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Stress-activated protein kinases are negatively regulated by cell density

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Stimulation by UV irradiation, TNFα, as well as PDGF or EGF activates the JNK/SAPK signalling pathway in mouse fibroblasts. This results in the phosphorylation of the N-terminal domain of c-Jun, increasing its transactivation potency. Using an antibody that specifically recognizes c-Jun phosphorylated at Ser63, we show that culture confluency drastically inhibited c-Jun N-terminal phosphorylation due to the inhibition of the JNK/SAPK pathway. Transfection experiments demonstrate that the inhibition occurs at the same level as, or upstream of, the small G-proteins cdc42 and Rac1. In contrast, the classical MAPK pathway was insensitive to confluency. The inhibition of JNK/SAPK activation depended on the integrity of the actin microfilament network. These results were confirmed and extended in monolayer wounding experiments. After PDGF, EGF or UV stimulation, c-Jun was predominantly phosphorylated in cells bordering the wound, which are the cells that move to occupy the wounded area. Thus, modulation of the stress-dependent signal cascade by confluency will restrict c-Jun N-terminal phosphorylation in response to mitogenic or chemotactic agents to cells that border a wounded area.

Keywords: c-Jun/JNK/SAPK pathway/mouse fibroblasts/protein kinase/stress activation

Introduction

Over the past few years, remarkable progress has been made in deciphering the signal transduction pathways that allow a cell to regulate gene expression in response to growth modulators. In fibroblasts, growth factors like EGF or PDGF activate their respective tyrosine kinase receptors, which in turn activate the Ras-dependent Raf/MEK/MAP kinase cascade of protein kinases. This signalling pathway triggers the activation of pre-existing transcription factors which in turn activate the transcription of a set of genes defined as immediate early or competence genes. The Jun and Fos transcription factors were among the first proteins whose synthesis was shown to be modulated rapidly after such stimuli (Lau and Nathans, 1987; Almendral *et al*., 1988; Herschman, 1991) and the c-*fos* gene became a paradigm for the study of growth factor-stimulated transcription. Increased c-*fos* transcription after mitogenic stimulation is mainly mediated by the phosphorylation of TCF and SRF bound to the serum response element (SRE) present in the c-*fos* promoter. Each of the three known TCFs (Elk-1, SAP-1 and SAP-2) are activated by phosphorylation on multiple S/T-P motifs which are targets of the ERK/MAPK pathway (Hipskind *et al*., 1991; Dalton and Treisman, 1992; Gilleet *et al*., 1992; Gille *et al*., 1995; Janknecht *et al*., 1993, 1995; Price *et al*., 1995). c-*fos* and c-*jun* transcription are also induced in response to inflammatory cytokines or situations that cause cellular stress such as UV irradiation or osmotic shock (Buscher *et al*., 1988; Devary *et al*., 1991). Such stress conditions activate two novel parallel cascades of protein phosphorylation resulting in the activation of two new subclasses of the MAPK superfamily, the JNK/SAPKs (JNK1/SAPKγ, JNK2/SAPKα and JNK3/SAPKβ) and P38 (P38 α, β and γ) (Hibi *et al*., 1993; Derijard *et al*., 1994; Freshney *et al*., 1994; Han *et al*., 1994; Kyriakis *et al*., 1994; Waskiewicz and Cooper, 1995). Substrates for these kinases include TCFs, which activate c-*fos* transcription, c-Jun and ATF2. JNK/SAPKs phosphorylate the murine c-Jun transactivation domain on serines 63 and 73 as well as the transactivation domain of ATF2 (Gupta *et al*., 1995, 1996; Livingstone *et al*., 1995). As a result of their phosphorylation, the transcriptional activity of the ATF2/c-Jun heterodimers increases, resulting in increased transcription of target genes (van Dam *et al*., 1995). Growth factors like EGF and PDGF also activate JNK, albeit much less efficiently than UVC (Minden *et al*., 1994). However, the exact nature of the cross-talk between the MAP kinase and JNK pathways is still unclear.

In vivo, cytokines and growth factors are released at a wound site when skin lesion occurs. Whereas TNFα or interleukin-1 are likely to be involved in the inflammatory phase following the wound, EGF and PDGF have been shown to enhance the healing process (Martin *et al*., 1992). These observations suggest a potential role for the MAP/SAP kinase transduction pathways in tissues after stress or under conditions where a proliferative response is needed, such as the wound healing process.

Cell–cell interactions, as well as contacts between cells and the extracellular matrix, play an important role in establishing the structure of the different tissues of the body and in tissue remodelling. Whereas epithelial cells establish strong intercellular connections that strengthen epithelial tissues, fibroblasts establish contacts with the

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extracellular matrix that they produce. Important advances have been made in characterizing the molecules and pathways involved in cell–cell and cell–matrix contacts. Focal adhesion structures are assembled at the position where the fibroblast establishes its contacts with the extracellular matrix (ECM). Integrins in the plasma membrane link the intracellular actin filaments to the ECM through a multiprotein complex including α-actinin, vinculin and talin among others. It is becoming clear that this system also participates in the control of cell growth and in its response to extracellular signals. The dynamics of the actin cytoskeleton was shown to be regulated by members of the Rho family of small G proteins. The Rho family is composed of the Rac, Rho and Cdc42 subfamilies. Rac subfamily members participate in the regulation of lamellipodia whereas Cdc42 subfamily members are involved in the formation of fillipodia, different actin-containing cytoskeletal structures (Ridley and Hall, 1992; Ridley *et al*., 1992; Kozma *et al*., 1995). The Rho subfamily is composed of Rho A, Rho B and Rho C, the former being involved in the formation of actin stress fibres and subsequently integrin-containing focal adhesion complexes (Hall, 1994). Surprisingly, it was demonstrated that the members of the Rho family were also involved in the stress kinase signalling pathways. It has been shown that Rho A, Rac 1 and Cdc42 are able to stimulate c-*fos* transcription via SRF. and that JNK can be activated by Rac1 and Cdc42 (Coso *et al*., 1995a,b; Hill *et al*., 1995; Minden *et al*., 1995).

In the present study, we have investigated the influence of cell density on the activation of the MAP/SAP kinase pathways in NIH 3T3 mouse fibroblasts.Using antibodies that specifically recognize c-Jun phosphorylated on Ser63, we show that when cells become confluent, the phosphorylation of c-Jun on Ser63 following UVC irradiation or growth factor stimulation is strongly inhibited. We demonstrate that this is due to inhibition of the activation of the stress kinase pathways (JNK/SAPKs and p38). In contrast, classical MAPK activation is not affected by cell density. By transfecting constitutively active forms of Cdc42 and Rac1, we show that this inhibition probably occurs at the same level as, or upstream of, these small G proteins. We also present evidence that the inhibition of JNK in confluent cells is dependent on the integrity of the actin cytoskeleton. Taken together, these results suggest a crucial role for cell–cell and cell–matrix contacts in the regulation of JNK and p38 kinase signal transduction pathways.

Results

Characterization of anti-phospho-c-Jun-specific antibodies

Phosphorylation of c-Jun is commonly studied using *in vivo* labelling techniques followed by peptide mapping. Although this approach allows the detection of the different phosphorylated forms of c-Jun and mapping of the phosphorylation sites, it does not allow c-Jun phosphorylation to be studied at the cellular level. To overcome this limitation we prepared monoclonal and polyclonal antibodies by immunizing mice or rabbits with a peptide corresponding to amino acids 57–68 of c-Jun. This peptide contained a phosphoserine at position 63 corresponding to one of the two phosphorylation sites of the transactivation domain of c-Jun that are targets for the JNK.

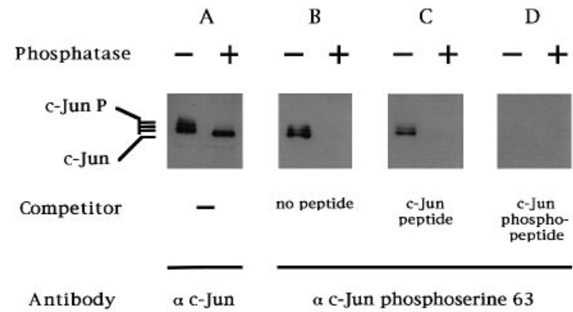

Fig. 1. Characterization of the anti-phospho-c-Jun antibody. When synthesized *in vitro* using a rabbit reticulocyte lysate system, c-Jun protein is phosphorylated on several sites including Ser63. We used this property to test our anti-phospho-c-Jun antibody.Western-blots were loaded with *in vitro* translated c-Jun treated with (+) or without (–) alkaline phosphatase. (**A**) The blot was incubated with an anti c-Jun antibody that recognized both phosphorylated and unphosphorylated c-Jun. (**B**) The same blot was incubated with the monoclonal antibody to phospho-c-Jun. (**C**) Anti-phospho-c-Jun was incubated at 37°C for 1 h with 1 mg/ml (a 10^5-fold excess relative to the antibody) of a non-phosphorylated peptide corresponding to amino acids 57–68 of murine c-Jun. The blot was then incubated using the same mix. (**D**) Anti phospho-c-Jun was incubated at 37°C for 1 h with 1 mg/ml (a 10^5-fold excess relative to the antibody) of a phosphorylated peptide corresponding to amino acids 57–68 of murine c-Jun. The blot was then incubated using the same mix.

A polyclonal antibody previously raised against a bacterially expressed N-terminal fragment of c-Jun (amino acids 1–58) recognized *in vitro* translated c-Jun (Figure 1A). The multiple bands detected arise from phosphorylation of N-terminal serine residues by kinases present in the reticulocyte lysate (Pulverer *et al*., 1991; Franklin *et al*., 1995). Alkaline phosphatase treatment of this protein preparation converted the multiple c-Jun bands into a single faster migrating band that was still recognized by the antibody (Figure 1A). In contrast, the monoclonal antibody raised against the phosphorylated c-Jun peptide only recognized the phosphorylated c-Jun protein (Figure 1B). Furthermore, as shown in Figure 1C and D, recognition of the phosphorylated c-Jun was competed by the phosphorylated immunizing peptide, but only slightly by the corresponding non-phosphorylated peptide due to the large excess of peptide (1×10^5-fold molar excess). Rabbit polyclonal antibodies raised against the same phosphopeptide and affinity purified showed a similar specificity (results not shown). The mono- or polyclonal antibodies did not recognize N-terminally phosphorylated Jun-D, even though the sequence around Ser63 is partially conserved in this protein (not shown). We note that Jun-B lacks homology to this part of c-Jun (Kallunki *et al*., 1994). Hence, our data clearly demonstrate that the antibodies we have raised specifically recognize c-Jun phosphorylated on Ser63. In the study described below, both monoclonal and polyclonal antibodies were used. However, the data presented correspond to the monoclonal antibody only.

Cell confluence inhibits N-terminal phosphorylation of c-Jun following UVC irradiation or serum stimulation

Contact inhibition of NIH 3T3 mouse fibroblasts is widely used to render these cells quiescent, and to study the

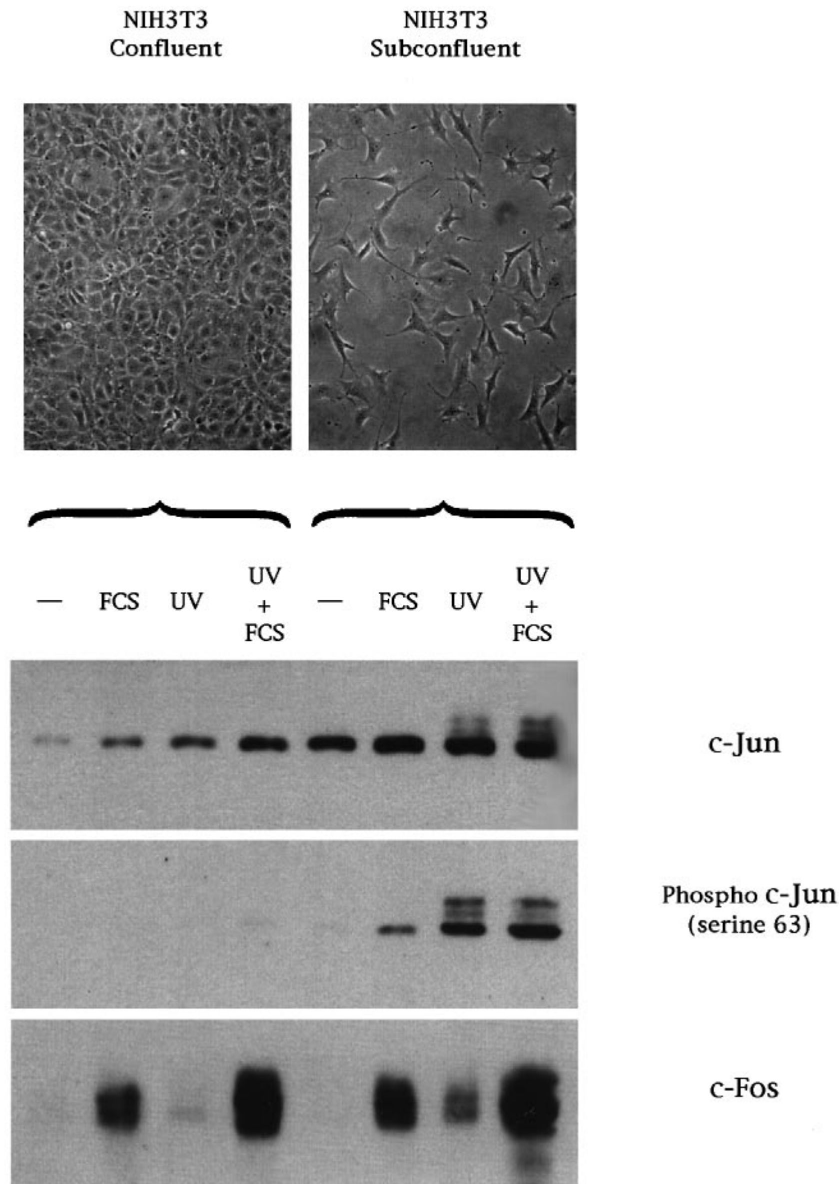

Fig. 2. Effect of cell density on c-Jun N-terminal phosphorylation. NIH 3T3 fibroblasts were seeded at different densities (10^6 per plate and 10^5 per plate) and allowed to grow for 4 days in DMEM containing 7% FCS. Cells seeded at the highest density became confluent within 2 days (NIH 3T3 confluent) and stayed confluent for two more days. Cells seeded at the lowest density reached a density of 10^6 per plate after 4 days in culture (NIH 3T3 subconfluent). Cells were then treated for 2 h with additional fresh 10% serum (FCS), 80 J/m^2 of UVC (UVC) or both (FCS + UVC). Total-cell extracts were prepared and loaded onto SDS–polyacrylamide gels. Western blots were incubated with antibodies to c-Fos, to phosphoserine 63 of murine c-Jun (phospho c-Jun Ser63) and to c-Jun.

signal transduction pathways involved in the establishment of quiescence or in the G_0 to G_1/S transition after addition of serum growth factors. In the present study we decided to investigate the effect of confluence on the activation of the different members of the MAPK superfamily in response to extracellular stimuli. Serum stimulation, UVC irradiation or their combined action are known to induce phosphorylation of serines 63 and 73 in the N-terminal transactivation domain of c-Jun by activation of the JNK/SAPK kinases (Hibi *et al*., 1993; Derijard *et al*., 1994). To follow the activity of these kinases, NIH 3T3 cells were plated at two different densities and cultured for 4 days in Dulbecco's modified Eagle's medium (DMEM) containing 7% fetal calf serum (FCS). At the end of this period, one set of plates had reached confluency while the other contained mostly sparse cells (Figure 2). First, we monitored the level of c-Jun protein in total-cell extracts adjusted to equal protein concentrations. In agreement with previous studies, the level of c-Jun protein is lower in confluent cells than in subconfluent cells (Pfarr *et al*., 1994; Lallemand *et al*., 1997) (Figure 2). Under both growth conditions, the level of c-Jun increases within 2 h of the addition of a 1/10 volume of extra fresh FCS or after UVC irradiation. The strongest increase was obtained when both stimuli were applied together to confluent cells. The same extracts were monitored for the expression of c-Fos. The protein was undetectable in non-stimulated confluent or subconfluent NIH 3T3 fibroblasts (Figure 2). Two hours after serum stimulation, c-Fos was easily detected in both confluent and subconfluent cells. c-Fos

was very weakly induced after UVC irradiation of confluent fibroblasts, whereas its induction was higher in subconfluent cells. Higher but similar levels were expressed at either cell density when UVC and serum were applied together (Figure 2). The most striking effect of the establishment of confluence was observed on c-Jun N-terminal phosphorylation. In confluent fibroblasts we could detect only a very low level of Ser63 phosphorylation 2 h after a combination of UVC irradiation and serum stimulation. In contrast, serum and UVC alone or both together induced a strong c-Jun phosphorylation in subconfluent cells. UVC induced a stonger c-Jun N-terminal phosphorylation than serum and we observed a slight synergy when the two stimuli were applied together (Figure 2). The three bands detected with the anti-c-Jun and anti-phospho-c-Jun antibodies correspond to protein containing different numbers of phosphoserine or phosphothreonine residues. Superposition of the radiograms show that even the major fastest migrating c-Jun band reacts with the anti P-Ser63 antibody, suggesting that a single phosphorylation is not sufficient to slow the mobility of c-Jun. Our analysis does not permit accurate measurement of the fraction of c-Jun that is phosphorylated on Ser63. However, the relative intensities of the three bands detected with both antibodies suggest that a considerable fraction of the c-Jun protein becomes phophorylated on Ser63 2 h after UV irradiation of subconfluent cells. In contrast, only a very low fraction of c-Jun is phosphorylated in confluent cells under these conditions. The failure to detect phosphorylated c-Jun in serum stimulated or UVC irradiated confluent cell extracts is not due to the lower amount of c-Jun protein or to a lack of sensitivity of our antibodies. We could still detect phosphorylated c-Jun by Western blot when we loaded 1/10 of the extracts from subconfluent UVC or serum treated cells that contain less c-Jun protein than extracts from confluent cells. Finally, an antibody that recognizes c-Jun phosphorylated on Ser73 gave similar results (data not shown). We could observe a low level of phospho-c-Jun at later time points (8 h after stimulation and later) in confluent cells after UVC irradiation. We believe that this signal may be linked to an apoptotic process in these cells triggered by the irradiation.

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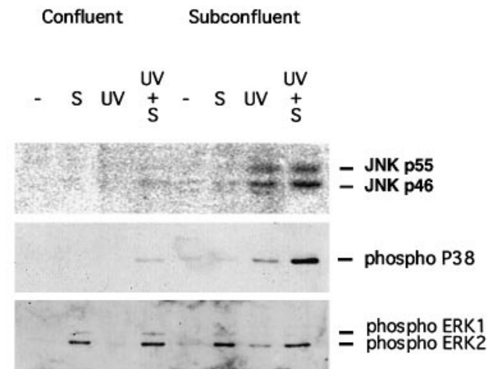

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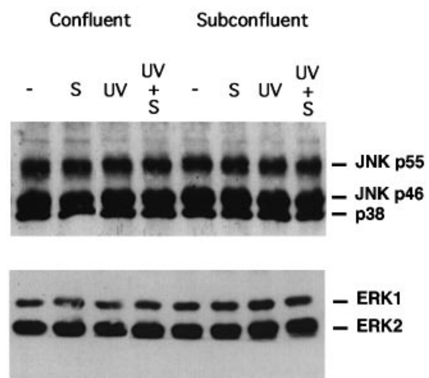

Fig. 3. Activation of JNK and p38 kinase but not ERKs is inhibited in confluent fibroblasts. Confluent and subconfluent NIH 3T3 fibroblasts were treated for 20 min with 10% serum (FCS), 80 J/m^2 of UVC (UVC) or both (FCS + UVC). (**A**) Total-cell extracts were prepared and loaded onto SDS–polyacrylamide gels, and in-gel kinase assays were performed using a GST c-Jun N-terminal fusion protein as a substrate for JNK p46 and p55. Activation of MAPK and p38 kinase was studied by immunoblotting using antibodies specific for their phosphorylated (activated) form. (**B**) Levels of ERK 2, JNK p46/55 and p38 in confluent and subconfluent cells before and after treatment with FCS or UVC, or both together (80 J/m^2) was visualized by immunobotting using specific antibodies.

Activation of JNK/SAPKs and p38, but not ERKs, is inhibited by confluency

The JNK/SAPK subfamily of stress-activated protein kinases are the major protein kinases involved in the phosphorylation of serines 63 and 73 of c-Jun, whereas ERK1 and ERK2 control in large part the activation of c-*fos* transcription through the phosphorylation of TCFs (Gille *et al*., 1992; Derijard *et al*., 1994). Since confluence inhibited c-Jun N-terminal phosphorylation while marginally affecting c-fos induction, we wondered whether the activation of these members of the MAP Kinase family might be differentially controlled by cell confluence. An in-gel kinase assay using a glutathione *S*-transferase (GST) c-Jun fusion protein as substrate revealed that confluency strongly inhibited activation of both 46 and 55 kDa isoforms of JNK by serum, UVC or both together (Figure 3A). In non-stimulated subconfluent cells we detect a weak basal level of JNK activity. Addition of 10% serum did not markedly modify JNK activity, while UVC alone produced an ~10-fold activation of JNK and a 15-fold activation was observed in combination with 10% serum. These data show that there is a slight synergy between UVC and serum for the induction of JNK activity in subconfluent cells. Under these conditions, the phosphorylation state of c-Jun (Figure 2) closely reflects the level of JNK activity and most probably the level of activity of JNK2 because c-Jun is known to be a better substrate for JNK2 than for JNK1 (Kallunki *et al*., 1994; Gupta *et al*., 1996) and because JNK3 expression is restricted to the nervous system (Martin *et al*., 1996). p38 is regulated in a similar manner to JNK/SAPK as shown by monitoring its phosphorylated active state with a phospho p38-specific antibody. Its activation by UVC or UV plus FCS is much stronger in subconfluent cells. This could explain why the c-Fos level is increased after UVC treatment predominantly in subconfluent cells. Under the same conditions, ERK2 activity is induced to similar levels in confluent or subconfluent cells when serum is added to the cultures as

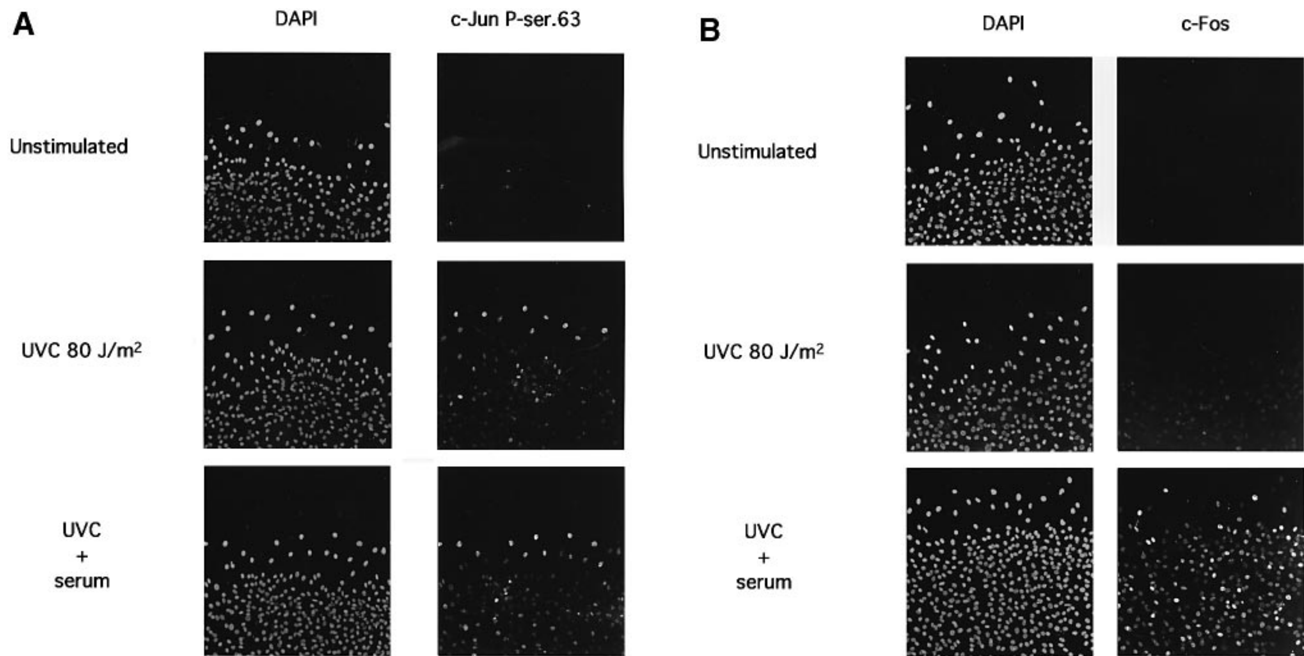

Fig. 4. (**A**) Phosphorylation of c-Jun after exposure of a wounded layer of fibroblasts to serum and UVC. NIH 3T3 cells were grown to confluence on coverslips and then wounded using a rubber policeman. Cells were allowed to grow into the wound for 16 h and were then treated with 80 J/m^2 of UVC or UVC plus 10% fresh FCS for 2 h. Fibroblasts were then stained with DAPI to visualize nuclei and with anti phospho-c-Jun (c-Jun P-Ser63). (**B**) c-Fos induction after exposure of a wounded layer of fibroblasts to serum and UVC. The experiment was carried out as described in Figure 4A, except that the cells were stained with an anti c-Fos antibody.

shown by an immunoblot using a phospho ERK-specific antibody. Furthermore, a slight induction of ERK phosphorylation can be detected after UV irradiation in subconfluent cells but not in confluent cells.The levels of the different kinases remain constant when cells become confluent, and during the next 48 h or after FCS and UVC treatment (Figure 3B). Thus, inhibition is not due to a downregulation of the quantity of the stress kinases but to a decrease in their specific activities. Nevertheless, we have observed a decrease in the level of the different MAP kinases after 4 days of confluence without changing growth medium (not shown). However, this decrease was not observed under the condition we used for this study.

In a wounded monolayer, phosphorylation of c-Jun in response to UVC or growth factors only occurs in migrating cells

In an organism, wound healing is a biological process which results in the repair of injured tissues. It is usually divided into three phases; inflammatory, proliferative and maturation. Cytokines and growth factors like TNF-α, IL-1, PDGF and EGF are released at the wounding site (Martin *et al*., 1992). TNF-α and IL-1 are involved in the systemic inflammatory response, while EGF and PDGF are involved in the stimulation of the mitogenic response. After a confluent monolayer of fibroblasts has been wounded, cells at the edge of the wound start to migrate into the empty space, re-enter the cell cycle and multiply to replace the missing cells. Sixteen hours after the wound, the culture contains both quiescent confluent cells and growing subconfluent cells. We decided to use this model to follow further the effect of serum stimulation and UVC irradiation on c-Jun phosphorylation in immunofluorescence experiments. This experimental system offers the advantage of looking at two populations of cells in the same preparation and under the same culture conditions, thus avoiding artefacts that may be due to changes in the composition of the medium caused by release or absorption of stimulatory or inhibitory factors by fibroblasts. We treated these cells with UVC radiation or a combination of UVC and fresh 10% FCS. Samples were stained with DAPI to visualize nuclear DNA and with specific antibodies. Very few phospho-c-Jun positive cells were detected in the non stimulated culture (Figure 4A). Upon UVC treatment, almost all the cells migrating into the wounded area became strongly positive. In addition, a few cells in the confluent area were also positive. Frequently, these cells were growing above the monolayer and should be considered as subconfluent. Combined UVC and serum treatment moderately increased the level of phospho c-Jun in the confluent area. Even so, this signal was much lower than that in the migrating cells. The nuclear DAPI signal of the migrating cells is higher than that of confluent cells since the first ones have re-entered the cell cycle and reached S or G_2 phase.

c-Fos synthesis was weakly induced by UVC irradiation (Figure 4B). Much stronger induction was obtained by the combined action of UVC and serum. Almost every nucleus became c-Fos positive under these conditions, whereas the phospho c-Jun signal was sparse. It is noteworthy that many cells of the forefront line only contained low levels of c-Fos after serum plus UVC stimulation (Figure 4B). These cells were the first to move into the empty space in response to wounding and might be refractory to a novel stimulation up to 16 h later. It was previously reported that wounding by itself causes rapid induction of c-*fos* transcription and accumulation of the protein as early as 30 min after wounding in the cells bordering the wound (Verrier *et al*., 1986; Martin and Nobes, 1992; Pawar *et al*., 1995). This induction was also

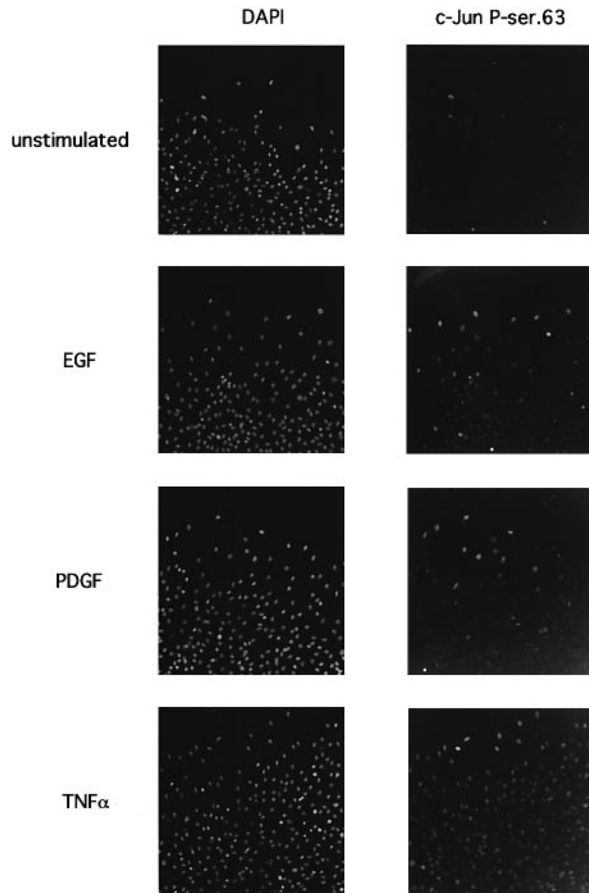

Fig. 5. Phosphorylation of c-Jun after exposure of a wounded layer of fibroblasts to EGF, PDGF and TNF. Wounding was performed as described in the legend to Figure 4, but cells were stimulated for 2 h using either EGF (100 ng/ml), PDGF (100 ng/ml) or TNFα (20 ng/ml) and stained with DAPI and anti-phospho-c-Jun.

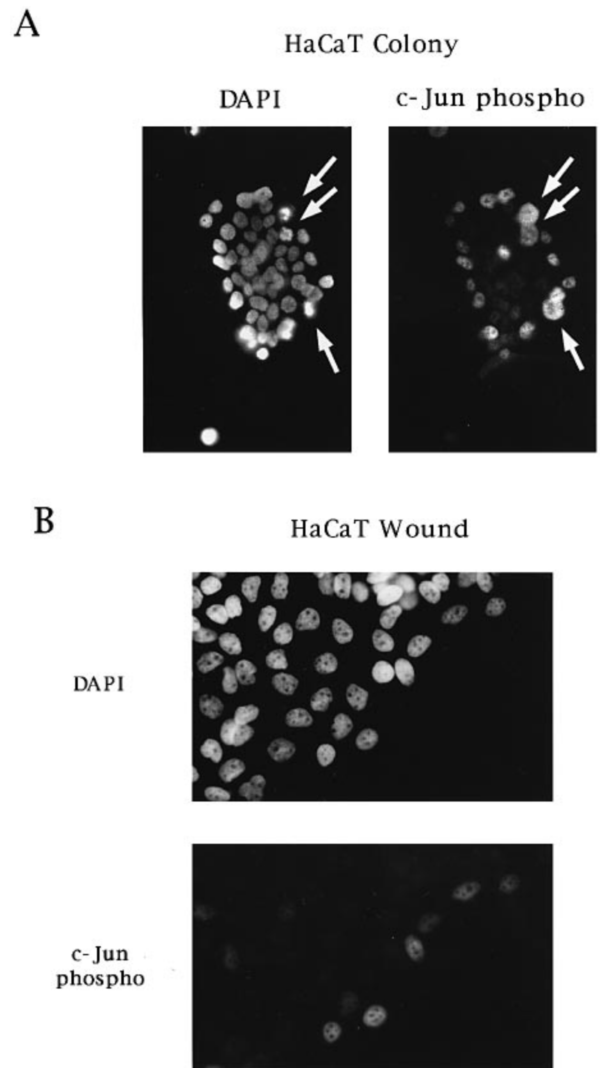

Fig. 6. Phosphorylation of c-Jun in HaCaT cells. HaCaT cells were grown on coverslips in DMEM medium containing 7% FCS. (**A**) Phosphorylated c-Jun was visualized on a typical HaCaT colony by immunostaining (c-Jun phospho) in the absence of any additional stimulation. Mitotic cells were identified by DAPI staining and are indicated with arrows. (**B**) A wound was performed on a confluent layer of HaCaT cells. Two hours later, cells were fixed, permeabilized and stained using anti-phospho-c-Jun antibody.

observed *in vivo*, after wounding mouse embryo (Martin *et al*., 1994), rat skin (Tsuboi *et al*., 1990) or rat corneal epithelium (Okada *et al*., 1996). In the two later cases, induction of c-*jun* mRNA was also observed. The absence of induction of c-Fos, 16 h after wounding in migrating cells in which c-Fos protein was present at least 3 h following the wound, may suggest that they became refractory to further c-Fos stimulation as mentioned above. This refractory state is likely to affect ERK 1/2 since these cells contain high levels of phospho-c-Jun after UV irradiation, a stress that activates essentially the JNKs and p38 (Figure 4A). Taken together, these results are consistent with those obtained by Western blot (Figure 2) and confirm the inhibition of JNK/p38 pathways by confluency.

Next we investigated the effect of pure growth factors and cytokines on the phosphorylation of c-Jun in the wounding model. Sixteen hours after wounding, cells maintained in normal growth medium were treated for 2 h with EGF, PDGF or TNFα, and then were stained with DAPI and anti-phospho-c-Jun antibody. After EGF or PDGF treatment, primarily subconfluent cells that were growing into the wound contained phosphorylated c-Jun (Figure 5). After TNFα treatment, cells colonizing the wound were all brightly stained using the anti-phospho-c-Jun antibody (Figure 5). However, most of the confluent cells were also stained although less brightly. The level of Ser63 phosphorylation observed in these experiments was similar to that obtained with serum but much lower than that obtained after 80 J/m^2 of UVC irradiation. These quantitative aspects correlate well with the increase in JNK activity observed under the same conditions (Minden *et al*., 1994a,b). Cells migrating into the wound were refractory to c-Fos stimulation by EGF, PDGF or TNFα treatment. c-Fos was induced in the confluent layer of cells with the highest signal following PDGF stimulation (not shown).

We examined further the relationship between c-Jun phosphorylation and confluency in a cell type other than fibroblasts. We followed the level of c-Jun phosphorylation in HaCaT cells, which are immortalised human keratinocyte, grown in medium containing serum. These cells exhibit strong cell–cell contacts and form colonies when seeded on tissue culture plates. Figure 6A shows a

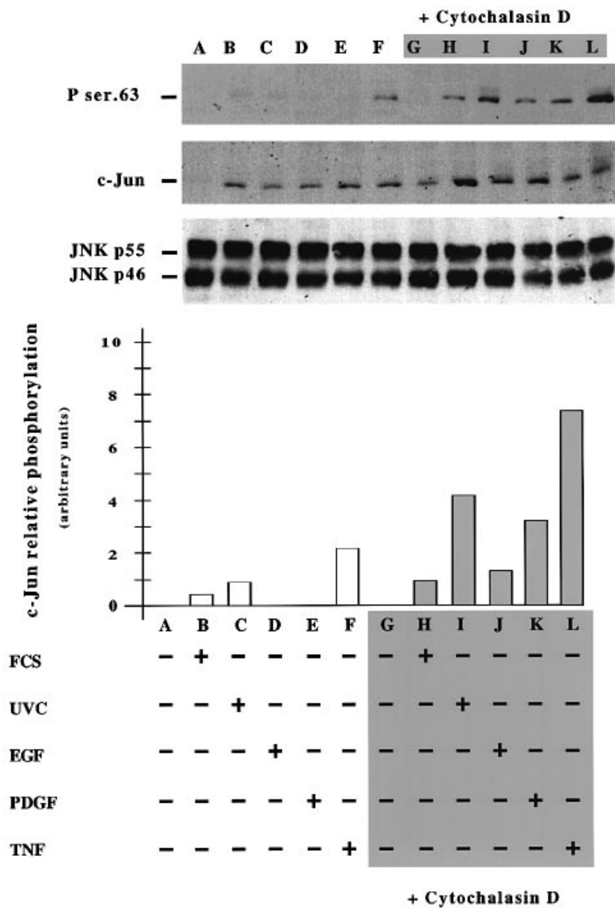

Fig. 7. Actin disruption restores inducibility of c-Jun N-terminal phosphorylation in confluent fibroblasts. Cells were treated with 2 μM cytochalasin D for 1 h or were left untreated prior to addition of 10% FCS, EGF (100 ng/ml), PDGF (100 ng/ml) or TNFα (20 ng/ml) for 2 h, or UVC irradiation (80 J/m^2). Total-cell extracts were then prepared and loaded onto SDS–polyacrylamide gels. Immunoblots were carried out using either anti c-Jun antibody, anti-phospho-c-Jun antibody or anti JNK p46 and p55 (Santa Cruz). The figure shows the result of one representative experiment, the results were reproduced three times.

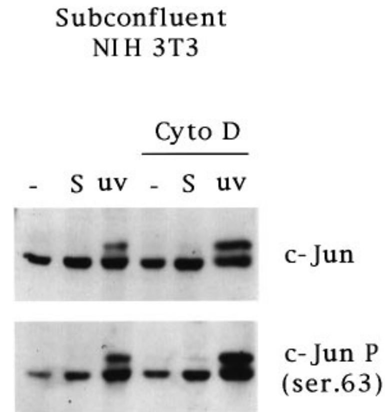

Fig. 8. Effect of actin disruption on the expression and phosphorylation of c-Jun in subconfluent NIH 3T3. Subconfluent cells were treated with 2 μM cytochalasin D for 1 h or were left untreated, prior to the addition of 10% FCS or UVC irradiation (80 J/m^2). Extracts were prepared 2 h after stimulation, and expression and phosphorylation of c-Jun were analysed by immunoblotting using specific antibodies.

typical colony under standard growth conditions after immunostaining for phospho c-Jun. Notably, in the absense of any external stimuli, cells at the periphery contain higher levels of phosphorylated c-Jun. Furthermore, mitotic cells (indicated by an arrow) also contain a high level of phospho c-Jun. When a confluent monolayer of HaCaT cells was wounded, we observed phosphorylated c-Jun in the cells at the margin as soon as 2 h after the wound (Figure 6B). These cells remained positive for labelling with anti phospho-c-Jun for at least 24 h after wounding (not shown). Thus, human keratinocytes present some similarities with fibroblasts as phosphorylated c-Jun can be detected predominantly in cells that are not completely surrounded by neighbours. Nevertheless, these cells contain phosphorylated c-Jun in the absence of additional external stimuli.

Disruption of the actin cytoskeleton relieves the inhibition of c-Jun phosphorylation in confluent cells

We observed that c-Fos expression is inducible by serum, by PDGF or EGF in confluent cells. These results demonstrate that cell surface receptors for EGF or PDGF are functional in confluent cells. These findings suggest that the inhibition of the signal transduction pathways leading to c-Jun N-terminal phosphorylation occurs downstream of tyrosine kinase receptors. Small G proteins that are involved in cytoskeleton remodelling, such as Rac and Cdc42, are also activators of JNK/SAPKs and p38 kinases. This dual role raised the possibility that state of the actin cytokeleton could be involved in the regulation of the Rac and Cdc42 signalling pathways. The actin stress fibre network changes dramatically when cells become confluent, with a redistribution of actin stress fibres and a diminution of F-actin (Bereiter and Kajstura, 1988). To test such a possible link, we treated confluent NIH 3T3 cells with cytochalasin D, an agent that disrupts actin fibres. We then UVC irradiated or added EGF, PDGF, TNFα or FCS to control or Cytochalasin D-treated cultures, prepared total-cell extracts and measured the level of c-Jun and phospho c-Jun by Western-blots. Cytochalasin D treatment in itself had no or very little effect on c-Jun phosphorylation on Ser63 and had no effect on the level of the Jun kinases. As shown in Figure 7, actin disruption potentiated the capacity of all the stimuli to induce Ser63 phosphorylation. In agreement with data mentioned before, TNFα induced c-Jun phosphorylation in confluent cells. This effect was increased in the presence of cytochalasin D (Figure 7). Cytochalasin D did not affect the induction of c-Fos by serum in confluent cells, demonstrating that the signal transduction pathways that lead to c-Fos activation are insensitive to the treatment (data not shown). These experiments clearly demonstrate that the actin cytoskeleton participates in the regulation of the cascade that links surface receptors to nuclear transcription factors by affecting Jun kinase activity but not their intracellular level. Nevertheless, the inhibition mediated by the actin cytoskeleton had less of an effect on the response to TNFα than to other stimuli.

Finally, the disruption of the actin cytoskeleton in subconfluent fibroblasts led to an increase both in the

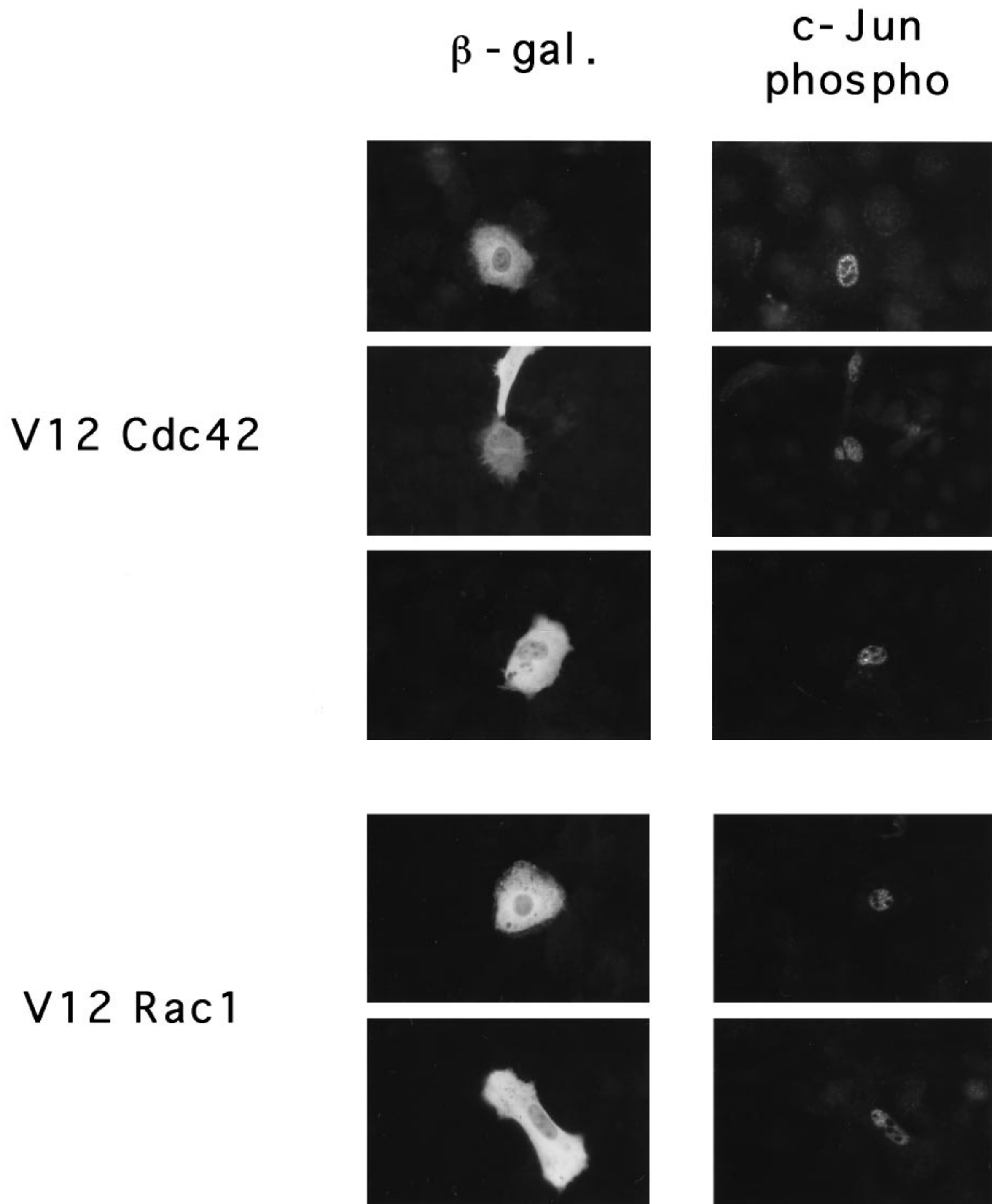

Fig. 9. Transfection of confluent fibroblasts with dominant active forms of Cdc42 or Rac1 results in phosphorylation of Ser63 of c-Jun. Confluent NIH 3T3 were transiently transfected using 1 μg of expression vector for β-galactosidase and 1 μg of expression vectors for dominant active Cdc 42 (V12 Cdc42) or Rac1 (V12Rac1). Forty eight hours after transfection, cells were fixed, permeabilized and subsequently stained using anti-β-galactosidase antibody (β-gal) (Sigma) and anti-phospho-c-Jun antibody (c-Jun phospho).

synthesis and the phosphorylation of c-Jun in response to serum or UV, as shown in Figure 8. However, when normalized for the increased synthesis of c-Jun (Figure 8, upper panel), the relative increase in phosphorylation of c-Jun is <1.5-fold. Thus, disruption of the cytoskeleton had a much more drastic effect (~5-fold increase) on c-Jun phosphorylation in confluent cells (Figure 7).

Activated Cdc42 and Rac1 induce phosphorylation of c-Jun Ser63 in confluent cells

Our data indicated that in confluent cells, growth-factor receptors such as the EGF or PDGF receptors could be activated by their ligands and activate the classical MAP kinase pathway. Thus, the inhibition of the JNK pathways must occur at an intermediate level and might be at any

of the different steps individually or in combination since we did not detect any difference in the level of endogenous Rac1 and Cdc42 between confluent and subconfluent fibroblasts (data not shown). We decided to transfect confluent cells with constitutively active forms of Cdc42 and Rac1 (V12Cdc42 and V12Rac1). We assumed that if the inhibition occured downstream of these small G-proteins, phosphorylation of c-Jun would not be induced by the activated G-proteins. After transfection of confluent cells with the active G-proteins we observed a strong phosphorylation of c-Jun in confluent cells, suggesting that the pathway downstream of Rac1 and Cdc42 was functional and could be activated (Figure 9). These data suggest that inhibition of the JNK cascade by confluency occured at the level of Cdc42 and Rac1 or upstream of them.

Discussion

During the last few years, the understanding of MAP Kinase signal transduction pathways has increased considerably. Three different sub-types of kinases (ERK/MAPK, SAPK/JNK and p38/HOG) have been grouped in one superfamily, sharing several common characteristics. In particular, these kinases share sequence similarity, phosphorylate similar S/T-P consensus motifs, are activated upon dual phosphorylation of threonine and tyrosine residues and constitute the final step of cascades that convert signals received at the cytoplasmic membrane into the activation of transcription factors. However, the emerging picture is that they belong to separate cascades that are activated independently, although a cross-talk exists between them. In the present study, we demonstrate that the activation of the different cascades during the response of fibroblasts to stress or mitogenic stimuli is strongly and differentially affected by the environment of these cells. UVC is unable to activate the stress kinase pathways (JNKs and p38) in confluent fibroblasts whereas it can activate them in subconfluent cultures. In contrast, confluency does not affect activation of the classical ERK pathway by serum. Consequently, c-Jun is much more efficiently phosphorylated on its N-terminal activating sites in subconfluent cells whereas c-Fos synthesis, a major target for the ERK pathway, can be induced to high levels in both confluent and subconfluent cultures. Stimulated with a much lower amplitude, the synthesis of c-Jun is also insensitive to confluency.

Wounding experiments with fibroblast monolayers further confirmed and extended our results. UV irradiation, and to a lesser extent PDGF, EGF or serum induced c-Jun phosphorylation in the migrating cells whereas c-Fos was induced in the confluent layer. The inhibition of JNK activation is not due to the downregulation of the growth factor receptors in confluent cells nor to the inhibition of their clustering, since EGF and PDGF are able to induce c-Fos expression in such cells. Furthermore, inhibition doesn't involve a decrease in the cellular level of the stress kinases themselves.These data prove that the inhibition of stress kinase pathways occurs at an intermediate level situated between the growth factor receptors and JNK/SAPK or p38.

A major morphological change observed when cells become confluent is the modification of the actin cytoskeleton (Bereiter and Kajstura, 1988). In a culture medium containing 7% FCS, confluency induces a redistribution but not the disruption of the actin fibres, which are no longer organized in stretches starting from focal adhesion complexes but appear more randomly distributed. Since actin organization is controlled by the Rho family of small G-proteins which also activate the stress kinases, we examined the effect of Cytochalasin D, a drug that depolymerizes actin. Addition of this drug did not induce c-Jun N-terminal phosphorylation and, more importantly, relieved the inhibition of stress kinase induction by confluency after stimulation by PDGF, EGF or UVC (Figure 7). On the contrary, disruption of actin in subconfluent cells had only a minor effect on the level of phosphorylation of c-Jun after serum or UVC stimulation (Figure 8). These observations demonstrate that the cytoskeleton is involved in the inhibition of stress kinase cascades by confluency at a level between growth factor receptors and the stress kinases. These data are supported by additional observations from our laboratory and others showing that induction of JNKs or phosphorylation of c-Jun occurs in cells with a disrupted actin cytoskeleton. We have recently observed that when HeLa or NIH 3T3 cells are blocked at their G_2/M transition with nocodazole, mitotic cells lacking actin fibres contain a detectable level of phosphorylated c-Jun, whereas G_2 phase cells in the same culture that preserve their actin fibres do not. Furthermore, when mitotic cells are released from the nocodazole block, phosphorylated c-Jun disappears with a kinetic that closely matches the reattachment of the cells to the plate and the reformation of actin fibres (L.Bakiri and D.Lallemand, unpublished observations). The phosphorylation of c-Jun can also be seen in mitotic HaCaT cells (Figure 6A). It was recently shown that the disruption of the interaction between MDCK cells and the ECM results in a rapid induction of JNK activity (Frisch *et al*., 1996). The cells also became rounded and loose their actin fibres. Finally, our data concerning human HaCaT keratinocyte cells show another situation where phosphorylation of c-Jun occurs naturally in cells that are not surrounded by neighbours. These cells behave somewhat differently than fibroblasts since they contain phosphorylated c-Jun under standard growth conditions and in the absence of stimulation with UVC. It is noteworthy that after addition of cytochalasin D to the culture, all the cells of a colony became positive for phosphorylated c-Jun (not shown), confirming the results obtained with fibroblasts. It is possible that in HaCaT keratinocytes, at the periphery of a colony or at the margin of a wound, the JNK pathway is constitutively stimulated by growth factors, but not in cells located at the centre of the colony or far from the wound margin where the inhibition is mediated, at least in part, by the cytoskeleton. Together with the results obtained with fibroblasts, these data argue for an important role for the regulation of the JNK pathway and the phosphorylation of c-Jun *in vivo* when a lesion occurs in a tissue.

Our finding that constitutively active cdc42 and Rac1 are both able to induce c-Jun phosphorylation in confluent fibroblasts demonstrates that the JNK cascade is functional downstream of cdc42 and Rac1 and that the inhibition by confluency occurs at a level situated between growth-factor receptors and small G-proteins. This could also

explain why UVC slightly induces ERK phosphorylation in subconfluent but not in confluent cells. This inhibition occurs upstream of a cross-talk between MAP and JNK pathways which could be mediated by MEKK1, a MAPKKK activated by stress, capable of inducing MEK1 activity and to a lesser extent ERK 1 and 2 activity (Lange-Carter *et al*., 1993; Minden *et al*., 1994). Furthermore, video microscopy observation of cultured fibroblasts clearly demonstrates that membrane ruffles and filipodia are continuously formed in subconfluent but not in confluent fibroblasts suggesting that cdc42 and Rac1 are inactive in the latter (D.Lallemand and S.Garbay, unpublished observations). Altogether, these data reinforce our conclusion that the cytoplasmic actin fibre network participates in the regulation of the JNK pathway. A possible scenario may involve the inhibition of the formation of GTP-bound small G proteins of the Rac/cdc42 family by the rigid cytoskeleton of confluent cells. It will be important to analyse this issue further by following the variations in activity of the corresponding exchange factors and GAP proteins. The findings that the induction of c-Jun N-terminal phosphorylation by TNFα is less sensitive to confluency suggests that either the activated TNFα receptor can overcome the block in Rho protein activation or that its input occurs downstream in the pathway.

We have next to consider our findings in the general context of AP1 activation by different stimuli. Even though we have followed, in the current study, the induction of c-Fos or c-Jun proteins, previous studies have clearly shown that this induction is dependent on transcriptional activation. It is not surprising that the induction of c-Fos synthesis by serum was not strongly affected by confluency. Many of the experiments that follow the induction of immediate early genes are done on starved confluent cultures of fibroblasts. Extensive analysis of the c-*fos* promoter/enhancer sequences has identified several elements that are involved in the response to serum growth factors. These include the SRE which binds SRF, an adjacent sequence element that recruits TCF, a member of the ets family in conjunction with SRF and the SIE element that binds members of the STAT family of transcription factors (Norman *et al*., 1988; Shaw *et al*., 1989; Treisman, 1990; Wagner *et al*., 1990; Hipskind *et al*., 1991). Each of these proteins integrates signals from more than one transduction pathway. For this reason, it is clear that the mere inhibition of JNK/SAPK activation will not abolish c-*fos* transcriptional activation. The c-*jun* promoter/enhancer sequences have been less extensively analysed. Although c-*jun* is considered as a paradigm of AP-1 activated genes, mutations in the two AP1 sites do not fully abolish promoter activity (Angel *et al*., 1988; Hattori *et al*., 1988; Unlap *et al*., 1992). We cannot totally exclude the possibility that a low level of c-Jun phosphorylation, below the limits of our immunological detection, occurs upon serum stimulation of confluent cells and participates in c-Jun transcriptional activation. Nonetheless, the amplitude of c-*jun* transcriptional activation is low in subconfluent cells after serum or UV treatment, while c-Jun is highly phosphorylated in these cells. Altogether, these results suggest that the contribution of the phosphorylated c-Jun/ATF2 complex to activation of the c-*jun* promoter may have been over-estimated. Other binding sites detected by *in vitro* and *in vivo* footprinting, but not yet well characterized, certainly contribute to c-*jun* transcriptional activation (Brach *et al*., 1992). For example, it was shown recently that myocyte enhancer factor (MEF) 2C, which binds and transactivates the c-*jun* promoter, is a target for p38 in monocytic cells (Han *et al*., 1995, 1997). But this mechanism cannot account for the induction of c-Jun protein that we observe in confluent cells after UVC treatment, as under these conditions p38 is not activated. Another potential candidate is BMK1/ERK5 that regulates serum-dependant induction of c-*jun* through MEF 2C (Kato *et al*., 1997). The observation that c-Jun phosphorylation is preferentially activated by PDGF and EGF in cells that are migrating suggests that phosphorylated c-Jun may be important for the activation of genes involved in tissue remodelling. It was shown that c-Jun regulates the expression of genes encoding proteins of the extracellular matrix (Mettouchi *et al*., 1994, 1997) and proteases involved in the remodelling of the matrix (Angel *et al*., 1987; Lee *et al*., 1987). TNFα induced c-Jun phosphorylation is less sensitive to confluency. This difference may also have important biological consequences (Liu *et al*., 1996). AP1 is known to participate in the transcriptional activation of cytokine/chemokine genes (Muegge *et al*., 1989; Hengerer *et al*., 1990; Kim *et al*., 1990). The activation of the AP-1 complex containing phospho c-Jun in dense fibroblasts surrounding a wound will enable the recruitment of immune cells from near by lymphoid organs. Altogether, these data clearly indicate that the nature of the target genes activated by membrane-triggered signalling will depend on cell–cell contacts.

Finally, it is interesting to note that massive c-Jun N-terminal phosphorylation has been shown to occur in cells that are induced to undergo apoptosis. It was shown to be an essential step in the onset of neuronal apoptosis upon withdrawal of survival factors (Watson *et al*., 1998). Disruption of cell–ECM contacts was also shown to induce apoptosis, defined as anoikis. Such treatment rapidly disrupts the actin cytoskeleton and induces JNK. Anoikis can be blocked by a dominant-negative JNKK mutant (Frisch *et al*., 1996). These studies raise the possibility that the control of JNK activation by cell–matrix or cell–cell contacts may also have consequences for the survival of cells under stress conditions.

Materials and methods

Cell culture

NIH 3T3 and HaCaT cells were grown at 37°C in 7% CO_2 in DMEM (Sigma) supplemented with 7% FCS. When indicated, NIH 3T3 cells were rinsed twice with PBS and then irradiated with UVC (80 J/m^2) in a minimal volume of phosphate-buffered saline (PBS) before culture medium was re-added with or without addition of 10% FCS. TNFα (20 ng/ml), PDGF (100 ng/ml) or EGF (100 ng/ml) were added to the culture medium before it was re-added to the plates.

Antibodies

The preparation of polyclonal antibodies to c-Jun and c-Fos was described previously (Lallemand *et al*., 1997). They were raised against GST fusion proteins spanning amino acids 1–58 of c-Jun and 220–314 of c-Fos. Mouse monoclonal antibodies were raised against a peptide corresponding to mouse c-Jun amino acids 57–68 with a phosphorylated serine at position 63. Positive clones were detected by an immunoenzymatic assay and propagated ascites. Specific antibodies were purified by affinity chromatography with the immunizing peptide linked to activated Sepharose.The affinities of the antibody for the phosphorylated

(4.0×10^{-11}) and unphosphorylated (1.0×10^{-7}) peptide were measured by ELISA assay. Affinities measured for the equivalent peptides of Jun-D were superior to 10^{-7}. The polyclonal antibodies were prepared by injection of the same coupled peptide and were purified according to the same procedure.

Western blotting

Whole-cell extracts were prepared by rinsing cultures grown on Petri dishes with cold PBS. Cells were harvested with a rubber policeman and centrifuged. The supernatant was removed and lysis buffer (50 mM Tris pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 0.5 μg/ml each of leupeptin, aprotinin and pepstatin A, and 1 mM PMSF; all reagents from Sigma) was then added directly to the pellet and vortexed strongly for 20 min at 4°C before centifugation at 40 000 *g* for 10 min. Protein concentration was determined using the Bradford assay. For immunoblots, equal amounts of protein were separated by 10% SDS–PAGE and transferred to nitrocellulose sheets (0.2 μm pore size; purchased from Bio-Rad). Blots were blocked in PBS containing 0.05% Tween (PBST) and 10% powdered milk, rinsed in PBST and incubated overnight at 4°C with the indicated primary antibody in PBST containing 10% FCS. Blots were again washed in PBST + 10% milk, and further incubated with horseradish peroxidase labelled secondary antibody (Amersham). After a final wash in PBST plus 200 mM NaCl the blots were developed according to the manufacturer's instructions (Amersham).

Immunofluorescence

Cells were grown on 18 mm square glass coverslips, rinsed with PBS and fixed for 30 min with 0.5% paraformaldehyde in PBS followed by permeabilization for 10 min in PBS containing 0.1% Triton X-100. The coverslips were rinsed briefly in PBST and incubated overnight at 4°C with the primary antibody solution containing PBST plus 10% FCS. Coverslips were then rinsed twice with PBST and incubated for 1 h at room temperature with the secondary antibody (fluorescein-coupled sheep anti-mouse IgG or Texas-red-coupled donkey anti-rabbit IgG; Amersham) in PBST plus 10% FCS. After one rinse in PBST containing DAPI and one rinse in distilled water the coverslips were mounted with an anti-bleaching glycerol mixture (Citifluor Ltd). We used a Zeiss Axiophot epifluorescence microscope with a 16× immersion oil objective. The image, given by the Hamamatsu C4880 cooled CCD camera connected to the Serie 150/151 hardware from Imaging Technology Inc., were acquired by the Khoros software package from Khoral Research Inc. In order to correct an uneven illumination from the mercury lamp, a shading correction was applied to the images.

In-gel kinase assay

Standard SDS polyacrylamide gels were polymerized in the presence of 100 μg/ml of protein substrate (GST–c-Jun amino acids 5–89 for JNK). Fifty micrograms of each extract were loaded. Following migration, the gel was soaked successively in 20% isopropanol in 50 mM HEPES pH 7.5 twice for 20 min, buffer H (50 mM HEPES pH 7.5 plus 5 mM β-mercaptoethanol) twice for 20 min, 6 M urea in buffer H plus 0.05% Tween 20 for 40 min, 3 M urea in buffer H plus 0.05% Tween 20 for 20 min, 1.5 M urea in buffer H plus 0.05% Tween 20 for 20 min, 0.75 M urea in buffer H plus 0.05% Tween 20 for 20 min, buffer H plus 0.05% Tween overnight at 4°C and buffer H plus 10 mM $MgCl_2$ for 20 min. The gel was then incubated in 4 ml of buffer H containing 10 mM $MgCl_2$ plus 20 mM ATP plus 100 μCi of [γ-^{32}P]ATP for 5 h at 30°C and washed extensively in 5% TCA, 1% sodium pyrophosphate. Finally, the gel was dried and exposed to either an autoradiography film (Kodak) or phospor-coated cassettes for 24–48 h. The cassettes were scanned using a PhosphorImager and the signals analysed using ImageQuant software (Molecular Dynamics).

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