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Expression of ring finger-deleted TRAF2 sensitizes metastatic melanoma cells to apoptosis via up-regulation of p38, TNFα and suppression of NF-κB activities

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Mechanisms underlying radiation and chemotherapy resistance, the hallmark of human melanoma, are not well understood. Here we demonstrate that expression levels of signal adaptor protein TRAF2 coincide with melanoma resistance to UV-irradiation. Altered TRAF2 signaling by a form of TRAF2, which lacks the ring finger domain (TRAF2ΔN), increases activities of p38 MAPK, ATF2, and the level of TNFα expression. Forced expression of TRAF2ΔN in HHMSX highly metastatic melanoma cells that lack Fas expression and thus utilize the TNFα-TNFR1 as the major apoptotic pathway sensitized cells to UV-induced apoptosis. An over twofold increase in degree of apoptosis was observed in TRAF2ΔN expressing cells that were treated with actinomycin D, anisomycin or with the radiomimetic drug neocarzinostatin. Sensitization by TRAF2ΔN is selective since it was not observed in response to either Taxol or cis-platinum treatment. TRAF2ΔN effects are primarily mediated via p38 since inhibition of p38 reduces, whereas activation of p38 promotes the level of UV-induced apoptosis. Conversely, activation of IKK attenuates the sensitization of melanoma by TRAF2ΔN, indicating that p38-mediated suppression of NF-κB activity is among TRAF2ΔN effects. Our finding identifies p38, TNFα and NF-κB among key players that efficiently sensitizes melanoma cells to UV-, ribotoxic (anisomycin) and radiomimetic chemicals-induced programmed cell death in response to aberrant TRAF2 signaling. *Oncogene* (2001) **20,** 2243–2253.

Keywords: p38; TRAF2; melanoma; apoptosis; radiation; anisomycin; actinomycin D

Introduction

The incidence of malignant melanoma, a tumor, which responds poorly to chemotherapy and irradiation, has exponentially increased over the past decade. At present, the nature of melanoma protection from radiation-induced apoptosis remains largely unknown, despite advances made in our understanding the biology of this tumor type (Meier *et al*., 1998). The ability to resist apoptosis, by re-arranging the apoptosis machinery, including Fas, TNFR1, DR-3, TRAIL-R1 and TRAIL-R2 (Ashkenazi and Dixit, 1998; Nagata, 1997) is characteristic of most tumor cells, including melanomas (Peli *et al*., 1999). Altered susceptibility to apoptosis was shown to include additionally increased expression of inhibitory apoptosis proteins (IAP's) that suppress caspase activity (Deveraux and Reed, 1999). Common to late-stage melanoma cells is the expression of a large subset of growth factors, cytokines and their respective receptors, which contribute to autocrine and paracrine regulation of their progression (Moretti *et al*., 1999). Among the latter are TNFα and TNFR1 whose interaction elicits either death- or survival-signaling cascades in which the signal adaptor TNFR-associated factor2 (TRAF2) plays a primary regulatory role (Hsu *et al*., 1996; Liu *et al*., 1996; Arch *et al*., 1998).

TRAF2 itself has been implicated in the activation of distinct signaling pathways, including the NF-κB and the p38/JNK cascades (Hsu *et al*., 1996; Liu *et al*., 1996; Arch *et al*., 1998; Natoli *et al*., 1997; Nishitoh *et al*., 1998). TNF-induced NF-κB activation can be mediated via the adapter receptor interacting protein (RIP), or by the NF-κB-inducing kinase (NIK) (Yuasa *et al*., 1998; Galibert *et al*., 1998; Kelliher *et al*., 1998; Lin *et al*., 1998; Malinin *et al*., 1997; Song *et al*., 1997). NIK activates a multiprotein catalytic complex (IκB kinase complex) that phosphorylates the NF-κB inhibitor, IκBα at serines 32 and 36 (Zandi *et al*., 1997). Phospho-IκBα is then targeted for proteasome-dependent degradation via the HOS/Skip1/Cullin1/Roc1 ubiquitin ligase complex (Maniatis, 1999; Fuchs *et al*., 1999; Tan *et al*., 1999), thus liberating NF-κB, which enters the nucleus and mediates NF-κB-dependent transcription. The other TRAF2-mediated pathway, which is independent of NIK, activates the c-Jun amino-terminal kinases/stress activated protein kinases (JNKs/SAPKs/p38), a family of Ser/Thr protein kinases regulated by extracellular signals. The Ser/Thr protein kinases stimulate transcription by

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phosphorylation and activation of a number of transcription factors, including c-Jun, activating transcription factor 2 (ATF2), p53, and ternary complex factor (TCF)/ELK2 (Fuchs *et al*., 1998; Ip and Davis, 1998; Minden and Karin, 1997). Activation of SAPK and p38 by TRAF2 is mediated by germinal center kinase (GCK), which associates with both TRAF2 and MEKK1 via its COOH-terminal regulatory domain (Yuasa *et al*., 1998).

Fas receptor cross-linking induces apoptosis in a caspase-dependent manner (Krammer, 2000), which has been also implicated in the cleavage and intracellular redistribution of MEKK1 (Deak *et al*., 1998) and MAPK/JNK activation (Goillot *et al*., 1997). Among possible mechanisms for JNK activation via Fas is interaction with Daxx (Yang *et al*., 1997), which disrupt the intramolecular inhibition of ASK1, leading to the activation of MKK7/JNK. JNK has been also implicated in the regulation of cell death through the Fas cascade by controlling the expression of Fas ligand (FasL), through a MEKK1-regulated element on FasL promoter (Faris *et al*., 1998). Interestingly, the activation of FasL is also NF-κB and AP1-dependent (Kasibhatla *et al*., 1998), pointing to a potential link between TRAF2 and FasL. Through its diverse downstream targets and respective kinases, TRAF2 has been identified as a critical component of the cell's ability to undergo programmed cell death in response to TNF treatment or UV-irradiation (Tobin *et al*., 1998).

In elucidating transcription factors that alter melanoma resistance to UV-irradiation we have identified CREB associated proteins (Yang *et al*., 1996) and ATF2 as factors that play an important role in acquiring this resistance (Ronai *et al*., 1998). ATF2 confers melanoma resistance to radiation via increased transcription and expression of TNFα which may elicit an anti-apoptotic signal in some late-stage melanoma cell lines. Forced expression of hypo-phosphorylated or transcriptionally inactive forms of ATF2, which suppress TNFα expression, is capable of sensitizing melanoma cells to radiation-induced apoptosis (Ivanov and Ronai, 1999). Furthermore, we have shown that via suppression of NF-κB transcriptional activity, ATF2 kinase – p38 down-regulates the expression of Fas, which is the primary apoptotic receptor utilized in most late-stage melanoma cells (Ivanov and Ronai, 2000). Thus, through coordinated increase in survival signal and decrease in pro-apoptotic signal (Fas), p38 and its effector ATF2 confer the resistance of human melanoma cells to radiation-induced FasL-Fas-mediated programmed cell death. These observations positioned p38 as a key regulator of the balance between TNFα-TNFR and FasL-Fas death signaling cascades, and suggest that modulating p38 activities may assist in sensitization of melanoma cells to chemo- and radiotherapy. Interestingly, the expression and activities of p38 upstream regulators, TRAF2 and GCK are altered during melanoma progression. Early-stage melanoma cells exhibit lower levels of TRAF2 and GCK expression in response to radiation, when compared with the more resistant and metastatic late-stage melanomas (Ivanov *et al*., 2000). Given the changes in TRAF2 expression during melanoma development and in light of the role of TRAF2 in the regulation of p38 catalytic activities, in the present studies we have explored the possible use of TRAF2 as a target to increase sensitivity of highly metastatic melanoma cells to radiation and chemical treatment.

Results

TRAF2 expression coincides with degree of sensitivity to UV-irradiation

Comparing five melanoma derived cell lines, which represent different stages in melanoma development, identified two (THX and WM1552) that had substantial lower levels of TRAF2 expression after UV-irradiation (Figure 1a). Altered TRAF2 expression in UV-treated melanoma cells does not reflect a general pattern of protein expression. For example, the expression of IκB, which is implicated in the regulation of melanoma ability to undergo cell death (Ivanov and Ronai, 2000), decreased 6 h after UV treatment, and increased back to basal levels 18 h after treatment (Figure 1a). Lower expression of TRAF2 after UV-treatment coincides with the higher amount of cells that underwent apoptosis following UV-irradiation (Figure 1b). Forced expression of TRAF2 in THX cells protected these cells from low-dose irradiation (data not shown). Similarly, forced expression of TRAF2 and GCK in the WM1552 cells efficiently increased the resistance and reduced level of apoptosis in response to UV-irradiation (Ivanov *et al*., 2000). These observations suggest that changes in TRAF2 expression coincides with altered resistance of melanoma cells to UV-irradiation.

Addition of antagonistic antibodies to growth medium enables to identify which of the apoptotic pathways, FasL-Fas- or TNFα-TNFR, mediates the apoptotic response following exposure to UV-irradiation. Whereas THX, WM1552 and HHMSX cells exhibited 30–50% decrease in cell death upon attenuating the TNFR-dependent pathway (using anti-TNFR1 Abs), LU1205 was the only melanoma cell line out of five tested here that showed noticeable (fourfold) decrease in cell death upon addition of the anti-Fas antagonistic antibodies (Figure 1c). Lack of a response to antagonistic Abs against Fas in the HHMSX cells following UV treatment (Figure 1c) coincided with almost undetectable levels of surface Fas expression (Figure 1d). When compared with other late-stage melanoma cells, HHMSX cells possess relatively low levels of the anti-apoptotic protein BclxL (Figure 1e).

Common to two (THX and WM1552) of the three melanomas, which exhibited TNFR-mediated cell death, is the higher sensitivity to UV-induced apoptosis and poor expression of TRAF2 in response to UV-irradiation. The latter coincides with inducible phosphorylation of p38 and increased TNFα expression

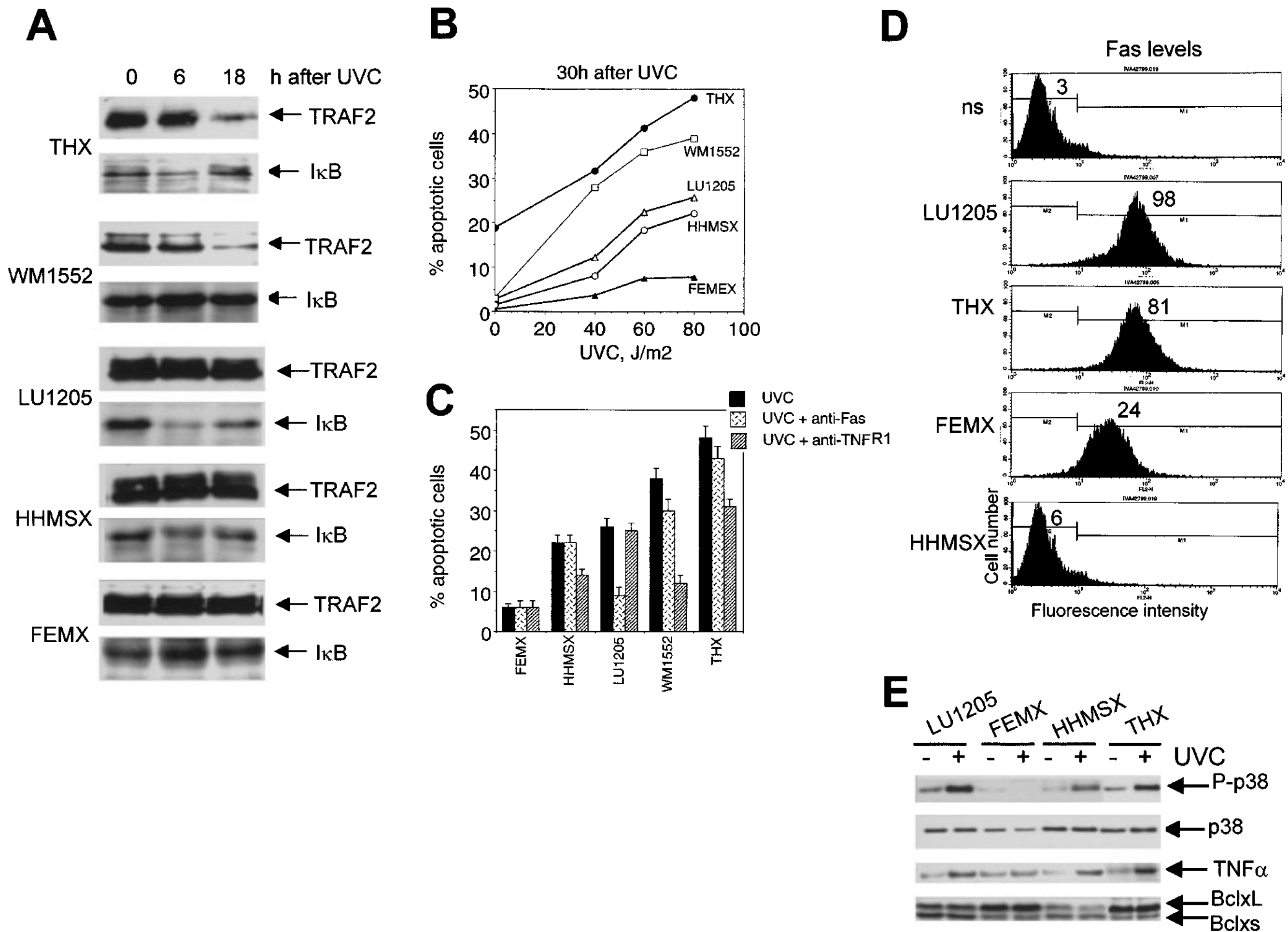

Figure 1 Level of TRAF2 expression coincides with resistance to UV-irradiation. (**a**) Western blot analysis of TRAF2 and IκB expression before and after UVC irradiation of indicated human melanoma cell lines. (**b**) Melanoma cells were UV-irradiated. Detection of apoptosis levels was performed by PI staining and the flow cytometry 30 h later, as described in Materials and methods. (**c**) Cell cultures were pretreated with mAbs against either Fas or TNFR1 2 h before UVC irradiation (80 J/m^2). Data shown represent three experiments performed in triplicates. (**d**) Cell surface expression of Fas was determined by anti-Fas-PE mAb and FACS analysis; ns: nonspecific staining. (**e**) Western blot analysis of Phospho-p38, p38, TNFα and BclXL levels before and 3 h after UV irradiation

following exposure to UV-treatment (Figure 1e and Ivanov *et al*., 2000). Conversely, the more resistant melanoma FEMX cells no longer exhibit p38 activation and TNFα expression in response to UV-treatment (Figure 1e). This observation points to an inverse correlation between the levels of TRAF2 expression, p38 activities, TNFα expression, and sensitivity of melanoma cells to radiation. This correlation was seen in three out of the four late stage aggressive melanoma cell lines tested, suggesting that in certain occasions, other signaling cascades may also prevail. As an upstream regulator of multiple stress kinases, including p38, and subsequent effectors, including TNFα, TRAF2 may play a central role in acquiring melanoma resistance to UV-treatment. To test this possibility we have selected to further characterize the highly metastatic HHMSX cells as a representative of late-stage melanoma cells, which constitutively express high levels of TRAF2 and are highly resistant to UV-irradiation and chemotherapy (data not shown).

TNFα expression is regulated by p38 and ATF2

The responsiveness to antagonistic Abs against TNFR and TNFα in HHMSX cells led us to further explore the regulation of TNFα expression in these cells. Monitoring luciferase activity driven by the TNFα promoter revealed noticeable basal activity, which increased (threefold) in response to UV-irradiation (Figure 2a). Point mutations within the CRE sequence (position −106) of the TNFα promoter (Rhoades *et al*., 1992) reduced basal activities and abolished their response to UV-irradiation. Mutation within the AP1-binding site (position −66) reduced degree of luciferase activity in response to UV-treatment, albeit to lesser extent than seen with the CRE mutant (Figure 2a). Pharmacological inhibitor of p38 catalytic activity, SB203508, was as efficient as the CRE mutation in abrogating the transcriptional activation mediated by TNF-Luc construct after UV-treatment. These results demonstrate that through the CRE site, p38 and its

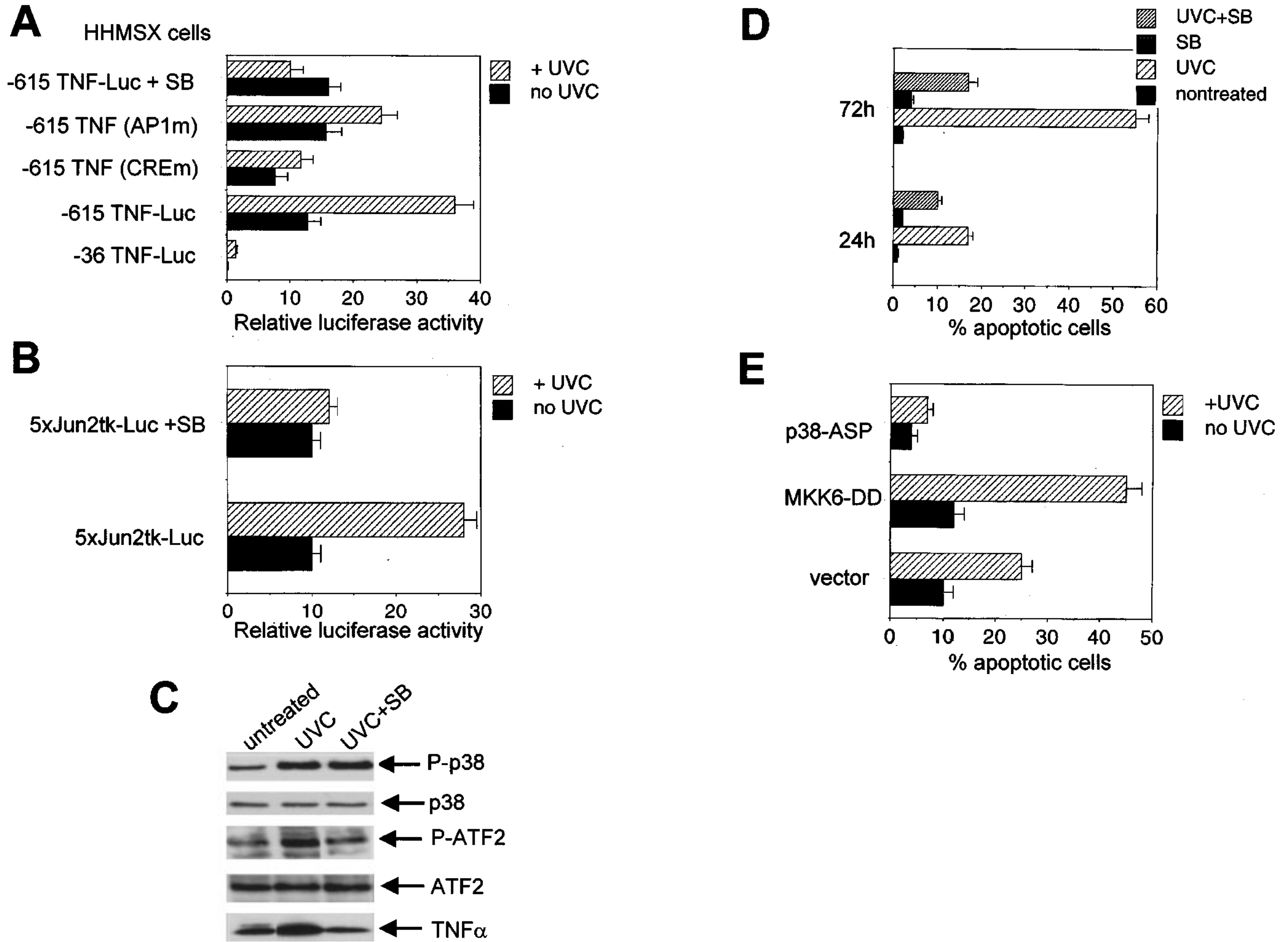

Figure 2 TNFα expression is regulated by p38 in HHMSX cells. (**a**) Role of p38 kinase and AP1- or CRE-elements and in regulation of TNFα promoter activity. HHMSX cells (5×10^5) were transiently co-transfected with the reporter construct −615 TNF-Luc or its mutated CREmut, and AP1mut variants (0.5 μg) in the presence of pCMV-β-gal (0.25 μg). Twenty-four hours after transfection cells were irradiated with UVC (60 J/m^2) and after an additional 16 h, cells were analysed for luciferase and β-galactosidase activity. Luciferase activity normalized to β-galactosidase is shown. (**b**) Effect of 5 μM SB203580 on UVC-induced luciferase activity directed by 5 × Jun2tk-Luc construct. (**c**) Western blot analysis of phospho-p38, phospho-ATF2 levels and TNFα expression in HHMSX cells following UVC irradiation in the absence or in the presence of 5 μM SB203580. (**d**) Apoptosis analysis of HHMSX cells was performed 30 h after UVC (60 J/m^2) irradiation as described in Materials and methods. (**e**) Degree of apoptosis upon forced expression of control empty vector, p38-ASP or MKK6-DD in the presence of pGFP before and after UV-irradiation (80 J/m^2)

substrates regulates TNFα transcription in the HHMSX melanoma cells. Indeed, HHMSX cells treated with the p38 inhibitor, SB203580, also exhibited an attenuated UV-induction of ATF2-c-Jun dependent reporter construct (5xJun2tk-Luc; Figure 2b). Inhibition of p38 catalytic activities was also reflected in reduced ATF2 phosphorylation and a concomitant decrease in expression of TNFα after UV-treatment (Figure 2c). Treatment of HHMSX cells with p38 inhibitor prior to UV-irradiation has lowered degree of UV-irradiation induced apoptosis from 17 to 10% and from 54 to 18%, 24 and 72 h after UV-treatment, respectively (Figure 2d), which reflects the fraction of apoptosis that is TNF-driven in these cells. Conversely, forced expression of a constitutive active form of p38 kinase, MKK6, led to a twofold increase in the degree of cell death in transfected cells, whereas dominant negative form of p38, p38-ASP, abolished UV-induced cell death (Figure 2e). These data suggest that TNFα expression in HHMSX cells is regulated primarily by p38/ATF2 and that inhibition of this pathway efficiently decreases the UV-induced apoptosis.

Expression of TRAF2ΔN increases activities of p38 and ATF2 and expression of TNFα after UV irradiation

Since HHMSX is among cells that exhibit stable expression of TRAF2 following UV-treatment, and since TRAF2 is among upstream regulators of p38 we have elucidated the possible role of TRAF2 in resistance of these melanoma cells to UV-induced apoptosis. To attenuate endogenous TRAF2 activities

we have used a truncated form of TRAF2, which lacks the first 87 aa that contains the ring finger domain (TRAF2ΔN) (Hsu *et al*., 1996). Expressions of TRAF2ΔN results in canonical suppression of NF-κB activity following TNFα or UVC treatment in several melanoma cell lines, including LU1205 cells (Ivanov *et al*., 2000). HHMSX cells that stably express the truncated form of TRAF2 were established and were characterized in comparison to control cells (neo), transfected with the empty vector pcDNA3. Among the HHMSX clones that stably express TRAF2ΔN we have selected the cells, which exhibited higher expression levels of TRAF2ΔN, for further characterization (Figure 3a). HHMSX/TRAF2ΔN cells exhibit noticeable expression of TNFR1 (Figure 3a). Expression of TRAF2ΔN increased and more noticeably prolonged the duration of UV-inducible levels of p38 and ATF2 phosphorylation (Figure 3b,c). Sustained p38 kinase activity and ATF2 transcriptional output in HHMSX/TRAF2ΔN cells coincided with a threefold increase in UV-inducible 5xJun2tk-Luc activity (which is regulated primarily by the ATF2/c-Jun heterodimers). Similarly, there was a twofold increase in UV-inducible TNFα promoter activity following UVC irradiation as monitored by the −615 TNF-Luc construct (Figure 3d). Elevated level of −615 TNF-Luc activity coincides with increased expression of TNFα after UV-treatment (Figure 3e). Conversely, UVC-dependent up-regulation of 2xNF-κB-Luc activity was attenuated in TRAF2ΔN expressing cells (Figure 3d). These observations suggest that expression of TRAF2ΔN results in up-regulation of p38/ATF2 activities and corresponding increase in TNFα-transcription and expression.

TRAF2ΔN sensitizes HHMSX cells to UV-induced apoptosis

Analysis of HHMSX response to UV-irradiation revealed that TRAF2ΔN expression increased fraction of cells that underwent apoptosis following exposure to UVC. Increased level of UV-induced apoptosis was attenuated upon addition of the antagonistic antibodies to TNFα (Figure 3f). A greater portion of melanoma cells underwent programmed cell death 64 h after UV-irradiation (41% of the neo-transfected *vs* 57% of those expressing the TRAF2ΔN (Figure 3f). TRAF2ΔN expression also increased degree of cell death in response to exogenous TNFα (10 ng/ml) and cycloheximide treatment (data not shown). These observations demonstrate that altered TRAF2 activity increases the level of UV-mediated cells death in melanoma cells, which utilizes TNFα-TNFR as the primary death pathway.

Inhibition of p38 or activation of IKK attenuates TRAFΔN-mediated sensitization of melanoma cells to UV-treatment

The finding that expression of TRAF2ΔN increases p38 activities led us to determine whether the pharmacological inhibitor of p38 will affect the sensitization observed in the melanoma cells studied here. Treatment of control (neo) HHMSX cells with SB203580 prior to their exposure to UV-irradiation attenuated the degree of UV-mediated apoptosis from 16 to 9% after 18 h and from 41 to 24% after 64 h (Figure 4a). The pharmacological inhibitor of p38 had greater inhibitory effect on the TRAF2ΔN expressing cells (where it decreased apoptosis from 26 to 10% after 18 h and from 57 to 30% after 64 h; Figure 4a). These data confirm that the primary signaling cascade that has been affected by the expression of TRAF2 lacking the ring finger domain and which contributes to the sensitization of melanoma cells to apoptosis is p38-dependent.

Given the ability of p38 to down-regulate the activity of NF-κB (Ivanov and Ronai, 2000), we have next determined the role of NF-κB in the sensitization of melanoma cells to UV-induced apoptosis in the presence of TRAF2ΔN. Forced expression of the constitutively active form of IKKβ (IKK^{S178E}) in HHMSX/TRAF2ΔN cells increased DNA binding activity of NF-κB heterodimers p65–p50 (Figure 4b; LU1205 cells were used as a positive control for NF-κB DNA-binding activity). Elevated NF-κB DNA binding activity coincided with a noticeable increase in NF-κB-mediated Luc activity (Figure 4c). Consequently, degree of apoptosis induced by UV-irradiation in the HHMSX/TRAF2ΔN cells co-expressing the IKKβ was abolished to basal levels seen prior to irradiation (Figure 4d). These data suggest that the sensitization of HHMSX melanoma cells to UVC irradiation by TRAF2ΔN is mediated via suppression of NF-κB activities.

TRAF2ΔN sensitizes HHMSX cells to apoptosis induced by anisomycin or actinomycin D

Given the relative modest changes seen in degree of UV-induced cell death upon TRAF2ΔN expression we have explored possible sensitization to other chemotherapeutic compounds. Treatment with Taxol arrested the melanoma cells in G2/M phase, but did not affect level of apoptosis in the TRAFΔN expressing cells (Figure 5a). Similarly, treatment with cis-platinum did not change level of cell death in TRAFΔN expressing cells (Figure 5a). Conversely, actinomycin D or anisomycin caused apoptosis, which further increased (2–2.5-fold) in cells that express TRAF2ΔN. Forty per cent of TRAF2ΔN expressing HHMSX human melanoma cells underwent apoptosis in response to anisomycin 18 h after treatment (Figure 5a). These results indicate that whereas sensitization by TRAF2ΔN is not affecting responsiveness of the melanoma cells to certain chemotherapeutic agents, there is greater sensitivity to treatments by ribotoxic drugs such as anisomycin. These results also indicate that a greater sensitization of melanoma cells by TRAFΔN expression is achieved by anisomycin compared with UV-irradiation. Of interest is that sensitization to actinomycin D (data not shown), but not to anisomycin-treatment could be attenuated by

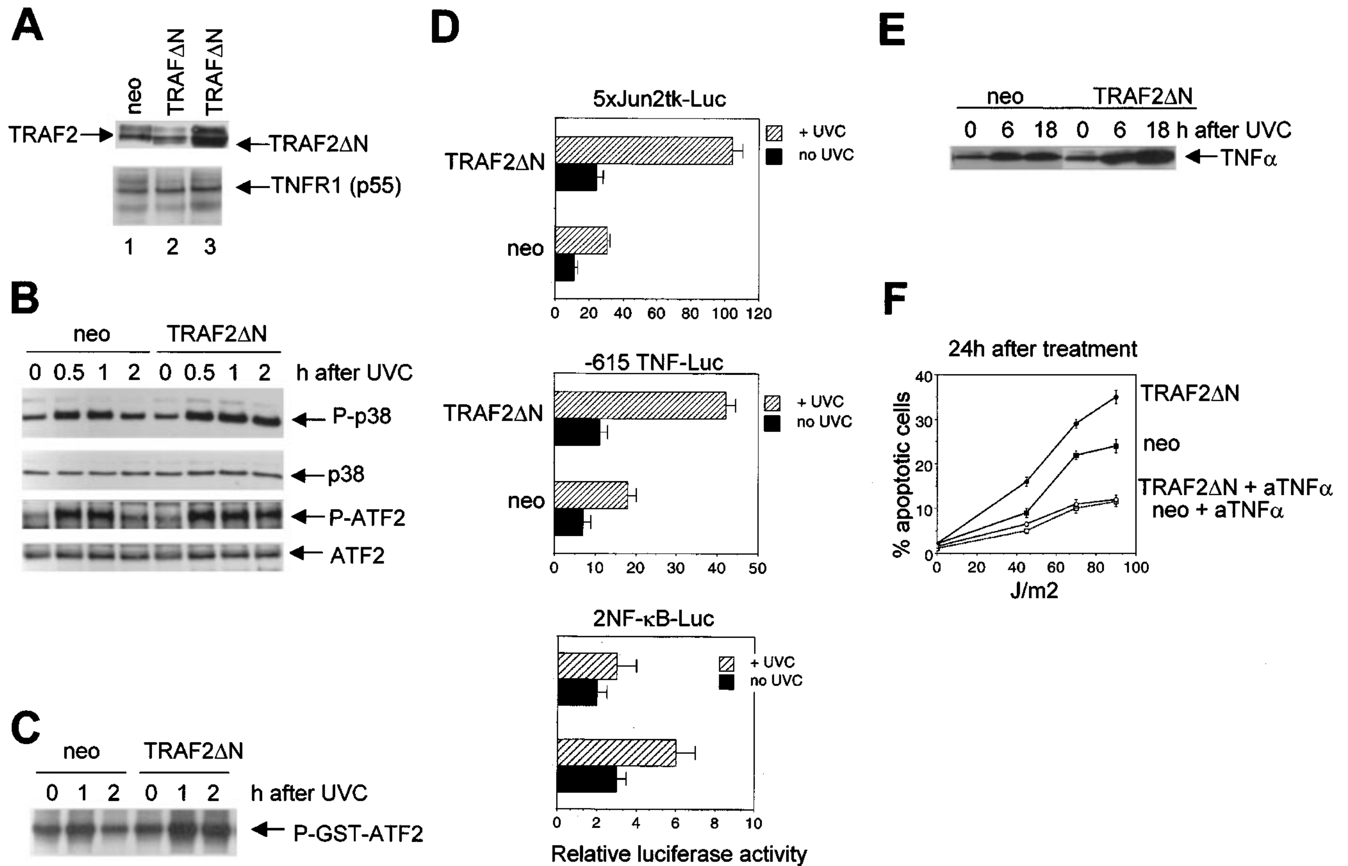

Figure 3 HHMSX/TRAF2ΔN cells possess prolonged and elevated p38/ATF2 activity, and ATF2-Jun-dependent transcription. (**a**) Western blot analysis was performed on cell extracts prepared from two HHMSX cell lines permanently transfected with TRAF2ΔN for detection of TRAF2ΔN. (**b**) Total cell extracts (75 μg) from HHMSX/TRAF2ΔN and HHMSX-neo (control) cells were isolated 0–2 h after UVC treatment and analysed by Western blotting using Abs against phospho-p38 MAPK, a control p38, phospho-ATF2 and control ATF2. (**c**) A solid-phase protein kinase assay was performed *in vitro* using GST-ATF2 immobilized on glutathione-agarose beads as a substrate and total protein extracts (10 μg) from HHMSX-neo and HHMSX/TRAF2ΔN cells isolated 0–2 h after UVC irradiation. (**d**) HHMSX-neo or HHMSX/TRAF2ΔN cells were transiently transfected with the indicated reporter constructs: 5×Jun2tk-Luc, −615 TNF-Luc or 2×NF-κB-Luc (0.75 μg) in the presence of 0.25 μg of pCMV-β-gal. Twenty-four hours after transfection cells were irradiated with UVC (60 J/m^2) and after an additional 16 h, proteins were prepared and used for the analysis of luciferase and β-galactosidase activity. The normalized ratio of luciferase to β-galactosidase activity is shown. (**e**) Western blot analysis of TNFα expression in HHMSX cells before and after UVC irradiation was carried out on total cell extracts (75 μg). (**f**) Degree of apoptosis levels of HHMSX-neo and HHMSX/TRAF2ΔN cells in response to different doses of UVC irradiation were determined 24 h after treatment. Data shown represents experiments that were reproduced over five times. Apoptosis of HHMSX cells that were subjected to UV-irradiation in the presence or absence of antagonistic anti-TNFα mAb (aTNFα) (5 μg/ml) is shown

the antagonistic antibodies to TNFα (Figure 5b). These observations suggest that the sensitization of melanoma cells to UV or actinomycin D by TRAF2ΔN expression, utilize different apoptotic regulatory pathways than the sensitization to anisomycin treatment.

TRAF2ΔN sensitizes HHMSX cells to apoptosis induced by the radiomimetic drug neocarzinostatin (NCS)

Important to the characterization of melanoma sensitization to treatment is to evaluate the effect of radiation, to which late-stage melanoma are notoriously resistant. To this end we used the radiomimetic drug NCS (Peixoto and Andreo, 2000). Treatment of melanoma cells with low doses (<500 ng/ml) did not alter degree of apoptosis (data not shown). At doses of 0.5 μg/ml or higher NCS caused a noticeable degree of apoptosis (9, 30 and 68% apoptosis in response to 0.5, 1 and 4 μg/ml, respectively; Figure 6). Forced expression of TRAF2ΔN efficiently increased the degree of apoptosis (from 9–18; 30–80 and from 68–100%; Figure 6). These finding illustrate the ability to sensitize a highly resistant melanoma cells to the radiomimetic drug NCS upon expression of TRAF2 that lacks the ring finger domain.

Discussion

The present study identifies TRAF2 as a target for sensitizing selective melanoma cells to UV irradiation, radiomimetic and ribotoxic drug treatment. In as much

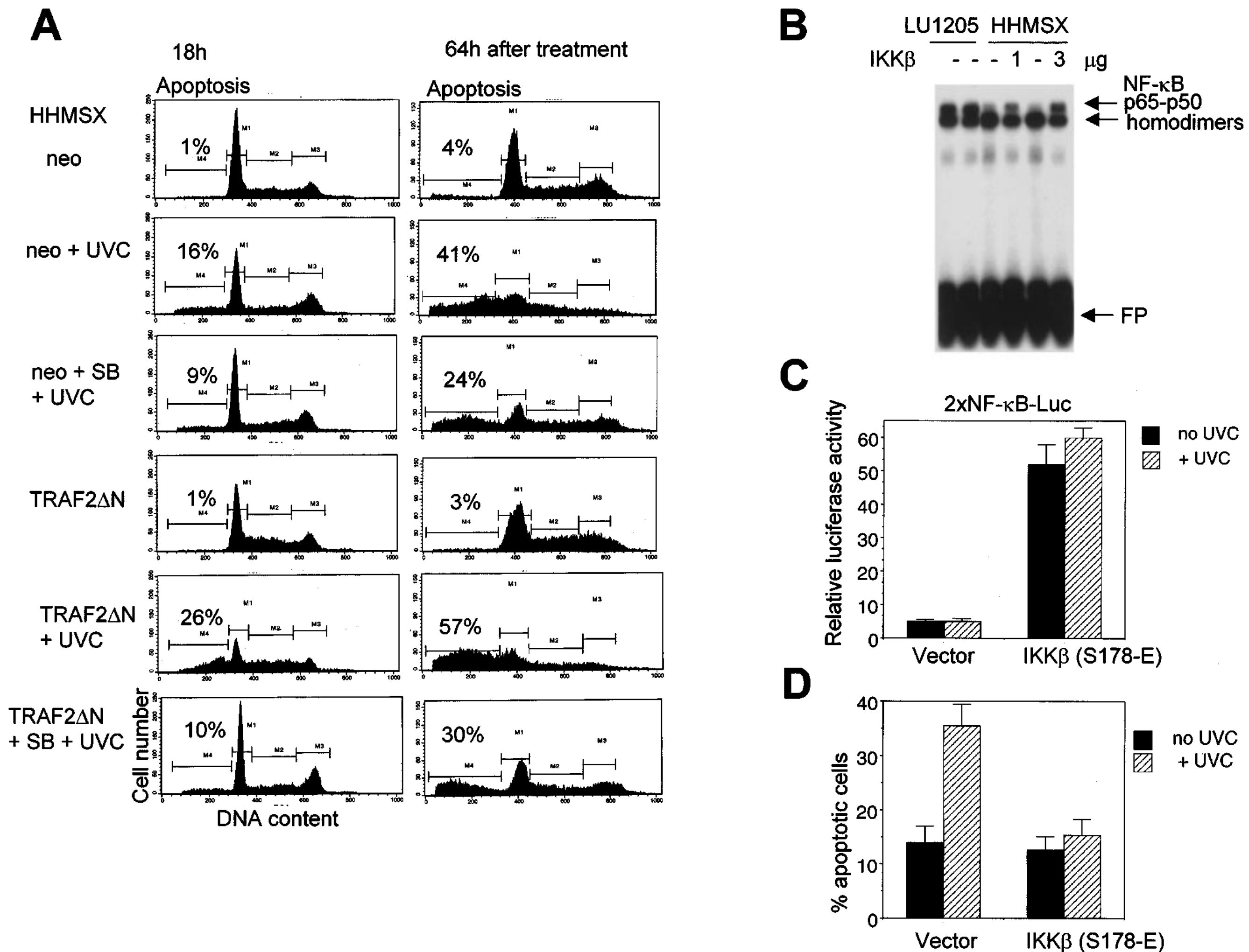

Figure 4 Inhibition of p38 or activation of NF-κB attenuates TRAF2ΔN sensitization of melanoma cells to apoptosis. (**a**) HHMSX-neo and TRAFΔN cells were treated with the pharmacological inhibitor of p38, SB203580 (5 μM) prior to UV-irradiation and changes in the degree of apoptosis was determined 18 and 64 h after irradiation. (**b**) and (**c**), Forced expression of IKKβ^{S178E} in TRAF2ΔN expressing melanoma cells was followed by analysis of NF-κB DNA binding activity by EMSA (**b**) and NF-κB-mediated luciferase reporter activity (**c**). (**d**) Degree of apoptosis in TRAF2ΔN expressing melanoma cells that were co-transfected with the constitutively active IKKβ^{S178E} construct and pGFP is shown

this finding highlights two novel observations. First, we show that altered TRAF2 activity efficiently sensitizes melanoma cells to radiation and thus identifies new target regulation of apoptosis in this tumor type. Second, we demonstrate that sensitization is also seen in response to anisomycin or actinomycin D, thus by identifying that ribotoxic compounds may serve as potentially effective drugs for combination treatment of melanoma tumors. Through the analysis of HHMSX cells we demonstrate that alteration of TRAF2 activities efficiently increase degree of radiomimetic and ribotoxic drug-induced apoptosis. HHMSX was selected out of five melanoma cell lines that we have studied here, of which three exhibit a constitutively high level of TRAF2 expression that is not altered in response to UV-irradiation. These observations coincide with the finding that melanoma cells that exhibit reduced levels of TRAF2 after UV-irradiation are more sensitive and reveal greater degree of cell death in response to UV treatment.

The ability to use ring finger-deleted form of TRAF2 to increase sensitivity to irradiation depends on the related changes in the degree of TNFα expression. Given the role of p38/ATF2 in the regulation of TNFα expression and since TRAF2ΔN increases p38 activities, the elevated expression of TNFα is expected to result in increased expression of TNFR1. Interestingly, TRAF2 deficiency in TRAF2$^{-/-}$ mice was also accompanied by substantial increase of TNFα expression (Yeh *et al.*, 1997; Tyers and William, 1999). Changes in TNFα expression alone may not suffice to increase sensitization to UV-irradiation, since cell ability to resist such treatment also depends on the nature of alternate apoptotic and anti-apoptotic pathways. For these reasons HHMSX, in which expression of Fas is almost undetectable, and in which TNFR1 is

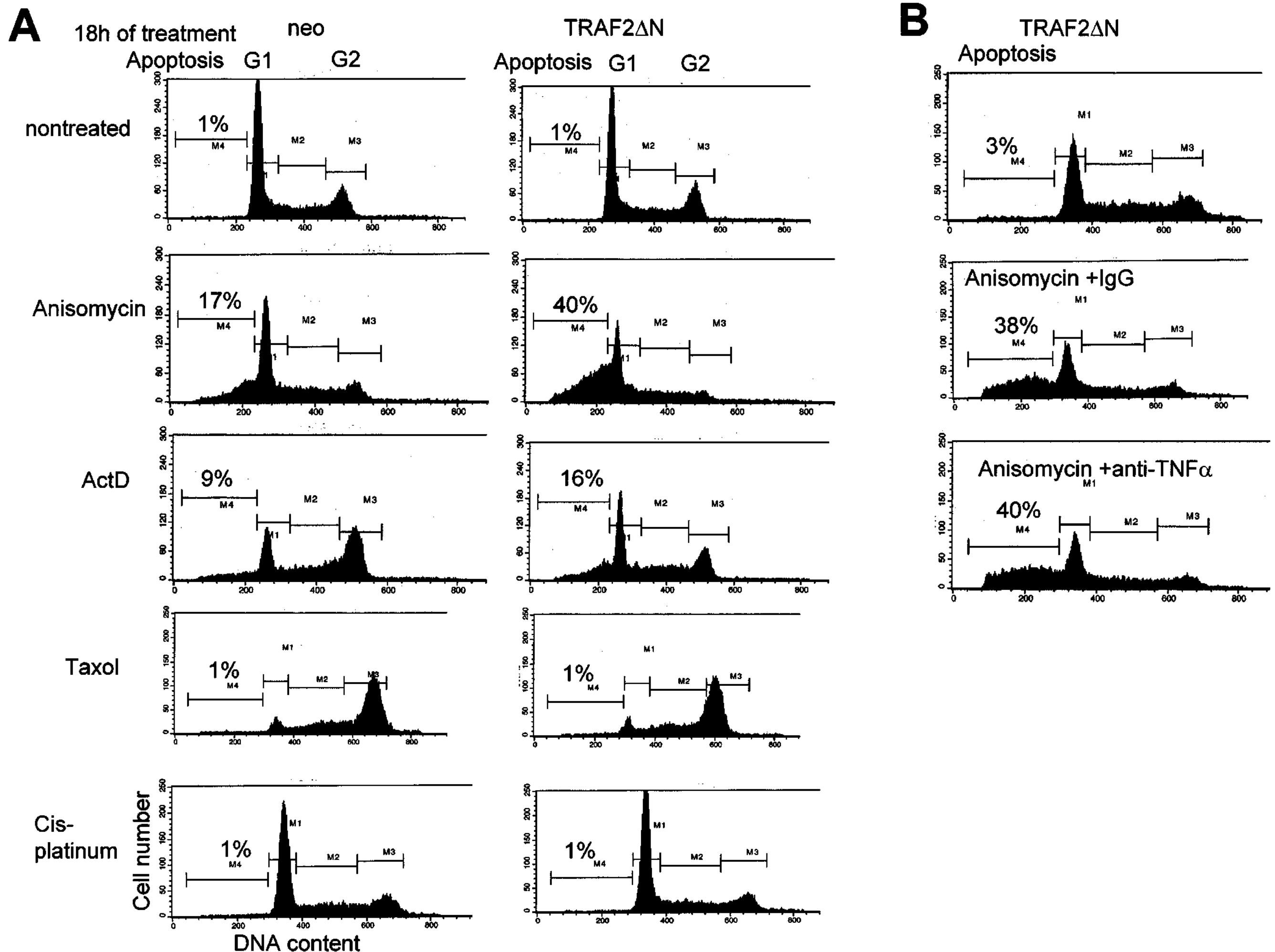

Figure 5 Effect of ribotoxic compounds and chemotherapeutic drugs on apoptosis of TRAFΔN expressing melanoma cells. (**a**) Levels of apoptosis in HHMSX neo and TRAFΔN expressing cells after exposure to anisomycin (10 μg/ml), actinomycin D (5 μM), cis-platinum (1.2 μM) or Taxol (5 μg/ml). In all cases analysis was performed 18 h after treatment. (**b**) Levels of apoptosis in HHMSX/TRAFΔN cells which were pretreated with normal IgG or anti-TNFα Ab before anisomycin treatment

present at moderate levels, serves as an ideal example. The lack of changes in Fas levels was best reflected by the observation that TNF-TNFR serves as the primary apoptotic pathway in response to UV treatment of these melanoma cells. In as much our finding suggest that the analysis of Fas and TNF expression may allow identifying melanomas which may be sensitized to treatment with selective radiomimetic or ribotoxic (anisomycin) drugs, upon modulation of TRAF2 activities.

What are the precise changes elicited by the TRAF2 construct that lacks ring finger are yet to be elucidated. TRAF2 may belong to the growing list of ring finger proteins, which possess E3 ligase activities (Tyers and Jorgensen, 2000). In as much, deletion of the ring would abrogate this activity, while preserving some of other TRAF2 functions. This may explain why TRAF2ΔN expression caused elevated activity of p38 signaling cascade as reported here. TRAF2ΔN ability to activate p38 requires ASK1/2, suggesting that the TRAF2-ASK-p38 pathway is activated upon TRAF2ΔN expression.

Further insight to the possible mechanisms by which TRAF2ΔN sensitizes HHMSX cells to radiomimetic and ribotoxic treatment comes from the finding that both the pharmacological inhibitor of p38 and the constitutively active form of IKKβ efficiently attenuated TRAF2ΔN-mediated sensitivity to UV-treatment. These findings are in line with the increase in p38 activity observed upon TRAF2ΔN expression, with the increased TNFR1 expression by p38 effector, ATF2 (Ivanov and Ronai, 1999), and with the downregulation of NF-βB activities by p38 (Zandi *et al*., 1997). Inhibition of NF-κB activity by adenoviral vector was also reported to sensitize melanoma cells to apoptosis (Bakker *et al*., 1999).

Sensitization of melanoma cells by anisomycin or actinomycin D with altered activities of p38/ATF2, achieved via TRAF2ΔN resembles the ability to sensitize tumor cells to ribotoxic stress using combina-

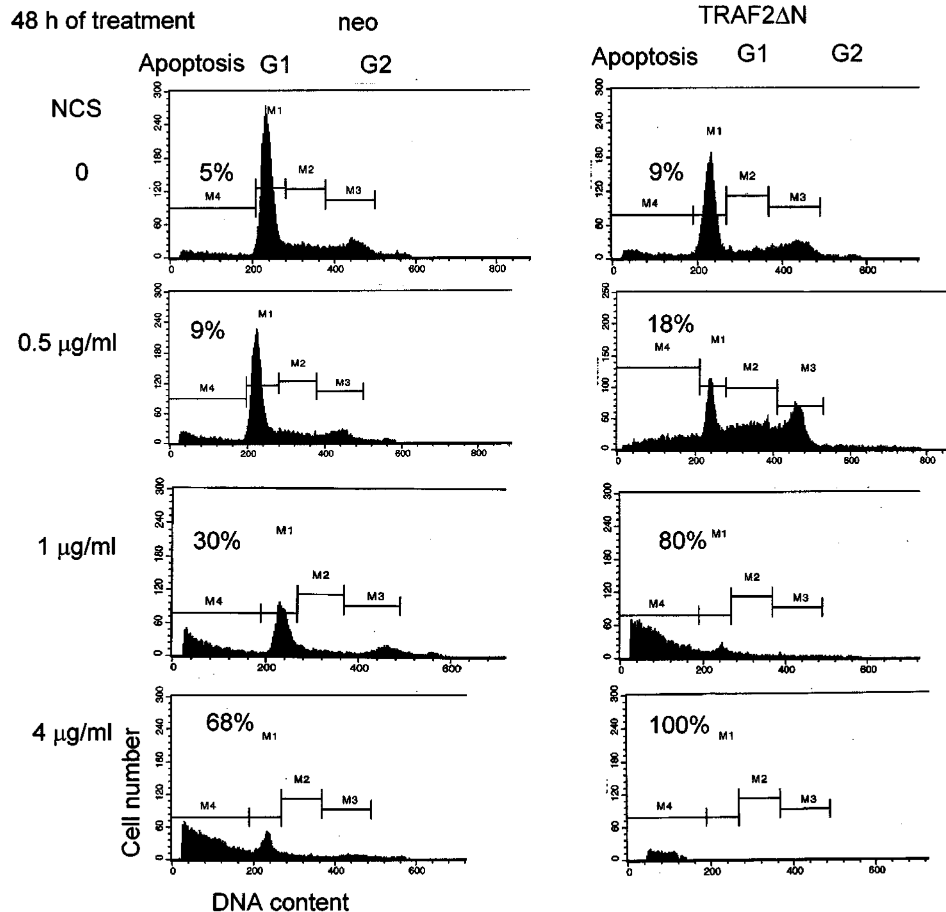

Figure 6 Effect of radiomimetic drug NCS on apoptosis of TRAFΔN expressing melanoma cells. HHMSX cells were subjected to treatment with the indicated concentrations of NCS and degree of apoptosis was determined after 48 h as detailed in Materials and methods

tion of anisomycin, histone deacetylase and cyclin dependent kinase inhibitors both in culture and in nude mice (Ruller *et al*., 1999). Given that alteration of apoptosis represents one of the crucial targets in tumor development, identifying additional targets, as shown here for TRAF2, points to new means one may consider for design of therapeutic means to treat this tumor type.

Materials and methods

Cell lines

Human melanoma LU1205 cells were maintained in MCDB153/L15 medium (4 : 1) supplemented with 5% fetal bovine serum, L-glutamine and antibiotics. In addition, medium for WM1552 early-phase human melanoma cells contained insulin (5 μg/ml). FEMX, HHMSX and THX, human melanoma-derived cells are maintained in RPMI1640 medium supplemented with 10% FCS. HHMSX/TRAF2ΔN cells were maintained in the same medium supplemented with G418 (200 μg/ml).

Chemicals

Actinomycin D, cycloheximide, anisomycin and paclitaxel (Taxol) were purchased from Sigma. The radiomimetic drug neocarzinostatin (NCS) was obtained from Kayaku Co. (Tokyo, Japan). The inhibitor of p38 (SB203580) was purchased (Calbiochem).

Stable transfection and selection

pcDNA3-neo (Invitrogen, Carlsbad, CA, USA) or pcDNA3-FLAG-TRAF2ΔN constructs encoding the truncated form of TRAF2 cDNA without the first 86 aa and (Relaix *et al*., 1998) were electroporated (230 V, 1050 μF) into HHMSX cells as previously described (Ronai *et al*., 1998). HHMSX-neo control and and HHMSX/TRAF2ΔN cell lines were created as a mixed population of G418-resistant clones.

Transient transfection and luciferase assay

Transient transfection of reporter constructs (0.75 μg) [$2\times$NF-κB-Luc, $5\times$Jun2tk-Luc, $5\times$TRE-tk-Luc and vector tk-Luc (van Dam *et al*., 1998), −615 TNF-Luc and its mutated variants (Rhoades *et al*., 1992)] together with the indicated expression vectors and pCMV-βgal (0.25 μg) into 5×10^5 LU1205 melanoma cells was performed using

Lipofectamine (GIBCO–BRL). Luciferase activity was determined using the Luciferase assay system from Promega (Madison, WI, USA). Values were normalized based on β-galactosidase levels. Constitutive active forms of IKK were previously described (Zandi *et al*., 1997).

Treatment and apoptosis studies of melanoma cells

Cells were exposed to UVC at indicated doses as previously described (Ivanov and Ronai, 1999). Cycloheximide (10 μg/ml; Sigma, St. Louis, MO, USA), SB203580 (1–10 μM) (Calbiochem, San Diego, CA, USA) and recombinant TNFα (10 ng/ml) (Pharmingen, San Diego, CA, USA) were used to treat melanoma cells. Antagonistic monospecific antibodies against human Fas (clone G254–274) and against TNFα (clone Mab1; Pharmingen, San Diego, CA, USA) or TNFR1 (R&D Systems, Minneapolis, MN, USA) were added at final concentrations of 1–10 μg/ml.

Apoptosis analysis was performed by flow cytometry of PI-stained cells as described previously (Nicoletti *et al*., 1991). The percentage of cells to the left of the diploid $G_{0/1}$ peak, characteristic of hypodiploid cells that have partially lost DNA, was calculated as percentage of apoptotic cells and is referred to in Results as per cent of apoptosis. Melanoma cells (5×10^5) were transiently cotransfected by expression vectors together with marker plasmid encoding Green Fluorescent Protein, pGFP (1 and 0.25 μg, respectively) using Lipofectamine (GIBCO–BRL). DNA fragmentation analysis of GFP positive cells was performed using cells fixed in 1% paraformaldehyde, treated with 70% ethanol, washed with PBS and stained with PI. Surface expression of Fas was determined using anti-Fas-PE Ab (PharMingen, CA, USA) and flow cytometric analysis.

Western blotting analysis

Cell lysates (50–100 μg protein) were resolved on 10% SDS–PAGE, transferred to nitrocellulose and processed according to the standard manual. The polyclonal Abs used were against p38 and phospho-p38 (Thr180/Tyr182), ATF2 and phospho-ATF2 (Thr71) from New England BioLabs. Polyclonal anti-TRAF2 and anti-BclXL antibodies were purchased from Santa Cruz Biotechnology. The primary Abs were used at dilution 1:1000 to 1:3000. The secondary Abs were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5000). Signals were detected using the ECL system (Amersham, Arlington Heights).

Kinase assay

Protein kinase assays were carried out using a fusion protein, GST-ATF2, as a substrate as previously described (Fuchs *et al*., 1998). Briefly, the GST-ATF2 fusion proteins (0.5 μg/assay) were bound to glutathione-sepharose beads before addition of melanoma proteins as the source of p38/JNK (10 μg/assay) in the presence of kinase buffer (20 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM NaF, 1 mM $NaVO_3$, 50 mM NaCl) at room temperature for 15 min. The beads were pelleted and washed extensively with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% β-MeOH, 0.2 mM phenylmethylsulfonyl fluoride and 5 mM benzamidine) before incubation with [γ-^{32}P]ATP (50 c.p.m./fmol; Amersham) in the presence of kinase buffer. Following extensive washing, the phosphorylated ATF2 was boiled in SDS sample buffer and the eluted proteins were run on a 15% SDS-polyacrylamide gel. The gel was dried and phosphorylation of the ATF2 substrate was determined by autoradiography, followed by quantification with a phosphorimager (BioRad).

Abbreviations

AP1: activator protein-1; ATF2: activating transcription factor 2; NF-κB: nuclear factor kappa B; IKK: inhibitor nuclear factor kappa B kinase; PI: propidium iodide; TNFα: tumor necrosis factor alpha; TNFR: tumor necrosis factor receptor; TRAF2: tumor necrosis factor receptor associated factor 2; GFP: green fluorescent protein; IκB: inhibitor NF-κB; MAPK: mitogen-activated protein kinase; MFI: medium fluorescence intensity; MKK: MAPK kinase; CHX: cycloheximide; NCS: neocarzinostatin.

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