinases in the cascade of events triggered by microtubule disruption is a task for future studies.

How does microtubule depolymerization induce adhesion-dependent tyrosine phosphorylation? And in general, how do cells transform cytoskeletal changes into specific biochemical reactions, such as protein phosphorylation? The possibility that the increase in the concentration of unpolymerized tubulin in the cytoplasm is involved in the regulation of adhesion-dependent signal transduction seems unlikely, because vinblastine — which, in contrast to nocodazole, decreases the levels of free tubulin by sequestering tubulin subunits into paracrystals [28] — exerts the same effects as nocodazole on tyrosine phosphorylation and the microfilament system. Thus, it appears that the destruction of microtubules, rather than the increase in free cytoplasmic tubulin, is the main factor that promotes integrin-dependent signaling.

Figure 5



Role of the ECM in nocodazole-induced protein tyrosine phosphorylation. (a–d) Immunofluorescence using PT66 anti-phosphotyrosine antibodies. (a,b) Control untreated cells, 6 h after seeding in serum-free medium; (c,d) identical cultures exposed to nocodazole for 30 min. (a,c) Cells attached to fibronectin-coated coverslips; (b,d), cells attached to poly-L-lysine-coated coverslips. Cells attached to poly-L-lysine form only sparse dot-like phosphotyrosine-containing structures in the absence (b) and presence (d) of nocodazole. Scale bar = 20 μm. (e) Analysis of major tyrosine phosphorylated proteins by immunoprecipitation of total cell lysate with the anti-phosphotyrosine antibody PY-20 and immunoblotting of the precipitate using another anti-phosphotyrosine antibody (PT-66). The cells were lysed 16 h after seeding on Petri dishes coated with fibronectin (FN), poly-L-lysine (PL), or concanavalin A (Con A). Nocodazole was added 20 min before cell lysis.

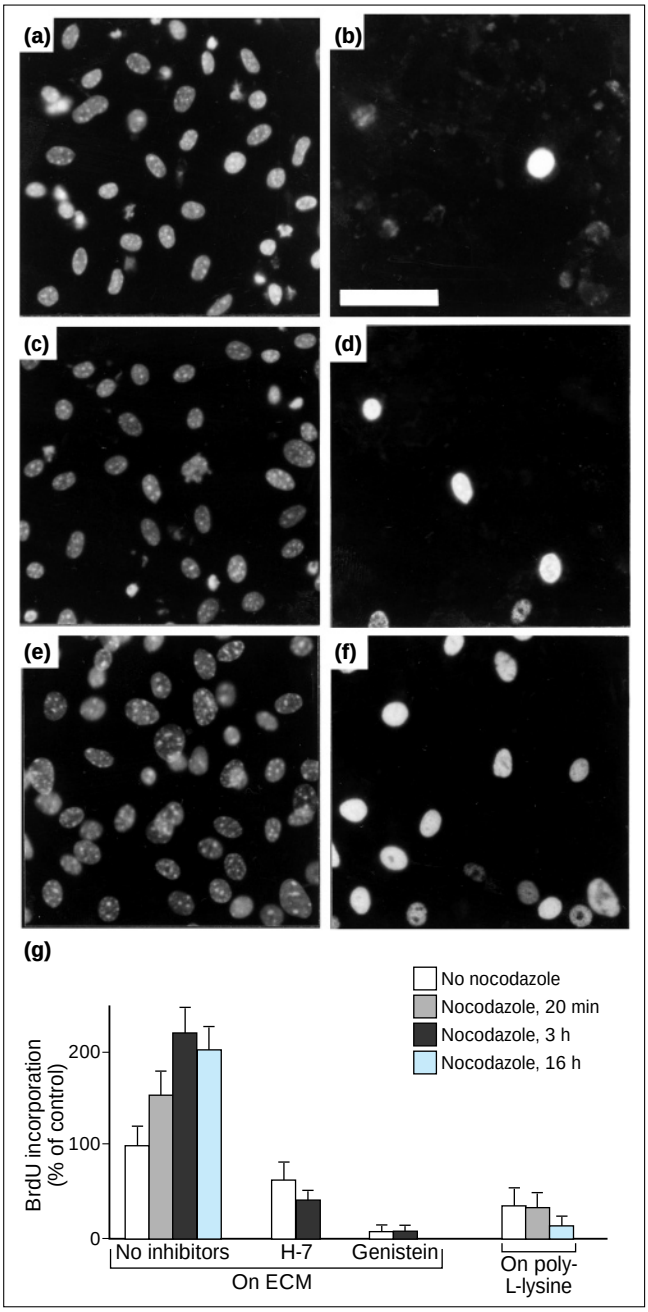
Several general mechanisms (not necessarily mutually exclusive) that may link microtubule destruction to the various cellular responses discussed above may be considered. First, the impairment of microtubules may block intracellular vesicular transport, and might affect signaling by blocking receptor internalization or recycling, for example [37]. Second, microtubule depolymerization may lead to the translocation of kinases or phosphatases normally associated with microtubules [37–39]. The most immediate cellular effect of microtubule disruption, however, seems to be generalized cell contraction [27] that was shown to be associated with an increase in the phosphorylation of the myosin regulatory light chain [40]. Thus, the disruption of microtubules might alter the force balance in the cell leading to increased tension at focal adhesions.

As previously mentioned, our data suggest that this increase in cell contractility is an indispensable step between microtubule disruption and the organization of focal adhesions and microfilament bundles. This notion is based on the fact that MLCK inhibitors, such as H-7 and KT5926, which block cell contractility, also inhibit nocodazole-induced tyrosine phosphorylation of focal adhesion proteins and assembly of focal adhesions and actin bundles. An additional requirement for a 'full effect' of microtubule disruption is the interaction between the cell and ECM components (such as fibronectin), indicating that integrin signaling demands the local tension exerted through actin on the integrin–ECM transmembrane complex.

We propose that microtubules normally counteract cell contractility, and thus microtubule disruption strongly increases the tension exerted by the actin cytoskeleton on the cell–ECM focal adhesions, as shown in Figure 7. These sites are highly enriched with tyrosine kinases and other signal transduction proteins and may function as 'signal transduction organelles' [3,4]. More specifically, one may regard focal adhesions as tension-sensing devices

[22], which convert cell contraction into protein modification events, such as tyrosine phosphorylation (Fig. 7). This signaling may also have a local effect in stimulating the recruitment of new structural and signal-transduction proteins, leading to the expansion of focal adhesions and the assembly of stress fibers. This is in line with the idea that isometric tension may promote the formation of actin microfilament bundles [32,41]. Recent data showing that cell stretching can stimulate tyrosine phosphorylation of FAK [42] also support this view.

Moreover, recent studies indicate that Rho-mediated induction of focal adhesions and stress fibers by a variety of growth factors also might operate *via* contractility regulation. First, it was shown that Rho and Rho-associated kinase inhibit the activity of myosin light chain phosphatase [43]. This might result in an increase in the myosin light chain phosphorylation and consequently stimulate cell contraction. In fact, it is known that Rho-activating growth factors (such as lysophosphatidic acid) as well as whole serum are potent inducers of cell contractility [44]. Second, Chrzanowska-Wodnicka and Burridge [45] showed that, in quiescent cells, the impediment of cell contractility by several myosin inhibitors prevented the effects of Rho on the formation of stress fibers and focal adhesions. These findings indicate that stimulation of cell contractility could be an important step in the Rho-dependent signal transduction pathway. The similarity between Rho-mediated effects of growth factors and the effects induced by microtubule disruption can be then explained by the fact that both types of treatment cause cell contraction.



Conclusions

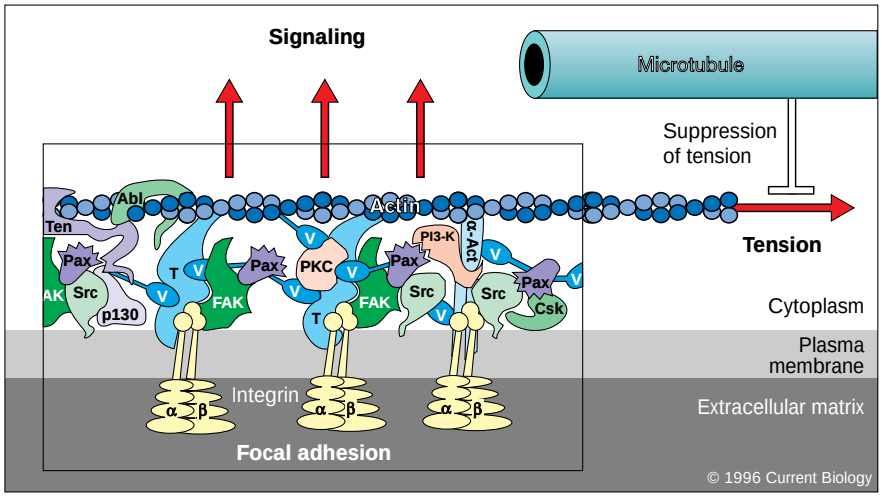
The results presented here show that the disruption of microtubules in quiescent 3T3 cells induces the assembly of focal adhesions and actin microfilament bundles, the tyrosine phosphorylation of FAK and paxillin, and subsequently promotes the G1–S transition. Both protein tyrosine phosphorylation and the assembly of actin bundles and adhesion plaques require cell adhesion to the ECM and do not occur when cell contractility is impaired. Moreover, ECM deprivation, or the addition of protein kinase inhibitors that block the early phosphorylation and assembly events, also inhibit the stimulation of DNA synthesis.

We have provided the first demonstration that cytoskeletal modulation, such as microtubule disruption, triggers integrin-dependent signaling in the absence of external growth factor stimulation. The results of our experiments with inhibitors of cell contractility indicate that nocodazole-induced contraction is an important element in triggering

Figure 6

Effects of microtubule disruption on DNA synthesis. Visualization of BrdU incorporation into DNA in control serum-starved cells (**a**,**b**), and in similar cells incubated for 16 h either with 10 μM nocodazole (**c**,**d**), or with 10 % serum (**e**,**f**). (a,c,e) DNA staining with DAPI; (b,d,f) indirect anti-BrdU antibody staining of cells from the same fields; (b) corresponds to (a), (d) to (c), and (f) to (e). Scale bar in (b) = 50 μm. (**g**) Measurements of the effects of kinase inhibitors and attachment to non-specific substrates on BrdU incorporation in control and nocodazole-stimulated cells. Cells were plated in serum-free medium on the coverslips coated either with poly-L-lysine alone, or with poly-L-lysine and fibronectin (ECM), treated with nocodazole for different periods and incubated overnight in serum-free medium. Alternatively, in the experiments with inhibitors, cells were grown for several days on coverslips in serum-containing medium, serum-starved for 24 h and then treated with nocodazole in the presence or absence of inhibitors. Inhibitors were added to the serum-free medium 30 min earlier and washed off 30 min later than nocodazole. BrdU incorporation was measured 16 h later.

Figure 7



Proposed model for the involvement of microtubules in the regulation of adhesion-dependent signaling. Focal adhesions are specialized sites where actin filaments are associated with the ECM *via* integrins and several structural linkers such as talin (T), vinculin (V) and α-actinin (α-Act). These cellular domains are also involved in signal transduction. Among the signaling proteins associated with focal adhesions are tyrosine protein kinases (FAK, Src family, Csk and Abl), their potential substrates (paxillin (Pax), tensin (Ten) and p130^{Cas}), serine/threonine kinase PKC and phosphatidylinositol 3-kinase (PI3-K) [4]. Our studies suggest that microtubules control integrin-dependent signaling. A possible mechanism of this control may be a microtubule-mediated suppression of contractility. The tension at focal adhesions developed by the actin system may affect adhesion-dependent signaling. This tension might be locally controlled by the state of microtubule assembly.

integrin-dependent signal transduction. Finally, we have shown that the disruption of microtubules induces a battery of cellular responses in ECM-adherent cells — including contraction, tyrosine phosphorylation, the assembly of focal adhesions and actin bundles, and the stimulation of DNA synthesis — which are remarkably similar to those induced by a variety of growth factors. We propose that mechanical tension may enhance tyrosine phosphorylation at focal contacts. This, in turn, may enhance the assembly of focal contacts and stress fibers, and ultimately generate specific signals which might have long-range and long-term effects on cell behavior and growth. One of the functions of the microtubule system might be to control these tensions either at the global or local level.

Materials and methods

Cells, substrates and culture conditions

Swiss 3T3 fibroblasts were used in all the experiments. The cultures were routinely maintained in Dulbecco Modified Eagle's Medium (DMEM), supplemented with 10 % bovine calf serum (HyClone Laboratories, Logan, Utah, USA) in a humidified atmosphere with 7.5 % CO_2 at 37 °C; trypsin–EDTA was used to subculture the cells. Confluent cultures grown in serum-containing medium were washed once with warm serum-free medium and incubated in serum-free medium for 16–48 h. These serum-starved cultures were used in most of the experiments. Alternatively, when the effect of extracellular matrices were studied, the cells were plated in serum-free medium on ECM-coated substrates. Coverslips or plastic culture dishes were incubated for 5–10 min at room temperature with a 0.1 mg ml^{-1} aqueous solution of poly-L-lysine (approximate molecular weight 115 kDa; Sigma Chemical Co.; catalogue reference P-1274). The coated surfaces were rinsed with water and allowed to dry for 1–2 h. For fibronectin coating the substrates were treated first with poly-L-lysine, as described above, and then briefly incubated in a 0.1 mg ml^{-1} solution of bovine plasma fibronectin (Sigma; catalogue reference F-1141) in PBS. After air-drying, the substrates were sterilized by UV treatment. To coat the substrates with concanavalin A (Sigma; catalogue reference C-2010) they were immersed in a 0.5 mg ml^{-1} sterile solution of the lectin in PBS and incubated overnight at 4 °C. The substrates were rinsed in serum-free medium before use. Confluent cultures were suspended by trypsin–EDTA treatment, washed in serum-free medium containing 5 mg ml^{-1} soybean trypsin inhibitor (Sigma; catalogue reference T-9003) and resuspended in serum-free medium. Cells were allowed to settle onto coated substrates, then treated with nocodazole or other drugs, and fixed (for immunofluorescence) or lysed (for immunoprecipitation and western blotting) at appropriate time points.

Nocodazole (methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) carbamate), vinblastine, taxol (paclitaxel), and the protein kinase inhibitors, genistein, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) and KT5926, were obtained from Sigma; bisindolylmaleimide (GF 109203X) was from Calbiochem. Stock solutions of vinblastine and H-7 were prepared in water; stock solutions of other compounds were prepared in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the medium in all experiments did not exceed 0.4 %

Immunofluorescence microscopy

Mouse monoclonal antibodies to vinculin (clone hVIN-1) and α-tubulin (clone DM 1A) were purchased from Sigma Israel Chemicals (Holon, Israel); the mouse monoclonal anti-paxillin antibody was from Transduction Laboratories (Lexington, USA). Rhodamine- and fluorescein-labeled phalloidin were from Molecular Probes (Eugene, USA). FITC- and TRITC-conjugated goat anti-mouse and anti-rabbit immunoglobulins were obtained from Jackson Laboratories (West Grove, USA). For regular fluorescent staining, the cells were permeabilized by brief treatment with 0.5 % Triton X-100 in 50 mM MES buffer (pH 6.0) and then fixed with 3 % paraformaldehyde. Immunolabelling and photographing were performed as described [32]. For immunofluorescence with anti-phosphotyrosine antibodies, the cells were simultaneously fixed and permeabilized in PBS containing 3 % paraformaldehyde and 0.5 % Triton X-100 for 2 min, postfixed in the 3 % paraformaldehyde for 20 min, and stained with the monoclonal PT66 anti-phosphotyrosine antibody (Sigma Israel Chemicals) using indirect immunofluorescence.

Western blotting and immunoprecipitation

Cells cultured on 50 mm Petri dishes were washed with PBS and dissolved in the Laemmli electrophoresis sample buffer supplemented with 1 mM sodium orthovanadate. Cell lysates were subjected to SDS–PAGE, electroblotted onto a nitrocellulose membrane (Hybond-C, Amersham, UK) and probed with the anti-phosphotyrosine

antibody PT66, followed by anti-mouse horseradish peroxidase-labeled antibody (Amersham). Immunoreactive bands were detected using enhanced chemiluminiscence, using reagents from Kirkegaard and Perry Laboratories (Maryland, USA), and RX film (Fuji Photo Film Co., Japan). For immunoprecipitation, cells cultured on the dish were lysed in 1 ml of non-denaturing solubilization buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 10 % glycerol, 1.5 mM $MgCl_2$, 1 mM sodium orthovanadate, 180 μg ml^{-1} aprotinin, 2 mM PMSF and 180 μg ml^{-1} leupeptin. Insoluble material was removed by centrifugation. Protein A–Sepharose CL-4B (Pharmacia, Sweden) was suspended in immunoprecipitation (IP) buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 0.1 % Triton X-100 and 10 % glycerol), incubated with rabbit anti-mouse antibody (Jackson Laboratories), washed with IP buffer and incubated with the anti-phosphotyrosine antibody PY20 (Santa Cruz Biotechnology, California, USA). After washing with IP buffer, the Sepharose beads covered with the anti-PY antibody were incubated with the cell lysates (containing equal amounts of total cell proteins) for 90 min at 4 °C. Precipitated proteins were extracted by boiling the washed beads in the electrophoresis sample buffer. They were then subjected to SDS–PAGE and either probed with anti-paxillin antibodies (Transduction Laboratories) using the immunoblotting procedure described above, or with rabbit polyclonal anti-FAK antibodies (UBI, Lake Placid, USA; catalogue reference 06-287) using horseradish peroxidase-labeled protein A (Amersham) instead of second antibody.

BrdU incorporation

The cells growing in serum-free medium were stimulated by the addition of serum or 10 μM nocodazole for different time intervals. BrdU (10 μM) was added 16 h later for 1 h. After incubation with BrdU, the cells were fixed/permeabilized in 0.5 % Triton X-100 and 4 % paraformaldehyde in PBS for 3 min, postfixed in PBS containing 4 % paraformaldehyde without Triton for 20 min, and treated with 2N HCl/0.5 % Triton X-100 for an additional 20 min. After thorough washing in PBS, BrdU incorporation was visualized by indirect immunofluorescence staining with anti-BrdU (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) and TRITC-labeled goat anti-mouse antibodies. In addition, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (2 μg ml^{-1}) to visualize the cell nuclei.

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