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ERK and PI3K negatively regulate STAT-transcriptional activities in human melanoma cells: implications towards sensitization to apoptosis

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Signal transducers and activators of transcription (STAT) proteins nuclear translocation and transcriptional activity are regulated by diverse protein kinases in response to extracellular stimuli by cytokines, growth factors and stress. Using two melanoma-derived cell lines that exhibit marked differences in basal activities of MAPKs and PI3K-AKT, we studied changes both in STAT activities and in their sensitization to apoptosis. Activating mutations of B-RAF (T1796A) and impaired expression of PTEN are detected in LU1205, but not in FEMX melanoma cells, and are reflected in high basal levels of expression and activities of MAPKs and PI3K-AKT. Treatment with either PD98059 (PD) or LY294002 (LY), the pharmacological inhibitors of MEK-ERK and PI3K, respectively, markedly increased GAS-Luc activity in LU1205, but not in FEMX cells. Tyrosine phosphorylation of STAT3/5 and of JAK2 also increased upon treatment of LU1205 cells with either PD or LY, suggesting that constitutive active MAPK and PI3K signals inhibit tyrosine phosphorylation of JAK/STATs. Treatment of FEMX and LU1205 with PD sensitized the cells to apoptosis, albeit by TNFα and TRAIL death cascades, respectively, indicating that additional yet distinct targets are affected by each signaling pathway. Indeed, the combination of LY and PD treatment synergistically increased the apoptosis of LU1205 and FEMX cells. Overall, whereas PI3K and MAPK down-regulate JAK-STAT signaling, additional targets are affected by these kinases and sensitizes melanoma to apoptosis via distinct death cascades.
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Introduction

Signal transducers and activators of transcription (STAT) proteins are dual function molecules that can be activated by diverse extracellular stimuli to transmit signals from the cell surface to the nucleus and directly participate in gene regulation (Ihle, 1995; Darnell, 1997). Activation of STATs results in expression of genes that control critical cellular functions (reviewed in Bowman *et al*., 2000; Bromberg and Darnell, 2000). Ligand-dependent activation of STATs is often associated with differentiation and/or growth regulation, whereas constitutive activation is often associated with growth disregulation. An increasing number of tumor-derived cell lines as well as samples from human cancers are reported to contain constitutively activated STAT proteins that are, in most cases, STAT3 and STAT5 (Gouilleux-Gruart *et al*., 1996; Weber-Nordt *et al*., 1996; Chai *et al*., 1997; Garcia *et al*., 1997; Garcia and Jove, 1998; Grandis *et al*., 2000; Levy and Gilliland, 2000). Abundant evidence strongly suggests that aberrant STAT3 signaling is an important process in malignant progression, but the regulation of STAT3 during tumorigenesis is not well understood.

The prerequisite for STAT transcriptional activities lies in its phosphorylation. Phosphorylation of STAT3 on Tyr 705 is mediated by Janus Kinases (JAKs; Darnell, 1997) and Rac1 (Simon *et al*., 2000), and results in its homo- or heterodimerization which enables nuclear localization and DNA binding (Darnell, 1997). Phosphorylation on Ser 727 alters its transcriptional activities (Wen *et al*., 1995; Wen and Darnell 1997) and is mediated by the serine threonine kinases including MAPK, JNK and p38 (Chung *et al*., 1997; Sengupta *et al*., 1998; Lim and Cao, 1999). Evidence is accumulating in support of PI3K's role in tyrosine phosphorylation of STATs (Sharfe *et al*., 1995; Yamauchi *et al*., 1998), possibly via members of the Src tyrosine kinase family that link cytokine receptors to PI-3 kinase through JAK (Takahashi-Tezuka *et al*., 1997).

Several mechanisms that inhibit JAK-STAT signaling have been described. They include dephosphorylation, proteolytic degradation, or association with inhibitory molecules (Haspel *et al*., 1996; Kim and Maniatis 1996; Shuai *et al*., 1996; Azam *et al*., 1997; Chung *et al*., 1997; Sengupta *et al*., 1998). Activation of PKC or PKA, for example, inhibits JAK-STAT signaling through mechanisms that are not completely understood (Sengupta *et al*., 1996). Members of the MAPK family, including ERK, elicited both positive and negative effects on JAK-STAT signaling (David *et al*., 1995; Fukada *et al*., 1996; Minami *et al*., 1996; Bonni *et al*., 1997; Czerniecki

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et al., 1997; Ihara *et al*., 1997; Boccaccio *et al*., 1998). The mechanisms underlying inhibition of STAT activities include both serine and tyrosine phosphorylation. Evidence in support of the possible effect of ERK on JAK activities is accumulating (Sengupta *et al*., 1998).

In earlier studies, we demonstrated that STAT cooperation with c-Jun results in silencing Fas receptor expression in advanced human melanomas (Ivanov *et al*., 2001). Further, we showed that PI3K-PKB elicits signals that inhibit both STAT3 and c-Jun transcriptional activities, which attenuates their inhibition of Fas promoter and results in concomitant upregulation of Fas receptor expression (Ivanov *et al*., 2002). Advanced melanoma often overrides PI3K because MAPK is constitutively active. In all, 15–25% of melanomas are mutated within the Ras oncogene, and B-RAF mutations were reported to occur at a high frequency (>60%) in primary melanoma samples and in corresponding cell lines (Brose *et al*., 2002; Davies *et al*., 2002). Together these mutations occur in about 80% of melanomas that exhibit high levels of MAPK activities. In addition, PI3K activities are also upregulated as a result of impaired PTEN expression. Since both PI3K and MAPK appear to play a central role in the regulation of STAT activities and consequently in the sensitivity of melanoma to apoptosis, we assessed possible changes in STAT transcriptional activities using melanoma cells that harbor constitutively active MAPK versus cells that do not. Here we demonstrate that ERK and PI3K both negatively regulate the transcriptional activities of STATs and establish new links between these signaling cascades, the regulation of STATs and sensitization to apoptosis in melanomas.

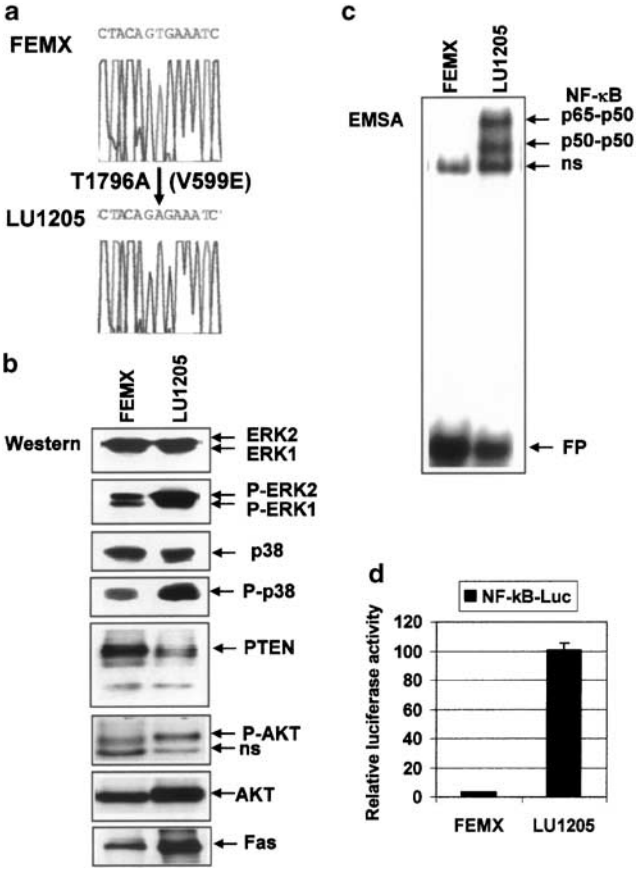

Figure 1 Differential activation of B-RAF and PI3K downstream targets in melanoma cells. (**a**) B-RAF-activating mutations in human melanomas. (**b**) Western blot analysis of total cell extracts (80 μg) from FEMX and LU1205 cells using primary antibodies to ERKs, phospho-ERKs, p38, phosho-p38, PTEN, AKT, phospho-AKT and Fas. (**c**) EMSA of nuclear NF-κB DNA-binding activity of FEMX and LU1205 cells. Two main NF-κB DNA-binding complexes are indicated; ns, nonspecific band. (**d**) NF-κB-dependent transcriptional activity in FEMX and LU1205 cells was determined after transient transfection of NF-κB-Luc construct

Results

Distinct activation of signaling cascades because of mutation in B-RAF and impaired PTEN in LU1205, but not in FEMX human melanoma cells lines

Analysis of B-RAF mutations in metastatic melanoma cell lines commonly used in our studies identified BRAF-activating mutation (T1796A) in LU1205, but not in FEMX melanoma cells (Figure 1a). Analysis of the corresponding kinase cascades revealed high basal levels of active (phosphorylated) forms of MAPK kinases (ERK and p38), suggesting that they are constitutively activated in LU1205 but not in FEMX cells (Figure 1b). In addition, LU1205 cells possessed very low basal levels of PTEN (Figure 1a and data not shown). Decreased PTEN expression was reflected in increased expression and activities of PI3K-AKT (Figure 1b). LU1205 cells were also found to exhibit high basal levels of nuclear NF-κB DNA-binding activity (Figure 1c) and corresponding NF-κB-dependent transcriptional activity (Figure 1d), which could be attributed to the constitutively active ERK and/or AKT kinases (Ozes *et al*., 1999; Jones *et al*., 2000; Dhawan and Richmond, 2002). LU1205 cells express relatively high levels of the Fas receptor (CD95/APO1; Figure 1b), which could be attributed to high basal levels of NF-κB (Figure 1c). These findings highlight the major changes in MAPK/PI3K signaling in the two melanoma cell lines, as reflected in key targets including NF-κB and Fas.

STAT3 transcriptional activities are negatively regulated by ERK

As STATs were shown to be affected by both ERK and PKB signaling cascades, we elucidated possible changes in STAT transcriptional activities in melanomas that could explain the marked differences in the activities of these signaling cascades.

Analysis of STAT3 phosphorylation in the two melanoma cell lines that exhibited marked changes in MAPK/PI3K activities revealed a higher basal level of Ser 727 phosphorylation in LU1205 cells but a relatively low level of Tyr 707 phosphorylation, which is required for STAT3 dimerization-dependent

nuclear import and transcriptional activities. STAT3 phosphorylation was notably less pronounced in FEMX cells (Figure 2a). Accordingly, STAT3 exhibited a low basal level of transcriptional activities, monitored using the 3xLy6E-Luc reporter construct containing three GAS elements of the Ly6E gene promoter (Wen *et al*., 1995). The analysis to detect STAT3 transcriptional activities in LU1205 cells revealed a low basal level of GAS-Luc activities induced upon treatment with PD98059, the pharmacological inhibitor of MEK-ERK, (fivefold) in LU1205, but not in FEMX cells (Figure 2b, c). Exogenous expression of ERK further decreased the relatively low level of basal GAS-Luc-dependent activities, while dominant-negative form of MEK (which suppress ERK activation) upregulated Gas-Luc. This is similar to what was seen after treatment with PD98059, although somewhat less pronounced because of the limited transfection efficiency of these melanoma cells (Figure 2b, c). These data suggest that constitutively active ERK signaling downregulates STAT transcriptional activities in LU1205 melanoma cells. To further elucidate the changes in STAT activity after treatment with MEK inhibitor, we tested the effect of IL6 on GAS-Luc activities. On its own, IL6 did not alter the activities of 3xLy6E-Luc in either melanoma cell line, further demonstrating that this signaling cascade is not functional in the melanoma cells studied here. In combination with PD98059, however, IL6 further increased Gas-Luc reporter activities in LU1205 cells and induced such activities in FEMX cells, albeit to a lesser extent (Figure 2b, c). These results suggest that the signaling pathway required for STAT transcriptional activities can be activated upon inhibition of the ERK kinases, albeit to different degrees in each of the melanoma cell lines.

Treatment with PD98059 markedly increased tyrosine phosphorylation of STAT3 in LU1205 cells, but not in

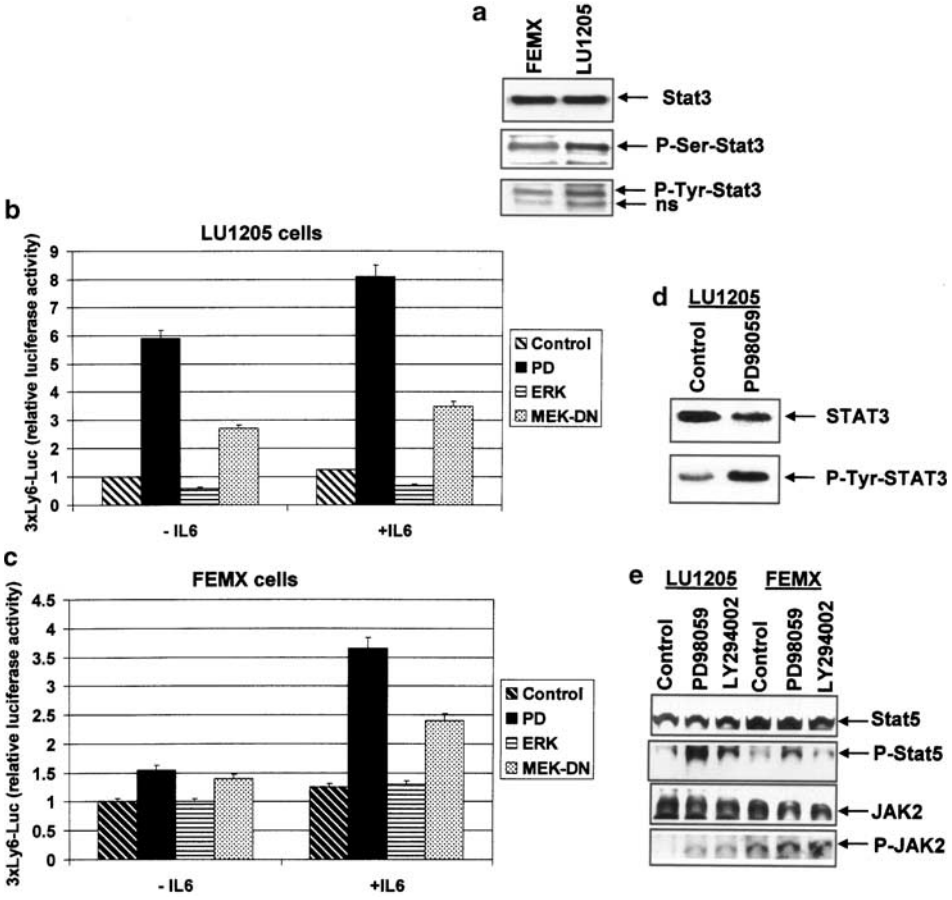

Figure 2 Effect of MEK inhibitor PD98059 on STAT3 and STAT5 phosphorylation and STAT-mediated promoter activity. (**a**, **d** and **e**) Western blotting of total cell extracts from FEMX and LU1205 cells using anti-STAT3, antiphospho-Ser-STAT3, antiphospho-Tyr-STAT3, anti-STAT5, antiphospho-STAT5, anti-JAK2 and antiphospho-JAK2 Abs. Cells were either untreated or treated with PD98059 (25 μM) or LY294002 as indicated. (**b**, **c**) Effect of PD98059 (25 μM) treatment, active ERK and dominant-negative MEK overexpression on the activity of 3xLy6E-Luc reporter construct in the presence or absence of IL6 (50 ng/ml). 5×10^5 FEMX or LU1205 cells were transiently transfected with 0.5 μg of 3xLy6E-Luc reporter construct plus 0.25 μg of pCMV-βgal in the presence of empty vector, ERK or MEK-DN expression vector (0.25 μg). At 16 h after transfection, PD98059 (25 μM) and IL6 (50 ng/ml) were added to the media. After an additional 12 h, luciferase and β-galactosidase activity in the cells was analysed and normalized luciferase activity was determined

FEMX cells (Figure 2d and data not shown). Unlike the changes in tyrosine phosphorylation, there was no change in the level of Ser phosphorylation on aa 727 (data not shown).

PD98059 treatment also effectively increased tyrosine phosphorylation of STAT5 in LU1205 cells and to a lesser degree in FEMX cells (Figure 2e). Given that ERK is a serine/threonine and not a tyrosine kinase, this finding suggests that ERK indirectly affects STAT tyrosine phosphorylation.

Since JAK2 is among the best-characterized kinases that phosphorylate both STATs on tyrosine residues, we next assessed changes in JAK phosphorylation that are expected to directly reflect its activity. FEMX exhibited basal phosphorylation of JAK2 that was not affected by the pharmacological inhibitors of ERK or PI3K, but treatment of LU1205 cells with these inhibitors caused a small yet noticeable increase in JAK2 phosphorylation (Figure 2e). These findings indicate that ERK may negatively influence JAK2 phosphorylation and attenuate its ability to phosphorylate STAT on the respective tyrosine residues.

As PD98059 was required in both melanoma cell lines to elicit IL6 effects, we next tested the possibility that levels of STAT proteins limit GAS-dependent transcription. Forced expression of STAT3 alone had a slight effect on GAS-Luc activities in each melanoma culture. However, in the presence of PD98059, expression of exogenous STAT3 resulted in a 40 fold increase in GAS-Luc activities in LU1205 cells, but not in FEMX cells (compare Figure 3a, b). These findings suggest that ERK-mediated inhibition of STAT3 cannot be overcome by overexpression of STAT3, but excess STAT3 enables stronger GAS-mediated transcription after attenuating ERK-dependent inhibition with PD98059.

Treatment of LU1205 cells with IL6 caused a twofold increase in STAT3-induced GAS-Luc activities, but further increased the degree of activation in PD98059-treated cells (up to 68-fold; Figure 3a). FEMX subjected to IL6 treatment exhibited a higher degree of GAS-Luc activities (up to 10-fold) which was increased (to 27-fold) upon treatment with PD98059 (Figure 3b). These results demonstrate that exogenous expression of STAT3 results in greater GAS-Luc activities in the FEMX cells and that the amount of STAT3 may, in part, limit GAS-Luc activities.

In light of the finding that exogenously expressed STAT3 can induce greater levels of GAS-Luc activities in FEMX cells than in LU1205 cells, we assessed whether expression of JAK2 would affect GAS-Luc activities. Forced expression of JAK2 resulted in less than a four fold increase in GAS-Luc activity in LU1205 cells, compared with a 15-fold increase in FEMX cells (Figure 3c). These results indicate that expression of JAK2 sufficed to rescue inhibition of STAT activities, albeit to a greater degree in FEMX melanoma cells. These findings further illustrate that inhibition of JAK-STAT activities takes place at different levels in LU1205 and FEMX cells.

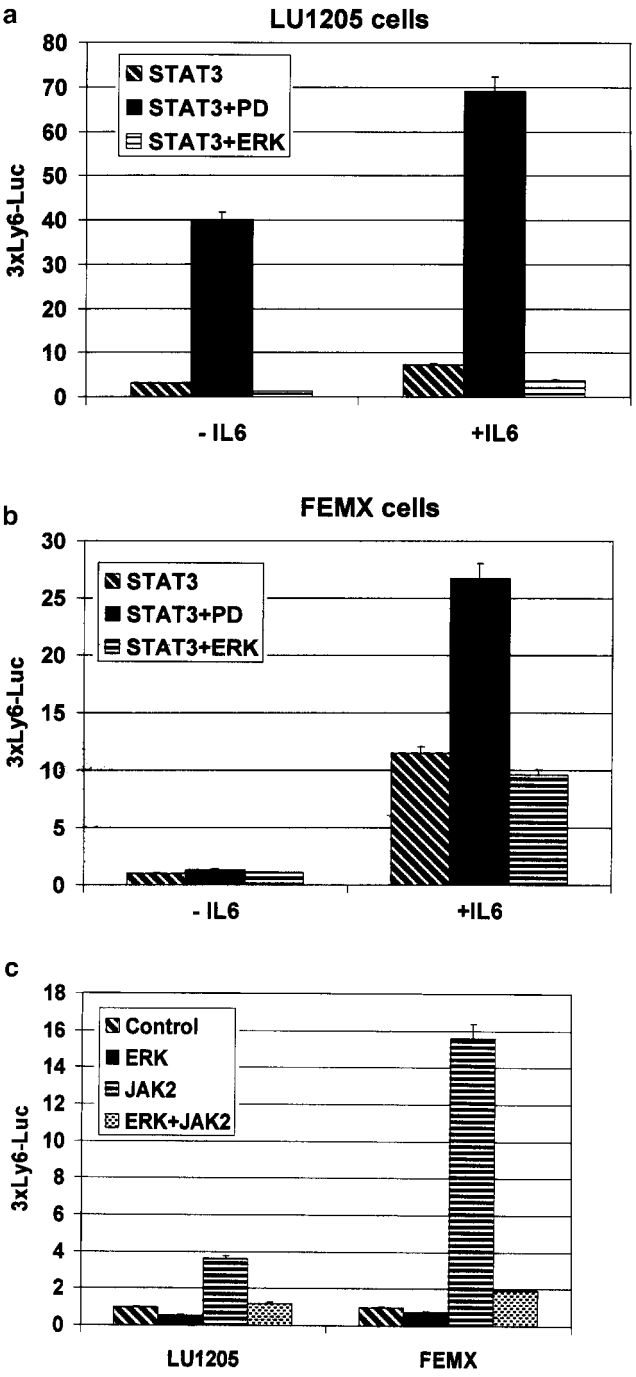

Figure 3 Effect of MEK inhibitor PD98059 and STAT3 overexpression on STAT-mediated promoter activity. (**a**, **b**) Effect of STAT3 overexpression, PD98059 (25 μM) treatment, on the activity of 3xLy6E-Luc reporter construct in the presence or absence of IL6 (50 ng/ml). 5×10^5 FEMX or LU1205 cells were transiently transfected with 0.5 μg of reporter construct, 0.25 μg of pCMV-βgal plus 0.25 μg of STAT3 expression. At 16 h after transfection, PD98059 (25 μM) and IL6 (50 ng/ml) were added to the media. After an additional 12 h, the cells were analysed for luciferase and β-galactosidase activity, and normalized luciferase activity was determined. **(c)** 5×10^5 FEMX or LU1205 cells were transiently transfected with 0.5 μg of reporter construct and 0.25 μg of pCMV-βgal in the presence of 0.25 μg ERK expression vector, JAK2 expression vector, or both. STAT-dependent reporter activity was determined as described above

PI3K elicits negative regulation of GAS-STAT as part of the MAPK pathway

LU1205 cells not only express mutant B-RAF, but also low levels of PTEN (Figure 1a). Inhibition of PI3K by LY appears to have effects on tyrosine phosphorylation of JAK and STAT5 similar to those seen with PD98059 (Figure 2e). Therefore, we further studied the nature of the PI3K-MAPK relation in the context of GAS-Luc activities. Forced expression of a constitutively active form of p110*, the catalytic subunit of PI3K, increased total levels of PI3K activities in melanoma cells, while LY294002 suppressed this increase (Krasilnikov *et al*., 1999). Forced expression of p110* did not change basal transcriptional activities of STAT3, but did efficiently attenuate the increase in GAS-Luc seen after treatment with PD98059 (Figure 4a). Overexpression of p85Δ (a dominant-negative form of PI3K) upregulated basal levels of STAT-dependent transcription, close to the degree reached after expression of MEK-DN (Figure 4a). These results suggest that like MAPK, PI3K elicits negative effects on the transcriptional activities mediated by GAS-Luc promoter sequences. These results are in agreement with our previous data (Ivanov *et al*., 2002) demonstrating that AKT, like ERK, may serve to limit the degree of STAT-dependent transcriptional activities. These findings also imply that PI3K and MAPK converge in their negative regulation of GAS-Luc activities.

In further addressing relative contributions of PI3K and MAPK to STAT activities, we tested the effect of PD98059 and LY294002 both alone and in combination on each of the two melanoma cultures. Treatment of the LU1205 cells with LY294002, the pharmacological inhibitor of PI3K, did not affect basal GAS-Luc activities, whereas treatment with PD98059 led to a seven fold increase (Figure 4b). Exogenously expressed STAT3 led to an additional increase (five fold) in GAS-Luc transcription proportional to the basal effect of the corresponding inhibitor (from 2- to 10-fold

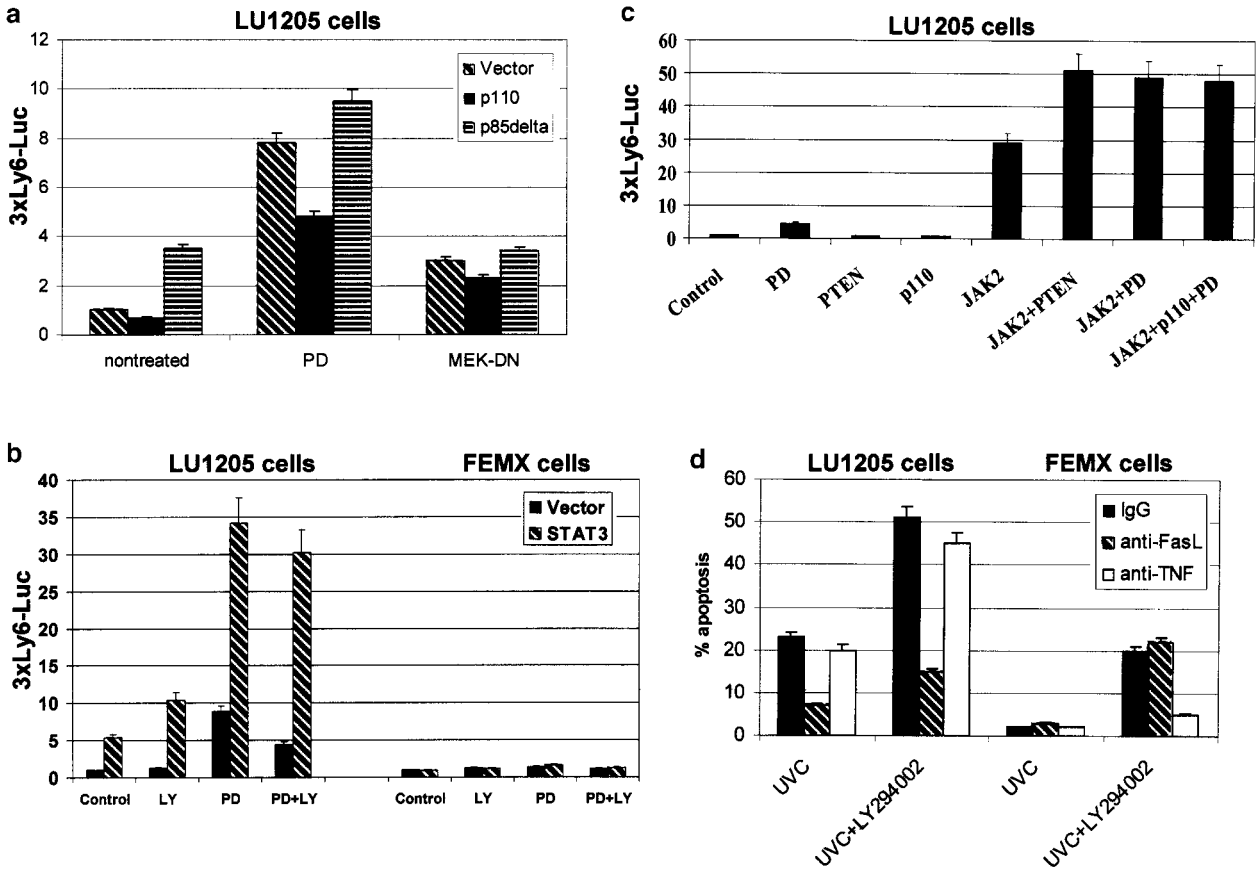

Figure 4 Effect of p110* and JAK2 overexpression on STAT-dependent promoter activity. (**a**) LU1205 cells were transiently transfected with 0.5 μg of 3xLy6E-Luc reporter construct plus 0.25 μg of pCMV-β-gal in the presence of 0.25 μg of PI3K p110 or p85 δ together with the empty vector or MEK-DN expression construct (0.25 μg). At 16 h after transfection, the cells were treated with PD98059, and after an additional 12 h were analysed for luciferase and β-galactosidase activity. (**b**) LU1205 and FEMX cells were transiently transfected with 0.5 μg of 3xLy6E-Luc reporter construct plus 0.25 μg of pCMV-β-gal in the presence or absence of 0.25 μg of STAT3 expression construct. At 16 h after transfection, the cells were treated with PD98059, LY294002 or their combination, and after an additional 12 h cells were analysed for luciferase and β-galactosidase activity. (**c**) Activity of 3xLy6E-Luc reporter construct was analysed after cotransfection with PTEN, p110 and JAK2 expression vectors. Treatment of the cells with PD989059 was performed under the same conditions as above. (**d**) FEMX and LU1205 cells were treated with 50 μM LY294002 1 h before irradiation with UVC (60 J/m^2) in the presence of normal mouse IgG, anti-FasL mAb (2 μg/ml) or anti-TNFα mAb (2 μg/ml). Apoptosis levels were determined after an additional 16 h using PI staining and flow cytometry

in LY-treated, and from 8- to 34-fold in PD98059-treated cells; Figure 4b). These results suggest that PI3K and MAPK operate on the same signaling pathway for activation of STAT-GAS activities. These observations also imply that MAPK is upstream of PI3K in this pathway, as it is capable of eliciting a greater effect on GAS-Luc activities. The latter conclusion is supported by the findings that p110 can attenuate PD98059 effects (Figure 4a), and that the combination of the two inhibitors does not result in additive or synergistic effects on GAS-Luc transcription (Figure 4b). Furthermore, forced expression of JAK2 sufficiently induces GAS-Luc activities in a manner that can be further increased upon the expression of PTEN, with effects similar to that of PD98059 (Figure 4c). Similar to activities observed in cells transfected with STAT3 (Figure 4b), the combination of LY294002 and PD98059 did not cause any additional increase in JAK2-mediated activation of GAS-Luc activities (Figure 4c). Together, these results suggest that constitutively high activities of ERK and PI3K-AKT in LU1205 cells inhibit STAT-transcriptional activities via their effects on JAK2. Neither treatment affected GAS-Luc activities in FEMX cells (Figure 4b), pointing to the role of impaired PTEN and MAPK signaling in LU1205 cells and suggesting that other mechanism(s) contribute to the regulation of STAT in FEMX cells.

Combined inhibition of PI3K and MAPK supersensitizes LU1205 and FEMX melanoma cells to apoptosis via distinct death cascades

As STAT3-GAS activities are inhibited, either PI3K or by MAPK, the level of FAS expression should increase because STAT3 is required for silencing the FAS promoter. Indeed, FAS expression was substantially higher in LU1205 cells, compared to the expression in FEMX cells (Figure 1b). Inhibition of PI3K or MAPK was expected to relieve STAT suppression and to result in decreased FAS surface expression, and this was observed in LU1205 cells, but not in FEMX cells (data not shown). However, treatment of LU1205 cells with LY294002 resulted in a marked increase in the degree of apoptosis after UV treatment (Figure 4d) because of upregulation of FasL levels (not shown). The sensitization of LU1205 to UV-induced apoptosis by LY294002 treatment could be attenuated by antibodies antagonistic to FasL but not to TNFα (Figure 4d). As occurred with LU1205 cells, treatment of FEMX cells with LY294002 resulted in their sensitization to apoptosis, albeit to a lower degree, and via the TNFα-TNFR1-mediated pathway rather than the FAS death cascade (Figure 4d).

Whereas treatment of LU1205 cells with PD98059 resulted in about 8% apoptosis (without any other stimuli), the combination of LY294002 and PD98059 caused a marked degree of apoptosis (46%) that could be attenuated by antibodies to TRAIL (Figure 5a). Similarly, combined treatment with PD and LY markedly increased the sensitization of FEMX cells to apoptosis (68%), primarily driven by the TNFα pathway, as revealed in the ability of antagonistic antibodies (to TNFα, but not to FasL or TRAIL) to attenuate this response (Figure 5b). These findings suggest that despite the marked difference among the two melanoma cultures in their responses to PI3K, MAPK and STAT activities, both are sensitized to apoptosis to a marked degree after exposure to a combination of the two inhibitors, albeit via alternate death cascade pathways. These findings indicate that MAPK and PI3K affect additional yet distinct targets in the two melanomas studied here, which are of critical importance in their sensitization to apoptosis.

Discussion

Recent advances in the understanding of genetic changes underlying melanoma development suggest that MAPK signaling is affected in over 80% of human melanomas as a result of mutation in either the Ras or the B-RAF genes, resulting in a constitutively high activity of corresponding MAPK, including ERKs. Here we demonstrate that constitutively high ERK activity suppresses the JAK-STAT signaling cascade. We further demonstrate that, like ERK, PI3K also elicits negative effects on JAK-STAT signaling. Our findings suggest that PI3K utilizes the same signaling cascade as ERK and may be affected by ERK, as p110 efficiently overrides the effect of the MEK-ERK inhibitor PD98059 (Figure 6). Inhibition of STAT transcriptional activities was associated with a decrease in its tyrosine phosphorylation, which could be rescued with treatment using the pharmacological inhibitor of MEK. Further, our data suggest that inhibition of STAT tyrosine phosphorylation is because of inhibition of JAK activities, as inhibitors of MAPK increased the level of JAK phosphorylation and, concomitantly, phosphorylation on STAT. Our results are in agreement with the finding of Sengupta *et al*. (1998), demonstrating a decrease in IL6 signaling and STAT3 activation by MAPK-ERK in human myeloma cells, thereby providing additional support for the notion that ERK can elicit inhibition rather than activation of the STAT signaling cascade. The ability of ERK to inhibit STAT signaling was also demonstrated in other studies (Kim and Baumann, 1999). Nevertheless, the present work adds the following important insights into our understanding of ERK-dependent inhibition of STAT: (a) our study demonstrates ERK inhibition of STAT in the physiological context of melanoma with a mutant B-RAF gene, thereby highlighting the consequences of this prevalent mutation for STAT activities; (b) our data suggest that ERK inhibition of STAT is mediated via targeting of JAK; (c) our results position PI3K as one of the components within the MAPK cascade, as p110 can override activation of STAT by PD98059.

The finding that ERK inhibits STAT via its down-regulation of JAK raises several interesting questions. The first question relates to the mechanisms underlying the inhibition of JAK by ERK. Our experiments

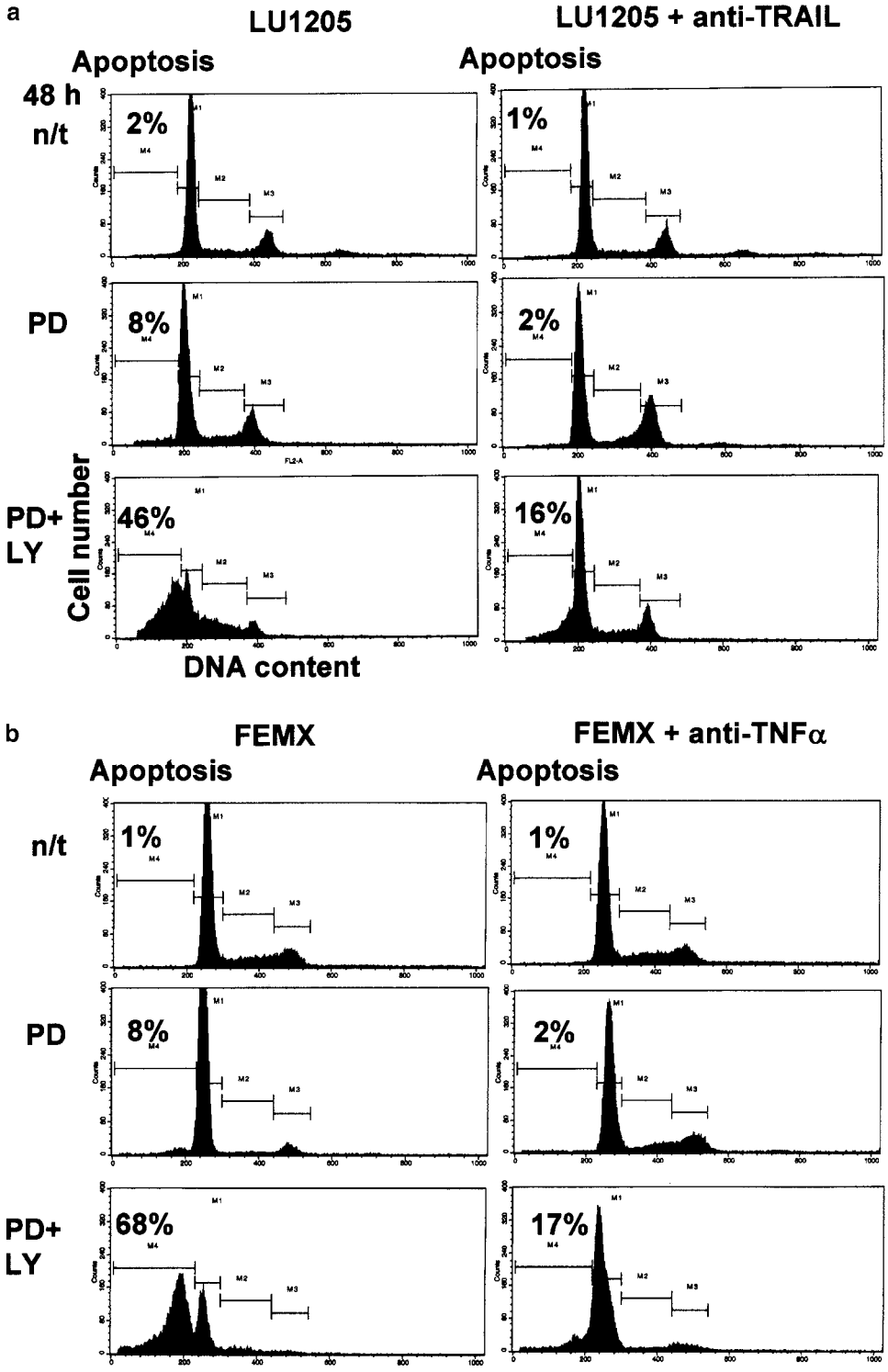

Figure 5 LY294002 and PD98002 inhibitors induce apoptosis of melanoma cells. (**a**, **b**) Effect of pretreatment with anti-TRAIL and anti-TNFα (2 μg/ml) Abs on LY294002 (50 μM), PD98002 (50 μM) or (LY294002 + PD98002)-induced apoptosis of LU1205 and FEMX cells. Apoptosis levels were determined 48 h after treatment using PI staining and flow cytometry

indicate that it is likely that ERK indirectly regulates the degree of JAK activity, although we cannot rule out effects on another kinase that may phosphorylate STAT3 on tyrosines. Associated inhibitors, SOCS (Cacalano *et al*., 2001), and associated phosphatase, CD45 (Irie-Sasaki *et al*., 2001), are among the possible candidates for affecting JAK activity by MAPK phosphorylation. Further studies will determine the target of the ERK activity that elicits inhibition of JAK and results in suppression of STAT3 activity.

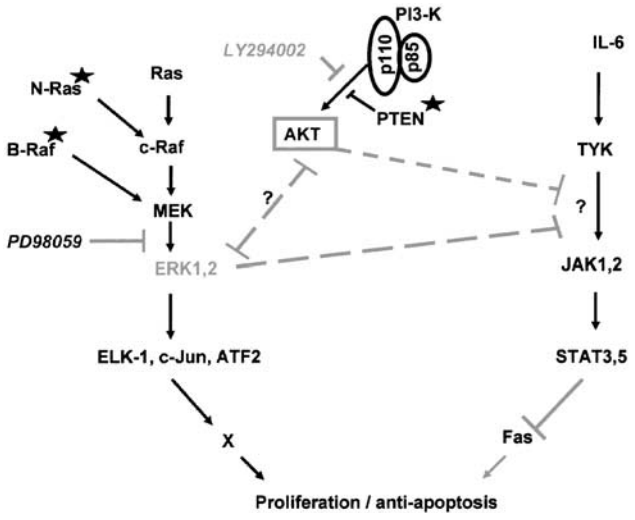

Figure 6 Chart outlines the signaling cascades that are modified in the course of melanoma development and their effect on STAT signaling and the sensitization of melanoma to apoptosis. Constitutively active MEK and downstream kinases, including ERK, elicit suppression of JAK-STAT transcriptional activities, congruent with suppression that is elicited by the PI3K pathway. Suppression is likely to converge the two signaling pathways either at the level of the actual kinases or their target (JAK or upstream component), as indicated by question marks. Suppression of STAT3 can be alleviated with the use of either dominant-negative kinases or their corresponding pharmacological inhibitors as indicated. Combination of these inhibitors also results in synergistic sensitization of the melanomas to apoptosis, probably becasue of the effect on multiple targets, illustrated in figure, in addition to the effects shown here for STAT

Inhibition of STAT activities coincides with an increased expression of Fas receptor, which is otherwise suppressed by the cooperation between STAT3 and c-Jun. Increased cell surface expression of Fas in LU1205 cells is expected to result in sensitization of the cells to Fas-mediated apoptosis. Owing to the low basal levels of endogenous FasL in these cells, such sensitization does take place after UV irradiation or upon addition of exogenous FasL. Nevertheless, it is expected that other targets downstream of the Fas death cascade would be impaired by high levels of MAPK/PI3K (Panka *et al*., 2001). LU1205 cells also possess high basal levels of p38 activity, which could also contribute to antiapoptotic signaling by p38 effect on NF-κB activity (Ivanov and Ronai, 2000). Clearly, inhibition of the ERK cascade had a limited effect on LU1205 or FEMX cells to spontaneous apoptosis. Further, inhibition of ERK signaling by the pharmacological inhibitor appears to sensitize LU1205 to spontaneous apoptosis via the TRAIL–TRAIL-R cascade, whereas the FEMX cells are sensitized via the TNFα-mediated pathway. This observation suggests that different changes in the signaling cascades occur in the two tumors and affect alternate death signaling cascades. LU1205 cells exhibit mutation in B-RAF and aberrant PTEN expression, yet, FEMX cells exhibit equal resistance to treatment as a result of changes that have yet to be identified. Nevertheless, in both cases, inhibition of MAPK alone has a limited effect, but inhibition of both the PI3K and the ERK cascades resulted in supersensitization of both melanoma cultures to apoptosis, albeit via alternate death pathways. Such sensitization clearly involved targets distinct in each of the two melanoma cell lines studied here and not involved in the common effect of the two inhibitors on JAK-STAT signaling. These observations imply that melanomas lacking B-Raf or Ras mutation may experience other changes that are either part of or affected by PI3K and MAPK, as found in the FEMX cells. Our conclusion is in agreement with studies performed on other tumor types that clearly implicate the role of the RAS-RAF-MEK-ERK pathway in tumor resistance to apoptosis, in part via the activation of the hepatocyte growth factor or PI3K signaling (von Gise *et al*., 2001; Edwards *et al*., 2002; Paumelle *et al*., 2002). As reported in the present study (see model in Figure 6), the roles of PI3K and B-RAF in STAT signaling suppression are part of a complex network of signaling cascades that converge and acquire melanoma resistance, but could be overcome after inhibition of both PI3K and MAPK pathways, resulting in supersensitization of these tumors to apoptosis.

Materials and methods

Cell culture

Human melanoma FEMX cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. LU1205 cells were maintained in MCDB153/L15 medium (4 : 1) supplemented with FBS, L-glutamine and antibiotics. Cells were grown at 37°C with 5% CO_2.

Transient transfection and luciferase assay

The luciferase reporter gene containing three GAS elements from the Ly6E gene promoter was previously described (Wen *et al*., 1995). Transient cotransfection of luciferase reporter gene together with different expression constructs (for STAT3, STAT3–phosphomutant, JAK2, PTEN, and the p110 subunit of PI3K and pCMV-βgal) into the cells was performed using Lipofectamine (Life Technology-BRL). 16 h after transfection, cells were treated with inhibitors. Luciferase activity was determined after an additional 12 h using the Luciferase assay system (Promega, USA) and normalized on the basis of β-galactosidase level in transfected cells. The active form of ERK and dominant-negative form of MEK (MEK-A) in pcDNA3 vector, active and dominant-negative forms of PI3K, p110* and p85Δ, respectively, were kindly provided by Dr Andrew Chan and used for cotransfection with the reporter $3 \times$ Ly6-Luc construct. MAPK inhibitor PD98059 (Calbiochem) was used at final concentrations of 25–50 μM. PI3K inhibitor LY294002 was used at a final concentration 50 μM.

Apoptosis analysis

Cells were exposed to UVC at 60 J/m^2 and treated with MAPK inhibitor PD98059 (25 μM) or PI3K inhibitor LY294002 (50 μM), and apoptosis levels were detected after 16–48 h by quantifying the percentage of hypodiploid nuclei undergoing DNA fragmentation to the left of the diploid $G_{0/1}$ peak. Flow-cytometric analysis was performed on a FACS Calibur flow

cytometer (Becton Dickinson) using the CellQuest program. Cell cultures were pretreated with Abs antagonistic to FasL, TNFα or TRAIL (2 μg/ml) in some experiments.

Western blot analysis

The cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na-orthovanadate and aprotinin (10 μg/ml). Cell lysates (100 μg protein) were resolved on 10% SDS–PAGE, transferred to a nitrocellulose membrane and processed according to standard methods. Primary antibodies (Abs) to phosphorylated and nonphosphorylated STAT3, p38 and ERKs, control Ab to AKT and Phospho-AKT (Ser473) Ab, and PTEN Ab were obtained from Cell Signaling (Beverly, MA, USA). Anti-JAK2 and anti-Phospho-JAK2 (Tyr1007, Tyr1008) Abs were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-Fas Ab from Pharmingen (San Diego, CA, USA) was also used. Anti-rabbit IgG conjugated to horseradish peroxidase were used as secondary antibodies. Signals were detected by ECL reagent (Amersham).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed for the determination of NF-κB DNA binding activity as previously described (Ivanov *et al*., 1995).

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