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Genetics

A role for Pak protein kinases in Schwann cell transformation

(neurofibrosarcoma/neurofibromatosis/Ras/Rac)

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ABSTRACT **Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder caused by loss of the NF1 gene, is characterized clinically by neurofibromas and more rarely by neurofibrosarcomas. Neurofibromin, the protein encoded by NF1, possesses an intrinsic GTPase accelerating activity for the Ras proto-oncogene. Through this activity, it is a negative regulator of Ras. The Pak protein kinase is a candidate for a downstream signaling protein that may mediate Ras signals because it is activated by Rac and Cdc42, two small G proteins required for Ras signaling. Here, we use Pak mutants to explore the role of Pak in Ras signaling in Schwann cells, the cells affected in NF1. Whereas an activated Pak mutant does not transform cells, dominant negative Pak mutants are potent inhibitors of Ras transformation of rat Schwann cells and of a neurofibrosarcoma cell line from an NF1 patient. Although activated Pak stimulated jun-N-terminal kinase, inhibition of Ras transformation by dominant negative Pak did not require inhibition of jun-N-terminal kinase. Instead, the Pak mutants appeared to inhibit transformation by preventing Ras activation of the ERK/mitogen-activated protein kinase cascade. These results have implications for our understanding of NF1 because a neurofibrosarcoma cell line derived from a patient with NF1 was reverted by stable expression of the Pak dominant negative mutants.**

Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder caused by loss of the NF1 gene, is characterized clinically by neurofibromas and more rarely by neurofibrosarcomas (1–3). Abnormalities in Schwann cells are thought to be responsible for both types of tumors (4, 5). Whereas the basis of the Schwann cell as the affected cell is not well understood, the underlying mechanism of transformation for both tumors is through the Ras signaling pathway. Neurofibromin, the protein encoded by NF1, possesses an intrinsic GTPase accelerating activity for the Ras proto-oncogene. Through this activity it is a negative regulator of Ras. Thus, loss of neurofibromin causes elevated levels of activated Ras, which leads to hyperactivation of downstream signals (6–10).

The best characterized Ras signals are through the mitogen-activated protein (MAP) kinase cascade. Ras binds and activates the serine/threonine kinase Raf, which leads to sequential activation of the MEK and ERK kinases (11). Ras also activates another MAP kinase family member, jun-N-terminal kinase (JNK), through a Raf-independent mechanism that is not well defined but known to require the small G proteins Rac and Cdc42 (12, 13).

A candidate for an effector of Rac is the serine/threonine kinase Pak ($p65^{pak}$) because it binds Rac, stimulates JNK, and can induce changes in the actin cytoskeleton similar to those induced by activated Ras and Rac (14–19). In yeast, members of the Rho/Rac family participate in Ras signaling (20), and the Pak homolog Ste20 mediates a signal required for filamentous growth through a Ras>Cdc42>Ste20 cascade (21).

These observations prompted us to determine whether Pak is required for Ras signaling in Schwann cells. We found that dominant negative mutants of Pak1 ($Pak1^{R299}$) specifically inhibited transformation by Ras of a rat Schwann cell line and markedly reduced the tumorigenic capacity of a neurofibrosarcoma cell line from an NF1 patient. Surprisingly, transformation inhibition correlated with ERK inhibition but not JNK inhibition. These observations suggest that a functional connection between Pak and ERK mediates a signal essential for Ras transformation.

MATERIALS AND METHODS

Plasmids. cDNA expression plasmids using the CMV promoter to express myc-tagged Pak1 and $Pak1^{R299}$ based on the plasmid pCMV6M (a modified version of pCMV5) have been described elsewhere (22). Human K-ras4B expression systems that use pZIP-NeoSV(x)1, a retrovirus vector (neomycin resistant) were a gift from C. Der (University of North Carolina, Chapel Hill) Expression of the inserted gene is regulated from the Moloney long terminal repeat promoter. v-Raf, Rac, JNK, and ERK plasmids have been described elsewhere (23).

Cell Culture and Transformation Assays. Schwann cells and ST88–14 cells were obtained from Thomas Glover, University of Michigan (8, 24). Schwann cells were grown in high glucose (4.5 g/liter) Mediatech DMEM purchased from Fisher Scientific, supplemented with 10% heat inactivated fetal bovine serum (Sigma), penicillin (100 units/ml), streptomycin (100 mg/ml), and 100 μM $ZnCl_2$, and kept at 37°C and 5% CO_2/95% air. ST88–14 cells were grown in RPMI 1640 purchased from Fisher Scientific, supplemented with 15% fetal bovine serum (Sigma), penicillin (100 units/ml), and streptomycin (100 mg/ml), and kept at 37°C and 5% CO_2/95% air. DNA transfections were performed by the calcium phosphate precipitation technique. Twenty micrograms total DNA (6.5 μg of each test DNA plus 7 μg of Pak DNA and the appropriate amount of pUC19 plasmid to bring the total to 20 μg) were mixed briefly with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2× *N,N*-bis(2-hydroxethyl)-2-aminoethanesulfonic acid (BES)-buffered saline and incubated 10–20 min at room temperature. The mixture was then added dropwise to a 50–60% confluent, freshly fed 100-mm dish of cells, swirled gently, and incubated 18–24 h at 37°C and 5% CO_2. Cells were

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Abbreviations: NF1, neurofibromatosis type 1; JNK, jun-N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; ERK, extracellular signal-regulated protein kinase; HA, hemagglutinin.

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washed twice with growth media, refed, and incubated for 24–48 h. Soft agar assays were performed as described (25). The 10^5 post-transfection cells were plated on 60-mm dishes. After 21–24 days, colonies were scored by inspection under a Nikon DIAPhot microscope using phase contrast and by staining the dishes with 0.5 μg/ml MTT (Sigma) overnight at 37°C. Transfection efficiency assays using pRSV as a β-galactosidase reporter have been described (23). Transfection efficiencies ranged from 1% to 3% and were not affected by any of the plasmids used in this study.

Stable Cell Lines Expressing Pak. To establish stable cell lines expressing Pak1, Pak1^{R299}, Pak1^{L83,L86,R299}, and Pak1^{L83,L86}, each construct was cotransfected with pCDNA3 into Schwann cells or ST88–14 cells. DOTAP liposomal transfections were performed according to manufacturer's protocol (Boehringer Mannheim). A total of 5 μg of DNA (4.5 μg of Pak and 0.5 μg of PCDNA3) and 40 μl of DOTAP reagent (μg/μl) were added to a 100-mm dish containing 3 ml of Opti-MEM (Life Technologies, Grand Island, NY). After 4–5 h, 7 ml of growth medium was added. Cells were split and plated on another dish 48–72 h after the addition of DNA. The transfected cells were selected in growth medium containing 400 μg/ml (for ST88–14 cells) or 500 μg/ml (for Schwann cells) of Geneticin (G418; GIBCO/BRL). Protein expression levels were determined by Western blot (immunoblot) analysis of G418-selected cell lysates using the anti-myc tag mAb 9E10 (Calbiochem) or anti-ERK antibodies (New England Biolabs). Blots were visualized with the procedure outlined in the enhanced chemiluminescence kit (Amersham).

JNK and MAP/ERK Kinase Assays. Transfections of Schwann cells were performed with DOTAP liposomal transfections. A total of 5 μg of DNA (1 μg of HA-JNK1 or HA-ERK1, 2 μg of each test DNA; when single plasmids were tested, and 2 μg of pUC19 plasmid) and 40 μl of DOTAP reagent were added to a 10-cm dish containing 3 ml of Opti-MEM. After 4–5 h, 7 ml of fresh growth medium was added. Approximately 48–72 h after addition of DNA, cells were washed with cold PBS and lysed in 40 mM Hepes (pH 7.4), 1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, and centrifuged at 12,000 $\times$ *g* for 25 min at 4°C (15). Extracts were incubated with HA-antibody (12CA5) and protein A beads for 3–3.5 h at 4°C. Precipitates were washed three times with lysis buffer and washed two times in 2X phosphorylation buffer. The precipitates were then incubated with 5 μg of GST-c-jun (for Jun kinase) or myelin basic protein (for ERK), 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 20 μM unlabeled ATP (final concentration) at 30°C for 30 min. Mixtures were washed three times with lysis buffer and two times with 2$\times$ phosphorylation buffer. Reactions were stopped by adding 2$\times$ SDS sample buffer and heating to 95°C. All of the experiments were performed at least three times with similar results.

Subcutaneous Tumor Xenografts. ST88–14 xenografts were derived from *in vitro* cell cultures using described methods (26). Log-phase cells grown in RPMI 1640 and 15% fetal bovine serum were harvested by trypsinization, washed, and centrifuged into a pellet. The cell pellet was resuspended in sterile 0.9% saline and Matrigel (Collaborative Biomedical Products, Bedford, MA). Aliquots were injected s.c. into the right flank of each nude mouse. Mice were observed for the appearance of nodular tumor growth. When the perpendicular diameter of each tumor reached 2 mm, the length and width of s.c. xenografts were measured every 3–4 days with vernier calipers (Manostat, New York). Tumor volume was calculated by the following formula: width^2 $\times$ length/2.

RESULTS

To evaluate the roles of Rac and Pak in Schwann cell transformation, we transfected Rac^{L61} into MT4H1, a T antigen-immortalized Schwann cell line known to be sensitive to Ras transformation, and then determined whether the transfected cells grew in soft agar (24, 27). Because Rac is known to cooperate with activated Raf (28), we also tested whether Rac could cooperate with Raf^{D340} to transform cells with high efficiency. Raf^{D340} is an activated Raf mutant that fails to transform cells by itself but cooperates with Rac to transform cells with high efficiency. As shown in Fig. 1*a*, Rac^{L61} transformed cells poorly and Raf^{D340} failed to transform cells altogether. However, when transfected together, they transformed cells with high efficiency. Furthermore, Rac^{N17}, a dominant negative mutant of Rac, inhibited transformation by Ras but not v-Raf, an oncogenic mutant (Fig. 1 *b* and *c*). These results suggest that Rac is essential for Ras transformation of Schwann cells.

Rac and the related protein Cdc42 bind and activate the serine threonine kinase Pak *in vitro*. To test whether Pak is involved in Ras transformation of Schwann cells, we cotransfected the activated and kinase-deficient Pak mutants with Ras. The hyperactive Pak, Pak1^{L83,L86}, was not oncogenic on its own (not shown). However, Pak1^{R299}, which lacks kinase activity, inhibited Ras transformation by $\approx$80% (Fig. 1*b*). Pak1^{R299} also inhibited Rac/Raf cooperation (data not shown). This mutant was not a general inhibitor of cell transformation because it failed to inhibit transformation by v-Raf, an oncogenic Raf mutant (Fig. 1*c*). To determine whether the inhibition we observed was caused by sequestering Rac/Cdc42, we tested inhibition by Pak1^{L83,L86,R299}, a mutant that lacks kinase activity and also fails to bind either Rac or Cdc42 (Fig. 1*b*). Pak1^{L83,L86,R299} was an equally effective inhibitor of Ras transformation as Pak1^{R299}, suggesting that Pak binds additional targets essential for Ras signaling, distinct from Rac and Cdc42. Ras was expressed at comparable levels in all transfections, as determined by Western blotting experiments (data not shown), suggesting that Pak mutants did not affect Ras expression.

We also developed rat Schwann cell lines that expressed similar levels of the various Pak mutants. As expected from the results described above, the cell line expressing the hyperactive Pak1^{L83,L86} showed no effects on transformation, and cell lines expressing the two dominant negative Pak mutants were highly resistant to transformation by Ras (Fig. 1*d*). Again, inhibition of transformation was not seen when v-Raf was substituted for Ras. These results suggest an essential role for Pak in Ras transformation of rat Schwann cells, although Pak itself is not an oncogene.

Two MAP kinases are activated by Ras, ERK, (Ras>Raf>MEK>ERK) and JNK (Ras>Rac>Pak>MEKK>SEK>JNK) (11). Whereas in most experimental systems Rac and Pak only activate JNK, under some conditions they also activate ERK (18, 29, 30). Such alternative inputs are to be required likely to maintain prolonged ERK activation (31). To determine the effect of Pak mutants on downstream Ras effector pathways for Rac and Ras, we measured kinase activities after cotransfection with the various plasmids (Fig. 2). K-Ras, Rac^{L61}, and Pak1^{L83,L86} activated JNK 18- to 25-fold relative to Pcmv6, a vector control (data not shown for Rac and Pak) when each was cotransfected with JNK. Pak1^{R299} inhibited activation by Ras and Rac 40–70%. Pak1^{L83,L86,R299} failed to inhibit either Ras or Rac activation of JNK (Fig. 2*a*; data not shown for Rac). v-Raf had no effect on JNK activity nor did it inhibit Pak1^{L83,L86} activation of JNK (not shown). Thus, our data suggest the presence of a Ras to Rac to Pak pathway for JNK activation and suggest that Pak1^{R299} but not Pak1^{L83,L86,R299} sequesters endogenous Rac family members to inhibit JNK activation. However, because Pak1^{L83,L86,R299} inhibited Ras transformation without inhibiting JNK, JNK inhibition is not essential for dominant negative Paks to inhibit Ras transformation.

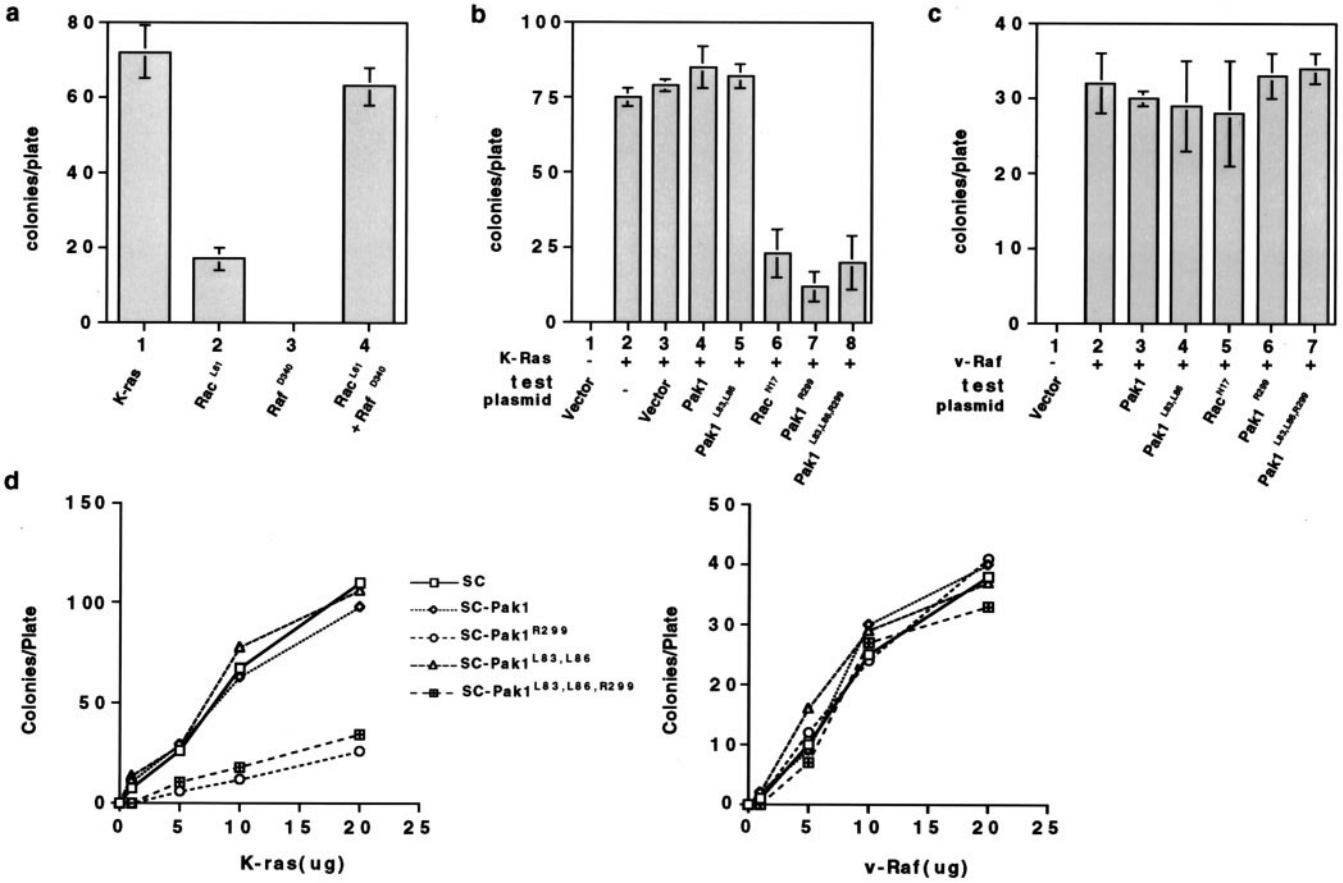

FIG. 1. A role for Rac and Pak in Schwann cell transformation. (*a*) Rac^{L61} cooperates with Raf^{D340} to transform rat Schwann cells. Schwann cells were transfected with the indicated plasmids, plated on soft agar, and then stained with thiazolyl blue [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] to visualize colonies. The data is presented as number of colonies per plate. The error bars indicate SDs. Data shown were representative of three independent experiments. (*b*) Pak^{R299} and $Pak^{L83,L86,R299}$ inhibit Ras transformation in soft agar. Data shown were representative of four independent experiments. (*c*) Pak^{R299} and $Pak^{L83,L86,R299}$ do not inhibit v-Raf transformation. Data shown were representative of two independent experiments. (*d*) Ras and Raf transformation of cell lines expressing mutant Pak1 proteins. Cell lines expressing Pak constructs were developed as described in *Materials and Methods* and tested for expression by Western blots (data not shown). The indicated amounts of K-ras or v-Raf were transfected into the stable cell lines, and transformation was scored in soft agar colony assays. Similar results were obtained in three independent experiments.

Next, we tested the effects of Pak dominant negative mutants on activation of ERK. Cotransfection of ERK with Ras and v-Raf, but not Rac^{L61}, stimulated ERK activity 19- to 33-fold over vector controls (Fig. 2 *b–d*). When the four Pak mutants were tested in this assay, we observed that Pak1 and $Pak1^{L83,L86}$ had no effect on ERK activity either by themselves or together with Ras (Fig. 2*b*) or v-Raf (Fig. 2*d*). However, Rac^{L61} and the hyperactive $Pak1^{L83,L86}$, both stimulated ERK activation by Raf^{D340}, the partially activated Raf mutant (Fig. 2*c*). When we tested the two kinase-deficient mutants $Pak1^{R299}$ and $Pak1^{L83,L86,R299}$, we found they inhibited Ras activation of ERK by 66 and 57%, respectively (Fig. 2*b*). Both Pak mutants also inhibited cooperative ERK activation by Rac^{L61}/Raf^{D340} and $Pak1^{L83,L86}/Raf^{D340}$ (data not shown). Neither Pakdominant negative mutant inhibited ERK activation by Raf (Fig. 2*d*). Thus, in all of the cases where Pak inhibited Ras transformation and Rac/Raf cooperative transformation, Pak also inhibited ERK activation.

The experiments described so far used rat Schwann cells immortalized with T-antigen (24), and the results were consistent with those we recently observed in the Rat-1 cell line (23). However, we have not demonstrated a role for Pak in NIH 3T3 cell transformation. To test the validity of the rat cell systems for evaluating the role of Pak in human neurofibrosarcomas, we determined the effects of Pak mutants on the ST88–14 cell line. This cell line was derived from a neurofibrosarcoma that developed in an NF1 patient. ST88–14 cells have reduced levels of neurofibromin, which leads to elevated levels of Ras-GTP. Furthermore, its transformed phenotype is reverted by expression of GAP or blocking Ras processing with farnesyl transferase inhibitors (8, 32). We made stable cell lines of ST88–14 expressing the various Pak mutants, confirmed expression through Western blots, and then tested for maintenance of the transformed phenotype (Fig. 3*a*). We also observed that levels of phosphorylated ERK were reduced in extracts from cells expressing the dominant negative Pak mutants although the levels of total ERK were not affected (Fig. 3*b*). Cells expressing the dominant negative Pak constructs were reduced in their soft agar plating efficiency by ≈80% (Fig. 3*c*).

To evaluate the effect of Pak mutations on tumor formation *in vivo*, we injected wild-type ST88–14 cells or ST88–14 transfected with wild-type Pak (Pak1), hyperactive Pak ($Pak1^{L83,L86}$), or dominant negative Pak constructs s.c. into the flanks of athymic (*nu/nu*) mice. At tumor injection doses of 5×10^6 cells, tumor growth was observed in 7 of 9 mice with wild-type or $Pak1^{L83,L86}$ transfectants. In contrast, no gross or histological evidence of tumors was observed in mice injected with the dominant negative Pak cell lines, $Pak1^{R299}$ and $Pak1^{L83,L86,R299}$. In subsequent experiments, 1 or 2×10^7 cells injected s.c. produced tumors in all animals. The growth rate for ST88–14 wild-type xenografts was unchanged by transfec-

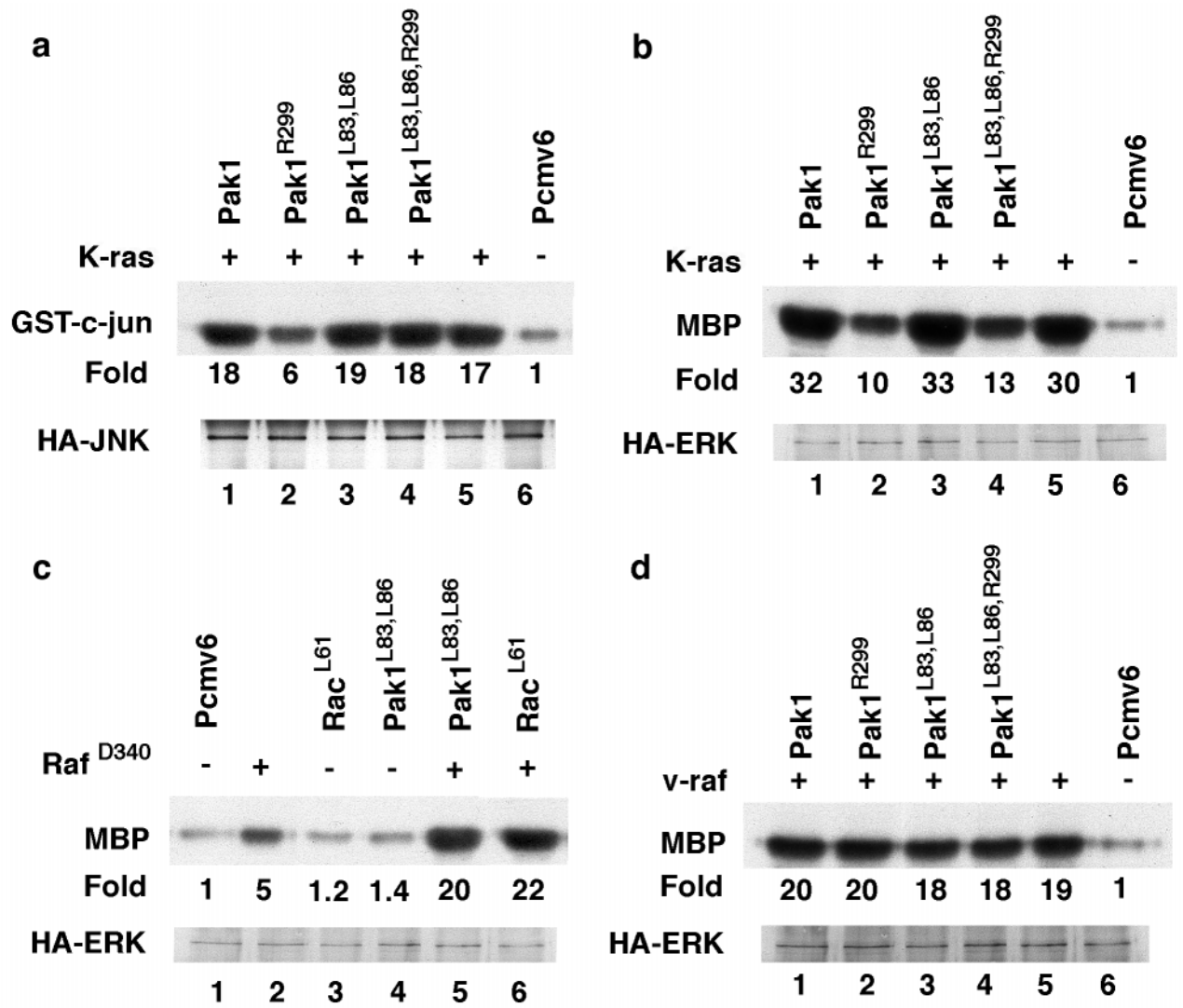

FIG. 2. Effect of Pak on activation of JNK and ERK. Schwann cells were cotransfected with either HA-JNK or HA-ERK and plasmids encoding the proteins shown. Fold indicates the fold increase in substrate phosphorylation over that occurring in the pcmv6 lanes, as determined through phosphorimager analysis. At the bottom of each panel is a Western blot showing expression of HA-JNK or HA-ERK, as indicated. MBP, myelin basic protein. Data shown are representative gels of three independent experiments. (*a*) Ras activation of JNK. (*b*) Ras activation of ERK. (*c*) Rac/Raf^{D340} and Pak/Raf^{D340} cooperative activation of ERK. (*d*) v-Raf activation of ERK.

tion with Pak1 or Pak1^{L83,L86} but was reduced when transfected with Pak dominant negative mutations Pak1^{R299} and Pak1^{L83,L86,R299} (Fig. 4*a*). The mean tumor volume-doubling time was reduced to 28% and 24.5% for Pak1^{R299} and Pak^{L83,L86,R299}, respectively, when compared with wild-type ST88–14 tumor growth. Histological examination of wild-type ST88–14 s.c. tumors showed spindle-shaped cells arranged in whorls and linear bands and a large number of mitotic figures (Fig. 4*b*). Pak1 and Pak1^{L83,L86} transfectants showed a relative preservation of the wild-type ST88–14 morphology and arrangement (Fig. 4 *c* and *d*). However, dominant negative Pak tumors showed a comparative increase in cytoplasmic volume, dispersed nuclear chromatin, a loss of fibrosarcomatous architectural organization, and few mitotic figures (Fig. 4 *e* and *f*). The experiments with ST88–14 cells correlated with those experiments described above with the rat Schwann cells, suggesting that Ras transformation of rat Schwann cells is an appropriate model system to study the role of Pak in neurofibrosarcomas.

DISCUSSION

Our data show that activated Pak is not an oncogene in Schwann cells; however, because mutants lacking kinase activity inhibit Ras transformation, Pak interacts with essential Ras signaling molecules. There are likely to be multiple Ras signaling proteins that Pak binds because a dominant negative Pak that fails to bind either Cdc42 or Rac is as effective at inhibiting cell transformation as one that binds. Our study uses dominant negative mutants, which are not true knockouts of Pak, and they may act through nonphysiological interactions and not by interfering with endogenous Pak. However, the observation that there are multiple interactions between Pak and Ras signaling components strengthens the conclusion that Pak itself mediates a signal essential for Ras transformation. Our studies also suggest that the relevant signal responsible for transformation inhibition is from Ras to ERK because both mutants that inhibit transformation also inhibit Ras activation of ERK; whereas, JNK activation only is inhibited with the mutant that binds Cdc42 and Rac. Furthermore, activated Pak cooperates with a partially activated Raf to stimulate ERK, although the stimulation is not sufficient to transform cells.

The central role of the Ras-Raf-MEK-ERK pathway in transformation has been established for many cell types, but other signals are required to maintain prolonged ERK activation (29, 31). Recent results suggest that a Rac to Pak signal may assist ERK activation by Raf. Dominant negative mutants

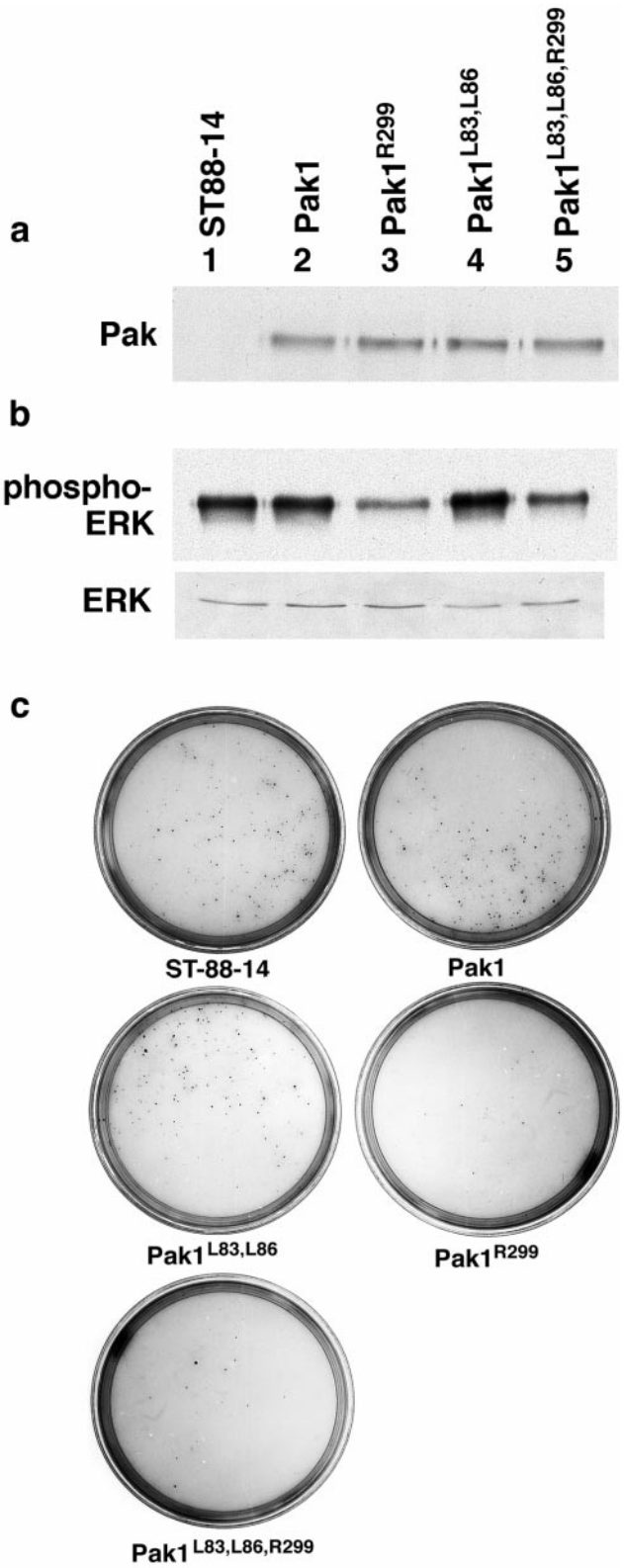

FIG. 3. Effect of Pak mutants on ST88–14, a neurofibrosarcoma cell line. (*a*) Western blot showing stable expression of Pak1 mutants in ST88–14 cells. 50 μg of extracts from ST88–14 cells were probed with antibody 9E10, which recognizes the myc tag on the Pak1 constructs. Pak1 is seen as a 65-kDa band. (*b*) Western blot of ERK with (*Upper*) phospho-specific ERK antibody and (*Lower*) ERK antibody. (*c*) Growth of cells expressing the dominant negative Pak constructs was reduced in soft agar; 1×10^5 cells were plated onto soft agar plates and then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to visualize colonies. Similar results were obtained in three independent experiments.

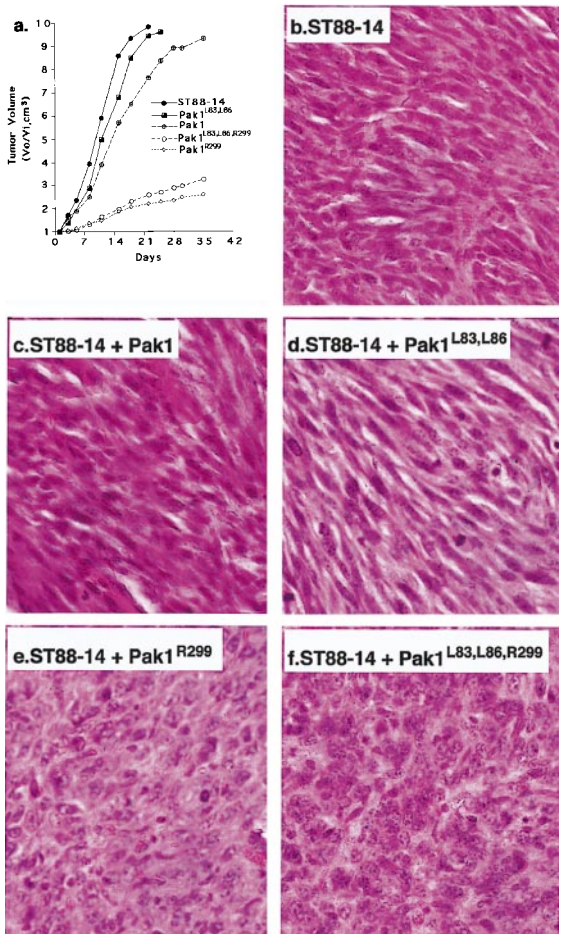

FIG. 4. Effect of Pak dominant negative mutations on ST88–14 human neurofibrosarcoma s.c. xenografts in nude mice. The growth rate for ST88–14 wild-type xenografts and xenografts of ST88–14 transfected with Pak1 and Pak1^{L83,L86} is greater than for xenografts of ST88–14 transfected with Pak1 dominant negative mutations Pak1^{R299} and Pak1^{L83,L86,R299} (*a*). Tumor volumes are expressed as the ratio of observed tumor volume (Vo) to the initial tumor (Vi) on the first day each tumor reached 0.04 cm^3, a reproducible and measurable volume. Each point represents the mean of five or more separate tumors. Representative tumor sections (hematoxylin/eosin-stained, 400X magnification) show spindle-shaped cells arranged in whorls and linear bands in wild-type ST88–14 (*b*), which are preserved relatively in ST88–14-Pak1 (*c*) and ST88–14-Pak1^{L83,L86} (*d*) transfectants but not in Pak-dominant negative ST88–14-Pak1^{R299} (*e*) or ST88–14-Pak1^{L83,L86,R299} (*f*).

of Rac and the Rac-binding domain of Pak inhibit ERK activation. Furthermore, Rac cooperates with Raf to activate ERK in transfection experiments, whereas Rac by itself activates only JNK (18). Pak likely mediates this signal because a membrane-targeted Pak can activate ERK modestly whereas activated Pak cooperates with Raf to strongly stimulate ERK (30, 33). In addition, we observe coactivation of ERK when activated Raf is cotransfected with activated Pak (Fig. 2*c*). Putting all of these data together, we propose that there is a Rac>Pak>ERK signal that is essential to maintain ERK activation and Ras transformation. The signal from Pak to ERK may be mediated through various intermediates such as MEK or Raf because both are stimulated and phosphorylated by Pak (33). There is also a Ras>Rac>Pak>JNK signal, but because Pak^{L83,L86,R299} does not inhibit Ras or Rac activation of JNK (Fig. 2*a*), it is likely that Rac uses other effectors as well as Pak to activate JNK.

Another way Pak may affect Ras transformation is through the actin cytoskeleton. Activated Pak mutants can alter the

actin cytoskeleton by inducing ruffling and disassembling stress fibers (22, 34). Both of these events are similar to those induced by activated Ras (35, 36). Our data suggest that blocking these actin events also may contribute to cell reversion in Schwann cells because nude mice xenografts of a neurofibrosarcoma expressing the dominant negative Paks lose their basic sarcoma architecture (Fig. 4).

Our previous study demonstrated that Pakdominant negative mutants inhibited Ras transformation in Rat-1 cells, but because the mutants had no effect on Ras transformation in NIH 3T3 cells, it was not clear that the observation could be extended to other cell systems (23). The reason that Pak failed to effect Ras transformation in the NIH 3T3 cells remains unclear, but one possibility is that NIH 3T3 cells rely more heavily on the Ras/Raf/ERK cascade than other cells; another possibility is that our Pak expression systems do not express Pak at levels sufficient to sequester interacting proteins. We favor the latter possibility because there is a well-documented role of Rac in NIH 3T3 cell transformation, suggesting that Pak^{R299} should inhibit transformation by sequestering Rac. Here, we report Pak-dominant negative mutants inhibit Ras transformation and ERK activation in two additional cell systems relevant to Ras-sensitive tumors. Furthermore, the sensitivity of an NF1 neurofibrosarcoma to Pak-dominant negative mutants suggests that Pak may be a target for novel antineoplastic drugs useful for treating Ras-dependent tumors (37).

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