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Focal Adhesion Kinase and Protein Kinase B Cooperate to Suppress Doxorubicin-Induced Apoptosis of Breast Tumor Cells

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ABSTRACT

Focal adhesion kinase (FAK) is up-regulated in a variety of cancers, including breast cancer, in association with poor disease prognosis. In the present study, we examined the role of FAK in the control of anticancer drug-induced apoptosis of mammary adenocarcinoma MTLn3 cells. Doxorubicin caused the formation of well defined focal adhesions and stress fibers early after treatment, which was later followed by their loss in association with the onset of apoptosis. Phosphorylation of FAK on tyrosine 397 decreased only during the onset of doxorubicin-induced apoptosis in a Bcl-2 and caspase-independent manner. Doxorubicin also caused an early activation of protein kinase B (PKB). Expression of the dominant-negative acting focal adhesion kinase-related nonkinase (FRNK) sensitized MTLn3 cells to apoptosis caused by doxorubicin. FRNK inhibited the doxorubicin-induced activation of PKB. In addition, inhibition of phosphatidylinositide-3 (PI-3) kinase with wortmannin inhibited the activation of PKB by doxorubicin. Both wortmannin and transient overexpression of the dual lipid/protein phosphatase and tensin homolog deleted on chromosome 10 enhanced doxorubicin-induced cell death. Altogether, these data fit with a model wherein FAK is involved in the doxorubicin-induced activation of the PI-3 kinase/PKB signaling route, thereby suppressing the onset of apoptosis caused by doxorubicin.

Adhesion to the ECM is essential for normal functioning of epithelial cells and provides survival signaling cues (Giancotti and Ruoslahti, 1999; Frisch and Screaton, 2001). In normal cells, detachment generally results in apoptosis, also termed anoikis (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997). However, tumor cells are often resistant to anoikis. This resistance is linked to constitutive activation of survival signaling pathways by oncogenic transformation that otherwise depend on cell adhesion (Streuli and Gilmore, 1999; McFall et al., 2001). These survival signals may confer resistance not only to anoikis but also to apoptosis induced by anticancer drugs due to an overlap in signaling pathways that control cell death. Indeed, adhesion to ECM proteins via the β1 integrin subunit protected small cell lung cancer cells against doxorubicin- and etoposide-induced apoptosis and protected breast cancer cells against paclitaxel-induced apoptosis (Sethi et al., 1999; Aoudjit and Vuori, 2001). This dysregulation of cell-ECM interaction-mediated signaling in tumor cells followed by the suppression of or resistance to apoptosis may well contribute to metastasis formation. Likewise, metastatic tumor cells may already be (partly) resistant to anticancer agent-induced apoptosis. Therefore, it is important to know how focal adhesion-dependent signaling is linked to protection against anticancer agent-induced apoptosis.

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ABBREVIATIONS: ECM, extracellular matrix; AV, Annexin V; CLSM, confocal laser scanning microscopy; FAK, focal adhesion kinase; FRNK, focal adhesion kinase-related nonkinase; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; Neo, neomycin-resistant cells; PI, propidium iodide; PI-3, phosphatidylinositide-3; PI-3K, phosphatidyl inositol 3-kinase; PY, phosphotyrosine; zVAD-fmk, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone; PKB, protein kinase B; α-MEM, α-modified minimal essential medium with ribonucleosides and deoxyribonucleosides; FBS, fetal bovine serum; APC, allophycocyanin; PBS, phosphate-buffered saline; RT, room temperature; PKCδ, protein kinase Cδ; TBP, bovine serum albumin and Triton X-100 in phosphate-buffered saline; HA, hemagglutinin; PIP_3, phosphatidylinositol 3,4,5-triphosphate; ROS, reactive oxygen species; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TSE+, Tris-HCl, sucrose, and EGTA containing dithiothreitol, leupeptin, aprotinin, sodium vanadate, sodium fluoride, and phenylmethylsulfonyl fluoride; FAT, fatty adhesion targeting; Y27632, *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride.

Adhesion to the ECM occurs through the extracellular domain of integrins, resulting in the recruitment and activation of several focal adhesion-associated proteins to the intracellular domains. One of these proteins is focal adhesion kinase (FAK), which, upon binding to the integrins, is autophosphorylated on tyrosine residue 397 (Schaller et al., 1994). This phosphorylation recruits and activates Src, and together, these two tyrosine kinases induce a cascade of tyrosine phosphorylations that regulate the activity and interactions of several adapter and signaling proteins at the focal adhesions (Schlaepfer et al., 1994), including paxillin, p130Cas, and Grb2 (Tachibana et al., 1997; Thomas et al., 1999). In addition, the FAK autophosphorylation domain interacts with phosphatidylinositide-3 kinase (PI-3K), which plays a central role in the activation of several downstream signaling pathways, including survival signaling (see below). Several reports indicate that FAK is involved in the survival signaling generated by adhesion to the ECM.

Amplification of the FAK gene has been observed in several tumor types, and the increase in FAK expression is associated with an increase in metastatic potential (Owens et al., 1995; Agochiya et al., 1999). This may be associated with protection against anoikis because experimental expression of constitutively active FAK decreased anoikis and enhanced tumor formation (Frisch et al., 1996). Conversely, inhibition of FAK function by FAK antisense oligonucleotides, microinjection of FAK antibodies, or expression of the C-terminal domain of FAK, which competes with FAK for focal adhesion localization, results in detachment and apoptosis (Hungerford et al., 1996; Xu et al., 2000). Moreover, FAK is cleaved by caspases during apoptosis induced by a variety of stimuli (Wen et al., 1997; van de Water et al., 2001); the resulting C-terminal domain of FAK resembles FAK-related nonkinase (FRNK) and acts as a dominant inhibitor of FAK, thereby further blocking FAK-mediated antiapoptotic signaling (Wen et al., 1997; Gervais et al., 1998). Although much is known about the role of FAK in the control of cell survival in general, little information is available on the potential role of the mechanism by which FAK interferes with the apoptosis caused by the anticancer drug doxorubicin in tumor cells.

A major survival signaling route is mediated by the activation of PI-3 kinase. Activation of PI-3 kinase results in the generation of the second messengers PI(3,4,5)P3 and PI(3,4)P2. These phospholipids recruit protein kinase B (PKB or Akt) to the plasma membrane, and subsequently, PKB is phosphorylated by phosphoinositide-dependent kinase. Activated PKB provides survival signaling by inactivation of a series of proapoptotic proteins, such as p21WAF, FKHR, Bad, and glycogen synthase kinase 3 (Datta et al., 1997). This PI3-kinase/PKB survival route can be inhibited by the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which dephosphorylates both PIP_3 lipids and the protein kinases Shc and FAK (Di Cristofano and Pandolfi, 2000). So far, little is known about the relationship between FAK and the PI-3 kinase/PKB route in the control of cytostatic-induced apoptosis of tumor cells. Because our preliminary experiments indicated that doxorubicin caused activation of the PKB signaling pathway, we further determined the potential relationship between FAK function and PKB-mediated survival signaling.

To study the relationship between FAK and the PI-3K/PKB pathway in the control of doxorubicin-induced apoptosis, we used the rat mammary adenocarcinoma cell line MTLn3. These cells are often used as a model to study molecular mechanisms of metastasis formation (Kiley et al., 1999) and responses to drug therapy both in vitro and in vivo (Welch et al., 1983; Huigsloot et al., 2001). We have characterized previously in detail the induction of apoptosis by doxorubicin in these cells (Huigsloot et al., 2001, 2002). Our current combined observations demonstrate that doxorubicin causes the early formation of stress fibers and focal adhesions in association with activation of PKB. Pharmacological and molecular biological approaches indicate that PKB and FAK cooperate to suppress doxorubicin-induced apoptosis.

Materials and Methods

Chemicals. α-Modified minimal essential medium with ribonucleosides and deoxyribonucleosides (α-MEM), fetal bovine serum (FBS), penicillin/streptomycin, Lipofectamine Plus, and Geneticin (G418 sulfate) were from Life Technologies (Rockville, MD). Collagen (type I, rat tail) was from Upstate Biotechnology (Lake Placid, NY). Doxorubicin, etoposide, propidium iodide (PI), and Rnase A were from Sigma (St. Louis, MO). Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) was from Bachem (Bubendorf, Switzerland). Annexin V was from Boehringer Mannheim (Basel, Switzerland). Hoechst 33258 was from Molecular Probes (Leiden, The Netherlands). Allophycocyanin (APC) was from Prozyme (San Leandro, CA). All other chemicals were of analytical grade.

Cell Culture. MTLn3 rat mammary adenocarcinoma cells were originally developed by Dr. D. R. Welch (Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, PA) and were used between passages 48 and 58. MTLn3 cells overexpressing Bcl-2 or empty vector (Bcl-2 and Neo cells, respectively) were obtained and characterized as described previously (Huigsloot et al., 2002). All cell lines were cultured in α-MEM supplemented with 5% (v/v) FBS (complete medium). For experiments, cells were plated at a density of 4×10^3 cells/cm^2 in Corning plates (Acton, MA) and grown for 3 days in complete medium supplemented. For transient transfection, subconfluent cells were transfected with pEGFP, pEGFP-FRNK, pEFGP-FAT, or GFP-PTEN wild type, GFP-PTEN mutant using Lipofectamine Plus reagents. tetFRNK-MTLn3 cells were created as described previously (van Nimwegen et al., 2005). Exposure to doxorubicin occurred 24 h after transfection. Cells were exposed to doxorubicin for 1 h in Hanks' balanced salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 25 mM HEPES, and 5 mM D-glucose, pH 7.4). After removal of doxorubicin, cells were allowed to recover in α-MEM containing 2.5% (v/v) FBS and penicillin/streptomycin for the indicated periods. In some experiments, cells were recovered in α-MEM containing 2.5% (v/v) FBS, penicillin/streptomycin, and 100 μM zVAD-fmk.

Determination of Cell Death. For Annexin V/propidium iodide (AV/PI) staining, cells were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 2H_2O$, and 1.4 mM KH_2PO_4, pH 7.4) containing 1 mM EDTA (PBS-EDTA) and subsequently trypsinized with 0.13 g/l trypsin in PBS-EDTA. Medium, washes, and cells were combined, centrifuged (5 min, 200*g*, 4°C), and the pellet was washed once with PBS-EDTA. Cells were allowed to recover from trypsinization in complete medium (30 min, 37°C). Externalized phosphatidylserine was labeled (15 min, 0°C) with APC-conjugated Annexin V in AV-buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, and 1.8 mM $CaCl_2 \cdot 2H_2O$, pH 7.4). Propidium iodide (2 μM) in AV-buffer was added 1 min before analysis by flow cytometry on a FACScalibur (BD Biosciences, Heidelberg, Germany).

For cell cycle analysis, trypsinized and floating cells were pooled, washed with PBS-EDTA, and fixated in 70% (v/v) ethanol (24 h, −20°C). After two washes with PBS-EDTA, cells were incubated

with PBS-EDTA containing 50 μg/ml Rnase A and 7.5 μM PI (45 min, RT) and subsequently analyzed by flow cytometry.

Immunoblotting. Attached cells were scraped in ice-cold TSE+ (10 mM Tris-HCl, 250 mM sucrose, and 1 mM EGTA, pH 7.4, containing 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Floating cells in the medium and in one wash of PBS were pelleted (5 min, 200*g*, 4°C) and pooled with scraped cells in TSE+. The protein concentration in the supernatant was determined using the BioRad protein assay using IgG as a standard. Fifteen micrograms of total cellular protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Etten Leur, The Netherlands). Blots were blocked with 5% (w/v) nonfat dry milk in 0.5 M NaCl, 20 mM Tris-HCl, and 0.05% v/v Tween 20, pH 7.4, and probed for active caspase-3 (CM-1; kindly provided by Dr. A. Srinivasan, DUN Pharmaceuticals, La Jolla, CA), PY (4G10; Upstate Biotechnology), PY^{397} FAK (polyclonal; Biosource Europe S.A., Nivelles, Belgium), FAK N-terminal antibody (77; BD Biosciences), FAK C-terminal antibody (polyclonal; Upstate Biotechnology), or PKCδ (δ14K; kindly provided by Dr. S. Jaken, Eli Lilly, Indianapolis, IN), PTEN (BD BioSciences, Heidelberg, Germany), PKB (polyclonal; Cell Signaling Technology, Beverly, MA), and $P\text{-}Ser^{473}$-PKB (polyclonal; Cell Signaling Technology), followed by incubation with secondary antibody containing horseradish peroxidase and visualization with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Uppsala, Sweden). For all Western blotting experiments, equal protein loading was verified by Ponceau S (blots) and Coomassie staining (gels) unless otherwise stated in the figure legends.

Immunocytochemistry. Cells were cultured on 12-mm collagen-coated glass coverslips and fixated in 4% (w/v) formaldehyde in PBS. Coverslips were blocked in TBP [0.5% (w/v) BSA and 0.1% (v/v) Triton X-100 in PBS, pH 7.4] (1 h, RT) and subsequently incubated with primary antibody in TBP (1 h at RT or overnight at 4°C). Coverslips were washed three times in TBP and incubated with Alexa488, Cy3-, or Cy5-conjugated secondary antibodies or 0.2 U/ml rhodamine 123-conjugated phalloidin (Molecular Probes, Eugene, OR) in TBP (1 h, RT). After washing with TBP, coverslips were incubated with 2 μg/ml Hoechst 33258 in PBS (15 min, RT), washed in PBS, and mounted in Aqua PolyMount (Polysciences, Warrington, PA). Primary antibodies used were P-Tyr (4G10, Upstate Biotech-

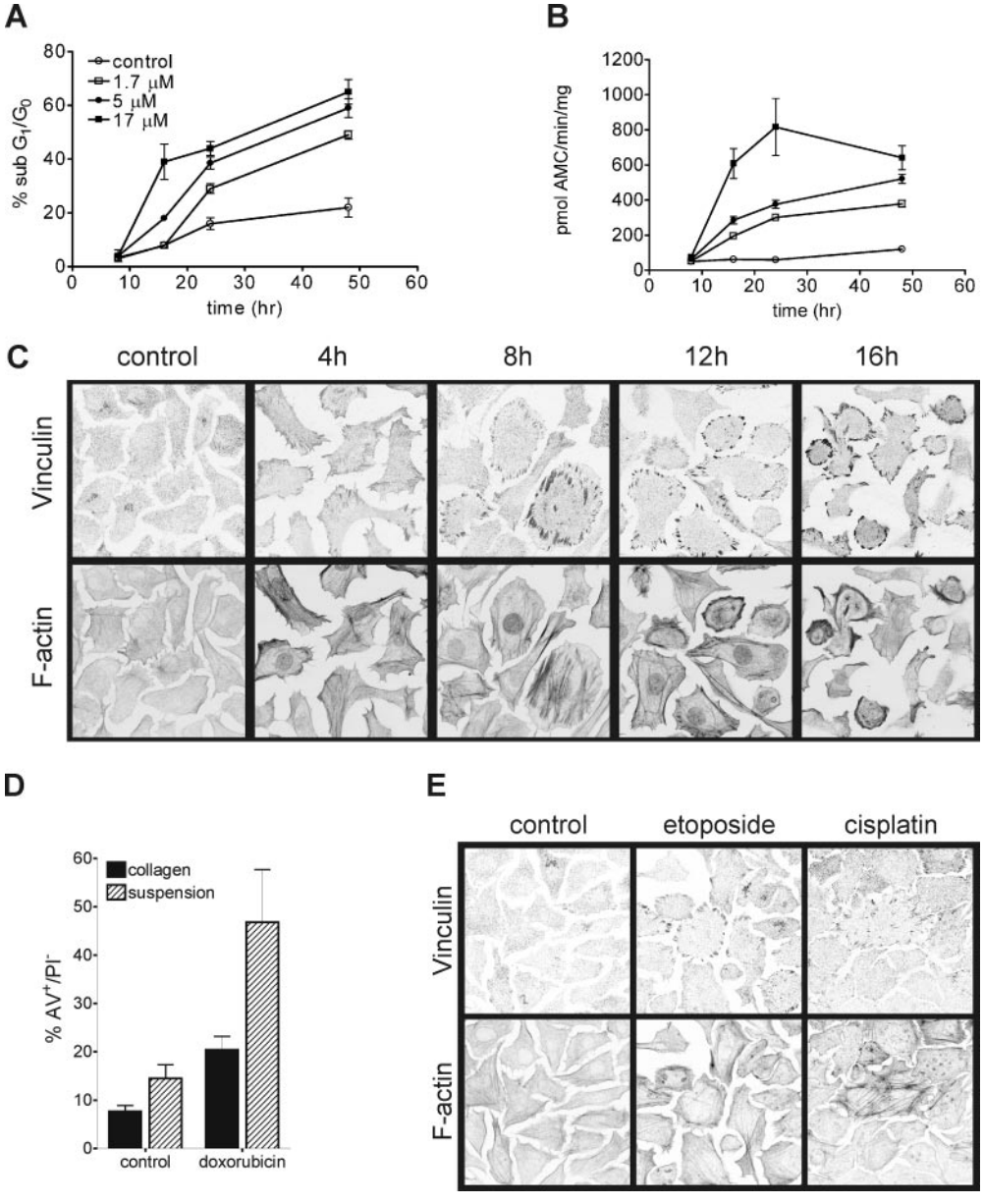

Fig. 1. Doxorubicin-induced focal adhesion formation occurs before caspase-3 activation and apoptosis. A, MTLn3 cells were exposed to vehicle (control) or different concentrations doxorubicin for 1 h and allowed to recover. At the indicated time points, cells were fixated, and the percentage of apoptosis was determined by flow cytometric analysis of cellular DNA content as described under *Materials and Methods*. B, caspase-3 activity was determined as described under *Materials and Methods*. C, attached cells were immunostained for vinculin and F-actin and pictures were taken using CLSM. MTLn3 cells were exposed to vehicle or 10 μM doxorubicin while kept in suspension or allowed to attach to collagen. D, after 8 h, the percentage of apoptosis was determined by flow cytometric analysis of cellular DNA content. E, MTLn3 cells were exposed to vehicle, 100 μM etoposide, or 100 μM cisplatin and immunostained for vinculin and F-actin, and pictures were taken using CLSM. Data shown (A, B, and D) are the mean of three independent experiments ± S.E.

nology) and vinculin (Sigma). Imaging occurred using a Bio-Rad Radiance 2100 MP confocal laser scanning system (Bio-Rad, Hercules, CA) equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60× Nikon objective (Nikon, Tokyo, Japan).

Statistical Analysis. Student's t test was used to determine whether there was a significant difference between two means ($p < 0.05$). When multiple means were compared, significance was determined by one-way analysis of variance ($p < 0.05$).

Results

Doxorubicin Causes Focal Adhesion Formation before Apoptosis. Treatment of the rat mammary adenocarcinoma MTLn3 cell line with the cytostatic doxorubicin resulted in a time- and concentration-dependent increase in apoptosis and activation of caspase-3 (Fig. 1, A and B). Doxorubicin-treated cells appeared more rounded up, indicative for cytoskeleton-mediated cell contraction before cell death. Doxorubicin increased the number of F-actin stress fibers (Fig. 1C), which was associated with more concentrated focal adhesions, as indicated by staining for the focal adhesion-associated protein vinculin (Fig. 1C). The F-actin organization and focal adhesion formation occurred early, between 8 and 12 h after exposure of the cells; after 16 h, these stainings were less pronounced, although these cells did not show signs of apoptosis (i.e., either positive for active caspase-3 and/or a fragmented nucleus). Because the onset of doxorubicin-induced apoptosis was associated with the loss of focal adhesions and F-actin stress fibers, abrogation of survival signaling generated by cell-ECM interaction may increase the effectiveness of doxorubicin-induced apoptosis. Indeed, when cells were kept in suspension, the extent of apoptosis caused by doxorubicin was strongly increased compared with cells allowed to attach to collagen (Fig. 1D). Next, we studied whether this effect of doxorubicin was more general. For this purpose, MTLn3 cells were treated with either etoposide or cisplatin, two cytostatics that cause apoptosis after 16 h in MTLn3 cells, a timeframe similar to that of doxorubicin (Huigsloot et al., 2001). Both etoposide and cisplatin caused the increased formation of focal adhesions and F-actin stress fiber formation (Fig. 1E).

Doxorubicin Decreases Protein Tyrosine Phosphorylation at Focal Adhesions Independent of Caspase-3 Activity. Our results indicated that cell adhesion to the ECM confers a survival signal to MTLn3 cells exposed to doxorubicin. Therefore, we next investigated whether doxorubicin itself induced a change in cell-ECM-dependent signaling preceding or during apoptosis. Adhesion-dependent signaling at focal adhesions is mediated by a number of tyrosine kinases (Giancotti and Ruoslahti, 1999), and many tyrosine-phosphorylated proteins are localized at focal adhesions. We first investigated the time course of tyrosine phosphorylation (PY) after treatment with doxorubicin. Up to 8 h after exposure to doxorubicin, no major changes in overall tyrosine phosphorylation were observed (Fig. 2A). However, at 16 h, tyrosine phosphorylation was decreased, and at 24 h, little tyrosine phosphorylation remained; the latter coincided with the onset of apoptosis (Fig. 2A, right) (Huigsloot et al., 2001).

To investigate whether the decrease in PY was associated with a loss of tyrosine phosphorylation at the focal adhesions, PY localization was determined by immunofluorescence in combination with F-actin staining (Fig. 2B). In control cells, a large portion of PY staining was located at focal adhesion-like structures at the cell periphery. At 8 h after exposure to doxorubicin, more pronounced focal adhesions were observed compared with control cells; the PY staining was also more intense. The newly formed focal adhesions were predominantly located at the ends of F-actin stress fibers. Because there was no overall increase in tyrosine-phosphorylation (Fig. 2A), the increase in PY staining at the focal adhesions is most likely due to complexation and/or concentration of tyrosine phosphorylated proteins at these signaling sites. At 16 h, cells were rounded up, and focal adhesions had become much smaller and less numerous, whereas F-actin fibers had

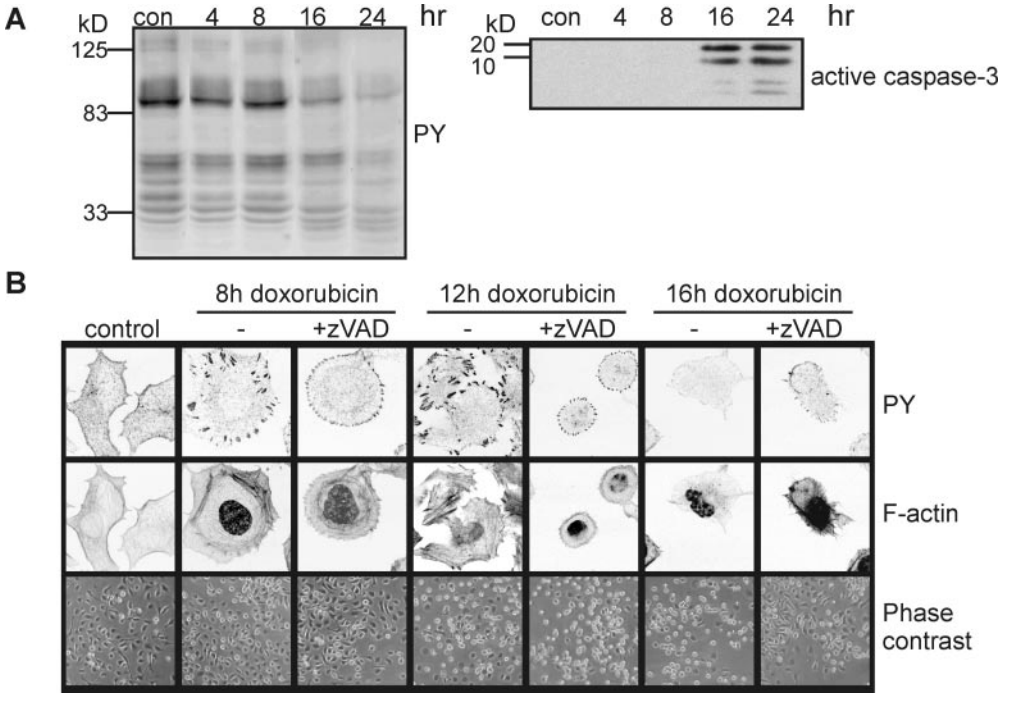

Fig. 2. Doxorubicin causes a decrease in tyrosine phosphorylation at focal adhesions concomitant with the activation of caspase-3. MTLn3 cells were exposed to vehicle (Con) or 17 μM doxorubicin for 1 h and allowed to recover for the indicated period before fixation. Cell lysates were immunoblotted against phosphotyrosine (A, left) and active caspase-3 (A, right). B, MTLn3 cells were fixated and stained for phosphotyrosine and F-actin. Where indicated, zVAD-fmk (100 μM) was added during the recovery of the exposed cells. Immunofluorescence was evaluated by CLSM, and phase-contrast images are included. Data shown are representative of three independent experiments.

disappeared. Hoechst staining indicated that the nuclei of these (attached) cells still seemed normal (data not shown). Inhibition of caspase-3 activity by the addition of the pan-caspase inhibitor zVAD-fmk inhibited doxorubicin-induced apoptosis (data not shown; Huigsloot et al., 2002) but could not prevent rounding of the cells or the disappearance of the large focal adhesions after 16 h (Fig. 2B).

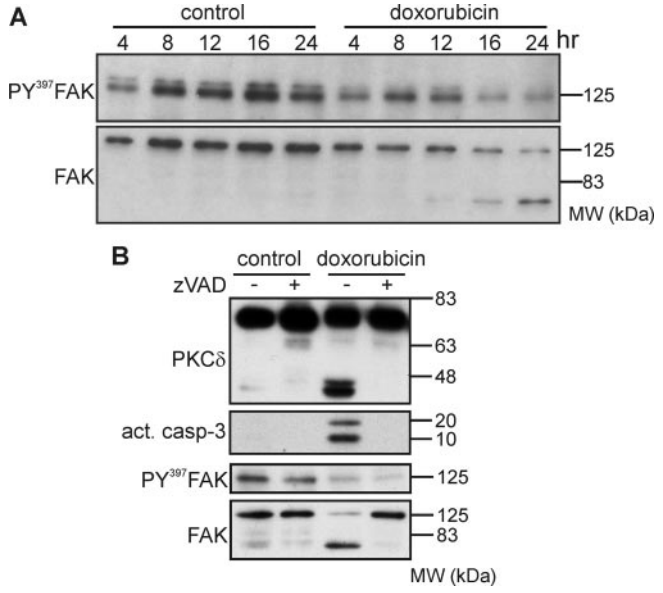

Fig. 3. Doxorubicin causes FAK dephosphorylation independent of caspase activation. MTLn3 cells were treated as described in Fig. 2 for the indicated period (A) or for 24 h (B), and where indicated, zVAD-fmk (100 μM) was added during the recovery period. Immunoblots were stained with antibodies directed against PY^{397}-FAK, FAK, PKCδ, and the active cleavage fragments of caspase-3 (act. casp-3). Data shown are representative of three independent experiments.

Doxorubicin-Induced Dephosphorylation of FAK Is Independent of Cleavage by Caspases. FAK is an important tyrosine-phosphorylated protein that localizes at focal adhesions and is involved in survival signaling. The most essential site of tyrosine phosphorylation on FAK is the autophosphorylation site Tyr^{397}, which is required for both kinase activity and the interaction with other signaling proteins (Schaller et al., 1994). Using a phosphorylation state-specific antibody, we found that doxorubicin caused dephosphorylation of Tyr^{397}-FAK after 16 and 24 h (Fig. 3A). The expression level of FAK decreased after 16 and 24 h, which was associated with the formation of a 75-kDa band. The formation of this cleavage product is most likely mediated by caspases, which are activated in a similar time course (Fig. 2A, bottom) (Huigsloot et al., 2001). The dephosphorylation and caspase-3-mediated proteolysis of FAK may be difficult to separate and may be cell stress-dependent (Kabir et al., 2002). Using zVAD-fmk, we excluded the possibility that the decrease in Tyr^{397} phosphorylation was caused by caspase-mediated cleavage of FAK. zVAD-fmk effectively inhibited apoptosis (Huigsloot et al., 2001) and caspase activation upon exposure of MTLn3 cells to doxorubicin; no active caspase-3 and PKCδ cleavage fragments were formed (Fig. 3B). zVAD-fmk also inhibited the cleavage of FAK, indicating that formation of the cleavage fragment of FAK was indeed due to a caspase-dependent process. Despite the inhibition of apoptosis and FAK cleavage, a decrease in tyrosine phosphorylation of FAK still occurred.

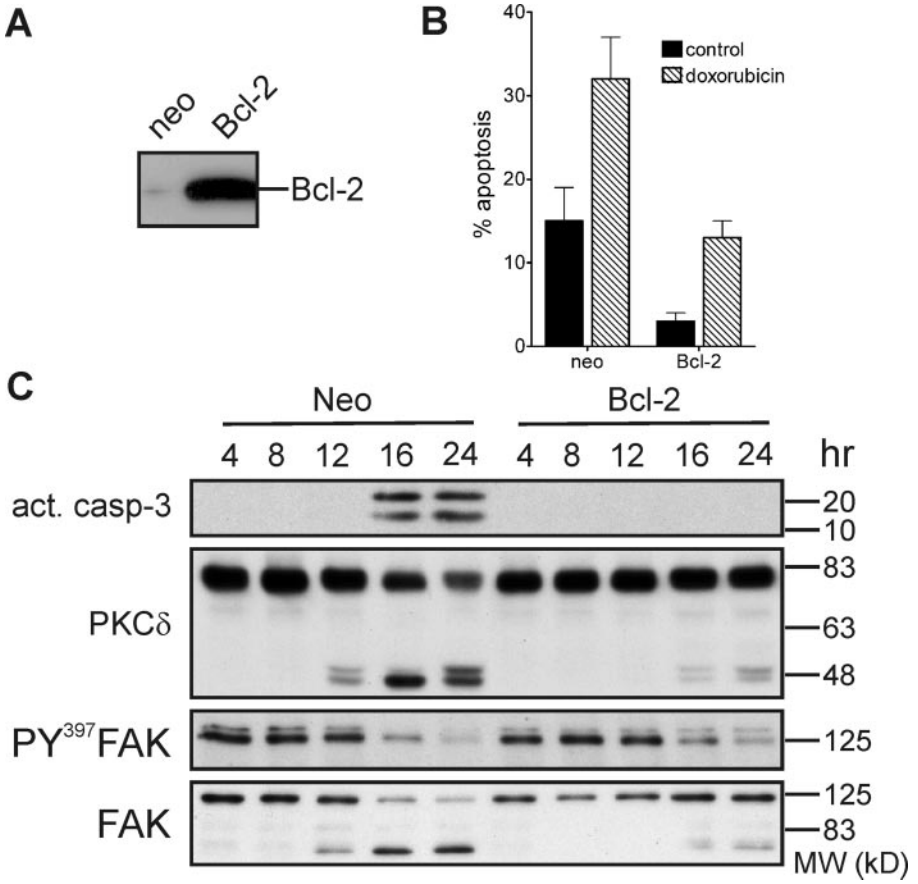

Fig. 4. Doxorubicin-induced FAK dephosphorylation occurs independent of the Bcl-2 control point of apoptosis. A, cell lysates of neomycin-resistant (Neo) and Bcl-2-overexpressing MTLn3 clones (Bcl-2) were immunoblotted against Bcl-2. Neo and Bcl-2 cells were exposed to 17 μM doxorubicin for one h and allowed to recover for the indicated period before fixation. B, apoptosis was evaluated using cell cycle analysis. C, total cell lysates were evaluated for active caspase-3, PKCδ cleavage, and FAK phosphorylation by immunoblotting. Data shown are representative of three independent experiments or the mean of three independent experiments ± S.E.

Because dephosphorylation of FAK occurs upstream of caspase activation, the question was raised whether FAK dephosphorylation occurs upstream of the mitochondrial commitment to apoptosis. Therefore, we used MTLn3 cells overexpressing Bcl-2 (Fig. 4A), which reduced apoptosis from 33% in Neo cells to 11% in Bcl-2 cells 24 h after exposure to 17 μM doxorubicin (Fig. 4B). Bcl-2 overexpression also prevented the activation of caspases by doxorubicin and the cleavage of PKCδ (Fig. 4C). It is noteworthy that FAK cleavage was also inhibited by Bcl-2 (Fig. 4C). In agreement with the persistence of dephosphorylation in the case of caspase-inhibition by zVAD-fmk, inhibition of caspase activation by Bcl-2 overexpression did not prevent the dephosphorylation of FAK occurring 16 h after exposure; FAK levels in Bcl-2 cells did not decrease at these time points. Together, these data suggest that dephosphorylation of FAK occurs upstream of the Bcl-2 checkpoint at the level of the mitochondria and independent of caspase activation and may contribute to the commitment to apoptosis in MTLn3 cells.

Loss of FAK Function by Expression of eGFP-FRNK Sensitizes MTLn3 Cells to Doxorubicin-Induced Apoptosis. Next, we further investigated the role of FAK in the protection against doxorubicin-induced apoptosis. For this purpose, we interfered FAK function by expression of the FRNK and the FAT domain of FAK. eGFP-FAK and eGFP were included as controls. At 24 h after transfection, eGFP was located diffusely throughout the cell, whereas the other constructs localized mainly at focal adhesions (data not shown; van de Water et al., 2001). To test whether FAK is required for cell survival of MTLn3 cells, we determined whether the expression of eGFP-FAK, eGFP-FRNK, or eGFP-FAT induced apoptosis. At 40 h after transfection, eGFP-positive cells were selected, and apoptosis was determined in these cells by additional staining with APC-coupled Annexin V and propidium iodide. Expression of eGFP-FRNK alone was sufficient to increase the percentage of Annexin V^+/PI^- (i.e., apoptotic) cells to 18%, whereas eGFP-FAT increased apoptosis to only 14% (Table 1). No significant effect on apoptosis was observed by eGFP-FAK. Next, we examined the effect of abrogation of FAK signaling by the expression of eGFP-FRNK on doxorubicin-induced apoptosis. Here, we used 10 μM doxorubicin, which allows for the identification of a synergistic effect. At 24 h after transfection of the different eGFP constructs, cells were exposed to doxorubicin, and after 16 h, the percentage of apoptotic cells was determined (Table 1). When eGFP-FRNK-transfected cells were exposed to doxorubicin, the percentage of apoptosis was even further increased, suggesting that functional FAK can suppress doxorubicin-induced apoptosis. Expression of eGFP-FAT was less effective, and full-length eGFP-FAK did not protect against doxorubicin, suggesting that FAK-mediated survival signaling was already maximal in MTLn3 cells. The apoptotic effect of eGFP-FRNK was concentration-dependent; only in a subpopulation of MTLn3 cells with high eGFP-FRNK expression increased apoptosis was observed (data not shown). Inhibition of FAK using eGFP-FRNK was not able to potentiate etoposide-induced apoptosis (data not shown). These data suggest that the expression of eGFP-FRNK abrogates survival signaling and thereby increases doxorubicin-induced apoptosis in MTLn3 cells.

TABLE 1

Expression of eGFP-FRNK enhances doxorubicin-induced apoptosis

MTLn3 cells were transiently transfected with eGFP, eGFP-FAK, eGFP-FAT, and eGFP-FRNK and subsequently exposed to 10 μM doxorubicin. Sixteen hours after exposure of the cells, the percentage of apoptotic cells was determined by annexin V/propidium iodide staining followed by flow cytometric analysis.

	Apoptosis	
	Control	Doxorubicin
	%	
eGFP	9.6 (±2.7)	16.2 (±1.6)
eGFP-FAK	10.1 (±2.9)	18.6 (±3.2)
eGFP-FAT	14 (±5.4)	21 (±3.3)
eGFP-FRNK	18.2 (±6.6)	26.7 (±4.1)

Doxorubicin Causes PKB Activation in a FAK-Dependent Manner. To investigate the FAK-mediated downstream-signaling events involved in the control of cell sur-

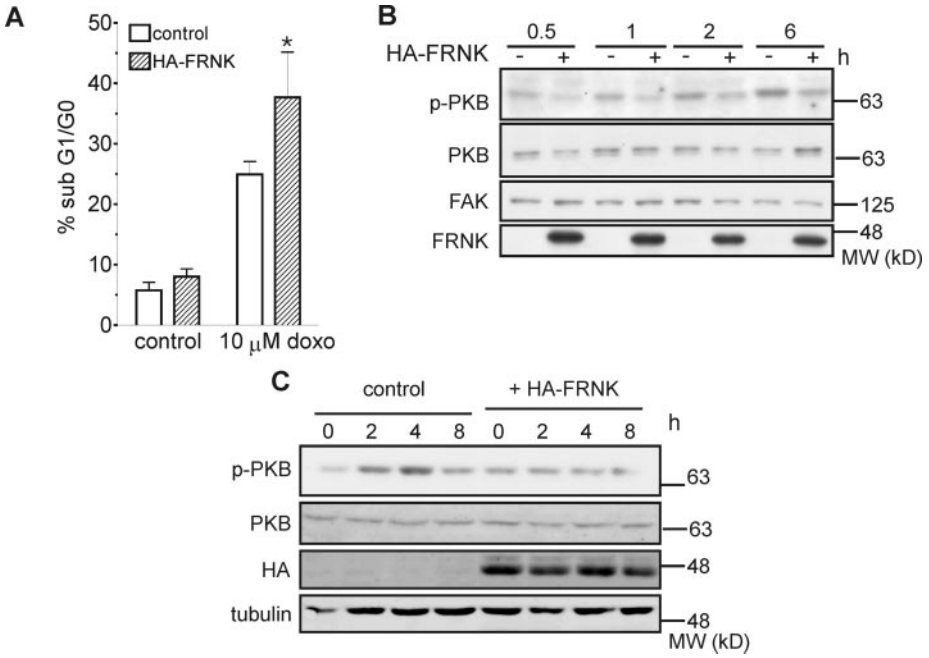

Fig. 5. Conditional expression of FRNK enhances doxorubicin-induced apoptosis and reduces activation of PKB. A, doxycycline-inducible HA-FRNK-expressing MTLn3 cells were treated for 24 h with doxycycline to express HA-FRNK and were exposed subsequently to 10 μM doxorubicin. The percentage of apoptosis was determined by cell cycle analysis. B, HA-FRNK-expressing and control cells were trypsinized, kept in suspension in the absence of serum for 1 h, and plated on collagen-coated plastic culture dishes. At the indicated time points, attached cells were immunoblotted for P-Ser^{473}-PKB (P-PKB), PKB, and FAK. Note the conditional expression of HA-FRNK after doxycycline pretreatment. C, HA-FRNK-expressing and control cells were exposed to 10 μM doxorubicin, and after different periods of recovery, cell lysates were immunoblotted for P-PKB, PKB, HA, and tubulin as a loading control. Data shown are representative for three independent experiments or the mean of three independent experiments ± S.E.

vival of breast tumor cells, we used an MTLn3 cell line that conditionally expresses HA-tagged FRNK in a doxycycline-dependent manner (tetFRNK-MTLn3). Expression of HA-FRNK inhibited endogenous FAK function because it reduced MTLn3 cell attachment, spreading, and cell migration (van Nimwegen et al., 2005). In these cells, expression of HA-FRNK itself did hardly affect cell survival, due to the relatively low expression of this inducible HA-FRNK compared with the transient-transfected eGFP-FRNK. Yet HA-FRNK potentiated the onset of doxorubicin-induced apoptosis (Fig. 5A). We further evaluated the cell adhesion-mediated signaling pathways that are controlled by FAK in MTLn3 cells. Therefore, tetFRNK-MTLn3 cells were allowed to adhere to collagen. We primarily focused on PKB because of its central role in cell-survival signaling. Adhesion of cells to collagen resulted in a rapid activation of protein kinase B (Fig. 5B). Expression of HA-FRNK delayed the attachment-mediated activation of PKB (Fig. 5B). No effect of HA-FRNK expression on the activation of extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase in response to attachment was observed (data not shown).

Next, we studied the relationship between PKB activity and doxorubicin-induced apoptosis. To our surprise, doxorubicin caused an activation of PKB at 2 and 4 h (Fig. 5C, control), which occurred slightly before the formation of stress fibers and focal adhesions. To test whether HA-FRNK also affected the doxorubicin-induced activation of PKB, cells were pretreated with doxycycline followed by exposure to doxorubicin. HA-FRNK expression did not affect the activity of PKB under control conditions (data not shown). However, it is important to note that in the HA-FRNK-expressing cells, PKB was not phosphorylated in response to doxorubicin (Fig. 5C, HA-FRNK). This suggests a role for FAK in the activation of the PI-3 kinase survival pathway in response to doxorubicin exposure of MTLn3 cells.

Inhibition of PI-3 Kinase and Overexpression of PTEN Enhance Doxorubicin-Induced Apoptosis. The above indicated that normal FAK function is required for doxorubicin-mediated activation of PKB. Therefore, we determined the involvement of PKB in the control of doxorubicin-induced apoptosis. For this purpose, we used both pharmacological and molecular biological approaches. Treatment of MTLn3 cells with an inhibitor of PI-3 kinase, wortmannin, completely prevented the phosphorylation of PKB (Fig. 6A). This inhibition of PKB phosphorylation was associated with enhanced apoptosis of MTLn3 cells after doxorubicin treatment, as measured by cell cycle analysis (Fig. 6B). In addition, we used the dual-protein/lipid-phosphatase PTEN as an alternative to prevent PKB activation. PTEN is a tumor suppressor gene that is mutated in various types of cancer, including breast cancer (Wu et al., 2003). PTEN dephosphorylates PIP_3, which is formed after activation of PI-3 kinase.

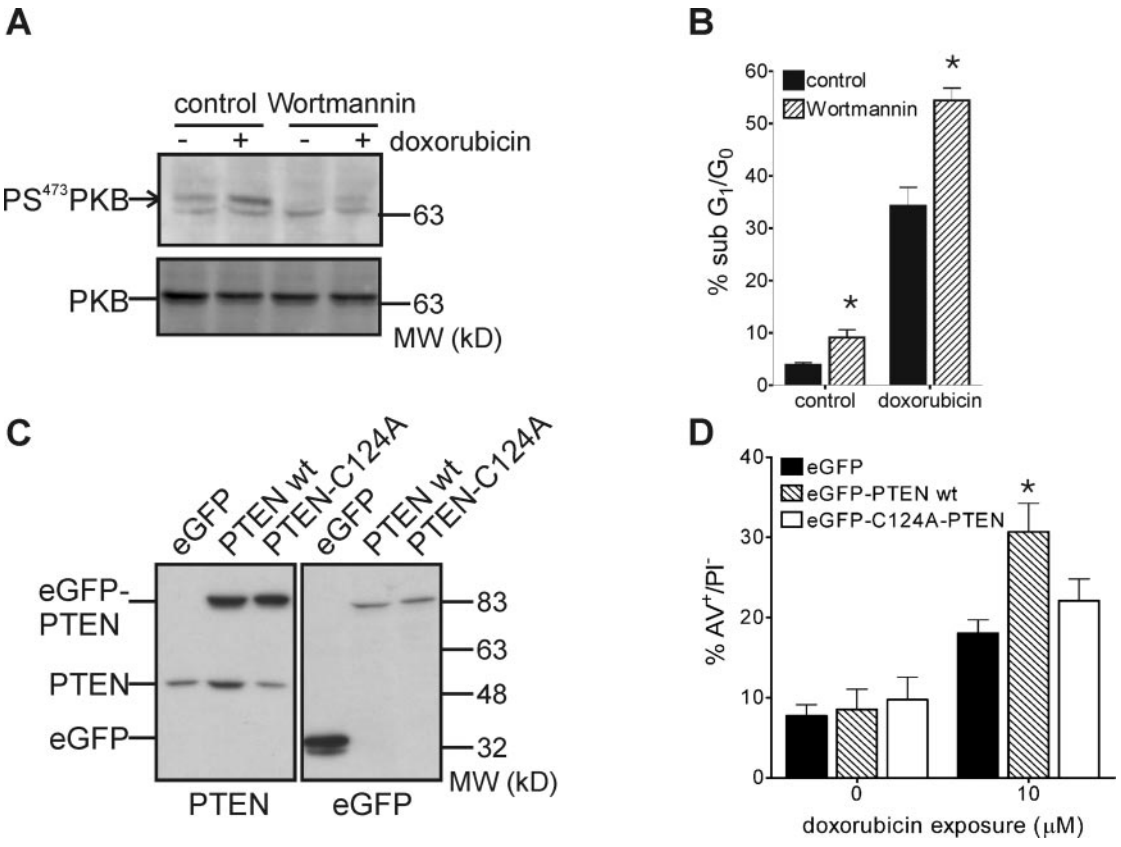

Fig. 6. Inhibition of PI-3K and overexpression of PTEN enhance doxorubicin-induced apoptosis. MTLn3 cells were exposed to 10 μM doxorubicin for 1 h and allowed to recover in the absence or presence of 10 nM wortmannin. After 8 h, cells were immunoblotted for PKB and P-PKB (A, upper band indicated by arrow), and after 16 h the percentage of apoptosis was determined by cell cycle analysis (B). MTLn3 cells were transiently transfected with eGFP, GFP-PTEN, or GFP-PTEN-C124A and 24 h after transfection, cell lysates were immunoblotted for PTEN (C, left) and eGFP (C, right). Transient transfected cells were exposed to 10 μM doxorubicin and allowed to recover for 16 h. D, the percentage of apoptosis in the eGFP-positive cells was determined by staining with Annexin V/propidium iodide followed by flow cytometric analysis. Data shown are representative of three independent experiments (A and C) or the mean of three independent experiments ± S.E. (B and D).

To study the effect of PTEN, cells were transiently transfected with GFP-PTEN wild type or GFP-PTEN-C124A, a phosphatase-inactive mutant. Immunoblotting using antibodies directed against PTEN and GFP confirmed the presence of the transfected proteins (Fig. 6C). Transfected cells were treated with doxorubicin, and the percentage of apoptotic cells was determined using Annexin-V-APC/PI staining followed by flow cytometric analysis. Overexpression of wild-type PTEN enhanced doxorubicin-induced apoptosis almost 2-fold; this effect was dependent on the phosphatase activity, because expression of the phosphatase-inactive mutant PTEN-C124A did not significantly affect doxorubicin-induced apoptosis. Both wild-type and mutant PTEN itself did not affect the viability of MTLn3 cells (Fig. 6D). PTEN can also dephosphorylate FAK on tyrosine residue 397 (Tamura et al., 1998). To exclude this possibility, we also determined the FAK-Y^{397} phosphorylation status in GFP-positive cells after transfection. Neither wild-type nor mutant PTEN affected FAK phosphorylation or the reorganization of focal adhesions (data not shown). These data provide evidence that the doxorubicin-induced signaling through the PI-3 kinase/PKB pathway suppresses the doxorubicin-induced apoptosis.

Discussion

In the present study, we examined the relationship between FAK and the activation of the PI-3 kinase/PKB survival signaling route in control of doxorubicin-induced apoptosis of mammary adenocarcinoma cells. Several important conclusions can be drawn. First, doxorubicin caused the reorganization of the F-actin network in association with increased focal adhesion formation, which is directly associated with the increased activation of PKB but not focal adhesion kinase. These cell biological changes are independent of activation of the apoptotic machinery, because increased expression of Bcl-2 or inhibition of caspase activity does not protect against it. Second, FAK function is involved in the maintenance of survival signaling in the MTLn3 cells, and inhibition of FAK function by expression of FRNK accelerates the onset of doxorubicin-induced apoptosis in association with an FRNK-mediated inhibition of PKB activation by doxorubicin. Third, the doxorubicin-induced apoptosis is suppressed by PI-3 kinase/PKB signaling because overexpression of PTEN enhances doxorubicin-induced apoptosis. The combined data support a paradigm in which doxorubicin causes an increased formation of focal adhesions; FAK is then involved in the doxorubicin-induced activation of the PKB signaling pathway, thereby suppressing the onset of Bcl-2-dependent apoptosis (Fig. 7).

Our data indicate a close relationship between FAK function and doxorubicin-induced PKB activation and suppression of apoptosis caused by doxorubicin. First, doxorubicin itself induced the activation of PKB, which correlated with the increased formation of stress fibers and focal adhesions. Expression of FRNK in MTLn3 cells inhibited the cell adhesion-mediated activation of PKB. Second, inhibition of PI-3 kinase using wortmannin blocked the doxorubicin-mediated PKB activation and sensitized cells to apoptosis. Third, overexpression of PTEN, which dephosphorylates PIP_3, also increased the sensitivity of MTLn3 cells toward doxorubicin. Although in prostate tumor cells, PY^{397} of FAK is also a target for PTEN (Tamura et al., 1998), no effect of PTEN overexpression on PY^{397}-FAK was seen. This excludes the possibility that the effect of PTEN is related to combined effect of PIP_3 and FAK dephosphorylation and further supports the role of PKB signaling in the control of doxorubicin-induced apoptosis. In addition, in LLC-PK1 cells, the nephrotoxicant dichlorovinyl-cysteine caused activation of PKB, which is inhibited by the dominant-negative acting deletion mutant FAT. Moreover, in these cells, PTEN overexpression also facilitates the onset of apoptosis (B. van de Water, M. de Graauw, R. B. Tijdens, unpublished data). These combined data sets suggest the existence of a general cellular stress-mediated signaling pathway through FAK that results in the activation of PKB, thereby providing survival signals.

Focal adhesions are important anchoring sites for the F-actin cytoskeleton, and many focal adhesion-associated proteins directly or indirectly regulate actin dynamics. We found that, initially, doxorubicin increased the bundling of F-actin into stress fibers, which was associated with the formation of larger focal adhesions with a concentration of tyrosine-phosphorylated proteins. A possible pathway leading to stress fiber formation is through the reduction of doxorubicin to a semiquinone free radical intermediate by complex I of the mitochondrial electron transport chain and subsequent generation of reactive oxygen species (ROS) (Wallace, 2003). A similar cytoskeletal response was found in endothelial and glioblastoma cells, which, upon exposure to the ROS-generating agent H_2O_2, formed stress fibers before an increase in tyrosine phosphorylation of FAK and paxillin (Sonoda et al., 1999). One of the proteins that is known to induce these specific cytoskeletal changes is p21Rho, a member of the Rho GTPase family (Ridley and Hall, 1992), suggesting that

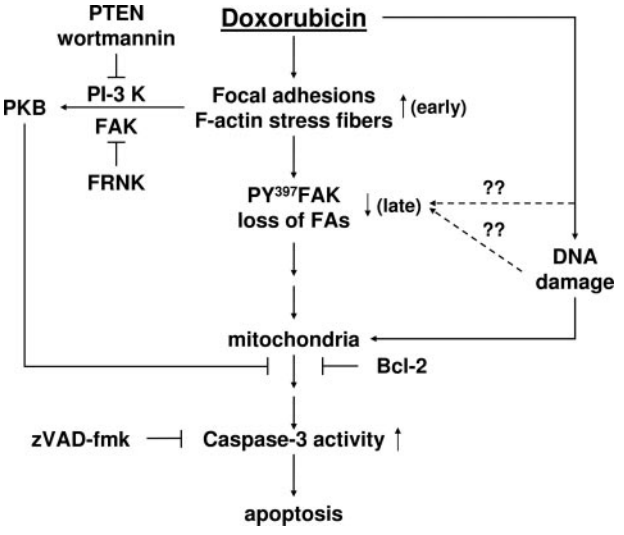

Fig. 7. Working model for doxorubicin-induced apoptotic signaling pathways in MTLn3 adenocarcinoma cells. An early response in doxorubicin-induced cell stress is increased focal adhesion and F-actin stress fiber formation in association with the activation of the PI-3 kinase pathway. Inhibition of FAK (by FRNK) or inhibition of PI-3 kinase (by PTEN or wortmannin) inhibits the activation of PKB and increases the apoptotic cell death. At a later stage, doxorubicin causes the loss of focal adhesions in association with dephosphorylation of PY^{397}FAK. These events occur in cooperation with classic doxorubicin-induced DNA damage responses. Apoptosis can be abrogated at the level of the mitochondria (Bcl-2) and at the level of caspase activation (zVAD-fmk) without affecting upstream reorganization of focal adhesions, FAK phosphorylation events, and actin reorganization.

p21Rho may be an intermediate in doxorubicin- and ROS-induced signaling. Indeed, pharmacological inhibition of a downstream effector of RhoA, Rho-kinase, using Y27632, inhibited F-actin stress fiber accumulation and focal adhesion organization in MTLn3 cells caused by doxorubicin; also, no effect of Y27632 on FAK dephosphorylation caused by doxorubicin was observed (M. J. van Nimwegen, S. Verkoeijen, and B. van de Water, unpublished observations). Tyrosine phosphorylation of FAK correlates with protection against apoptosis, suggesting that doxorubicin initially promotes survival signaling, which is subsequently overridden by pro-apoptotic signaling. After the initial increase, tyrosine phosphorylation strongly decreased 16 h after exposure, suggesting that doxorubicin-induced cytotoxicity involves several signaling pathways with differential kinetics. The decrease in tyrosine phosphorylation at focal adhesions occurred concomitant with the disruption of the F-actin network, suggesting a direct relationship between tyrosine phosphorylation and cytoskeletal organization. Such a decrease may be directly related to the potential inactivation of c-Src activity in the process leading to the onset of apoptosis, as has been described for transforming growth factor-β1-induced apoptosis (Park et al., 2004).

Our data indicate that interference of the function of FAK by expression of eGFP-FRNK abrogated the FAK-mediated suppression of apoptosis in adherent cells. These data are consistent with those obtained by other studies: expression of either FAT in fibroblasts or the C-terminal domain of FAK, which is similar to FRNK, in breast cancer cells caused apoptosis (Ilic et al., 1998). In addition to these studies, we now show that FAK is also involved in the suppression of apoptosis induced by the anticancer agent doxorubicin in breast cancer cells. This suggests that these two stress-response pathways may converge. For treatment of adherent tumor cells, the protective function of FAK was only demonstrated for cisplatin in FAT-expressing glioblastoma cells (Jones et al., 2001), and a link to the PI-3 kinase/PKB pathway was not demonstrated. Induction of apoptosis of BT474 by the FAK C-terminal domain was inhibited by epidermal growth factor receptor, which was associated with activation of the PI-3 kinase/PKB route (Golubovskaya et al., 2002). We found that overexpression of full-length FAK did not provide protection against doxorubicin-induced apoptosis. This suggests that endogenous FAK is present in excess in MTLn3 cells and that the expression of either focal adhesion-associated binding partners and/or downstream adapter or signaling proteins limit the extent of FAK-induced survival signaling. In that case, exogenously added FAK would be unable to amplify FAK-mediated survival signaling. This is in contrast to the findings in HL-60 leukemia cells, which express little endogenous FAK and normally do not adhere, and in which overexpression of FAK protected against hydrogen peroxide- or etoposide-induced apoptosis (Sonoda et al., 2000).

FRNK-mediated apoptosis was clearly dependent on its expression levels. In our inducible tetFRNK-MTLn3 cell line, expression of FRNK itself did not affect apoptosis, whereas inhibition of FAK potentiated doxorubicin-induced apoptosis. This indicates that at these levels of FRNK expression, growth factor receptors are able to mediate survival signals that compensate the decrease in FAK-mediated survival signals.

The degradation of FAK was a late event that occurred in parallel with dephosphorylation of FAK. Only when apoptosis was prevented by the inhibition of caspase activity with zVADfmk, we could define doxorubicin-mediated FAK dephosphorylation as a process that does not require caspase activation. Overexpression of Bcl-2, as shown previously, prevented doxorubicin-induced apoptosis but did not prevent dephosphorylation of FAK. Therefore, doxorubicin-induced dephosphorylation of FAK is a process that occurs upstream of the mitochondrial checkpoint of apoptosis. In a recent study in opossum kidney cells, ATP depletion rapidly (within 15 min) caused caspase-dependent fragmentation of FAK, which was inhibited by caspase-3 (Mao et al., 2003). Moreover, in primary porcine aortic endothelial cells, staurosporine but not cycloheximide caused a rapid dephosphorylation of FAK that preceded the onset of apoptosis (Kabir et al., 2002). These data indicate that the regulation of FAK dephosphorylation and degradation is dependent on the cell type and cellular stress conditions. Our data indicate that FAK dephosphorylation by doxorubicin occurs independent of caspase activity.

In conclusion, doxorubicin caused an early activation of PKB, which is dependent on the function/localization of FAK at focal adhesions. Both inhibition of FAK with FRNK or inhibition of the PI-3K/PKB pathway with wortmannin or PTEN increased the sensitivity of MTLn3 cells toward doxorubicin-induced apoptosis. Doxorubicin also caused an early formation of F-actin stress fibers and focal adhesions, followed by dephosphorylation of PY^{397}FAK and loss of focal adhesions. Abrogation of the apoptotic pathway was possible at the level of the mitochondria by overexpression of Bcl-2 and at the level of the executioner caspases by the addition of zVAD-fmk. These data suggest that pharmacological inhibition of FAK and/or the PI-3K/PKB pathway may represent a possible means to enhance the treatment of cancer with classic anticancer drugs such as doxorubicin.

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