Regulation of Fas Expression by STAT3 and c-Jun Is Mediated by Phosphatidylinositol 3-Kinase-AKT Signaling*

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Cooperation between STAT3 and c-Jun results in suppression of Fas Receptor (FasR) transcription, which is often seen in advanced human tumors. To identify requirements for STAT3-Jun cooperation, we elucidated the role of protein kinases that affect both transcription factors. The phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway was found capable of down-regulating both STAT3- and c-Jun-dependent transcription, resulting in derepression of FasR transcription. Conversely, inhibition of PI3K-AKT signaling via the specific pharmacological inhibitor LY294002 up-regulated AP1/Jun- and STAT-dependent transcriptional activities, resulting in suppression of the FasR promoter activities and decreased FasR surface expression. PI3K-AKT's ability to affect FasR transcription was not observed in c-*jun* null fibroblasts, suggesting that c-Jun is required for PI3K/AKT-mediated regulation of FasR transcription. Interestingly, the dominant negative form of Rac1 (RacN17) was also efficient in relieving FasR expression, suggesting that the increase in FasR expression following AKT stimuli could be mediated via AKT ability to elicit suppression of Rac1, which in turn decreases JNK activities and c-Jun phosphorylation. Overall, our findings demonstrate that through its negative effects on both c-Jun and STAT3, the PI3K-AKT pathway disrupts cooperation between c-Jun and STAT3, which is required for silencing the FasR promoter, resulting in increased expression of surface FasR and concomitant sensitization to FasL-mediated programmed cell death.

Deregulation of cell proliferation and suppression of apoptosis meet the essential requirements for neoplastic development and progression. Understanding the multiple regulatory elements that are impaired in human tumors highlights the complexity of controlled cell growth while pointing to targets that may be impaired during tumor development. Both normal and tumor cells use the PI3K-AKT[1] (protein kinase B) as survival

pathways that utilize several critical cellular effectors as substrates and modulators of the cell's ability to undergo apoptosis (1–3). These include phosphorylation-dependent inhibition of proapoptotic signals of proteins such as BAD, caspase 9, and the family of forkhead transcription factors (4–6). Additionally, AKT enhances survival signals via activation of NF-κB-dependent expression of anti-apoptotic genes, including FLIP and c-IAPs, and suppresses mitochondrial pathways required for apoptosis (7–9). Certain tumors increase AKT expression, activity, or both as a means of escaping programmed cell death. For example, AKT was found to be overexpressed in breast cancer cell lines and in ovarian and pancreatic cancers and amplified in gastric adenomas (10–13). Expression of downstream antiapoptotic factors, such as inhibitors of apoptosis (IAPs), is altered in human tumors; ML-IAP is an example for a member of the family of IAPs, which is preferentially expressed in human melanoma (14). Expression of survivin and Bcl2 are also modified in the course of tumor development (15, 16). Apaf1, a cell death effector that acts with cytochrome *c* and caspase 9 to mediate stress-dependent apoptosis, is inactivated in 40% of malignant melanomas, thereby providing another example for a modification of an apoptotic cascade that acquires malignant melanoma with chemoresistance (17).

Changes in the proapoptotic signaling were also documented during tumor development and progression. Each of the six major cell death pathways, TNFR1, FasR, TRAIL-R1, TRAIL-R2, DR3, and DR6, were reported to undergo certain changes in the course of tumor progression (18). Fas Receptor (Fas, CD95/Apo-1) signaling appears to serve as a primary death cascade in human melanomas (19). Upon interacting with Fas Ligand, FasR forms a complex with the Fas-associated death domain protein, which directly binds and activates caspase 8, resulting in the induction of apoptosis (20, 21). FasR ligation also induces a rapid and transient tyrosine phosphorylation, which coincides with PI3K/AKT activation and is required for Fas mediated apoptosis (22). Tyrosine phosphorylation was found essential for FasR-mediated apoptosis (23, 24).

Several studies point to the relationship between pro- and antiapoptotic cell death signaling (*i.e.* PI3K-AKT and Fas). PI3K is among signaling pathways implicated in the regulation of FasR responses (25). UV irradiation-mediated activation of PI3K signaling via epidermal growth factor receptors in human skin provides an example for physiological stimuli that utilize the PI3K/AKT cascade to affect the degree of damage-induced cell death (26). UV, via generation of hydrogen peroxide, induces AKT phosphorylation (27). Overall increase in basal levels of PI3K/AKT activity coincided with tumor cell ability to exhibit resistance to UV and ionizing radiation (28, 29).

Impaired Fas signaling is frequently observed during tumor progression and has been attributed in most cases to downregulation of Fas expression (30, 31). Loss of Fas function has been implicated in increased resistance of tumors to apoptosis

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; AKT^{myr}, myristoylated AKT; GFP, green fluorescent protein; JNK, Jun N-terminal kinase; STAT3, signal transducer and activator of transcription 3; IAP, inhibitor of apoptosis; FasR, Fas receptor; WT, wild type; TNF, tumor necrosis factor; CHX, cycloheximide; PE, phycoerythrin; PTEN, phosphatase and tensin homology deleted on chromosome 10.

induced by chemical and physical stimuli and is thought to represent one of the mechanisms that enable such tumors to escape immune surveillance as well as to acquire the metastatic phenotype (32–37). Whereas regulation of *fas* transcription is mediated by both constitutive and inducible regulatory pathways, down-regulation of Fas expression in advanced human melanoma-derived cell lines was found to be mediated by cooperation of STAT3 with c-Jun, a process that can be most clearly identified in advanced tumors. Interference with such cooperation through the use of dominant negative forms of either STAT3 or c-Jun efficiently restored transcription and surface expression of FasR (38). In our search for signaling pathways that could control such cooperation, we discovered the role of PI3K/AKT, which we report here.

Concomitant with elevated PI3K-AKT activities in human tumors is increased survival, motility, and metastatic capacity (39–44). Here we demonstrate that AKT is also capable of altering the activities of transcription factors that play important roles in regulating FasR expression. In demonstrating that AKT signaling plays an important role in controlling FasR expression, we document a novel layer of regulation that links the PI3K-AKT pathway with Fas-mediated death in tumor cells, which represents an important mechanism in the control of tumor development and progression.

EXPERIMENTAL PROCEDURES

Materials—PD98059, SB203580, AG490, and LY294002 were obtained from Calbiochem. Final concentrations used were 50 μM PD98059, 10 μM SB203580, 50 μM AG490, and 50 μM LY294002.

Cell Lines—Human melanoma cells were kindly provided by Drs. M. Herlyn and O. Fodstad and maintained in culture as previously described (45, 46). Embryonic stem cells E14 (*STAT3+/+*) and E14 clone 3–2 (*STAT3+/−*) were kindly provided by Dr. D. Levy of New York University; these cultures were propagated by standard methods on monolayers of mitomycin C-treated mouse fibroblasts (47). Normal mouse fibroblasts (c-*jun*+/+), (c-*jun*−/−) fibroblasts were a kind gift of Dr R. Wisdom; mouse melanoma cells were kindly provided by L. Owen-Schwab and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Plasmid Constructs—The Fas promoter reporter constructs (−1.7 kb Fas-Luc and −460 Fas-Luc) were previously described (48). Mutations within the GAS or AP1 site and deletion of the 24-bp sequence containing both AP1 and GAS elements were generated using the QuikChange kit (Stratagene) and were previously described (38). The expression plasmid pIRES-STAT3β, which encodes the human STAT3β (49), was kindly provided by Dr. R. Jove. WM9, WM793, and LU1205 cells stably transfected with STAT3β were previously described (38). The expression vector of permanently active AKT (HA-AKT-myr) was kindly provided by Dr. P. Tsichlis. The expression vectors of active form of PI3K (p110 subunits) was obtained from Dr. L. Williams (50). The dominant negative form of PI3K (p85Δ subunits) was previously described (51); PTEN expression vector (52) was kindly provided by Dr. A. Chan. WM9 cells were permanently transfected with p110, p85Δ, AKT-myr, or PTEN expression vectors. Transfected cultures were subjected to selection in G418 (200 μg/ml) or puromycin (2 μg/ml) as indicated under "Results," and a mixed population of resistant cells was analyzed.

Transient Transfection and Luciferase Assay—The luciferase reporter gene containing five TRE elements from the c-Jun promoter (5×Jun2-tk-Luc) or three GAS elements from the Ly6E gene (3×Ly6E-Luc) as target sequences for c-Jun and STAT3, respectively, were previously described (38, 53). Transient transfection of different reporter constructs (0.5 μg) together with expression vectors and pCMV-βgal (0.25 μg) into 5×10^5 melanoma cells was performed using LipofectAMINE (Invitrogen). Proteins were prepared for β-galactosidase and luciferase analysis 18 h after transfection. Luciferase activity was determined using the luciferase assay system (Promega) and normalized based on β-galactosidase levels.

Treatment and Apoptosis Studies—Cells were exposed to UVC at 60 J/m^2 as previously described (54). FasL (25–50 ng/ml) was used in combination with cycloheximide (10 μg/ml). Apoptosis was assessed by quantifying the percentage of hypodiploid nuclei undergoing DNA fragmentation (55). Surface expression of Fas was determined using anti-Fas-PE Ab (Pharmingen). Flow cytometric analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson) using the CellQuest program.

Transient Transfection and GFP Assay—Melanoma cells (5×10^5) were transiently co-transfected with expression vectors together with marker plasmid encoding green fluorescent protein (pGFP; 1 and 0.25 μg, respectively) using LipofectAMINE (Invitrogen). 24 h after transfection, surface Fas expression in GFP-positive cells was determined by staining with PE-anti-Fas Ab and flow cytometry. For apoptosis studies, cells were irradiated with UVC (60 J/m^2) 24 h after transfection and 18 h later were stained with propidium iodide and analyzed by flow cytometry.

Western Blot Analysis—Cell lysates (50–100 μg of protein) were resolved on 10% SDS-PAGE and processed according to the standard protocols. The Abs used were anti-phospho-AKT (Ser^{473}), anti-phospho-JNK (Thr^{183}/Tyr^{185}), anti-phospho-c-Jun (Ser^{73}), anti-phospho-STAT3 (Tyr^{705}), and anti-phospho-p38 (Thr^{180}/Tyr^{182}), and corresponding control Abs were obtained from Cell Signaling and used at dilutions of 1:1000 to 1:3000. The secondary Abs (anti-rabbit or anti-mouse) conjugated to horseradish peroxidase (dilution 1:5000). Signals were detected using the ECL system (Amersham Pharmacia Biotech).

Oligonucleotides and Protein Binding Assay—Double-stranded biotinylated oligonucleotides used in this study were derived in wild type and mutant configurations from the Fas promoter sequences: WT (Biotin-AAT GCC CAT TTG TGC AAC GAA CCC TGA CTC CTT CCT), mutated GAS, and AP1 sites (Biotin-AAT GCC CAT TTG TGC TTC GAA CCC AAA CTC CTT CCT). Nuclear proteins were prepared, as previously described (56). Nuclear proteins (400 μg) were incubated with biotinylated oligonucleotides in solution followed by coupling of oligonucleotides and their bound proteins to Streptavidine-agarose (Stratagene); bead-bound material was subjected to extensive washes before specifically associated proteins were eluted in SDS-loading buffer and separated on SDS-PAGE followed by Western blotting with Abs against phospho-STAT3 (Tyr^{705}), phospho-c-Jun (Ser^{73}), and corresponding control Abs.

RESULTS

The PI3K/AKT Pathway Positively Regulates FasR Expression via Suppression of STAT3 and c-Jun—Cooperation between STAT3 and c-Jun suppresses Fas receptor (FasR) transcription and subsequent FasR expression on the cell surface, as often seen in advanced tumors. To elucidate mechanisms underlying the cooperation between two ubiquitously expressed transcription factors that results in silencing FasR transcription and expression, we explored the possible role of protein kinases, which affect both STAT3 and c-Jun. To this end, we monitored the effect of pharmacological inhibitors of the PI3K-AKT pathway (LY294002) and the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (PD98059) as well as of mitogen-activated protein kinase p38/JNK (SB203580) and JAK (AG490) on cell surface expression of Fas receptor (FasR) (Fig. 1A). Of the inhibitors used, only LY294002 resulted in a marked decrease (60%) in surface FasR expression in WM9 and WM793 cells and to a lesser extent (20%) in LU1205 cells. These results imply that the PI3K/AKT signaling cascade positively regulates expression of FasR and that inhibition of this pathway results in decreased FasR expression on the cell surface. Additionally, a notable decrease of surface Fas expression after LY294002 treatment was observed for the early melanoma cell lines WM1650 and WM35 (not shown). It is of interest that LU1205 cells also exhibited increases in FasR levels in response to inhibition of p38/JNK, suggesting that p38/JNK negatively regulates FasR expression in these cells. Western blot analysis confirmed down-regulation of total Fas levels in these cell lines (not shown). The three melanomas selected for the initial analysis included early phase melanoma cells WM9 and WM793 and the late phase melanoma LU1205 cells. Of these cell lines, LU1205 was found to express a truncated form of the PTEN protein indicative of mutated PTEN (data not shown). Mutated PTEN is expected to result in constitutively active PI3K/AKT signaling, which would explain the limited inhibition observed in the LU1205 cells after LY294002 treatment.

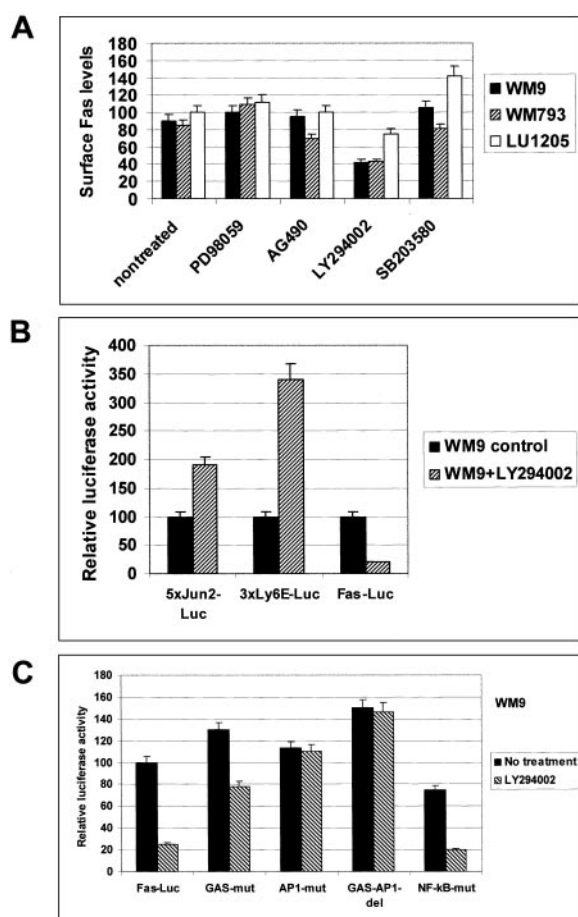To further explore the role of PI3K/AKT in regulating FasR

FIG. 1. **The PI3K inhibitor LY294002 down-regulates surface Fas expression and Fas promoter activities in human melanoma cell lines.** *A*, effect of inhibition of distinct signaling pathways by PD98059 (50 μM), AG490 (50 μM), LY294002 (50 μM), and SB203580 (5 μM) on Fas surface expression in the indicated melanoma cell cites. Cell surface expression was determined by flow cytometric analysis (monitored via MFI). *B*, LU1205 and WM9 melanoma cells were transfected with 5×Jun2-Luc (a reporter construct specific for AP-1-dependent transcription), 3×Ly6E-Luc (a reporter specific for STAT-dependent transcription), and −460 Fas-Luc in the presence of pCMV-β-gal. Cells were untreated (control) or treated with LY294002 (50 μM) for an additional 6 h. The data shown reflect changes over the control nontreated cultures. In all cases, the normalized ratio of luciferase activity to β-galactosidase is shown. *C*, WM9 cells were transiently transfected with −460 Fas-Luc and mutated variants of this construct in the presence of pCMV-β-gal. LY294002 (50 μM) was added to the cultures 16 h after transfection for an additional 6 h before cells were harvested and proteins were analyzed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown. *D*, analysis of STAT3 and c-Jun expression and phosphorylation in WM9 and WM793 melanoma cells. Proteins were prepared from the melanoma cells under mock treatment or 2 h after exposure to LY29004 and subjected to analysis with the corresponding antibodies as indicated. The *arrows* point to the positions of the respective proteins. *E*, cell surface Fas expression of the indicated melanoma cells (WM9, LU1205, and WM793 cells, and derivatives of these cell lines that had been stably transfected with STAT3β) was determined by staining with an anti-Fas-PE mAb followed by flow cytometric analysis. *Filled histograms* represent nonspecific (*ns*) staining with mouse Ig-PE. *Empty histograms* represent control and LY294002-treated cells, as indicated. The *numbers* reflect MFI values. *F*, STAT3 tyrosine phosphorylation in WM9 cells. Analysis of tyrosine phosphorylation was carried out as indicated in *D*, using proteins prepared from WM9 control or cells that constitutively express STAT3β. The *arrows* point to the positions of the STAT3α and -β forms. The *image* was enlarged to enable better separation of the α and β forms.

expression, we monitored changes in the STAT- and AP1/Jun-dependent transcriptional activities. Using the luciferase reporter gene that is under the control of AP1/c-Jun (5×Jun2-tk-

Luc) or STAT (3×Ly6E-Luc) target sequences, we observed an increase (2–3-fold) in the level of STAT and Jun-dependent transcriptional activities in both WM9 and LU1205 cells after LY294002 treatment. Increased Jun and STAT transcriptional activities coincided with a 50–80% decrease in FasR-Luc activities (Fig. 1*B*). These findings suggest that PI3K elicits negative regulatory effects on both STAT and c-Jun transcription factors, as inhibition of this signaling cascade results in increased transcriptional activities of both transcription factors and concomitant suppression of the FasR promoter.

Using WT and mutated forms of the FasR promoter, we further monitored the effect of PI3K/AKT signaling on FasR promoter activity. FasR promoter mutated on the GAS element (which serves as the target sequence for STAT proteins) exhibited a 40% inhibition in FasR-Luc activity compared with the 80% inhibition seen with the WT promoter following LY294002 treatment (Fig. 1*C*). This finding suggests that the GAS element contributes to PI3K-mediated derepression of FasR transcription. Mutation within the AP1 site completely abolished the ability of LY294002 to suppress FasR transcription, suggesting that AP1 is essential for PI3K/AKT-mediated derepression of FasR promoter activity. Similarly, deletion of the AP1 and GAS elements from the FasR promoter sequences attenuated LY294002's ability to suppress FasR transcription, resulting in increased FasR transcription in the WM9 melanoma cells. Unlike AP1 and GAS elements, the NF-κB site did not appear to play a role in the regulation of FasR promoter by AKT/PI3K, since mutations of NF-κB binding site did not affect LY294002's ability to suppress FasR promoter activities (Fig. 1*C*). Together, this analysis reveals that PI3K/AKT utilizes transcription factor binding to the AP1 and GAS motives on the FasR promoter to mediate derepression of the promoter activities in these melanoma cells.

Analysis of STAT3 and c-Jun phosphorylation revealed a noticeable increase in the serine phosphorylation of both c-Jun and STAT3 in cells that were treated with LY294002 (Fig. 1*D*). Since an increase in serine phosphorylation of both transcription factors is a prerequisite for their transcriptional activities, this observation reveals how silencing of FasR is alleviated in the presence of the pharmacological inhibitor of PI3K. It is important to note that tyrosine phosphorylation of STAT3 was altered in one of the two melanomas tested here, suggesting that it may not reveal the primary mechanism for the changes seen upon LY29004 treatment (Fig. 1*D*). As indicated in former studies, it is sufficient to alter one of the two transcription factors in order to positively affect FasR transcription (38).

Along these lines, in earlier studies, we demonstrated the ability to increase FasR surface expression upon constitutive expression of dominant negative STAT3 or c-Jun (38). Treatment of melanoma cells that constitutively express STAT3β, the dominant negative form of STAT3, which efficiently increased FasR expression, with the pharmacological inhibitor of PI3K, efficiently suppressed FasR surface expression in all three melanoma lines (Fig. 1*E*). This finding provides additional support for the role of STAT3 as a target for PI3K regulation of FasR expression.

To further assess the effect of LY29004 on STAT3β, we have monitored possible changes in the tyrosine phosphorylation of STAT3β under these experimental conditions. LY29004 caused an additional increase in the levels of STAT3 (α and β forms) without affecting the levels of STAT3 tyrosine phosphorylation in control WM9-neo cells (*lanes 1–3* in Fig. 1*F*). Time-dependent increase in STAT3 forms without a corresponding increase in their tyrosine phosphorylation resulted in decreased FasR expression (Fig. 1*E*). Forced expression of STAT3β was accompanied by tyrosine phosphorylation of STAT3α and -β forms
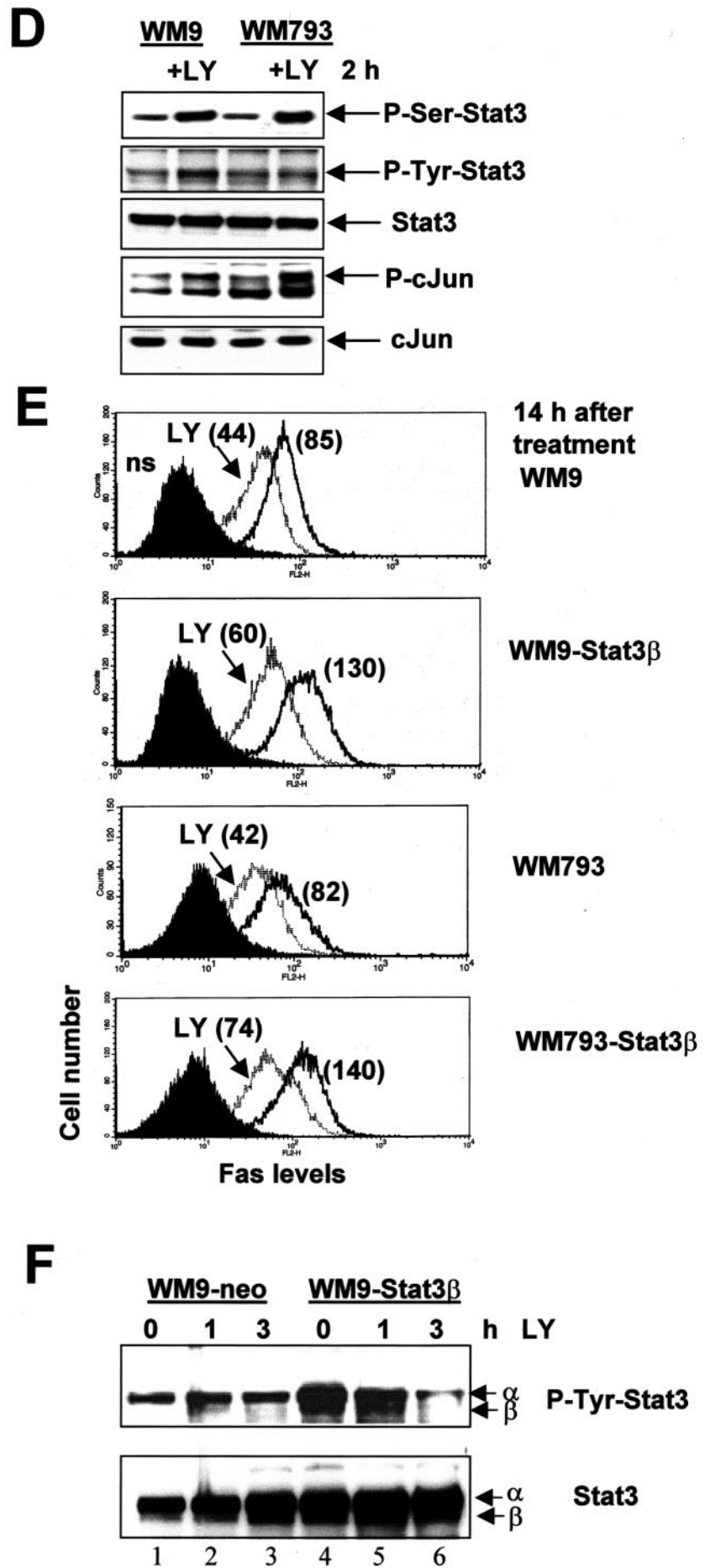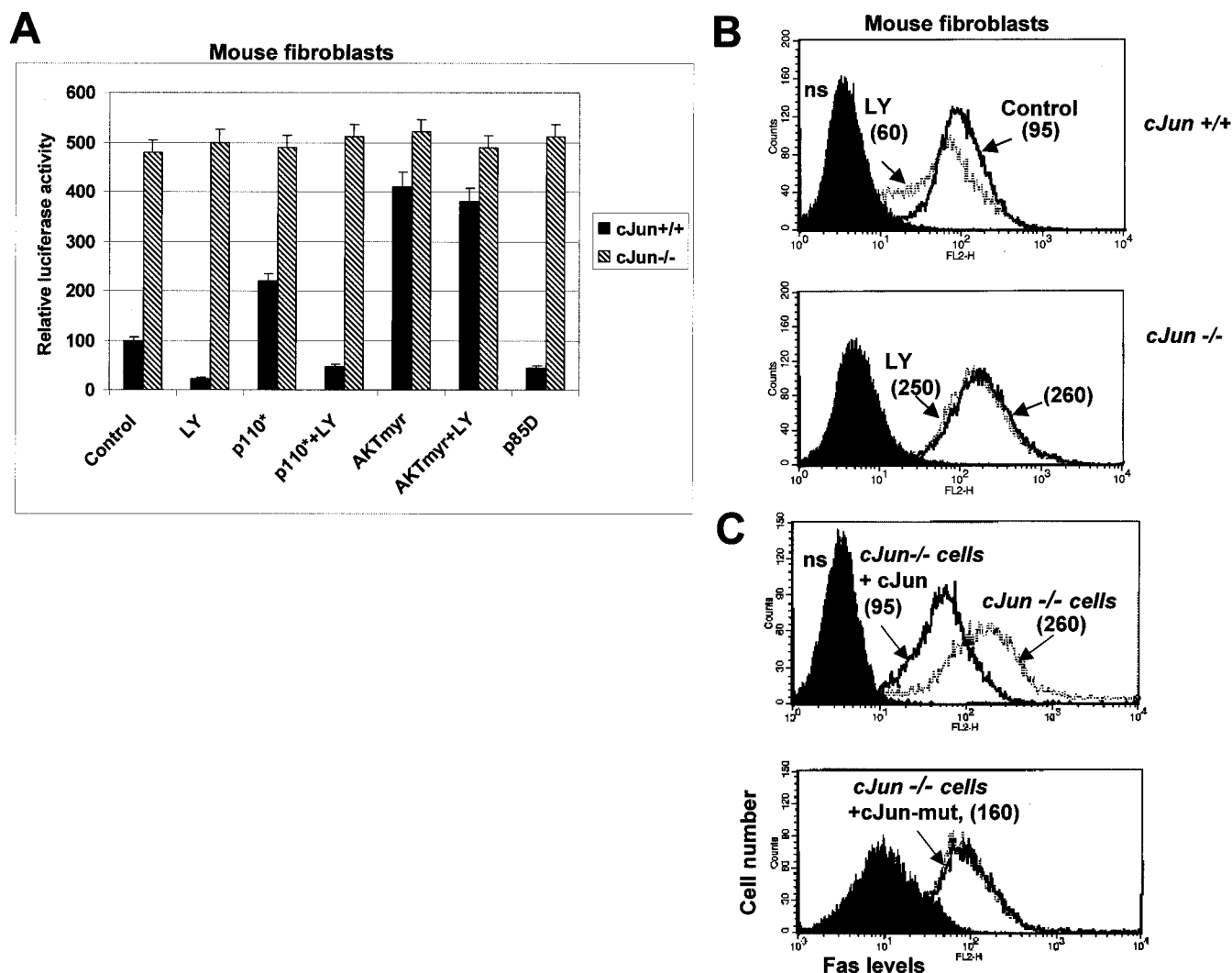
FIG. 1—*continued*

FIG. 2. **Effect of LY294002 on Fas expression requires c-Jun.** *A*, PI3K (p110*) or AKT^{myr} expression up-regulates Fas promoter activity in normal mouse *(cJun$^{+/+}$)* fibroblasts. Mouse fibroblasts *(cJun$^{+/+}$ or cJun$^{-/-}$)* were transiently cotransfected with −460 Fas-Luc and expression vectors encoding active forms of PI3K (p110*), AKT^{myr}, or the dominant negative form of PI3K (p85Δ). 16 h after transfection, LY294002 (50 μM) was added to the cultures for an additional 6 h. Then cells were analyzed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown. *B*, LY294002 failed to down-regulate surface Fas expression in c-*jun*$^{-/-}$ mouse fibroblasts but down-regulated Fas levels in normal c-*jun*$^{+/+}$ fibroblasts. Cell surface Fas expression was determined by staining with an anti-Fas-PE mAb followed by flow cytometric analysis. The *numbers* reflect MFI values. *C*, c-*jun*$^{-/-}$ fibroblasts were transfected with empty vector (pBabe-puro), c-Jun or mutated c-Jun-(63,73A) expression vectors. Puromycin-resistant cells were selected. Surface Fas expression was determined by staining with an anti-Fas-PE mAb and flow cytometric analysis. MFI levels are indicated. *ns*, nonspecific band.

(Fig. 1*F*, *lane 4*) and increased FasR expression (Fig. 1*E*), probably due to squelching of STAT3 by STAT3β, thereby decreasing cooperation with c-Jun, which is required for silencing of FasR transcription. Treatment of STAT3β-transfected cells with LY29004 decreased tyrosine phosphorylation of both forms of STAT3, despite an increase in their expression (Fig. 1*F*, *lanes 5* and *6*), and resulted in decreased FasR expression (Fig. 1*E*), similar to what was observed in the WM9 cells that were subjected to LY29004 treatment without STAT3β expression (Fig. 1*F*, *lanes 2* and *3*, and Fig. 1*E*). These data suggest that tyrosine phosphorylation of STAT3β is required to enable its inhibitory effect on STAT3 and its ability to relieve FasR suppression.

c-Jun Is Essential for PI3K/AKT Regulation of the FasR Promoter—The finding that the AP1-mutated form of the FasR promoter did not respond to PI3K inhibitor led us to directly explore the role of c-Jun in PI3K/AKT regulation of the FasR promoter. For this purpose, we used *c-Jun* null cells (57). Whereas LY294002 elicited suppression of the FasR promoter in normal mouse fibroblasts, forced expression of a constitu-

tively active form of PI3K (p110*) or AKT (AKT^{myr}) results in efficient increase in FasR-Luc activities, while p85Δ (dominant negative form of PI3K) negatively affected this activity, further pointing to the positive role of PI3K/AKT in the regulation of the FasR promoter. However, neither LY294002, p110*, AKT, or p85Δ were able to alter the high basal levels of the FasR promoter activities and surface FasR expression seen in the c-*jun*$^{-/-}$ fibroblasts (Fig. 2, *A* and *B*). These findings suggest that c-Jun is indeed a key element in PI3K's ability to regulate FasR transcription.

To determine whether c-Jun must be phosphorylated in order to affect FasR expression, we transfected the c-*jun*$^{-/-}$ cells with either WT or phosphoacceptor mutant forms of c-Jun. The WT form of c-Jun efficiently reduced the level of FasR expression to a mean fluorescence intensity (MFI) of 95 (Fig. 2*C*). Since transfection of the empty vector was sufficient to decrease basal FasR expression to 160 MFI, the relative decrease that should be noted upon c-Jun expression is from 160 to 95 MFI. In contrast to the effect of WT c-Jun, c-Jun mutated on its phosphoacceptor sites did not have any effect on FasR expres-
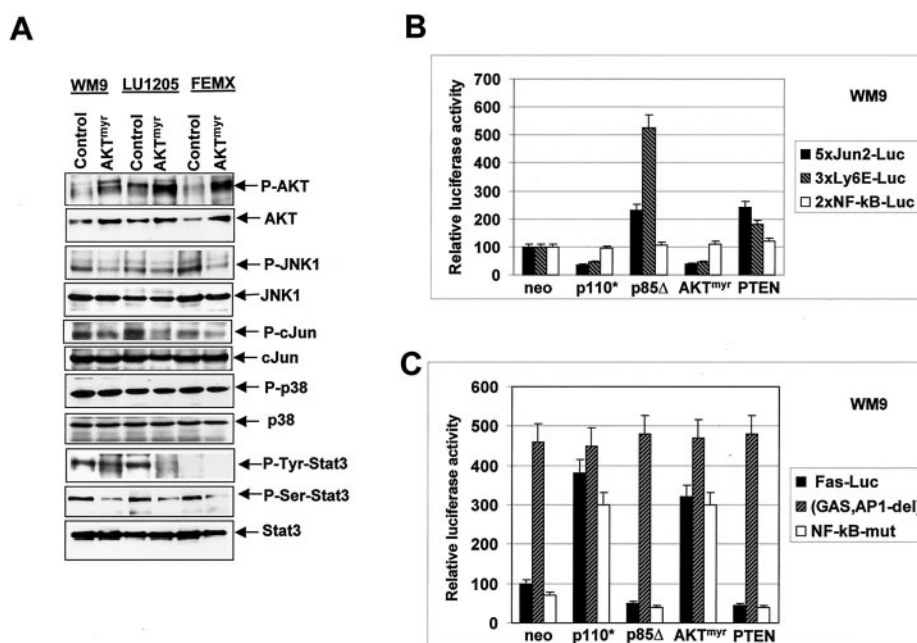
FIG. 3. **Melanoma cell lines stably transfected with the active form of AKT (AKT^myr) exhibit suppression of JNK activity, c-Jun phosphorylation, and an increase in Fas surface expression.** *A*, Western blot analysis of the indicated melanoma cell lines transfected with an empty vector or AKT^myr. Anti-P-AKT, control anti-AKT, anti-P-c-Jun, anti-c-Jun, anti-P-p38, anti-p38, anti-Tyr(P)-STAT3, and anti-STAT3 antibodies were used. *B* and *C*, WM9 cell lines stably expressing PI3K p110*, AKT^myr, p85Δ, and PTEN were established; control WM9-neo cells were stably transfected with empty vector pcDNA3. The indicated cell lines were additionally cotransfected with −460 Fas-Luc and its mutated variants (*C*) or with 5×Jun2-Luc, 3×Ly6E-Luc, or 2×NF-κB-Luc constructs (*B*) in the presence of pCMV-β-gal. 16 h after transfection, cells were analyzed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown. *D*, surface Fas expression of the indicated melanoma cell lines. Control Fas levels of WM9-neo cells (110 MFI) are indicated by the *open black histogram*, whereas changes in Fas surface levels upon expression of the p110*, AKT^myr, p85Δ, or PTEN are depicted as *gray open histograms*, with the respective MFI values indicated. *E*, binding of STAT3 and c-Jun to AP1-GAS sites decreases in cells that express active AKT. Biotinylated oligonucleotides containing GAS-AP1 element sites as present on the *fas* promoter (*fas* oligo) in WT or double mutants (as indicated) were incubated with nuclear proteins of WM9 cells or of WM9-AKT^myr cells. Biotinylated oligonucleotide-bound proteins were captured on avidin-agarose beads and washed, and GAS-AP1-bound proteins were eluted and analyzed on Western blotting with the control or phosphospecific Abs to c-Jun and STAT3. The relative positions of the corresponding proteins are shown. *ns*, nonspecific band.

sion, since the level of MFI was equal to those observed upon expression of the empty vector (Fig. 2*C*). These findings suggest that c-Jun must be phosphorylated on Ser[63]/Ser[73] to be able to reduce the FasR expression level.

AKT Mediates c-Jun Suppression via Down-regulation of JNK Activity and c-Jun Phosphorylation—The finding that c-Jun must be phosphorylated on Ser[63]/Ser[73] if it is to possess the capability of altering FasR transcription pointed to a plausible connection between PI3K and JNK, which is the primary c-Jun kinase in PI3K-dependent transcription of the FasR promoter. To determine whether AKT alters JNK/Jun phosphorylation, we established three melanoma cultures that stably express AKT^myr. Analysis of protein extracts to determine the effect of AKT^myr revealed a noticeable increase in expression and phosphorylation levels of AKT in each of the three melanoma lines (Fig. 3*A*). Increased AKT activity coincided with a decrease in the high basal levels of JNK, but not p38, phosphorylation (commonly seen in these melanomas) and c-Jun, as well as STAT3 serine phosphorylation (Fig. 3*A*). These findings suggest that AKT down-regulates JNK activity and consequently c-Jun and STAT3 phosphorylation. AKT-expressing melanoma cells also exhibited a marked increase in FasR transcription (Fig. 3*C*). Like AKT^myr, p110* also increased Fas-Luc activity. In contrast, p85 in its dominant negative form (p85Δ) and PTEN led to a marked decrease in Fas-Luc activities (Fig. 3*C*). It is important to note that forced expression of either AKT^myr or p110* elevated the transcriptional activities of the FasR promoter mutated on the NF-κB site (Fig. 3*C*), suggesting that the primary regulators of the FasR promoter by AKT are c-Jun and STAT3. Indeed, level of c-Jun and STAT3 transcriptional activities were inversely correlated with those observed

for the FasR promoter activity. AKT^myr and p110* expression led to a decrease in c-Jun and STAT transcriptional activities monitored via the 5×Jun2-Luc and GAS-Luc constructs, respectively. In contrast, their transcriptional activities increased in response to PTEN and even more markedly following expression of the dominant negative form of p85 (Fig. 3*B*). These data suggest that AKT's ability to increase FasR transcription depends on down-regulation of JNK and consequently c-Jun activities. Along with the changes in Jun and STAT transcription were changes in the level of FasR surface expression. Forced expression of either AKT^myr or p110* increased FasR cell surface expression (Fig. 3*D*). Conversely, forced expression of a dominant negative form of p85 as well as PTEN caused a marked decrease in the expression of cell surface FasR (Fig. 3*D*). In all cases, changes in the cell surface expression of FasR coincided with altered FasR promoter activity.

Changes in STAT3 and c-Jun phosphorylation and transcriptional activities following AKT^myr treatment was also monitored at the level of FasR promoter-bound proteins. With the aid of biotinylated oligonucleotides containing the GAS-AP1 element, as found on the FasR promoter (WT or mutated), we monitored changes in binding of STAT3 and c-Jun. An association of phosphorylated c-Jun and STAT3 to the FasR promoter-driven oligonucleotide was observed in normally growing WM9 cells. In cells that express AKT^myr, there was a noticeable decrease in binding of phosphorylated as well as the nonphosphorylated forms of both transcription factors (Fig. 3*E*). This observation suggests that inhibition of STAT3 and c-Jun phosphorylation by AKT affects their association on the FasR promoter, which is required to elicit FasR transcriptional silencing. Overall, these findings establish a link between PI3K/AKT
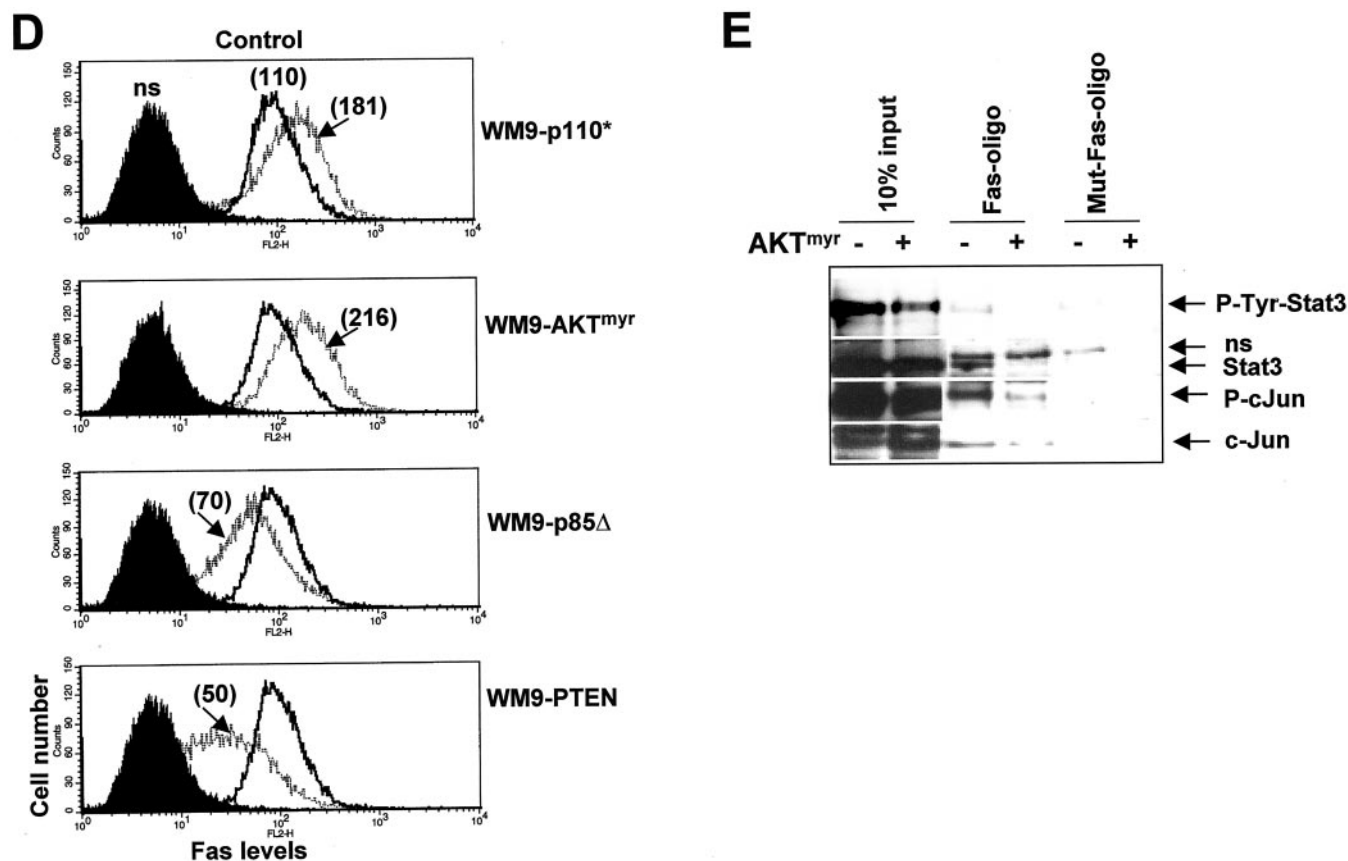
FIG. 3—*continued*

and JNK/Jun in the negative regulation of FasR promoter activities and, consequently, the FasR cell surface expression levels.

AKT's ability to alter FasR cell surface expression is independent of caspase activities, since pretreatment of WM9 melanoma cells with zVAD-fmk (a universal caspase inhibitor) before the addition of LY294002 did not attenuate LY294002-mediated down-regulation of Fas surface levels. Similarly, treatment of the WM9 melanoma cells with rapamycin to suppress mTOR, a rapamycin-sensitive protein kinase that is regulated by the PI3K-AKT pathway and which participates in the general control of translation in the cell, did not alter PI3K effects on Fas surface expression (data not shown), further indicating that the PI3K-AKT pathway regulates mainly the FasR transcriptional activity.

TNFα Increases FasR Expression via AKT Activation—Characteristic of late phase melanoma cells is their constitutive production of cytokines, enabling their autonomous growth. We tested a classic cytokine, TNFα, with regard to its effect on FasR surface expression. Treatment of late stage melanoma LU1205 cells with TNFα resulted in an increase in the phosphorylated form of AKT (Fig. 4A). TNFα also caused a marked increase in the level of FasR cell surface expression that could be effectively attenuated upon treatment with the pharmacological inhibitor of AKT, LY294002 (Fig. 4B). Given the effects of TNFα on NF-κB activities, we also monitored changes in FasR expression upon expression of IκBΔN, an IκB form that lacks the amino-terminal domain required for its phosphorylation targeted degradation, thereby rendering IκB highly stable, which consequently inhibits NF-κB transcriptional activities. Forced expression of IκBΔN slightly decreased the level of FasR expression and partially blocked the ability of TNFα to increase FasR expression. However, despite expression of IκBΔN, TNFα-mediated increase of FasR surface expression

was still attenuated by treatment with LY294002 (Fig. 4C). These data suggest that relevant cytokines are capable of elevating FasR expression in an AKT-dependent and NF-κB-independent manner. These findings point to the physiological relevance of AKT-mediated changes in FasR transcription and cell surface expression.

We next monitored possible changes in apoptosis of LU1205 cells before and after pretreatment with TNFα. Upon exposure to FasL and cycloheximide (CHX), there was a marked increase in apoptosis levels from 1% to 58% (Fig. 4D). Pretreatment with TNFα (10 ng/ml), which induces AKT activity, combined with subsequent exposure to FasL and CHX further increased level of apoptosis to 70%; pretreatment with TNFα also elevated the level of CHX-induced apoptosis from 4 to 15%. Increased Fas surface expression upon TNFα treatment coincides, albeit not linearly, with actual apoptosis levels.

AKT-mediated Increase in FasR Expression Sensitizes Selective Melanomas to FasL-mediated Death—An increase in FasR surface expression results in sensitization of the melanoma cells to apoptosis by the FasL-Fas death signaling cascade (38). In light of the observation that AKT mediates an important regulatory role in FasR transcription and expression, we have elucidated the changes in apoptosis elicited by AKT following different external stimuli. The mouse melanoma cell lines K1735 and SW1 provide a convenient system to monitor changes in FasR expression and their effects on cell death (33, 38). Expression of STAT3 or Jun in their dominant negative forms efficiently restored FasR expression and sensitization of these cells to FasL-mediated death. On the basis of the identification of AKT as a key component in the regulation of FasR expression, we established puromycin-resistant clones that stably express AKT^myr (Fig. 5A). K1735 cells that express AKT^myr exhibit reduced activities of c-Jun and, to a lesser degree, of STAT3 transcriptional activities, which coincided with in-
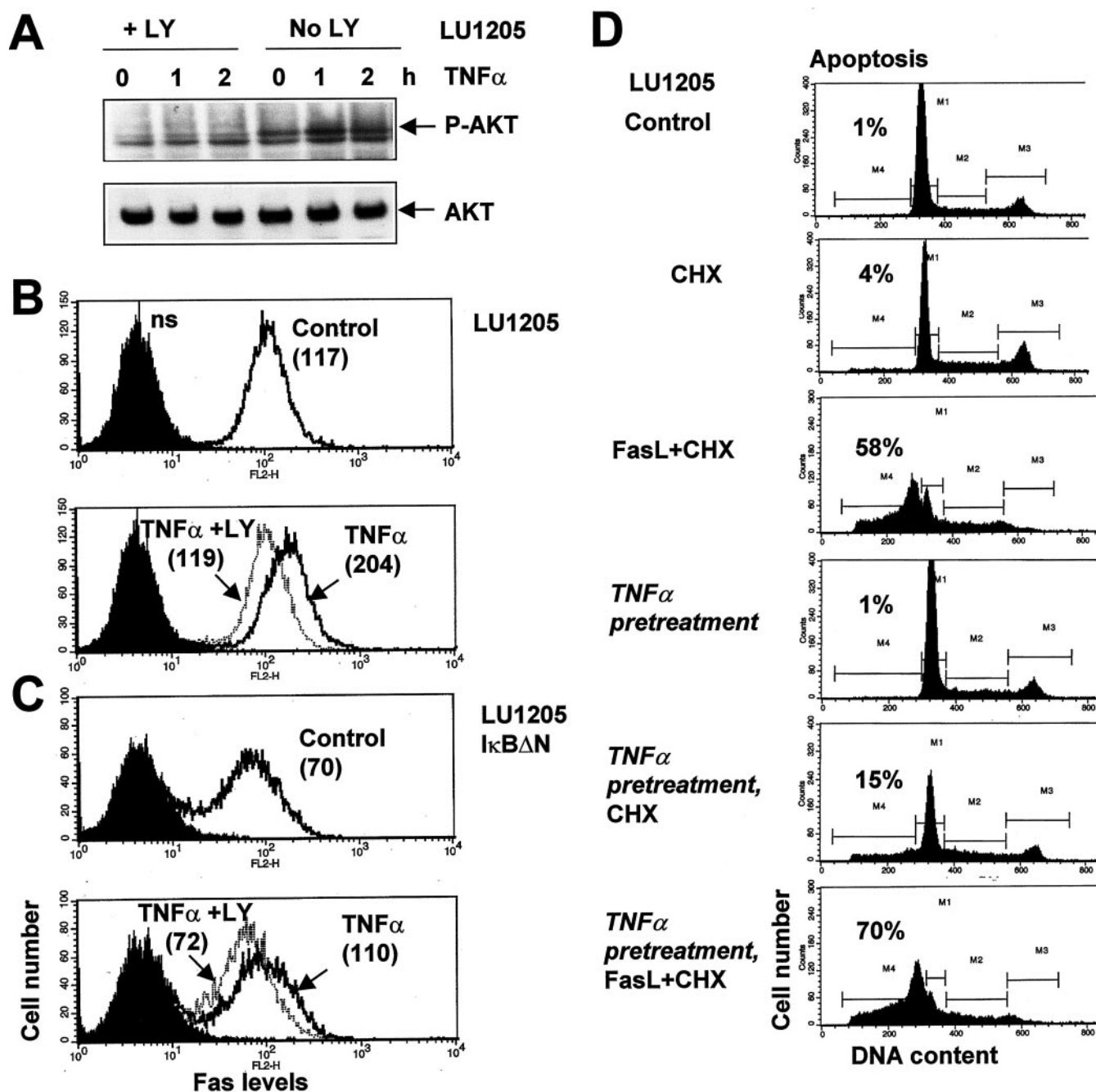
FIG. 4. **TNFα induces endogenous AKT activity and up-regulates Fas surface expression in LU1205 melanoma cells.** *A*, Western blot analysis of AKT activity following TNFα (10 ng/ml) treatment in the presence (added to cultures 1 h before TNFα) or absence of LY204002 (50 μM). *B* and *C*, Fas surface expression of LU1205 and LU1205-IκBΔN cells was determined before or 20 h after TNFα (10 ng/ml) treatment in the presence or absence of LY294002 (50 μM). MFI is indicated. *D*, apoptosis levels in LU1205 cells that were pretreated with TNFα (10 ng/ml) for 18 h and subsequently treated with FasL (50 ng/ml) and CHX (10 ng/ml).

creased FasR transcription (Fig. 5*B*). K1735 cells that express AKT^myr exhibited an over 4-fold increase in the expression of FasR on the cell surface (Fig. 5*C*). This finding coincides with the observations made in the human melanoma cells, where AKT^myr reduced Jun and STAT3 activities and elevated FasR transcription and Fas cell surface expression. Increased FasR expression sensitized these cells to FasL and CHX (from 14 to 33% apoptosis) but not to thapsigargin, an inhibitor of endoplastic reticulum-dependent Ca^{2+}-ATP required for apoptosis (58) (Fig. 5*D*). Similar data were obtained with the SW1 cells, where AKT expression resulted in elevated FasR expression and sensitization to FasL-mediated but not thapsigargin-mediated death (not shown). These observations suggest that increased FasR expression due to AKT activities is sufficient to sensitize selective melanoma cell lines to FasL-induced apoptosis.

Unlike the responses seen in the K1735 or SW1 mouse melanoma cells, AKT-increased FasR surface expression elicited protection from apoptosis in response to FasL, UV, thapsigargin, or the pharmacological inhibitors of PI3K or mitogen-activated protein kinase in both WM9 (Fig. 5*E*) and LU1205 cells (not shown). These data suggest that despite changes in FasR surface expression, AKT is capable of attenuating FasL-mediated apoptosis in a selective set of melanoma cell lines. It is likely that attenuated Fas-mediated apoptosis could be attributed to the effects of AKT signaling on the processing of
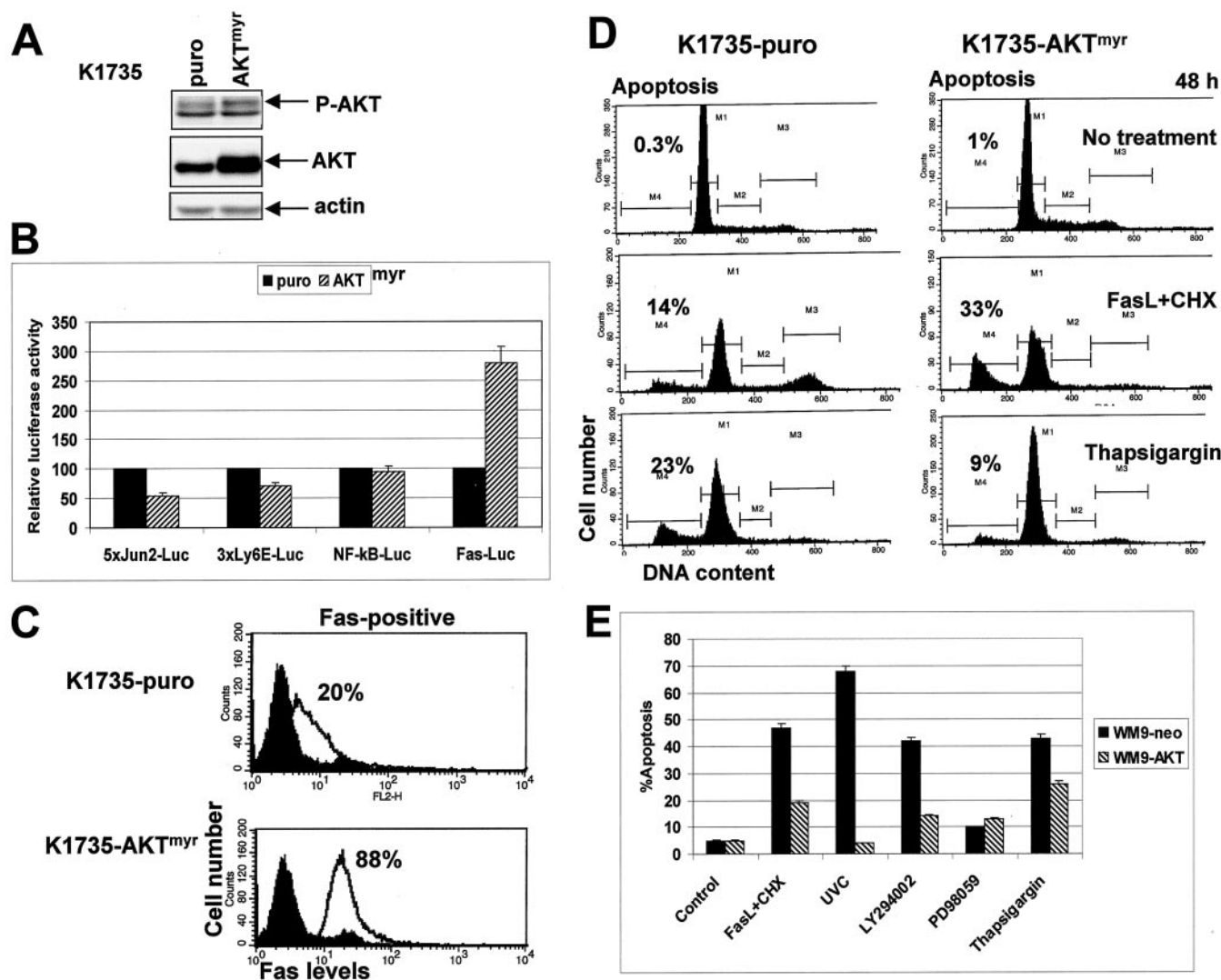
FIG. 5. **AKT overexpression up-regulates Fas levels and affects FasL-mediated apoptosis in melanoma cell lines.** *A*, Western blot analysis of K1735 melanoma cell lines stably transfected with empty vector (pBabe-puro) or AKT[myr]. Anti-P-AKT, control anti-AKT, and anti-actin Abs were used. *B*, effects of AKT[myr] overexpression on AP1-dependent, STAT-dependent, or NF-κB-dependent reporter activity and on Fas promoter activity. The normalized ratio of luciferase/β-galactosidase activity is shown. *C*, Fas cell surface expression in control and AKT[myr]-transfected melanoma cell lines was determined by fluorescence-activated cell sorting analysis. The percentage of Fas-positive cells is indicated. *D*, induction of apoptosis in K1735-puro and K1535-AKT[myr] cell lines following exposure to the indicated treatments. Analysis was carried out 48 h after treatment. *E*, protection from FasL-mediated apoptosis in WM9 cells that express a constitutively active form of AKT. Effects of UVC irradiation (60 J/m^2) FasL (50 ng/ml) and CHX (10 μg/ml) were determined 24 h after treatment. The effects of LY294002 (50 μM), PD98052 (50 μM), and thapsigargin (100 nM) on apoptosis induction were determined 48 h after treatment of WM9 cells that express the constitutively active form of AKT. *ns*, nonspecific band.

caspases and/or other regulators of apoptosis (59).

Inhibition of Rac1 Increases FasR Expression—Among the primary candidates for mediating AKT effects on JNK and Jun as well as on STAT3 is Rac1. Earlier studies established the ability of AKT to down-regulate the activity of Rac1, primarily through phosphorylation on Ser[71] (60). To directly assess the role of Rac1 as the critical link between AKT and FasR, we used the dominant negative form of Rac1, Rac1N17 (61). Forced expression of Rac1N17 in normal fibroblasts but not in *jun* null fibroblasts resulted in a marked increase in FasR surface expression (Fig. 6). Melanoma cells that express Rac1N17 also exhibited a marked increase in the expression of surface FasR, suggesting that Rac1 elicits negative regulation of FasR expression. Forced expression of Rac1N17 in ES cells also resulted in elevated FasR expression, which was less pronounced in the ES cells that lack one of the STAT3 alleles (and characterized by lower levels of STAT3 protein) (Fig. 6), indicating that Rac1 utilizes STAT3 to silence FasR expression. Both

STAT3- and c-Jun activities were demonstrated to be positively regulated by Rac1 (62, 63). This finding coincides closely with the notion that activity of Rac1 isoforms is often induced in late stage tumors, which are expected to exhibit loss of FasR expression. In all, these results suggest that Rac1 may serve as an intermediate in AKT-mediated suppression of FasR transcription.

DISCUSSION

Advanced tumors often exhibit reduced or complete loss of FasR expression, which coincides with greater resistance to apoptosis and is believed to enable tumors to escape immune surveillance (64). We previously identified cooperation between STAT3 and c-Jun as the mechanism that underlies the loss of FasR transcription and concomitant cell surface expression. Since both STAT3 and c-Jun are ubiquitously expressed, the present study was aimed at understanding the mechanism that enables their cooperation, since it appears to take place in
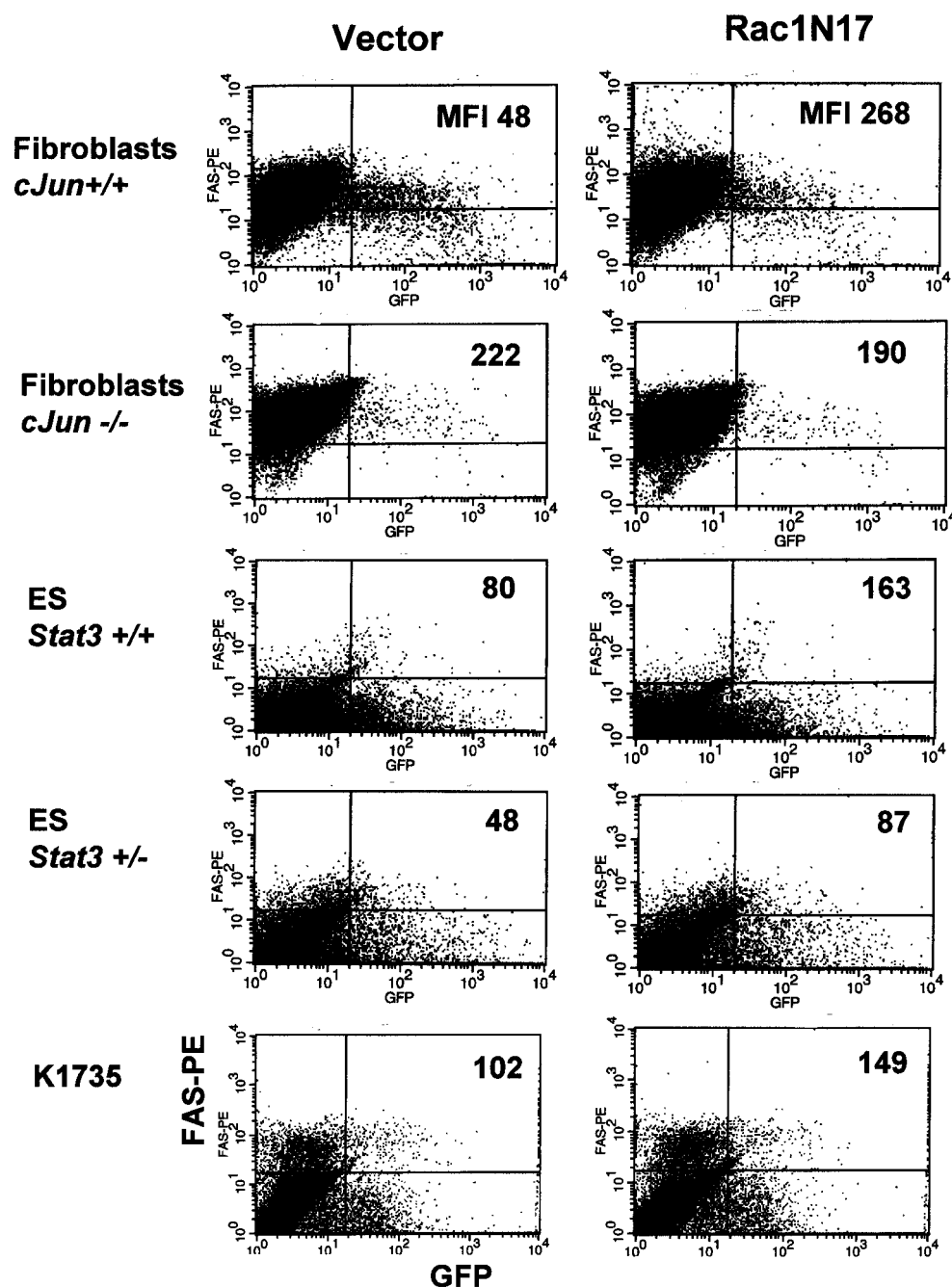
FIG. 6. **RAC1N17 increases FasR surface expression.** The indicated cell lines were transfected with the dominant negative form of Rac1, Rac1N17, and GFP. 24 h later, cells were subjected to fluorescence-activated cell sorting analysis to monitor FasR surface expression in GFP-positive cells of control (empty vector) or Rac1N17-transfected cells.

advanced tumors. Our studies, which relied on analysis of intermediate stage melanoma tumor-derived cell lines, led us to identify, albeit surprisingly, the role of the PI3K/AKT signaling pathway in the regulation of FasR expression. Indeed, tumor-derived cell lines including LU1205, WM9, and WM793 exhibit relative high levels of FasR and noticeable levels of AKT in its active form. Constitutively active forms of either AKT or p110, the catalytic subunit of PI3K, further increased FasR transcription, monitored via FasR-luciferase activity, with a concomitant increase in FasR expression on the cell surface. Inhibition of the PI3K/AKT pathway via either the dominant negative form of p85, the regulatory subunit of PI3K, or PTEN or via the pharmacological inhibitor LY294002 resulted in efficient suppression of FasR transcription and cell surface expression. These observations clearly demonstrate

the role of PI3K and AKT in the positive regulation of FasR transcription. Further support for the role of PI3K and AKT in FasR-mediated apoptosis was shown in several cell systems, although the prevailing mechanism was not clearly defined (65).

Further analysis revealed that AKT up-regulates FasR transcription via down-regulation of STAT3 and c-Jun transcriptional activities. First, the level of serine-phosphorylated forms of Jun and STAT3 decreases upon AKT treatment, which coincided with decreased binding of the phosphorylated Jun and STAT3 to the FasR promoter sequences *in vitro*. Noticeable changes in tyrosine phosphorylation of STAT3 were only found with the exogenously expressed form of STAT3β, suggesting that AKT may increase the activity of this dominant negative form of STAT3, thereby providing an additional mechanism to

explain inhibition of FasR transcription. Next, AP-1 and STAT-dependent transcription of the luciferase reporter gene decreased in this case, further pointing to the decrease in transcriptional activities of the two proteins. Changes in FasR promoter activities were primarily mediated by c-Jun and STAT3, since promoter sequences that were mutated in the AP1 site or from which AP1 and GAS elements had been deleted no longer responded to AKT signaling. The importance of c-Jun for AKT-mediated derepression of FasR expression is best illustrated in *c-Jun* null fibroblasts, where the constitutively high level of FasR expression was no longer affected by AKT. Further, a fully functional form of c-Jun was required to decrease FasR expression, since the phospho-mutant form failed to mediate these changes.

Taken together, these observations establish the important role of c-Jun and STAT3 phosphorylation by their upstream kinases for the regulation of FasR expression. JNK, which is the primary candidate for Jun phosphorylation, is down-regulated upon AKT expression. Along those lines, AKT was previously reported to elicit down-regulation of the stress kinase p38, highlighting another pathway that is subject to negative regulation by AKT (66). The link between AKT and JNK led us to explore the role of Rac1, which is the target for AKT phosphorylation on Ser[71], resulting in Rac1 suppression (60). Indeed, inhibition of Rac1 activities was as efficient in increasing FasR expression as constitutively active forms of AKT or p110. These observations led us to propose that AKT may mediate its effects on JNK via suppression of Rac1 activities. The ability of Rac1 to elicit activation of STAT3 and c-Jun via SEK/MKK4 was demonstrated (61, 62, 67–71), suggesting that suppression of Rac1 activities is expected to reduce STAT3 and c-Jun transcriptional activities.

Although Rac1 was shown capable of altering both Tyr and Ser phosphorylation of STAT3 (62), our experiments did not reveal that in the melanomas examined here there is an effect on basal levels of Tyr phosphorylation of STAT3; rather, there is a notable effect on the Ser phosphorylation of both STAT3 and c-Jun. This suggests that the primary mechanism by which AKT is capable of altering FasR transcription could be attributed to the serine phosphorylation of c-Jun and STAT3, both are required for their transcriptional activities. The latter is also supported by the notion that an inverse relationship between AKT and STAT3 phosphorylation was found in human cancer cells (72). Our findings are also in line with former studies, which demonstrated that the effect of PI3K may not be limited to Tyr phosphorylation of STAT3, since Ser phosphorylation (of STAT1) was also reported (73). Furthermore, Rac1-dependent Ser phosphorylation of STAT3 was also reported (67, 74). Finally, AKT does affect tyrosine phosphorylation of exogenously expressed STAT3β, thereby providing some additional mechanistic insight to the relief of FasR expression in these cells. The effect on STAT3β but not endogenous STAT3 Tyr phosphorylation could be attributed to the amount expressed, sensitivity of detection, or other possible causes, which will be the subject for further studies. It is important to emphasize that impaired STAT3 transcriptional activity may not always depend on the status of its Tyr phosphorylation (75).

The emerging model based on our studies suggests that PI3K/AKT signaling utilizes Rac1 to down-regulate JNK and consequently c-Jun as well as STAT3 transcriptional activities, which otherwise cooperate to mediate suppression of FasR transcription. As a result of AKT signaling and subsequent Rac1 suppression, the cooperation between STAT3 and c-Jun is impaired, thereby resulting in relief of FasR transcription and increased FasR cell surface expression. According to this model, the increase in AKT signaling, which takes place in

response to cytokines, UV (26), or altered PI3K signaling, will result in elevated FasR expression; it is also expected that elevated FasR will result in a concomitant sensitization of cells to FasL-mediated death. This was indeed found to be the case in some (K1735 and SW1) but not in other melanomas (WM9, LU1205, and FEMX). The differences among the two sets of melanomas studied here points to the possible existence of a switch in AKT effector(s) or interference by other signaling cascades, which alter the susceptibility to FasR-FasL-mediated apoptosis. Interference of AKT-mediated increase of FasR is expected to take place during tumor development and progression by any of the multiple signaling cascades that are linked to or interfere with AKT signaling. For example, oncogenic Ras was shown capable of inhibiting FasR expression via its effect on AKT/PI3K pathways (76, 77). Similarly, growth factors that are known activators of PI3K/AKT signaling, including hepatocyte growth factor and epidermal growth factor, were shown to be capable of down-regulating FasR-mediated death (78–80). Further, a key component in the regulation of PI3K-AKT is PTEN, which is either mutated or down-regulated in many tumor types including melanomas (81–84). The relationship between PTEN and Fas-dependent apoptosis is also illustrated by the finding that *PTEN*+/− mice exhibit impaired Fas responses, which could be restored upon the use of PI3K inhibitors (81).

Another plausible target for changes that are expected to override the AKT effect on FasR expression is c-*kit*; mutations of c-*kit* have been identified in a variety of malignancies including melanomas and were recently shown to result in constitutive activation of STAT3 (86). Thus, tumors that harbor mutant c-*kit* are also expected to exhibit down-regulation of FasR via activated STAT3, which could override the effect of AKT signaling. Finally, one must also consider other regulatory events, which take place between Fas transcription and cell surface expression (*i.e.* FasR trafficking). Among those, Par4, which is altered in human prostate tumors, was recently found to affect FasR trafficking (87).

Although we expect that K1735 and SW1, which exhibit sensitization to FasL-mediated cell death following AKT-dependent increase in FasR expression, represent a set of tumors, the protection by AKT seen in the other melanomas raises a question as to the possible physiological significance of elevated FasR expression. First, it is likely that activation of AKT by cytokines and DNA damage, as reported for epidermal growth factor receptor and UV irradiation, serves to increase FasR as a part of cell ability to undergo apoptosis, provided that other AKT targets that serve antiapoptotic functions are suppressed. Under constitutive expression of FasR, the scenarios that could be envisioned include the possibility that elevated FasR in parallel with protection from FasR-mediated death may serve to lure the immune system. Along these lines, constitutive expression of FasL in multiple myeloma cells was proposed as a potential mechanism of tumor-induced suppression of immune surveillance (88). Alternatively, it is possible that these changes serve other functions required for the aggressively progressing tumors, such as improved vascularization and consequent angiogenic potential. Indeed, elevated vascularization has been reported in tumors that overexpress AKT (89), and AKT-expressing SW1 tumors *in vivo* exhibit a marked increase in vascularization (data not shown). In contrast, inhibition of STAT3 or c-Jun, which abolish cooperation among the two protooncogenes to silence FasR, results in restored FasR expression and decreases tumor growth *in vivo*, due to an increased degree of apoptosis (not shown).

Elevated levels of Rac1 or its isoform expression, which is often reported to take place in human tumors, including human

melanomas, is expected to also alter the activities of Rac1 effectors, including JNK and STAT3 kinases, resulting in increased cooperation between STAT3 and c-Jun that inhibits the activities of the FasR promoter. Inasmuch, Rac1 may also serve as a focal point for the signaling cascade reported here. Further, Rac1 has been implicated as an important target of diverse signaling cascades including Rho, Ras, and c-*kit*, each of which has been shown to undergo modifications during the human tumor development and progression, including melanomas (90–93). Interestingly, the pathway identified in the present study may be part of a feedback regulatory loop, since STAT3 was reported capable of activating the PI3K cascade (94). Similarly, Rac2 was shown capable of stimulating AKT in primary mast cells (95), and the Rac1 ability to protect epithelial cells against ankiosis was shown to depend on its activation of PI3K/AKT (85). The existence of a positive feedback loop suggests that via STAT3 or other Rac effectors AKT activation may result in either inhibition of Rac1 (as one would expect to take place upon its phosphorylation on Ser^{71}) or constitutive activation, under which AKT-mediated suppression of STAT3 and c-Jun may no longer be seen. It is expected that part(s) of this feedback loop mechanism will be impaired in advanced tumors.

REFERENCES

1. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) *Biochem. J.* **335,** 1–13
2. Kandel, E. S., and Hay, N. (1999) *Exp. Cell Res.* **253,** 210–229
3. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes Dev.* **13,** 2905–2927
4. Pastorino, J. G., Tafani, M., and Farber, J. L. (1999) *J. Biol. Chem.* **274,** 19411–19416
5. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G., and Cohen, P. (1999) *J. Biol. Chem.* **274,** 17179–17183
6. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
7. Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* **276,** 18934–18940
8. Suhara, T., Mano, T., Oliveira, B. E., and Walsh, K. (2001) *Circ. Res.* **89,** 13–19
9. Plas, D. R., Talapatra, S., Edinger, A. L., Rathmell, J. C., and Thompson, C. B. (2001) *J. Biol. Chem.* **276,** 12041–12048
10. Bellacosa, A., Feo, D. D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Panici, P. B., Mancuso, S., Neri, G., and Testa, J. R. (1995) *Int. J. Cancer.* **64,** 280–285
11. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3636–3641
12. Thompson, F. H., Nelson, M. A., Trent, J. M., Guan, X. Y., Liu, Y., Yang, J. M., Emerson, J., Adair, L., Wymer, J., Balfour, C., Massey, K., Weinstein, R., Alberts, D. S., and Taetle, R. (1996) *Cancer Genet. Cytogenet.* **87,** 55–62
13. Staal, S. P. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5034–5037
14. Vunic, D., Stennicke, H. R., Pisabarro, M. T., Salvesen, G. S., and Dixit, V. M. (2000) *Curr. Biol.* **10,** 1359–1366
15. Grossman, D., Kim, P. J., Schechner, J. S., and Altieri, D. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 635–640
16. Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. (2000) *Lancet* **356,** 1728–1733
17. Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y. A., Cordon-Cardo, C., and Lowe, S. W. (2001) *Nature* **409,** 207–211
18. Daniel, P. T., Wieder, T., Sturm, I., and Schulze-Osthoff, K. (2001) *Leukemia* **15,** 1022–1032
19. Mouawad, R., Khayat, D., and Soubrane, C. (2000) *Melanoma Res.* **10,** 461–467
20. Nagata, S. (1999) *Annu. Rev. Genet.* **33,** 29–35
21. Krammer, P. (2000) *Nature* **407,** 789–795
22. Gulbins, E., Hermisson, M., Brenner, B., Grassme, H. U., Linderkamp, O., Dichgans, J., Weller, M., and Lang, F. (1998) *Pflugers Arch.* **435,** 546–554
23. Hehner, S. P., Hofmann, T. G., Ratter, F., Droge, W., and Schmitz, M. L. (1999) *Eur. J. Biochem.* **264,** 132–139
24. Simon, H. U., Yousefi, S., Dibbert, B., Hebestreit, H., Weber, M., Branch, D. R., Blaser, K., Levi-Schaffer, F., and Anderson, G. P. (1998) *Blood* **92,** 547–557
25. Holmstrom, T. H., and Eriksson, J. E. (2000) *Crit. Rev. Immunol.* **20,** 121–152
26. Wan, Y. S., Wang, Z. Q., Shao, Y., Voorhees, J. J., and Fisher, G. J. (2001) *Int. J. Oncol.* **18,** 461–466
27. Huang, C., Li, J., Ding, M., Leonard, S. S., Wang, L., Castranova, V., Vallyathan, V., and Shi, X. (2001) *J. Biol. Chem.* **276,** 40234–40240
28. Cataldi, A., Zauli, G., Di Pietro, R., Castorina, S., and Rana, R. (2001) *Cell. Signal.* **13,** 369–375
29. Krasilnikov, M., Adler, V., Fuchs, S. Y., Dong, Z., Haimovitz-Friedman, A., Herlyn, M., and Ronai, Z. (1999) *Mol. Carcinogen.* **24,** 64–69
30. Owen-Schaub, L. (2001) *Clin. Cancer Res.* **7,** 1108–1109
31. Decaudin, D., Beurdeley-Thomas, A., Nemati, F., Miccoli, L., Pouillart, P., Bourgeois, Y., Goncalves, R. B., Rouillard, D., and Poupon, M. F. (2001) *Exp. Cell. Res.* **268,** 162–168
32. Hug, H. (1997) *Biol. Chem.* **378,** 1405–1412
33. Owen-Schaub, L. B., van Golen, K., Hill, L., and Price, J. E. (1998) *J. Exp. Med.* **188,** 1717–1723
34. Shin, M., Park, W., Kim, S., Kim, H., Kang, S., Song, K., Park, J., Dong, S., Pi, J., and Oh, R. (1999) *Am. J. Pathol.* **154,** 1785–1791
35. Koomagi, R., and Volm, M. (1999) *Int. J. Cancer.* **84,** 239–243
36. Mottolese, M., Buglioni, S., Bracalenti, C., Cardarelli, M. A., Ciabocco, L., Giannarelli, D., Botti, C., Natali, P. G., Concetti, A., and Venanzi, F. M. (2000) *Int. J. Cancer.* **89,** 127–132
37. Mullauer, L., Gruber, P., Sebinger, D., Buch, J., Wohlfart, S., and Chott, A. (2001) *Mutat. Res.* **488,** 211–231
38. Ivanov, V. N., Bhoumik, a., Krasilnikov, M., Raz, R., Owen-Schaub, L. B., Levy, D., Horvath, C. M., and Ronai, Z. (2001) *Mol. Cell* **7,** 517–528
39. Zhou, H., Li, X. M., Meinkoth, J., and Pittman, R. N. (2000) *J. Cell Biol.* **151,** 483–494
40. Sheng, H., Shao, J., and DuBois, R. N. (2001) *J. Biol. Chem.* **276,** 14498–14504
41. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* **20,** 1626–1638
42. Miwa, W., Yasuda, J., Murakami, Y., Yashima, K., Sugano, K., Sekine, T., Kono, A., Egawa, S., Yamaguchi, K., Hayashizaki, Y., and Sekiya, T. (1996) *Biochem. Biophys. Res. Commun.* **225,** 968–974
43. Ruggeri, B. A., Huang, L., Wood, M., Cheng, J. Q., and Testa, J. R. (1998) *Mol. Carcinog.* **21,** 81–86
44. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999) *J. Biol. Chem.* **274,** 21528–21532
45. Ivanov, V. N., Kehrl, J. H., and Ronai, Z. (2000) *Oncogene* **19,** 933–942
46. Ivanov, V. N., and Ronai, Z. (2000) *Oncogene* **19,** 3003–3012
47. Raz, R., Lee, C., Cannizzaro, L., d'Eustachio, P., and Levy, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2846–2851
48. Chan, H., Bartos, D. P., and Owen-Schaub, L. B. (1999) *Mol. Cell. Biol.* **19,** 2098–2108
49. Catlett-Falcone, R., Landowski, T., Oshiro, M., Turkson, J., Levitzki, A., Salvino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J., and Nunez, G. (1999) *Immunity* **10,** 105–115
50. Klippel, A., Kavanaugh, W. M., Pot, D., Williams, L. T. (1997) *Mol. Cell. Biol.* **17,** 338–344
51. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) *Cell* **89,** 457–467
52. Tolkacheva, T., and Chan, A. M. (2000) *Oncogene* **19,** 680–689
53. Wen, Z., Zhong, Z., and Darnell, J., Jr. (1995) *Cell* **82,** 241–250
54. Ivanov, V. N., and Ronai, Z. (1999) *J. Biol. Chem.* **274,** 14079–14089
55. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) *J. Immunol. Methods* **139,** 271–279
56. Ivanov, V. N., and Nikolic-Zugic, J. (1997) *J. Biol. Chem.* **272,** 8558–8566
57. Wisdom, R., Johnson, R. S., and Moore, C. (1999) *EMBO J.* **18,** 188–197
58. Muthukkumar, S., Nair, P., Sells, S. F., Maddiwar, N., Jacob, R. J., and Rangnekar, V. M. (1995) *Mol. Cell. Biol.* **15,** 6262–6272
59. Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001) *Mol. Cell. Biol.* **21,** 5299–5305
60. Kwon, T., Kwon, D. Y., Chun, J., Kim, J. H., and Kang, S. S. (2000) *J. Biol. Chem.* **275,** 423–428
61. Hall, A. (1999) *Br. J. Cancer* **80,** 25–27
62. Faruqi, T. R., Gomez, D., Bustelo, X. R., Bar-Sagi, D., and Reich, N. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9014–9019
63. Girardin, S. E., and Yaniv, M. (2001) *EMBO J.* **20,** 3437–3446
64. Ungefroren, H., Voss, M., Bernstorff, W. V., Schmid, A., Kremer, B., and Kalthoff, H. (1999) *Ann. N. Y. Acad. Sci.* **880,** 243–251
65. Hausler, P., Papoff, G., Eramo, A., Reif, K., Cantrell, D. A., and Ruberti, G. (1998) *Eur. J. Immunol.* **28,** 57–69
66. Gratton, J. P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., and Sessa, W. C. (2001) *J. Biol. Chem.* **276,** 30359–30365
67. Schuringa, J. J., Decker, L. V., Vellenga, E., and Kruijer, W. (2001) *J. Biol. Chem.* **276,** 27709–27715
68. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146
69. Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995) *Cell* **81,** 1147–1157
70. Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J. S. (1996) *J. Biol. Chem.* **271,** 27225–27228
71. Kaminuma, O., Deckert, M., Elly, C., Liu, Y. C., and Altman, A. (2001) *Mol. Cell. Biol.* **21,** 3126–3136
72. Page, C., Huang, M., Jin, X., Cho, K., Lilja, J., Reynolds, R. K., and Lin, J. (2000) *Int. J. Oncol.* **17,** 23–28
73. Nguyen, H., Ramana, C. V., Bayes, J., and Stark, G. R. (2001) *J. Biol. Chem.* **276,** 33361–33368
74. Schuringa, J. J., Jonk, L. J., Dokter, W. H., Vellenga, E., and Kruijer, W. (2000) *Biochem. J.* **347,** 89–96
75. Masashi Fukuzawa, M., Araki, T., Adrian, I., and Williams, J. G. (2001) *Mol. Cell* **7,** 779–788
76. Peli, J., Schroter, M., Rudaz, C., Hahne, M., Meyer, C., Reichmann, E., and Tschopp, J. (1999) *EMBO J.* **18,** 1824–1831
77. Fenton, R. G., Hixon, J. A., Wright, P. W., Brooks, A. D., and Sayers, T. J. (1998) *Cancer Res.* **58,** 3391–3400

78. Suzuki, A., Hayashida, M., Kawano, H., Sugimoto, K., Nakano, T., and Shiraki, K. (2000) *Hepatology* **32,** 796–802
79. Xiao, G. H., Jeffers, M., Bellacosa, A., Mitsuuchi, Y., Vande Woude, G. F., and Testa, J. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 247–252
80. Gibson, S., Tu, S., Oyer, R., Anderson, S. M., and Johnson, G. L. (1999) *J. Biol. Chem.* **274,** 17612–17618
81. Di Christofano, A., Kotsi, P., Peng, Y. F., Cordon-Cardo, C., Elkon, K. B., and Pamdolfi, P. P. (1999) *Science* **285,** 2122–2125
82. Poetsch, M., Dittberner, T., and Woenckhaus, C. (2001) *Cancer Genet. Cytogenet.* **125,** 21–26
83. Hyun, T., Yam, A., Pece, S., Xie, X., Zhang, J., Miki, T., Gutkind, J. S., and Li, W. (2000) *Blood* **96,** 3560–3568
84. Zhou, X. P., Gimm, O., Hampel, H., Niemann, T., Walker, M. J., and Eng, C. (2000) *Am. J. Pathol.* **157,** 1123–1128
85. Coniglio, S. J., Jou, T. S., and Symons, M. (2001) *J. Biol. Chem.* **276,** 28113–28120
86. Ning, A. Q., Li, J., McGuinness, M., and Arceci, R. J. (2001) *Oncogene* **20,** 4525–4536
87. Chakraborty, M., Qiu, S. G., Vasudevan, K. M., and Rangnekar, V. M. (2001) *Cancer Res.* **61,** 7255–7263
88. Villunger, A., Egle, A., Marschitz, I., Kos, M., Bock, G., Ludwig, H., Geley, S., Kofler, R., and Greil, R. (1997) *Blood* **90,** 12–20
89. Jiang, B. H., Zheng, J. Z., Aoki, M., and Vogt, P. K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1749–1753
90. Wen, S., Stolarov, J., Myers, M. P., Su, J. D., Wigler, M. H., Tonks, N. K., and Durden, D. L. (2001) *Proc. Natl. Acad. Sci.* U. S. A. **98,** 4622–4627
91. Joneson, T., and Bar-Sagi, D. (1999) *Mol. Cell. Biol.* **19,** 5892–5901
92. Yamaguchi, Y., Katah, H., Mori, K., and Negishi, M. (2001) *J. Biol. Chem.* **276,** 18977–18983
93. Timokhina, I., Kissel, H., Stella, G., and Besmer, P. (1998) *EMBO J.* **17,** 6250–6262
94. Pfeffer, L. M., Mullersmann, J. E., Pfeffer, S. R., Murti, A., Shi, W., and Yang, C. H. (1997) *Science* **276,** 1418–1420
95. Yang, F. C., Kapur, A. J., Tao, W., Kim, C., Borneo, J., Breese, R., Marshall, M., Dinauer, M. C., and Williams, D. A. (2000) *Immunity* **12,** 557–568