c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase family. It has become clear that JNK does not only have a role in induction of stress responses but also in processes such as cell movement. In this report we demonstrate that JNK activity is necessary for platelet-derived growth factor (PDGF)-BB-induced chemotaxis of primary foreskin fibroblasts and in other cell types. PDGF-BB stimulation was found to lead to activation of JNK with a maximum after 30 min. Inhibition of JNK reduced Ser¹⁷⁸ phosphorylation of the focal adhesion component paxillin. Paxillin phosphorylation at this site has been shown to be involved in the dynamics of focal adhesions and consequently cell migration. Moreover, we observed localization of JNK to the actin-dense membrane ruffles induced by PDGF-BB stimulation both using immunofluorescence staining and green fluorescent protein-tagged JNK. This suggests a role for JNK at the leading edge of the cell compatible with a function in cell migration. Furthermore, we show that phosphatidylinositol 3-kinase (PI 3-kinase), which has an established role in PDGF-stimulated cell migration, is necessary for PDGF-induced activation of JNK. In conclusion, JNK is a critical component downstream of PI 3-kinase that may be involved in PDGF-stimulated chemotaxis presumably by modulating the integrity of focal adhesions by phosphorylating its components.

Platelet-derived growth factor (PDGF)² is a mitogen for connective tissue cells. The PDGF family consists of four protein chains that form five biologically active dimers (PDGF-AA, -AB, -BB, -CC, and -DD) (1). PDGF isoforms exert their cellular effects via binding to two tyrosine kinase receptors, i.e. PDGF receptors that binds PDGF-A, -B, and -C chains, and PDGFRβ that binds PDGF-B and -D chains. Ligand binding induces dimerization and autophosphorylation of PDGF receptors. Phosphorylated tyrosine residues function as docking sites for signal transduction proteins with SH2 domains (2). Proteins interacting with PDGF receptors include SHP2, PI 3-kinase, Src family kinases, Grb2, and Shc (3). PDGF stimulates cell proliferation and has also been shown to be a chemoattractant for several cell types including fibroblasts, smooth muscle cells, neutrophils, and monocytes (reviewed in Ref. 4).

Cell migration is a complex process that in a simplified view can be broken down into four major parts; first the cell extends a protrusion in the direction of movement (lamellipodium), second the lamellipodium attaches to the substratum by forming focal adhesions, third the contractile force of the actin cytoskeleton moves the cell body, and finally the rear of the cell detaches from the surface. Perturbations in any of these processes will result in defective cell migration.

The mitogen-activated protein kinase (MAPK) family is evolutionary conserved and all eukaryotic cells possess multiple MAPK pathways. The MAPK family can be subdivided into three major groups, i.e. extracellular signal-regulated kinase (Erk), p38, and c-Jun N-terminal kinase (JNK). JNK is activated by many different factors, including epidermal growth factor, PDGF, transforming growth factor-β, tumor necrosis factor, in addition to cellular stress (5). There is an increasing amount of evidence placing MAPKs in a central position for cell migration (6). However, the requirement for specific MAPKs appears to vary depending on cellular system and chemoattractant used. One possibility is that coordinated spatial and temporal activities of different types of MAPK are needed for correct migration, but the relative contributions may differ between cell types.

JNK has frequently been suggested to have essential roles in inflammation, differentiation, and apoptosis (7, 8). Moreover, there is accumulating data indicating a role for JNK in cell migration. In fact, inhibition of JNK reduces migration in several cell types (9, 10). It has been suggested that JNK regulates migration by phosphorylating paxillin, which is a component of the focal adhesions (10). Expression of a mutant form of paxillin (S178A) that cannot be phosphorylated by JNK significantly reduced the ability of the cell to migrate in a cell culture wound healing assay. There are data indicating a role for JNK in PDGF-mediated migration of vascular smooth muscle cells (11), human adipose tissue-derived mesenchymal stem cells (12), and osteoblastic cells (13). However, the molecular mechanism by which JNK mediates chemotaxis has not been described.

In the present study, we investigated the role of JNK in PDGF-BB-mediated chemotaxis using primary foreskin fibroblasts.
blasts (AG01523) but also PDGFRβ transfected porcine aortic endothelial (PAE) cells and two glioblastoma cell lines (T98G and A172). Our results show that JNK is activated by PDGF-BB in a PI 3-kinase-dependent manner and is essential for chemotaxis. Furthermore, we have demonstrated that PDGF-BB stimulation results in Ser\(^{178}\) phosphorylation of paxillin in a JNK-dependent manner. In addition, we could detect JNK in the actin-dense membrane ruffles at the leading edge of the cell. We propose that JNK may be a down-stream effector of PI 3-kinase important for PDGF-induced cell migration, possibly through paxillin Ser\(^{178}\) phosphorylation.

**EXPERIMENTAL PROCEDURES**

*Reagents*—Recombinant human PDGF-BB was generously provided by Amgen (Thousand Oaks, CA) and STI571 by Novartis Pharma AG (Basel, Switzerland). SP600125 and L-JNK11 were purchased from Alexis Biochemicals (Läufelfingen, Switzerland), PD098059 from New England Biolabs (Beverly, MA), LY294002 from Biomol (Plymouth Meeting, PA), and SU6656 and Calphostin C were from Calbiochem. The following antibodies were used for immunoprecipitation, immunoblotting, and immunofluorescence: monoclonal mouse phosphospecific anti-SAPK/JNK (number 9255), rabbit phosphospecific anti-c-Jun (number 9164), and rabbit monoclonal anti-c-Jun (number 9165) were from Cell Signaling Technology (Beverly, MA), rabbit anti-JNK (sc-571) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-phospho-Ser\(^{178}\)-paxillin (44-1026G) from BIOSOURCE (Camarillo, CA), monoclonal mouse anti-paxillin (number 610051) from BD Transduction Laboratories (Lexington, KY), and monoclonal anti-vinculin (V4505) from Sigma.

*Cell Culture*—AGO1523 foreskin fibroblasts (Coriell Institute for Medical Research, Camden, NJ) were cultured from passages 11–19 were cultured in Quantum 333 (PAA Laboratories). PAE cells expressing the wild-type PDGFRβ have been described previously (14). T98G, A172, and PAE cells were cultured in Ham’s F-12 medium (Sigma) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine (Sigma), and 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma). For starvation, cells were washed and incubated in Ham’s F-12 medium containing 0.1% fetal bovine serum.

*Adenoviral Infection of AGO1523 Fibroblasts*—Generation of the wild-type and kinase-dead recombinant JNK adenoviruses has been described previously (15). Subconfluent AG01523 fibroblasts were infected with adenoviruses at a multiplicity of infection of 100 in complete medium 24 h prior to starvation, when the virus-containing medium was replaced by starvation medium and incubated overnight. Under optimal conditions ~80% of the cells were infected as determined by green fluorescent protein (GFP) autofluorescence.

*Immunoprecipitations and Western Blotting*—Subconfluent AG01523 cells were starved as described above and incubated for 1 h with inhibitors at the indicated concentrations and thereafter stimulated with 20 ng/ml PDGF-BB for the indicated periods of time. Cells were washed with ice-cold phosphate-buffered saline and lysed (1% Triton, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.4, 1 mM Pefabloc, 1% Trasylol, 10 mM NaF, 1 mM sodium orthovanadate). Extracts were clarified by centrifugation, and protein concentration was determined by the BCA protein assay system (Pierce). Equal amounts of lysates were incubated with the indicated antibody, according to the manufacturer’s instructions. The immunoprecipitates were collected on protein A- or G-Sepharose beads. The beads were washed three times in lysis buffer and boiled with SDS sample buffer containing dithiothreitol. Immunoprecipitates or equal amounts of total cell lysates were analyzed by SDS-PAGE. For Western blotting, samples were electro-transferred to polyvinylidene difluoride membranes (Immobilon P), which were blocked in 5% milk powder (anti-phospho-JNK and anti-phospho-c-Jun) or 1% bovine serum albumin (anti-phospho-paxillin) in phosphate-buffered saline solution containing 0.1% Tween 20. Primary antibodies were used at concentrations and buffers recommended by the suppliers and incubated overnight in the cold. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (both from Amersham Biosciences), and proteins were visualized using ECL. Western blotting detec-
tion systems from either Roche Applied Science on a cooled charge-coupled device (CCD) camera (Fuji, Minami-Ashigata, Japan). For detection of total amount of proteins after analysis of phosphorylation status, membranes were stripped with 0.4M NaOH for 10 min at room temperature, blocked, and incubated with the corresponding antibodies.

**Chemotaxis**—A chemotactic response was assayed using a 96-well microchemotaxis chamber (NeuroProbe, Gaithersburg, MD). A polycarbonate membrane (polylevylpyrrolidone-free, pore size, 8.0 μm) was coated with 50 μg/ml fibronectin (BD Biosciences) before use. PDGF-BB was added to the lower chamber, and cells were added to the upper chamber at 3 × 10^5 cells/well. After 4 h, non-migratory cells on the upper membrane surface were mechanically removed, and the cells that had traversed and spread on the lower surface of the filter were fixed and stained with Giemsa. The migrated cells were quantified with cooled CCD camera (Fuji, Minami-Ashigata, Japan). Experiments were performed in quadruplicate.

**Immunofluorescence Microscopy**—AG01523 foreskin fibroblasts grown on glass coverslips were fixed in 2% paraformaldehyde and then permeabilized in 0.2% Triton X-100 in phosphate-buffered saline for 5 min. Cells were then blocked by incubation for 1 h in 10% fetal bovine serum and then incubated with 5% fetal bovine serum and JNK antibody overnight at 4 °C. The slides were then washed three times, incubated with fluorescein isothiocyanate-conjugated phallolidin (Sigma) and either TRITC-conjugated swine anti-rabbit IgG antibody or TRITC-conjugated rabbit anti-mouse IgG antibody for 1 h at room temperature, and then washed again three times. GFP was detected by its autofluorescence. Coverslips were mounted on glass slides using Fluoromount-G (Immunkemi, Sweden), and to visualize the fluorescence, a Zeiss microscope was used.

**RESULTS**

**Effect of Low Molecular Weight Inhibitors on PDGF-mediated Chemotaxis**—To elucidate the mechanism(s) by which PDGF induces chemotaxis, we investigated the involvement of various kinases in this process employing a panel of low molecular weight kinase inhibitors. Analysis of PAE/PDGFRβ cells using a 96-well microchemotaxis chamber revealed that inhibition of the Erk pathway (PD98059), Src family kinases (SU6656), or protein kinase C (Calphostin C) had no significant effect on chemotaxis (Fig. 1A). In contrast, inhibition of PI 3-kinase (LY294002), p38 (SB203580), or JNK (L-JNKI1) MAP kinases displayed an efficient inhibition (Fig. 1A). PI 3-kinase and p38 have been reported to have important roles in chemotaxis (16–18). Notably, if PDGF was placed in both the upper and lower chamber at

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**FIGURE 2. JNK inhibition reduces PDGF-induced chemotaxis.** The AG01523 fibroblasts were pretreated for 1 h with the indicated concentrations of SP600125 (A), L-JNK11 (B), or infected with adenoviruses encoding GFP-JNK, GFP-JNK-DN, or GFP 48 h prior to the experiment (C). Cells were plated on a fibronectin-coated polycarbonate membrane (pore size: 8.0 μm) on a 96-well microchemotaxis chamber. The cells were then allowed to migrate for 4 h toward 20 ng/ml PDGF-BB placed in the lower chamber. The membrane was then fixed in ethanol and cells that had migrated through the membrane stained with Giemsa. The intensity of the staining was measured using a CCD camera. Data are plotted as mean of quadruplicate samples with one standard deviation indicated. Data represent one experiment of three performed.
equal concentration there was no cell migration through the membrane, suggesting that we indeed measured chemotaxis and not chemokinesis (data not shown). To assess whether the effect of MAP kinase inhibition was cell type specific, we performed a similar experiment using primary human fibroblasts (AG01523). Also in these cells, inhibition of p38 or JNK (1-JNK11 and SP600125) resulted in complete loss of chemotaxis, whereas interference with the Erk MAP kinase pathway did not have as dramatic effect (Fig. 1B). Also in primary fibroblasts, inhibition of PI 3-kinase inhibited chemotaxis. It is interesting to note that for the AG01523 fibroblasts, 10% fetal calf serum was not chemotactic. In conclusion, our data suggest that also JNK has an important role in PDGF-induced chemotaxis.

**JNK Inhibitors as Well as Dominant-negative JNK Reduce PDGF-BB-induced Chemotaxis**—To strengthen our finding that JNK plays a central role in PDGF-mediated chemotaxis, we performed a dose-response study using SP600125, which is a competitive ATP inhibitor selective for JNK on primary human AG01523 fibroblasts. Fig. 2A shows that this inhibitor interferes with chemotaxis in a dose-dependent manner. Similar results were obtained using another JNK inhibitor denoted 1-JNK11 (Fig. 2B), which is an inhibitory peptide that interferes with the interaction between JNK and substrates. Thus, using two inhibitors that target JNK through distinct mechanisms, i.e. blocking kinase activity or interfering with the substrate interaction, we could demonstrate dose-dependent reductions in chemotaxis. Neither of these inhibitors had any significant effect on the low level of migration observed in the absence of ligand (data not shown). Because chemical inhibitor may have nonspecific effects we also investigated the role of JNK in PDGF-mediated cell migration using adenoviral constructs expressing a dominant-negative form of JNK. We infected AG01523 fibroblasts with adenoviruses encoding GFP-tagged wild-type JNK (Ad-JNK), dominant-negative JNK (Ad-JNK-DN), or GFP (Ad-GFP). All constructs were readily expressed, and we observed a significant inhibition of PDGF-induced chemotaxis in the presence of Ad-JNK-DN but not in the presence of Ad-JNK or Ad-GFP (Fig. 2C), supporting our findings with the chemical inhibitors.

**PDGF-BB Activates the JNK Pathway in a PI 3-Kinase-dependent Manner**—To further investigate the role of JNK in PDGF-mediated chemotaxis, we next sought to define how PDGF induces JNK activation. We first characterized the time course of JNK activation following PDGF-BB stimulation. As shown in Fig. 3, JNK phosphorylation increased with time and peaked at 30 min of PDGF-BB stimulation. Since we and others (16, 17, 19) have shown that PI 3-kinase is critical for chemotaxis, we investigated if PI 3-kinase activity is important for JNK activation by incubating cells with the chemical inhibitors LY294002 or wortmannin. Indeed, inhibition of PI 3-kinase interfered with PDGF-BB-mediated JNK activation (Fig. 4). In summary, activation of JNK downstream of PDGFRβ occurs with relatively slow kinetics and requires, at least in part, PI 3-kinase activity.

**JNK Mediates Phosphorylation of Serine 178 in Paxillin**—Studies from Huang et al. (10) have indicated a role for JNK-mediated paxillin phosphorylation in regulation of focal adhesion dynamics and hence cell migration. Therefore, we investigated the effect of JNK inhibition on PDGF-induced paxillin phosphorylation. Indeed, we observed a dose-dependent
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FIGURE 6. JNK co-localizes with actin-dense membrane ruffles induced by PDGF-BB. AG01523 fibroblasts were serum-starved overnight and stimulated with 20 ng/ml PDGF-BB for 30 min before staining for filamentous actin (A–C) or JNK (D–F). C and F are enlargements of the area of filamentous actin-JNK co-localization from B and E, respectively. G is a negative control with only secondary antibody. One representative experiment is shown out of three performed.

reduction in PDGF-BB-induced paxillin Ser\textsuperscript{178} phosphorylation after treatment with SP600125 (Fig. 5). In conclusion, PDGF-BB stimulation regulates JNK-dependent Ser\textsuperscript{178} phosphorylation of paxillin.

JNK Co-localizes with Actin-dense Membrane Ruffles in PDGF-BB-stimulated Cells—To shed further light on the function of JNK in PDGF-mediated chemotaxis, we performed immunofluorescence staining for JNK in AG01523 fibroblasts. We observed a general staining of JNK throughout the resting cells (Fig. 6D). After PDGF-BB stimulation JNK staining was seen in the actin dense membrane ruffles (Fig. 6B, C, E, and F). In addition, we observed an increased nuclear JNK staining, as expected (Fig. 6E). Moreover, we infected AG01523 fibroblasts with adenoviruses encoding GFP-tagged JNK (Ad-JNK) or GFP (Ad-GFP) and analyzed the subcellular localization of these proteins after PDGF-BB stimulation. We found that GFP-JNK was localized to the membrane ruffles after PDGF-BB stimulation (Fig. 7B, C, E, and F). In contrast, GFP alone where distributed throughout the cell irrespectively of PDGF-BB stimulation (Fig. 7G–L).

JNK Is Required for Migration of Glioblastoma Cell Lines—To investigate the generality of our finding, we determined whether JNK activity is also essential for migration of glioblastoma cells. Therefore, we analyzed the migration of T98G and A172 cells toward PDGF-BB in the presence or absence of SP600125. As shown in Fig. 8, both cell lines migrated toward PDGF-BB, and we could detect robust inhibition of migration in the presence of SP600125. Both glioblastoma cell lines had a tendency to migrate in the absence of PDGF-BB in the lower chamber. This migration could be blocked by STI-571 or SP600125 (Fig. 8) and is possibly due to the presence of an autocrine loop involving PDGF isoforms. Hence, JNK activity is critical for PDGF-BB-induced chemotaxis also in two glioblastoma cell lines.

DISCUSSION

The pathways that mediate the chemotactic response of PDGFRs are complex and not fully understood at the molecular level. Several studies have implicated phospholipase C\textgamma, PI 3-kinase and p38 MAP kinase (reviewed in Ref. 4). In the present study, we have identified JNK MAP kinase as an important component in the machinery regulating chemotaxis in AG01523 fibroblasts and two glioblastoma cell lines in response to PDGF-BB. In agreement with earlier results, we could show that PI 3-kinase and p38 are also involved in this process.

Using two distinct types of JNK inhibitors, SP600125, a competitive ATP inhibitor, and L-JNK11, a peptide inhibitor blocking binding between JNK and substrates, we could demonstrate a dose-dependent inhibition of chemotaxis. In an \textit{in vitro} kinase assay using SP600125, the IC\textsubscript{50} for MKK4 was reported to be ten times greater than that of JNK (0.4 and 0.04 \textmu M, respectively), whereas other kinases had a significantly larger IC\textsubscript{50} values (>25-fold) (20). Thus, it is possible that SP600125 also to some degree inhibits MKK4 which is an upstream activator of JNK. As is the case with all inhibitor studies there is also a possibility that other pathways are affected. Therefore, we confirmed our results by expressing an adenoviral construct encoding a dominant-negative form of JNK in AG01523 fibroblasts.

Persistent activation of JNK has been correlated with the induction of apoptosis, whereas transient activation may have other functions depending on which other pathways are activated in the cell (7). In line with this notion, we observed a transient phosphorylation of JNK in response to PDGF-BB. The kinetics of JNK phosphorylation was relatively slow with a peak phosphorylation after 30 min; in comparison, the other MAP kinases Erk and p38 are maximally activated within 2–5 min after PDGF-BB stimulation (21). Interestingly, we detected the strongest immunostaining of JNK in the actin-dense membrane ruffles after 30 min of PDGF-BB stimulation, which coincides with the peak activation of JNK. Similar results were obtained using adenoviral mediated expression GFP-tagged JNK. These results suggest that active JNK is localized to the actin ruffles at the cell edge.
Next we attempted to define the molecular mechanism by which JNK is activated downstream of PDGFRβ. In a recent study from Marinissen et al. (22), RhoA was demonstrated to activate JNK through the action of ROCK. However, this pathway appears not to be involved in PDGF-BB-mediated JNK activation, since a ROCK inhibitor did not block JNK activation (data not shown). It has been reported that for some receptor systems PI 3-kinase is important for JNK activation, possibly through activation of Rac or Cdc42 (23, 24). Therefore, we investigated the role of PI 3-kinase in the PDGF-BB-induced JNK activation. Indeed, inhibition of PI 3-kinase, using two different chemical inhibitors, i.e. LY294002 and wortmannin, abolished the PDGF-BB-mediated JNK phosphorylation. Thus, JNK may be an important component downstream of PI 3-kinase involved in chemotactic signaling.

The role of JNK in cell migration is not clear, but one suggestion is that JNK phosphorylates the focal adhesion component paxillin and thereby controls cell movement (10). In agreement with the study from Huang et al. (10), we observed that also PDGF-BB-mediated phosphorylation of Ser178 in paxillin was dependent of JNK activity. In addition, we observed localization of JNK in actin-dense membrane ruffles, suggesting a role for JNK at the leading edge of the cell, which is rich in focal contacts necessary for forward propulsion. It is likely that perturbation of the focal adhesion dynamics will have severe affects on the ability of the cell to migrate. Therefore one may speculate that JNK may exert it role in cell migration by modulating focal adhesion integrity, e.g. by phosphorylating key components such as paxillin. Moreover, it has been suggested that c-Jun is necessary for PDGF-induced migration of vascular smooth muscle cells (11). Similarly, c-Jun is also involved in the migration of keratinocytes (25). In the latter case it was shown that c-Jun was required to establish an autocrine loop of epidermal growth factor stimulation needed for cell migration.

It is possible that JNK contributes to PDGF-induced cell migration through regulation of gene expression, since we found that blocking protein synthesis using cycloheximide inhibited chemotaxis of AG01523 fibroblasts toward PDGF-BB (data not shown). Hence, it appears that JNK have functions required for cell migration in actin ruffles, and possibly also in the production of new proteins, either by controlling gene transcription or translation of pre-existing mRNA.

Glioblastoma multiforme is an aggressive human brain tumor for which currently no efficient treatment exists. One characteristic of the glioblastoma tumor is that it invades surrounding brain tissue (26). Moreover, glioblastomas often have autocrine loops involving PDGF isoforms (27), which may contribute to the invasive phenotype. Interestingly, we observed an inhibition of the PDGF-BB-stimulated chemotaxis in two glioblastoma cell lines after inhibition of JNK. Thus, inhibition of JNK activity might be a strategy to reduce the invasive properties of glioblastoma tumors.

In conclusion, this study demonstrates that JNK is a critical component in the cell migration machinery employed by PDGFRβ in several cell types. We show that PI 3-kinase activity is necessary for PDGF-BB-mediated JNK activation leading to its translocation to the nucleus but also to actin-dense membrane ruffles. In addition, the focal adhesion component paxillin is phosphorylated on Ser178 in a JNK-dependent manner.
after PDGF-BB stimulation. Thus JNK may directly modulate the integrity of focal adhesions and in this way affect cell migration.

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FIGURE 8. JNK is essential for PDGF-BB-induced chemotaxis of glioblastoma cells. The T98G and A172 glioblastoma cell lines were serum-starved overnight and pretreated for 1 h with SP600125 or STI571 at the concentrations indicated and then plated on a fibronectin-coated polycarbonate membrane (pore size: 8.0 μm) in a 96-well microchemotaxis chamber. The cells were then allowed to migrate for 4 h toward 10 ng/ml PDGF-BB and placed in the lower chamber. The membrane was then fixed in ethanol, and cells that had migrated through the membrane were stained with Giemsa. The intensity of the staining was measured using a CCD camera. Data are plotted as mean of quadruplicate samples with one standard deviation indicated. Data represent one experiment of four performed.

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