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c-Jun N-terminal kinase regulates lamellipodial protrusion and cell sheet migration during epithelial wound closure by a gene expression-independent mechanism

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Abstract

c-Jun N-terminal kinase (JNK) is emerging as an important regulator of cell migration. Perturbing the JNK signaling pathway with three structurally and mechanistically distinct inhibitors that selectively target either JNKs themselves or the upstream mixed-lineage kinases, we found dramatic inhibition of membrane protrusion and cell sheet migration during wound closure in Madin–Darby canine kidney (MDCK) epithelial cell monolayers. Extension of lamellipodia is blocked from the earliest times after wounding in the presence of JNK pathway inhibitors, whereas assembly of non-protrusive actin bundles at the wound margin is unaffected. Inhibitors of the other mitogen-activated protein kinase (MAPK) pathways, the extracellular signal-regulated kinase and p38 MAPK pathways, only have comparatively weak or marginal inhibitory effects on wound closure. Multiple splice variants of both JNK1 and JNK2 are expressed in MDCK cells, and JNK1 and JNK2 are rapidly and transiently activated upon wounding. Phosphorylation of c-Jun does not appear relevant to MDCK wound closure, and membrane protrusion directly after wounding is not affected by inhibitors of RNA or protein synthesis. While most known substrates of JNK are transcription factors or proteins regulating apoptosis, our data indicate that JNK regulates protrusion and migration in a gene expression-independent manner and suggest an important cytoplasmic role for JNK in the control of cell motility.

Keywords: c-Jun N-terminal kinase; Mitogen-activated protein kinase; Membrane protrusion; Lamellipodia; Epithelial cell sheet migration; Wound closure

Wound closure in cultured Madin–Darby canine kidney (MDCK) epithelial cell monolayers is driven by collective migration of a continuous sheet of cells [1]. Cell sheet migration, while less studied than the migration of individual cells such as fibroblasts and neutrophils, is central to processes such as embryonic morphogenesis, tissue repair, and invasion of certain carcinomas and vascular tumors (for reviews see [2,3]). MDCK epithelial cells are well suited for studies of cell sheet migration because, like epithelia in vivo, they maintain their cell–cell contacts after wounding while providing the technical advantages of a cell culture system for investigating questions about the mechanism and coordination of collective cell migration. During wound closure, MDCK cells migrate as a coherent sheet with none of the cells breaking away to migrate as individuals, unlike wounded monolayers of less cell–cell adhesive cell types.

MDCK cell sheet migration is regulated by the small GTPase Rac, with active protrusive force generation distributed from the wound edge to multiple rows of cells behind it in the cell sheet [1]. Rac is a member of a protein family that includes Cdc42 and Rho, the prototype member (for reviews see [4,5]). Both Rac and Cdc42 regulate actin dynamics and plasma membrane protrusion, although they control different kinds of actin assembly

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and protrusion. Specifically, activation of Rac isoforms leads to membrane ruffling and extension of lamellipodia, broad sheet-like membrane protrusions that drive cell migration and are formed by assembly of cross-linked filamentous actin (F-actin) networks at the cell's leading edge along with cell attachment to the substratum (for reviews see [6–12]). Cdc42 controls cell polarity and extension of filopodia, thin finger-like protrusions formed by assembly of parallel actin bundles. Rho isoforms are involved in the formation of contractile actomyosin bundles and stress fibers, as well as focal adhesion assembly. Rac-dependent actin polymerization and lamellipodial protrusion drive wound closure in MDCK cell monolayers, with little filopodial protrusion observed, and neither Cdc42 nor Rho is required for MDCK wound closure [1]. However, Cdc42 and Rho make advancement of the margin more even and regular by stabilizing and organizing the wound edge, with the contribution of Rho arising from its importance for assembly of non-protrusive actin bundles parallel to the free wound-edge plasma membrane [1]. These marginal actin bundles are dispensable for MDCK wound closure but may help to distribute force from more protrusive cells in the cell sheet to their neighbors at the margin.

A range of downstream effectors of Rac and Cdc42 signaling are known, including c-Jun N-terminal kinase (JNK) [13–17]. JNKs, also known as stress-activated protein kinases, constitute one of three subgroups of mitogen-activated kinases (MAPKs), along with classical MAPKs or extracellular signal-regulated kinases (ERKs) and p38 MAPKs (for reviews see [18,19]). We investigated the potential role of the JNK and other MAPK pathways in epithelial cell protrusion and migration during MDCK wound closure. We found that JNK activity is required for wound closure and lamellipodial protrusion using selective inhibitors of the JNK pathway. Inhibitors of p38 MAPK and MAPK/ERK kinase (MEK) only have relatively weak or marginal inhibitory effects on wound closure. Multiple JNK1 and JNK2 splice variants are expressed in MDCK cells, and both JNK1 and JNK2 are rapidly and transiently activated upon wounding above their basal levels. Phosphorylation of the transcription factor c-Jun does not appear to be involved in MDCK wound closure, and the effects of the JNK pathway inhibitors on lamellipodial extension directly after wounding are independent of any inhibition of gene expression, suggesting an important cytoplasmic role for JNK in the regulation of cell migration.

Materials and methods

Materials. SP600125, SB202190, SB203580, PD98059, U0126, and Mowiol 4-88 were purchased from Calbiochem. Compound 3 (4-[2,7]phenanthrolin-9-yl-phenol) was obtained from Merck, while CEP-11004 was from Cephalon. Polyclonal rabbit antibodies against JNK1 (C-17) and JNK2 (N-18), mouse monoclonal anti-JNK2 (D-2), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody were all purchased from Santa Cruz Biotechnology. HRP-conjugated donkey anti-rabbit IgG antibody was from Amersham Biosciences. *Taq* DNA polymerase and Prime RNase inhibitor were purchased from Eppendorf. RQ1 RNase-free Dnase, Moloney murine leukemia virus reverse transcriptase, and random hexamers were from Promega. Protein A–Sepharose beads were purchased from Sigma–Aldrich. [γ-^{32}P]ATP ($\sim$6000 Ci/mmol) was from MP Biomedicals. Glutathione *S*-transferase (GST)-c-Jun (1–79, human) was bacterially expressed and purified as previously described [20–23].

Cell culture. MDCK cells (CCL-34, American Type Culture Collection) were grown in minimum essential medium (Invitrogen) supplemented with 10% newborn calf serum (Biowhittaker) at 37 °C and 5% CO_2.

Wound closure and lamellipodial protrusion assays. MDCK cells were plated at a density of 5×10^4 cells/well on 24-well tissue culture plates in growth medium. The medium was changed to serum-free medium 36 h before the start of all experiments, except as noted. Inhibitors from stock solutions in dimethyl sulfoxide (DMSO) or ethanol were added to the confluent MDCK cell cultures with fresh medium. Carrier solvent alone was added to control cultures at corresponding concentrations. After 30-min pre-incubation, cell monolayers were wounded using ultramicro pipet tips to generate oval-shaped wounds with an initial open area of $\sim$0.5 mm^2, as previously described [24]. Phase-contrast images were acquired at intervals after wounding using a Zeiss Axiovert 25 inverted microscope with a Roper Scientific CoolSNAP-Pro charge-coupled device (CCD) camera and Roper Scientific RS Image software. In separate experiments, the number of lamellipodia at the wound margin was counted. Cell viability at the end of each experiment was confirmed by the trypan blue dye exclusion assay, as well as by phase-contrast microscopic observation of cell morphology, noting any signs of excessive cell rounding or detachment. None of the compounds were used at or above concentrations where there was any indication of toxicity. Subsequent morphometric analysis was done using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on an Apple Power Macintosh computer, as previously described [24].

F-actin staining. MDCK cells were plated on glass coverslips in 12-well tissue culture dishes at 1×10^5 cells/well and grown to confluence. Medium was changed to serum-free medium 36 h prior to inhibitor treatment, and cell monolayers were wounded using ultramicro pipet tips another 30 min following treatment. Cells were then fixed 3 h later with 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS, stained with 50 nM tetramethylrhodamine isothiocyanate-conjugated phalloidin (rhodamine–phalloidin) in PBS, and then washed twice with PBS. Coverslips were mounted on glass slides in Mowiol 4-88 (0.1 g/ml) mounting medium and then examined on a Zeiss Axiovert 200 inverted microscope using a 40× Plan Neofluar objective. Fluorescence images were captured with a Zeiss AxioCam HR CCD camera using Improvision OpenLab imaging software.

Experiments involving Western blot analysis. MDCK cells were plated on 100-mm diameter tissue culture dishes at 6×10^5 cells/dish, grown to confluence, and then shifted to serum-free medium 36 h before wounding. Numerous wounds were made in a checkerboard pattern across each dish using ultramicro pipet tips, with a spacing of $\sim$5 mm between wounds. At different times after wounding, cells were washed with PBS and lysed with a buffer containing 10 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 0.5% NP-40, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 50 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM benzamidine. Lysates were

sonicated and then clarified by centrifugation. Protein was quantitated using the Coomassie Plus protein assay (Pierce Biotechnology), and concentrations were equalized before sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Resolved proteins were blotted to polyvinylidene fluoride overnight at 4 °C in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol) and then blocked with 5% Carnation nonfat dried milk in TTBS (25 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 0.2% Tween 20). Membranes were incubated with primary antibody, washed with TTBS, incubated with HRP-conjugated secondary antibody, and again washed with TTBS. Immunoreactive bands were visualized with the Enhanced Chemiluminescence Western blotting detection system, according to the manufacturer's instructions (Amersham Biosciences).

Reverse transcriptase-polymerase chain reaction. The JNK isoform-specific primers used have been previously reported [25], with the exception of the JNK1α1 and JNK1α2 primers for which the following sequences were used: 5′-TGCCACAAAATCCTCTTTCC-3′ (JNK1α1 and JNK1α2 forward primer), 5′-TGCTGCACCTGTGCTAAAG-3′ (JNK1α1 reverse primer), and 5′-GGATGCTGAGAGCCATTGAT-3′ (JNK1α2 reverse primer). Each of the 10 primer sets selectively amplifies a different JNK isoform ([25] and sequencing of reverse transcriptase-polymerase chain reaction (RT-PCR) products, data not shown), with the exception of the JNK2α2 primer pair, which has been shown to also weakly amplify JNK2α1 [25]. Primers were synthesized by Sigma-Genosys. Total RNA was extracted from confluent unwounded MDCK cell monolayers on 100-mm diameter tissue culture plates using the RNeasy kit (Qiagen). RT-PCR was performed using the QuantumRNA 18S rRNA internal standard from Ambion for relative quantitative PCR, according to the manufacturer's instructions. After 27 PCR cycles, which was in the linear range for all amplifications, and then gel electrophoresis, the intensity of products was determined by band densitometry. The JNK products for each reaction were normalized to their internal 18S rRNA standard products.

JNK immunoprecipitation and kinase assay. MDCK cells were plated, grown to confluence, shifted to serum-free medium, and wounded as described above for experiments involving Western blot analysis. At the indicated times after wounding, cell monolayers were washed with PBS and lysed with a buffer containing 25 mM Hepes, pH 7.6, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM EDTA, 1 mM DTT, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM benzamidine. Following clarification by centrifugation, 500 μg of total lysate protein was incubated with anti-JNK1 (C-17) or anti-JNK2 (N-18) antibody overnight at 4 °C and absorbed onto protein A–Sepharose beads for 1 h at 4 °C. Immunoprecipitates were washed with lysis buffer and then kinase buffer (20 mM Hepes, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM benzamidine). Kinase activity was assayed in kinase buffer with 4 μg GST-c-Jun, 40 μM ATP, and 20 μCi [γ-^{32}P]ATP (~6000 Ci/mmol) for 30 min at 37 °C and terminated by the addition of an equal volume of 2× SDS–PAGE sample buffer. The samples were subjected to SDS–PAGE, followed by autoradiography.

Results

Inhibitors of the JNK pathway potently inhibit wound closure

Selective small molecule inhibitors of the JNK pathway (SP600125, Merck compound 3 and CEP-11004) potently inhibit MDCK wound closure when added prior to wounding (Figs. 1A–D). We also found that wound closure is inhibited when SP600125 added 1 h after wounding, though not as strongly (Fig. 1E). The rate of closure subsequent to post-wounding addition of inhibitor is ~2 times faster than pre-wounding addition of inhibitor to the same concentration. MDCK wound closure occurs at the same rate in the presence (Fig. 1A) or absence of serum (Figs. 1B–I), and SP600125 has similar inhibitory effects in both cases (Figs. 1A and B), although effective concentrations are higher in the presence of serum, as often the case for hydrophobic small molecules due to binding of serum proteins like serum albumin. To eliminate any possible contribution of growth factors or other serum components to JNK activation, which could complicate analysis in later experiments, most experiments were performed in serum-free conditions, unless otherwise noted.

Inhibitors of p38 MAPK and MEK have only weak effects on wound closure

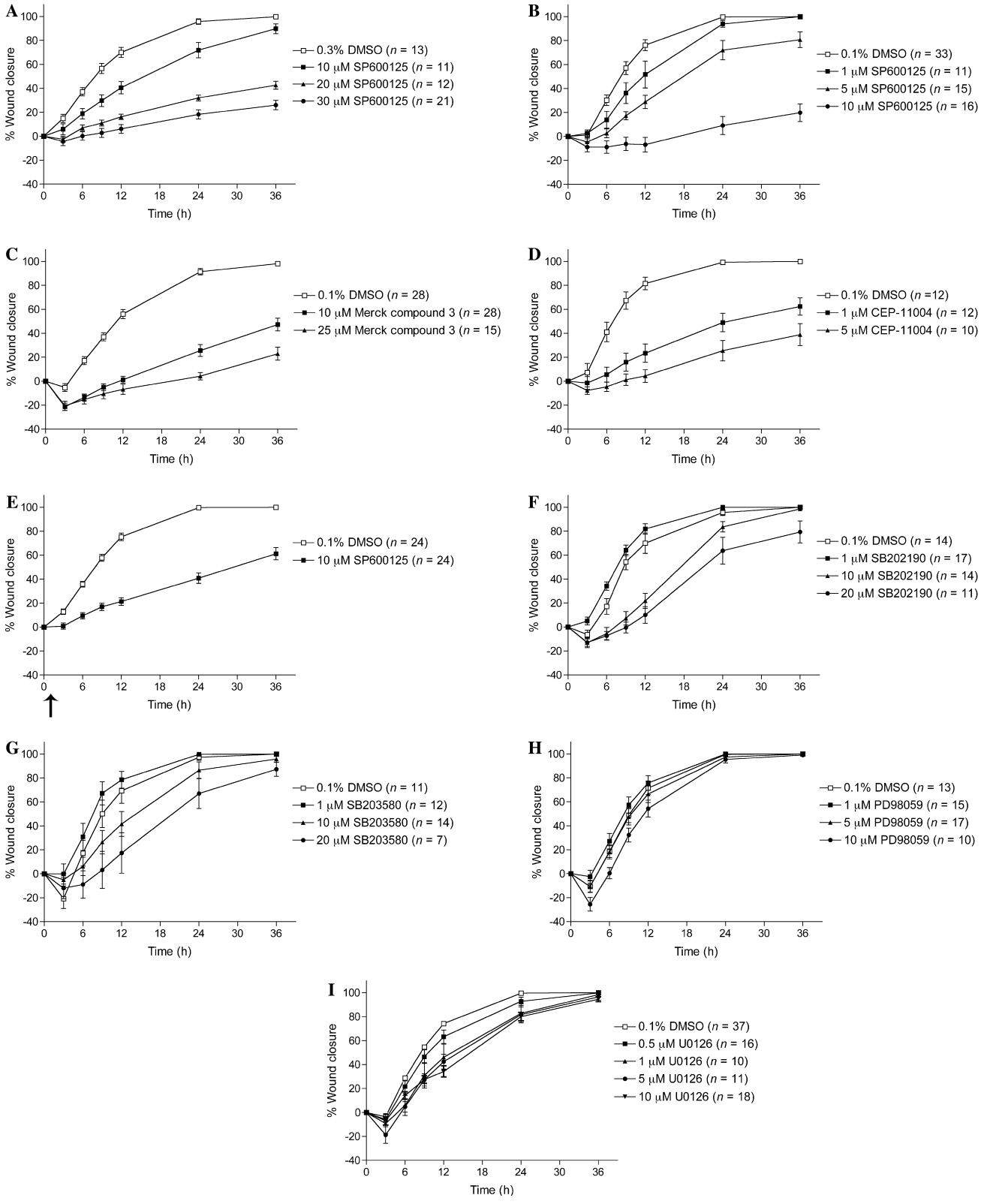
In contrast to the JNK pathway inhibitors, the p38 MAPK inhibitors SB202190 and SB203580 have weaker inhibitory effects on wound closure (Figs. 1F and G), while the MEK inhibitors PD98059 and U0126 have little effect under serum-free conditions (Figs. 1H and I). Similar results were obtained for all of the inhibitors in the presence of serum; however, the MEK inhibitors have a clearer though still relatively weak inhibitory effect on wound closure at higher concentrations (70 μM for PD98059 and 50 μM for U0126) that are cytotoxic in serum-free conditions (data not shown).

JNK pathway inhibitors block membrane protrusion during wound closure

Lamellipodial extension following wounding is strongly inhibited by the JNK pathway inhibitors (Figs. 2 and 3),

Fig. 1. Inhibition of the JNK signaling pathway blocks wound closure in MDCK epithelial cell monolayers. Percent wound closure is depicted as a function of time for the following treatments at the concentrations indicated. (A) SP600125 (JNK inhibitor) in serum-containing medium. The following treatments were done in serum-free medium: (B) SP600125; (C) Merck compound 3 (JNK inhibitor); (D) CEP-11004 (MLK inhibitor); (E) SP600125 added 1 h after wounding, as indicated with arrow (for all other treatments, inhibitors were added 30 min before wounding); (F) SB202190 (p38 MAPK inhibitor); (G) SB203580 (p38 MAPK inhibitor); (H) PD980059 (MEK inhibitor); and (I) U0126 (MEK inhibitor). Similar results were obtained for all of the inhibitors in serum-containing medium, although the MEK inhibitors have a more unambiguous but still relatively weak inhibitory effect on wound closure at higher concentrations (70 μM for PD98059 and 50 μM for U0126) that are cytotoxic in serum-free conditions (data not shown). The concentrations of carrier solvent in the controls correspond to the highest solvent concentration used in that experiment. Values are means ± standard error of the mean (SEM; *n*, number of separately treated wounds with one wound per well on multiwell plates), derived from at least three independent replicate plates for each treatment in this and subsequent figures.

including at the earliest times measured in both serum-containing (Fig. 3A and data not shown) and serum-free media (Figs. 3B–D). When SP600125 is added at 1 h after wounding, subsequent protrusions are also blocked (Fig. 3E). Higher levels of protrusion are observed in the absence of serum than in its presence, but SP600125 potently



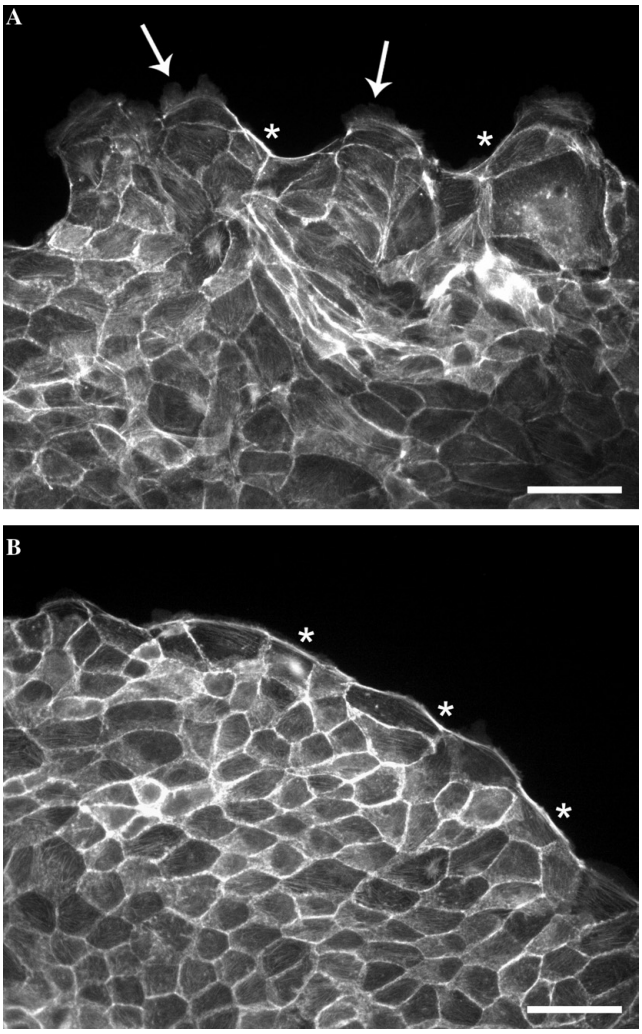


Fig. 2. Inhibitors of the JNK pathway inhibit lamellipodial protrusion but not assembly of non-protrusive actin bundles at the wound margin. Wounded MDCK cell monolayers were fixed, permeabilized, and stained for F-actin with rhodamine-phalloidin at 3 h after wounding in serum-free medium in the presence of 0.1% DMSO alone (A) or 10 μM SP600125 (B). Representative protruding lamellipodia are indicated with arrows. Representative non-protrusive marginal actin bundles are indicated with asterisks. Scale bars, 50 μm.

inhibits protrusion in both cases. The post-wounding formation of non-protrusive marginal actin bundles is unaffected by treatment with SP600125 (Fig. 2).

Inhibitors of gene expression do not affect early membrane protrusion following wounding

The protein synthesis inhibitor cycloheximide also inhibits wound closure in both serum-free (Fig. 4A) and serum-containing media (data not shown), although less rapidly and dramatically than the JNK pathway inhibitors. The inhibitory effect of cycloheximide becomes more pronounced at later times, with the closure rate falling gradually. Similar results were obtained with other protein synthesis inhibitors (puromycin and anisomycin), although the degree of inhibition of wound closure tends to be less than with cycloheximide, consistent with less complete inhibition of tritiated leucine incorporation into trichloroacetic acid-insoluble material at subtoxic concentrations (data not shown). In addition, treatment with the RNA synthesis inhibitor actinomycin D also weakly inhibits wound closure (data not shown). The inhibitory effects of these gene expression inhibitors on membrane protrusion are not seen until at least 3 h after wounding, and even then initially weakly (Fig. 4B and data not shown), whereas inhibitors of the JNK pathway strongly inhibit lamellipodial protrusion from the earliest times measured after wounding (Fig. 3).

Expression of different JNK isoforms

Anti-JNK1 immunoreactivity in MDCK cell lysates appears at ~46 kDa by Western blot analysis, with only very slight immunoreactivity at ~54 kDa (Fig. 5A). Anti-JNK2 immunoreactivity appears at both ~46 and ~54 kDa, with the ~54 kDa band being stronger (Fig. 5B). RT-PCR reveals clear expression of all four JNK1 and two of the JNK2 splice variants (JNK2α1 and JNK2α2), while JNK2β1, JNK2β2, JNK3α1, and JNK3α2 are barely or only weakly detectable (Fig. 5C).

Rapid and transient activation of JNK upon wounding

Both JNK1 and JNK2 kinase activities transiently increase above their basal levels by 15 min after wounding of MDCK cell monolayers, based on in vitro kinase assays with JNKs immunoprecipitated at different times after wounding (Figs. 6A and B). The kinase activities then appear to decline to below basal levels by 1 h post-wounding before returning to basal levels by 3–6 h and remaining there for the duration of subsequent closure (data not shown). We looked at phospho-JNK levels before and after wounding of monolayers by Western blot analysis and immunofluorescent staining using multiple anti-phospho-JNK antibodies (G-7 monoclonal antibody from Santa Cruz Biotechnology and different polyclonal antibodies against phospho-Thr183/Tyr185 JNK residues from Biosource International, Cell Signaling Technology, and Promega) but found no consistent changes in JNK phosphorylation state upon wounding (data not shown).

c-Jun phosphorylation after wounding and treatment with inhibitors

We found that endogenous c-Jun is phosphorylated on Ser63 with a peak of phosphorylation at 15 min after wounding, returning to the pre-wounding baseline state by 1 h and remaining there for the rest of closure, based on Western blot analysis of MDCK cell lysates

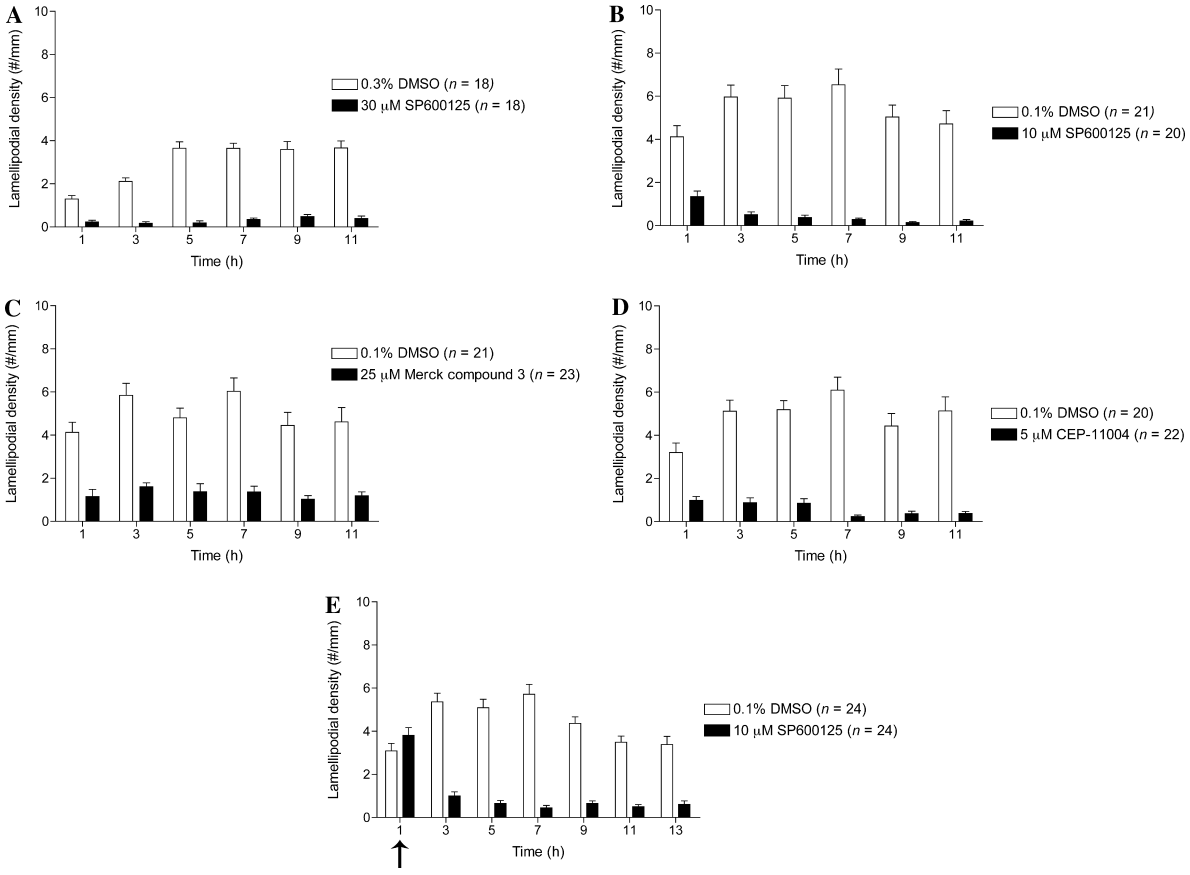


Fig. 3. JNK pathway inhibitors block extension of lamellipodia at the wound margin at all times following wounding. Density of lamellipodial protrusions (number of lamellipodia at the wound margin divided by margin perimeter length; mean with SEM; *n*, number of separately treated wounds) at the times indicated following wounding after treatment at the indicated concentrations with SP600125 in serum-containing medium (A) or the following in serum-free medium: (B) SP600125, (C) Merck compound 3, (D) CEP-11004 or (E) SP600125 added 1 h after wounding immediately before 1-h data collection, as indicated with arrow (for all other treatments, inhibitors were added 30 min before wounding).

with the KM-1 anti-phospho-c-Jun monoclonal antibody from Santa Cruz Biotechnology (data not shown). However, this rapid and transient phosphorylation of c-Jun upon wounding is not inhibited by JNK pathway, p38 MAPK or cyclin-dependent kinase (CDK) inhibitors, nor by simultaneous combined treatment with inhibitors of all three of these pathways. In contrast, treatment with MEK inhibitors completely abrogates this phosphorylation, reducing the state of c-Jun phosphorylation at 15-min post-wounding to the baseline.

Surprisingly, we found that treatment of both unwounded and wounded MDCK cell monolayers with any of the JNK pathway inhibitors (SP600125, Merck compound 3 or CEP-11004) actually results in a dose-dependent increase in the level of phosphorylation of c-Jun on Ser63. This hyperphosphorylation of endogenous c-Jun following treatment with JNK pathway inhibitors is inhibited by p38 MAPK inhibitors in unwounded monolayers and at every time point after wounding except at 15 min post-wounding. We verified that the JNK pathway inhibitors are active for inhibition of anisomycin-induced c-Jun phosphorylation in A549 adenocarcinoma cells. Moreover, we found that SP600125 and Merck compound 3 both potently inhibit the in vitro kinase activity toward recombinant GST-c-Jun of both JNK1 and JNK2 immunoprecipitated from wounded MDCK cell monolayers.

Discussion

There is growing evidence that MAPK pathways are involved in the regulation of cell motility. However, there is enormous diversity among different epithelial cell types in the importance of each pathway, which varies with the specific cell and migratory stimulus. JNK appears to play a role in some but not all forms of epithelial cell migration, with similar disparity in the involvement of the other MAPKs from particular epithelial cell to cell. The JNK signaling pathway is clearly important in *Drosophila* dorsal closure ([26–28]; for review see [29]), an embryonic morphogenetic process involving epithelial cell sheet movement (for re-

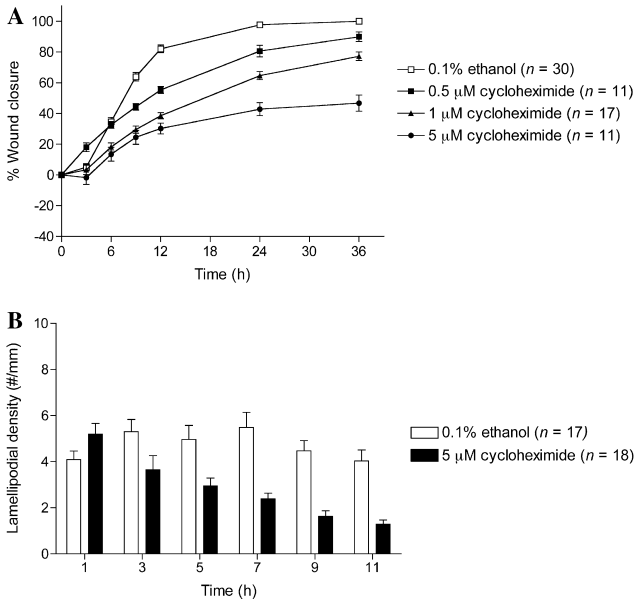


Fig. 4. Inhibition of gene expression slows wound closure but does not affect lamellipodial protrusion and migration in the early stages of closure. Percent wound closure in the presence or absence of the protein synthesis inhibitor cycloheximide is shown in serum-free medium (A). Density of lamellipodial protrusions at the wound margin is depicted in the presence or absence of cycloheximide at times indicated following wounding in serum-free medium (B). Values are means with SEM for *n* separately treated wounds. Similar results were obtained with other protein synthesis inhibitors (puromycin and anisomycin) and the RNA synthesis inhibitor actinomycin D (data not shown).

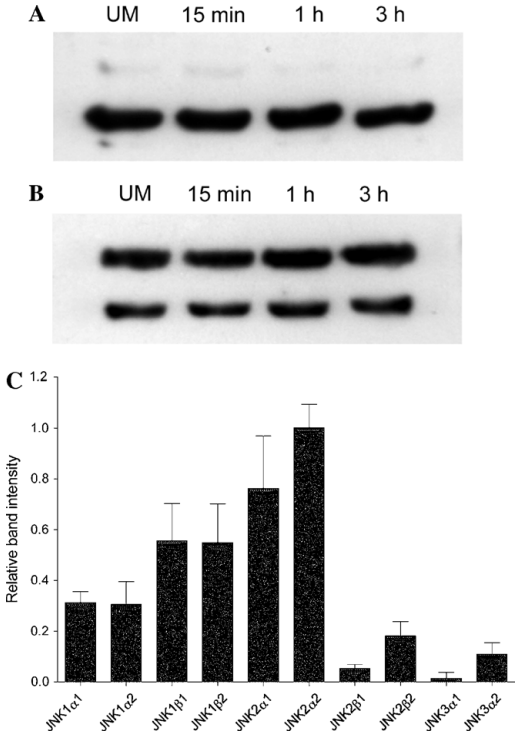


Fig. 5. Expression of JNK isoforms in MDCK cells. Western blot analysis with anti-JNK1 (A) or anti-JNK2 (B) antibody using MDCK cell lysates prepared from unwounded monolayers (denoted by UM) and as a function of time after wounding in serum-free medium, as indicated. Autoradiograms are representative of at least three independent experiments. (C) Relative RT-PCR product intensities (each JNK product intensity divided by 18S rRNA internal standard product intensity), from RT-PCR using isoform-specific primer pairs (see Materials and methods) to amplify cDNAs from RNA extracted from an unwounded monolayer. Results are normalized values (means ± SEM) for four experiments under identical reaction, gel-loading and analysis conditions.

views see [30–32]), and *Drosophila* wound healing [33], as well as migration of a number of cultured cell types [34–40]. Deletion of the two main *Jnk* genes (*Jnk1* and *Jnk2*) in mice results in an embryonic lethal phenotype with failure of neural tube closure [41]. Mice with no *Jnk1* and a single allele of *Jnk2* exhibit defects in embryonic optic fissure and eyelid closure ([42]; for review see [29]). Furthermore, MEK kinase 1 (MEKK1), an upstream activator of JNK signaling, has also been implicated in eyelid closure and epithelial cell migration ([43,44]; for review see [29]). In contrast, cell migration in other epithelial systems appears to instead more involve the p38 MAPK [45–52] and/or ERK pathways [45,50,52–58].

JNKs are activated in response to diverse stimuli, including various environmental stresses, through phosphorylation by MAPK kinases 4 and 7, which are themselves activated by mixed-lineage kinases (MLKs) and certain other MAPK kinase kinases like MEKK1 (for reviews see [59–61]). MLK3 interacts with and is activated by Rac and Cdc42 [62–64], as appears the case for MLK2 also [65]. Rac and Cdc42 may activate MEKK1 [66,67], although probably not through a direct interaction [68]. In addition, the p21-activated kinases (PAKs), well-characterized direct effectors of Rac and Cdc42, can activate the JNK pathway (for

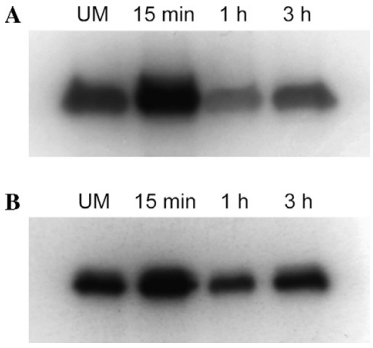


Fig. 6. JNK1 and JNK2 are rapidly and transiently activated upon wounding. Kinase activity was assessed in vitro following immunoprecipitation of JNK1 (A) or JNK2 (B) from MDCK cell lysates prepared from unwounded monolayers (denoted by UM) and as a function of time after wounding in serum-free medium, as indicated. Autoradiograms are representative of at least five independent experiments.

review see [69]). However, PAK1 activity has been shown to be not required for wound closure in MDCK cell monolayers, although it is necessary for contact

inhibition of MDCK cell growth [70]. Another direct Rac target involved in regulation of the JNK pathway is the "Plenty of SH3s" protein, an Src homology 3 domain-containing scaffold protein [71,72].

SP600125 [73] and Merck compound 3, [74] directly target JNK, whereas CEP-11004 inhibits the MLKs upstream of JNK [75], and these three compounds are structurally unrelated. It is therefore unlikely that their dramatic inhibitory effects on membrane protrusion and cell sheet migration in MDCK cells (Figs. 1A–E) result from inhibition of other kinases not in the JNK pathway due to poor selectivity and coincidentally similar profiles of kinase inhibition. p38 MAPK inhibitors have only a weak inhibitory effect in both serum-free (Figs. 1F and G) and serum-containing media (data not shown). Moreover, we found that MEK inhibitors have little effect on wound closure even at concentrations just below cytotoxic levels in serum-free conditions (Figs. 1H and I). However, we did observe a real but still relatively weak inhibitory effect in serum-containing medium at higher inhibitor concentrations, doses that are toxic in serum-free conditions (data not shown), roughly consistent under these conditions with another recent study [76]. Taken together, our data strongly suggest that the inhibitory effects of the JNK pathway inhibitors indeed arise from inhibition of the JNK pathway and not just from non-specific inhibition of an indeterminate host of kinases.

Inhibition of lamellipodial extension by JNK pathway inhibitors (Figs. 2 and 3) does not result from general effects on the actin cytoskeleton. Formation of non-protrusive marginal actin bundles is not inhibited (Fig. 2), indicating that the compounds do not affect gross cytoskeletal dynamics but specific cytoskeletal and/or adhesion processes involved in membrane protrusion and cell migration.

The best-characterized substrate of JNK is c-Jun, which is activated by JNK and forms a component of activator protein-1 transcription factors. We found that c-Jun is rapidly and transiently phosphorylated on Ser63 upon wounding of MDCK cell monolayers without involvement of JNK (data not shown). A number of instances of JNK-independent phosphorylation of the N-terminal region of c-Jun in vivo are known. Phosphorylation of c-Jun in response to certain stimuli can be inhibited by MEK inhibitors in embryonic fibroblasts [77] and PC12 pheochromocytoma cells [78]. In addition, phosphorylation of c-Jun during neuronal apoptosis in response to DNA-damaging agents is blocked by CDK inhibitors but not affected by inhibitors of the JNK pathway [79]. We tested whether these other kinases or p38 MAPK was possibly involved in the phosphorylation of c-Jun at 15 min after wounding of MDCK cell monolayers. Only inhibition of MEK had any effect, completely blocking this post-wounding phosphorylation and suggesting the involvement of the MEK-dependent kinase ERK. Since MEK inhibitors have little effect on wound closure, these data further argue that c-Jun is not relevant to wound closure in this system. In addition, the seemingly paradoxical hyperphosphorylation of c-Jun on Ser63 following treatment of MDCK cells with JNK pathway inhibitors is inhibited by co-treatment with p38 MAPK inhibitors, suggesting that inhibition of JNK signaling in the MDCK system induces a p38 MAPK-dependent stress response resulting in c-Jun phosphorylation. It may be pertinent that SP600125 can also activate the cyclic AMP response element-binding protein in a p38 MAPK-dependent manner in mouse pancreatic β cells [80].

The effects of JNK pathway inhibitors on membrane protrusion are distinct from those of RNA or protein synthesis inhibitors (Fig. 4B and data not shown), making it even more likely that a non-transcriptional JNK substrate is relevant in this case. Furthermore, transient phosphorylation of c-Jun at 15 min precedes by hours the first evidence that new gene expression may be required to sustain further membrane protrusion. The later progressive effects of the gene expression inhibitors on protrusion and migration are probably due to run down of general protein levels. Recent reports do suggest that c-Jun is important for proper keratinocyte migration and eyelid closure in the mouse [81,82], as well as fibroblast motility [35]. In addition, c-Jun mediates the JNK-dependent expression of *decapentaplegic*, a member of the transforming growth factor superfamily involved in *Drosophila* dorsal closure [83–86]. However, our data are inconsistent with a role for c-Jun as a relevant JNK substrate in lamellipodial extension during MDCK wound closure.

A number of other JNK substrates are known, although most of these are either other transcription factors (for reviews see [60,61]) or proteins involved in apoptosis (for reviews see [87,88]). The relevant substrate of JNK at least in the early stages of MDCK wound closure appears to have a cytoplasmic function and be directly linked to membrane protrusion and cell migration. There are two substrates of JNK known that have demonstrated potential connections to protrusion and motility. Spir was identified in a yeast two-hybrid screen as a JNK-interacting protein and was shown to be a phosphorylation target of JNK [89]. Spir may also interact with Rho-family small GTPases [90], contains Wiskott–Aldrich syndrome protein (WASP) homology 2 domains (potential binding sites for monomeric actin) and an acidic domain (potential binding site for the actin-related protein 2/3 or Arp2/3 complex, which nucleates new actin polymerization), binds monomeric actin, and has cellular activities consistent with a role in actin organization [89–91]. Members of the WASP family are modulators of de novo actin nucleation through the Arp2/3 complex and are regulated by Cdc42 or Rac, various signaling adaptor proteins, phos-

phoinositides, and/or phosphorylation (for reviews see [92–97]). Therefore, it is conceivable that Spir is involved in stimulating new actin polymerization upon activation by JNK, although this remains to be shown. It has also recently been demonstrated that JNK phosphorylates the focal adhesion adaptor protein paxillin, modulating the turnover of cell-substratum adhesions [34].

There are 10 JNK isoforms, resulting from alternative splicing of three genes, with five isoforms of ~46 kDa (JNK1α1, JNK1β1, JNK2α1, JNK2β1, and JNK3α1) and five of ~54 kDa (JNK1α2, JNK1β2, JNK2α2, JNK2β2, and JNK3α2) [98,99]. There is limited information on the relative abundance of each splice variant in different tissues; however, JNK1 and JNK2 are ubiquitously expressed, while JNK3 is restricted predominantly to brain, heart, and testis [98,100–105]. There is evidence that different JNK isoforms have different substrate affinities and specificities [98,106–110]. We found that multiple splice variants of both JNK1 and JNK2 are expressed in MDCK cells (Fig. 5).

Both JNK1 and JNK2 are rapidly and transiently activated upon wounding (Fig. 6). The kinase activities then appear to decrease to below basal levels before recovering to and remaining at basal levels for the rest of wound closure (Fig. 6 and data not shown). Interestingly, upon exposure of MDCK cell islands to hepatocyte growth factor/scatter factor (which induces cell scatter, another form of epithelial cell motility wherein cells lose cell–cell contacts, undergoing an epithelial–mesenchymal-like transition, and move as individuals and not as a continuous sheet), JNK phosphorylation is initially weakly stimulated but then repressed to below basal levels in a MEK- and MAPK phosphatase-dependent manner [111]. Following wounding of MDCK cell monolayers, rapid JNK activation may be required to initiate cell migration. Alternatively, JNK activity may be necessary permissively for cell migration during wound closure, and this possibility is not mutually exclusive with the previous one. While wound closure is still inhibited when JNK pathway inhibitors are added an hour after wounding (Fig. 1E), subsequent wound closure is not as strongly inhibited as when treatment is started before wounding. These results suggest that both the initial high JNK activity and the subsequent lower activity may be important for MDCK wound closure. The precise roles that JNK isoforms and their relevant substrates play in membrane protrusion and cell sheet migration during epithelial wound closure remain to be established.

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