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Elevated p21-Activated Kinase 2 Activity Results in Anchorage-Independent Growth and Resistance to Anticancer Drug–Induced Cell Death[1]

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Abstract

p21-Activated kinase 2 (PAK-2) seems to be a regulatory switch between cell survival and cell death signaling. We have shown previously that activation of full-length PAK-2 by Rac or Cdc42 stimulates cell survival, whereas caspase activation of PAK-2 to the proapoptotic PAK-2p34 fragment is involved in the cell death response. In this study, we present a role of elevated activity of full-length PAK-2 in anchorage-independent growth and resistance to anticancer drug–induced apoptosis of cancer cells. Hs578T human breast cancer cells that have low levels of PAK-2 activity were more sensitive to anticancer drug–induced apoptosis and showed higher levels of caspase activation of PAK-2 than MDA-MB435 and MCF-7 human breast cancer cells that have high levels of PAK-2 activity. To examine the role of elevated PAK-2 activity in breast cancer, we have introduced a conditionally active PAK-2 into Hs578T human breast cells. Conditional activation of PAK-2 causes loss of contact inhibition and anchorage-independent growth of Hs578T cells. Furthermore, conditional activation of PAK-2 suppresses activation of caspase 3, caspase activation of PAK-2, and apoptosis of Hs578T cells in response to the anticancer drug cisplatin. Our data suggest a novel mechanism by which full-length PAK-2 activity controls the apoptotic response by regulating levels of activated caspase 3 and thereby its own cleavage to the proapoptotic PAK-2p34 fragment. As a result, elevated PAK-2 activity interrupts the apoptotic response and thereby causes anchorage-independent survival and growth and resistance to anticancer drug–induced apoptosis.

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Introduction

Tissue homeostasis requires the coordinated regulation of proliferation, cell survival, and programmed cell death. Many cancer cells acquire the capability to evade cell death induced by physiological signals or by anticancer drugs. The molecular mechanisms for such an increased resistance to programmed cell death are largely unknown, but it seems that dysregulation of proapoptotic and antiapoptotic signaling pathways is involved. Among the signaling molecules that regulate cell survival and cell death is p21-activated kinase 2 (PAK-2). It is a member of the PAK family of serine/threonine–specific protein kinases. p21-Activated kinases are activated in response to different receptor signaling pathways, including growth factor receptors, G-protein–coupled receptors, and integrin receptors, to regulate cell shape and motility as well as cell survival, cell growth, and programmed cell death.

The mammalian PAK family consists of six members that are divided into two groups according to biochemical and structural features [1–3]. Group 1 consists of PAK-1 (α-PAK), PAK-2 (γ-PAK), and PAK-3 (β-PAK). PAK-1 and PAK-3 are tissue-specific with the highest levels in brain, whereas PAK-2 is ubiquitous. Group 2 consists of the more distantly related PAK-4, PAK-5, and PAK-6. Groups 1 and 2 have

Abbreviations: 4-HT, 4-hydroxy tamoxifen; EGFP, enhanced green fluorescent protein; ER, hormone-binding domain of estrogen receptor; IAP, inhibitor of apoptosis; PAK, p21-activated kinase; PARP, poly (ADP-ribose) polymerase; XIAP, X-linked inhibitor of apoptosis

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distinct biochemical and structural properties and seem to serve different cellular functions [3]. Group 1 PAKs can be activated by binding of active GTP-bound p21 GTPases such as Rac and Cdc42 and through GTPase-independent mechanisms including binding of bioactive lipids, protein interaction–dependent translocation, and phosphorylation by other protein kinases [1,2]. The ubiquitous PAK-2 is unique among the PAK family; it is also activated through proteolytic cleavage by caspases, which generates the constitutively active PAK-2p34 fragment [4–7]. Activated full-length PAK-2 has been shown to stimulate cell survival and to protect cells from proapoptotic signals [8]. Similar antiapoptotic functions have been shown for PAK-1 and PAK-4 [9–11]. In contrast, cleavage of PAK-2 by caspases and generation of the constitutively active PAK-2p34 fragment induce a cell death response [4,6,12,13]. The opposing effects of full-length PAK-2 and caspase-activated PAK-2p34 might be explained by differential targeting. Caspase-activated PAK-2p34, but not full-length PAK-2, translocates to the nucleus [12].

Elevated PAK-1 and PAK-2 activity is present in several human breast cancer cell lines and surgical breast cancer samples [14–16]. Expression of the protein kinase inhibitor domain of PAK-2 inhibits hyperactive PAK-1 and PAK-2 and suppresses proliferation in MDA-MB435 cells [14]. Inhibition of PAK-1 by overexpression of a kinase-deficient mutant decreases motility and invasion of highly invasive MDA-MB435 human breast cancer cells, whereas overexpression of constitutively active PAK-1 mutants increase motility, invasion, and anchorage-independent growth of noninvasive MCF-7 human breast cancer cells [16,17]. In addition, *PAK-2* is among 186 genes that are differentially expressed in breast cancer cells and normal mammary epithelial cells and are strongly associated with the risk of breast cancer metastasis and mortality [18]. Therefore, elevated PAK signaling seems to contribute to the malignant phenotype of breast cancer.

To characterize the role of elevated PAK-2 activity in breast cancer, we have generated a conditionally active form of PAK-2 by fusion with the estrogen receptor hormone–binding domain to avoid artifacts resulting from continued expression of an active PAK mutant. Conditionally active PAK-2 was introduced into Hs578T human breast cancer cells that have low endogenous PAK-2 activity levels. Conditional activation of PAK-2 stimulates loss of contact inhibition and anchorage-independent growth of Hs578T cells. Furthermore, conditional activation of PAK-2 suppresses cell death of Hs578T cells in response to the anticancer drug cisplatin and downregulates activation of caspase 3 and its own caspase cleavage to the proapoptotic PAK-2p34 fragment. Our results reveal a novel mechanism by which elevated PAK-2 activity promotes malignant growth and resistance to apoptosis of human breast cancer cells. This is highly significant because PAK-2 activity is frequently elevated in human breast cancer.

Materials and Methods

Reagents

The antibody specific for PAK-2 (γPAK-V19) and a C-terminal antibody that detects PAK-1, PAK-2, and PAK-3 (αPAK-C19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-PAK–2(Thr-402), phospho–PAK-2(Ser-141), and phospho–PAK-2(Ser-192/197) as well as antibodies for cleaved caspase 3(Asp-175), cleaved caspase 6(Asp-162), cleaved caspase 7 (Asp-198), cleaved caspase 9(Asp-315), cleaved caspase 9(Asp-330), and cleaved poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Danvers, MA). The antibody for enhanced green fluorescent protein (EGFP) Living Colors was from Clontech (Moutain View, CA). The phospho-histone 2B(Ser-14) antibody was from Upstate Biotechnology (Billerica, MA). The β-actin antibody, 4-hydroxy tamoxifen (4-HT), and myelin basic protein were from Sigma (St. Louis, MO). Secondary antibodies conjugated with horseradish peroxidase, SuperSignal West Pico chemiluminescence reagent, and Gelcode Blue Staining Reagent were from Pierce (Rockford, IL). [γ-^{32}P]ATP was obtained from Perkin-Elmer (Waltham, MA). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA). Kits for plasmid DNA isolation were obtained from QIAGEN (Valencia, CA). TransIT-LT1 transfection reagent was from Mirus (Madison, WI). Hoechst 33342 was from Molecular Probes (Carlsbad, CA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). The cDNA for the hormone binding domain of the murine estrogen receptor (ER) was provided by Drs. Zhongdong Huang and J. Michael Bishop (University of California, San Francisco). The matched surgical samples of cancer and normal breast tissue were collected at the Froedert Memorial Lutheran Hospital in Milwaukee, WI, under a protocol approved by the Human Research Review Committee of the Medical College of Wisconsin.

Molecular Cloning

pRetro-IRES-Zeo was generated by replacing the EGFP domain of pRetroIRES-EGFP [12] with a zeocin resistance gene. The hormone-binding domain of the murine ER was added to the C-terminus of the constitutively active PAK-2L106F mutant [13] in the pKoz/EGFP vector [19], and EGFP-PAK-2L106F-ER was subcloned into the pRetroIRES-Zeo vector. A mutation in the ER domain renders it unresponsive to estrogen, which is frequently found in fetal bovine serum added as growth supplements to cell culture media. However, the mutant ER domain is still responsive to the artificial ligand 4-HT [20].

Cell Culture, Transfection, Retroviral Transduction, and Treatments

Phoenix Ampho packaging cells, Hs578Bst human epithelial cells, and Hs578T, MCF-7, and MDA-MB435 human breast cancer cells were obtained from American Tissue Culture Collection (Rockville, MD). Phoenix Ampho cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen/Life Technologies, Carlsbad, CA), and Hs578Bst, Hs578T, MCF-7, and MDA-MB435 cells were grown in DMEM/F12 (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen/Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2.

Amphotropic retroviruses were obtained by transient transfection of the retroviral vector into the packaging cell line Phoenix Ampho using TransIT-LT1 transfection reagent and OPTI-MEM (Invitrogen/Life Technologies). The culture medium was replaced at 24 hours after transfection, and retrovirus-containing medium was collected at 48 hours after transfection and filtered through a 0.45-μm filter. Hs578T cells were grown to approximately 10% to 20% confluency in 100-mm culture dishes and then transduced by the addition of 2 ml of retrovirus-containing medium from packaging cells in the presence of 4 μg/ml polybrene. The culture medium was replaced at 24 hours after transfection and culture medium containing 500-μg/ml zeocin was added at 48 hours to select stable Hs578T cell populations for pRetroIRES-Zeo-EGFP-PAK-2L106F-ER.

To analyze loss of contact inhibition, cells were grown to confluency. To activate EGFP-PAK-2L106F, 100 nM 4-HT was added as indicated, and cells were incubated for indicated periods. To analyze anchorage-independent growth, cells were seeded in 0.4% soft agar in DMEM/F12 on top of 0.8% soft agar and covered with another aliquot of 0.8% soft agar. To activate EGFP-PAK-2L106F, 100 nM 4-HT was added as indicated, and cells were incubated for indicated periods. Images were acquired with a Nikon Eclipse TE2000-U microscope (Nikon Instruments, Inc, Melville, NY) and a Roper Scientific Photometrics CoolSnap ES camera using Metamorph 6.2 software (Roper Scientific, Tucson, AZ).

Western Blot

For treatment, cells were grown to 70% to 90% confluency and treated with indicated concentrations of taxol or cisplatin for the indicated periods. To activate EGFP-PAK-2L106F, cells were incubated with 100 nM 4-HT. Cells were lysed in modified RIPA buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μM MG-132, 25 mM NaF, 25 mM β-glycerophosphate, and 200 μM sodium vanadate). Cleared lysates were prepared by centrifugation for 15 minutes at 12,000*g*. For acid extraction of histones, the pellet, which includes the chromatin, was resuspended in hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μM MG-132, 25 mM NaF, 25 mM β-glycerophosphate, and 200 μM sodium vanadate), adjusted to 0.2 M HCl, and incubated on ice for 30 minutes. Extracted histones were cleared by centrifugation for 15 minutes at 12,000*g*. Protein concentrations were determined by a Bradford assay using bovine γ-globulin as a protein standard. Western blots were performed with cleared lysates (15-30 μg) or acid-extracted histones (1-2 μg) by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, transferred onto polyvinylidene membranes, and detected by chemiluminescence using horseradish peroxidase–conjugated secondary antibodies and SuperSignal Pico reagent. X-ray film exposures were converted into digital images using a Umax scanner (Techville, Inc, Dallas, TX), and images were processed using Adobe Photoshop 6.0.

In-gel Protein Kinase Assays

In-gel assays were performed with 0.1 mg/ml of myelin basic protein copolymerized in the separating gel of 11% SDS–polyacrylamide gels [21]. Cell lysates were prepared as described above. Cell lysates (30 μg) were separated by electrophoresis in the substrate-containing gels. After electrophoresis, gels were washed twice for 1 hour in 50 mM Tris-HCl, pH 8.0, 20% 2-propanol, once for 1 hour in 50 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol (buffer A), and denatured twice for 1 hour in buffer A containing 6 M guanidine-HCl at room temperature. Renaturation was performed with five changes of buffer A containing 0.04% Tween 40 for 16 to 24 hours at 4°C. Phosphorylation was carried out in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.4 mM EGTA, 30 mM 2-mercaptoethanol, and 50 μM [γ-^{32}P]ATP (500 cpm/pmol) for 1 hour at room temperature. Excess of [γ-^{32}P]ATP was removed by washing in 5% trichloroacetic acid and 1% sodium pyrophosphate. Gels are stained with Gelcode Blue, dried, and subjected to autoradiography. X-ray film exposures were converted into images using a Umax scanner, and images were processed using Adobe Photoshop 6.0.

Cell Death Assay

Cells were grown to 50% to 70% confluency and treated with 50 nM taxol or 50 μM cisplatin for the indicated periods. To activate EGFP-PAK-2L106F, cells were incubated with 100 nM 4-HT. Cells were stained with 10 μg/ml Hoechst 33342 for 10 minutes and analyzed by fluorescence microscopy. To determine levels of programmed cell death at least 500 cells were counted and analyzed for apoptotic chromatin condensation and fragmentation.

Cell Viability Assay

Cell viability in response to stress stimulants was determined with the CellTiter 96 AQueous One Solution Cell Proliferation Assay. Quadruplicate cell samples were seeded at 10,000 cells per well in 96-well plates and incubated for 16 to 24 hours. Cells were treated with 50 nM taxol or 50 μM cisplatin for the indicated periods. To activate EGFP-PAK-2L106F, cells were incubated with 100 nM 4-HT. Cell viability was measured by addition of the MTS tetrazolium compound. During a 2-hour incubation at 37°C, MTS was converted to the colored formazan, which was detected at 490 nm with a plate reader. Background absorbance was corrected by subtraction of blanks with an equal volume of growth medium. Background caused by cellular debris was corrected by subtraction of sample absorbance at the reference wavelength of 630 nm.

Results

Treatment of Human Breast Cancer Cells with the Anticancer Drugs Cisplatin and Taxol Causes Caspase Activation of PAK-2 and Cell Death

We have shown previously that caspase activation of PAK-2 induces a cell death response [12]. To examine whether anticancer drug–induced cell death of human breast cancer cells involves caspase activation of PAK-2, Hs578T, MDA-MB435, and MCF-7, human breast cancer cells were treated with cisplatin or taxol, which cause DNA damage or mitotic arrest, respectively. Cell lysates were analyzed by Western blot with a C-terminal anti-PAK antibody that detects PAK-1 and PAK-2 as well as caspase-activated PAK-2p34 (Figure 1). Treatment with cisplatin or taxol resulted in caspase cleavage of PAK-2 and generation of PAK-2p34 in Hs578T and MDA-MB435 cells, but cisplatin was more effective than taxol in generating PAK-2p34. Treatment with cisplatin but not taxol caused caspase activation of PAK-2 in MCF-7 cells. Because caspase 3 has been shown to cleave PAK-2 *in vitro* [7], we analyzed the activation of caspase 3 in response to cisplatin and taxol using an antibody specific for cleaved, active caspase 3. Treatment with cisplatin or taxol resulted in the activation of caspase 3 in Hs578T and MDA-MB435 cells, but cisplatin was more effective than taxol. As expected, treatment with cisplatin or taxol did not cause activation of caspase 3 in caspase 3–deficient MCF-7 cells [22]. Because caspase activation of PAK-2 correlates with activation of caspase 3, it seems that caspase 3 is the main caspase that cleaves PAK-2 to the proapoptotic PAK-2p34 fragment in Hs578T and MDA-MB435 cells. However, the fact that caspase activation of PAK-2 occurs in caspase 3–deficient MCF-7 cells in response to cisplatin shows that other caspases are capable to cleave PAK-2. Caspases 8 and 10 have both been shown to cleave PAK-2 and generate the PAK-2p34 fragment but to a lesser degree than caspase 3 [23].

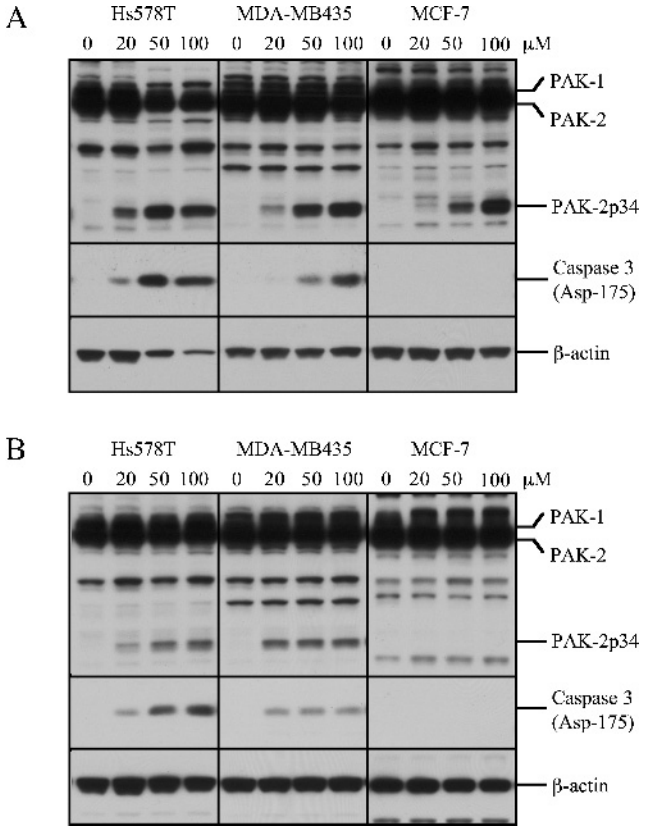

Figure 1. Caspase activation of PAK-2 and activation of caspase 3 in human breast cancer cells in response to the anticancer drugs cisplatin and taxol. Treatment of Hs578T, MDA-MB435, and MCF-7 human breast cancer cells with (A) 20, 50, and 100 μM cisplatin or (B) 20, 50, and 100 nM taxol for 24 hours. Adherent and detached cells were harvested and lysed. Cell lysates were analyzed by Western blot with a C-terminal anti-PAK antibody. Activation of caspase 3 was determined with an antibody specific for cleaved caspase 3. Positions of PAK-1, PAK-2, caspase-activated PAK-2p34, and cleaved caspase 3 are indicated at the right. Equal protein loading was verified by Western blot with an anti–β-actin antibody, but late apoptotic cell lysates such as of Hs578T cells with 50 and 100 μM cisplatin showed decreased β-actin levels.

Because Hs578T, MDA-MB435, and MCF-7 human breast cancer cells showed more caspase activation of PAK-2 in response to cisplatin than taxol, we compared the effects of cisplatin and taxol on cell viability and apoptosis of these breast cancer cells (Figure 2). Treatment with 50 μM cisplatin resulted in a marked reduction of cell viability of Hs578T cells at 24 hours and a further decline to almost zero at 48 hours (Figure 2*A*). In comparison, MDA-MB435 and MCF-7 cells were relatively less susceptible but showed reduced cell viability after 48 hours of cisplatin treatment. Treatment with 50 nM taxol reduced viability of Hs578T and MDA-MB435 cells at 48 but not at 24 hours, whereas taxol did not reduce viability of MCF-7 cells at 24 or 48 hours (Figure 2*B*). Histone 2B is phosphorylated at Ser-14 in response to a variety of apoptotic stimuli in different cell lines. Phosphorylation of histone 2B at Ser-14 has been associated with apoptotic chromatin condensation and cell death [24]. Because histone 2B has been used as a model substrate of PAK-2, we determined whether caspase-activated PAK-2p34 phosphorylates histone 2B at Ser-14 and if apoptotic histone 2B phosphorylation correlates with caspase activation of PAK-2 in response to 50 μM cisplatin or 50 nM taxol (Figure 2*C*). Recombinant PAK-2p34 but not kinase-deficient PAK-2p34K278R phosphorylated histone 2B at Ser-14 *in vitro*. Furthermore, histone 2B was phosphorylated at Ser-14 in Hs578T and MDA-MB435 cells in response to the anticancer drugs cisplatin or taxol. Cisplatin caused markedly more apoptotic histone 2B phosphorylation than taxol in Hs578T cells, whereas taxol treatment caused slightly more apoptotic histone 2B phosphorylation than cisplatin in MDA-MB435 cells. Apoptotic histone 2B phosphorylation was not observed in MCF-7 cells that lack caspase 3 and generated less or no PAK-2p34 in response to 50 μM cisplatin or 50 nM taxol. Therefore, apoptotic histone 2B phosphorylation correlates with caspase activation of PAK-2 indicating that caspase-activated PAK-2p34 directly or indirectly mediates phosphorylation of histone 2B at Ser-14 in Hs578T and MDA-MB435 cells in response to cisplatin or taxol.

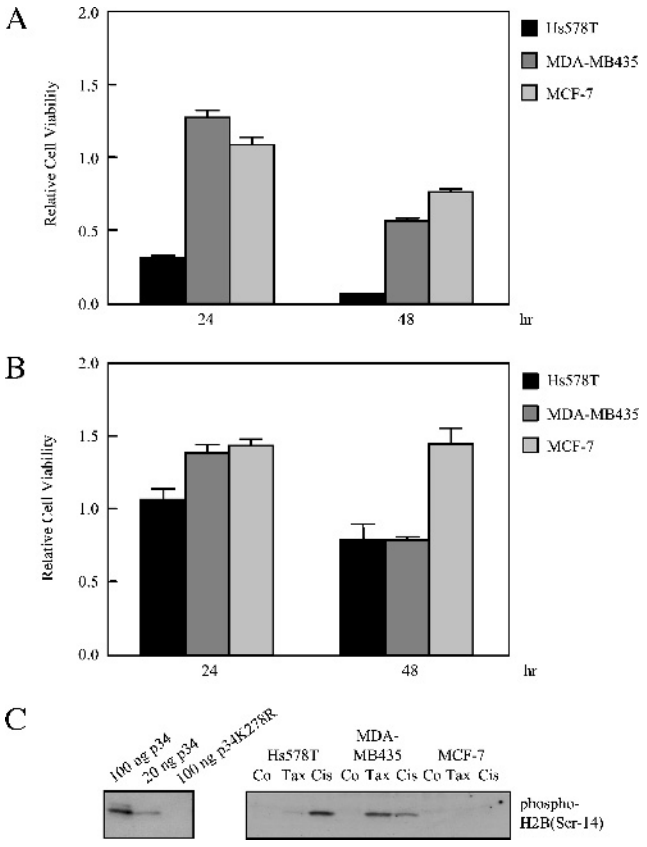

Figure 2. Effects on cell viability and apoptotic histone 2B phosphorylation in human breast cancer cells in response to the anticancer drugs cisplatin and taxol. Hs578T, MDA-MB435, and MCF-7 cells were grown in 96-well plates and treated for 24 or 48 hours with (A) 50 μM cisplatin or (B) 50 nM taxol. MTS assays were performed with quadruplicate samples and relative cell viability is shown as mean values and SDs in comparison to viability of cells before treatment, which was set arbitrarily at 1. (C) Purified histone 2B was incubated with recombinant GST-PAK-2p34 (100 or 20 ng) or kinase-deficient GST-PAK-2p34K278R (100 ng) for 30 minutes at 30°C in the presence of Mg^{2+} and ATP; Hs578T, MDA-MB435, and MCF-7 human breast cancer cells were treated with 50 nM taxol (Tax) or 50 μM cisplatin (Cis) for 24 hours or left untreated as controls (Co), and histones were acid-extracted from cell lysates. Phosphorylation of histone 2B (H2B) at Ser-14 was analyzed by Western blot with an anti–phospho(S14)H2B antibody.

Caspase Activation of PAK-2 and Activation of the Caspase Cascade in Human Breast Cancer Cells in Response to Treatment with the Anticancer Drugs Cisplatin and Taxol

Because caspase activation of PAK-2 correlated with cell death of Hs578T, MDA-MB435, and MCF-7 human breast cancer cells in response to the anticancer drugs cisplatin and taxol, we compared effects of cisplatin and taxol on the activation of the caspase cascade and caspase activation of PAK-2 in these breast cancer cells (Figure 3). Cells were treated with 50 μM cisplatin or 50 nM taxol for 0, 8, 16, 24, 32, and 48 hours. Caspase activation of PAK-2 was determined by Western blot with a C-terminal anti-PAK antibody that detects PAK-1 and PAK-2 as well as caspase-activated PAK-2p34. Activation of the initiator caspase 9 and the effector caspases 3 and 7 was determined by Western blot using antibodies specific for cleaved fragments of these caspases and the caspase substrate PARP. In response to cisplatin, caspase activation of PAK-2 occurred the most rapid and at highest levels in Hs578T cells, whereas MDA-MB435 cells showed slower caspase activation of PAK-2 and lower levels of caspase-activated PAK-2p34, and MCF-7 cells showed very little caspase-activated PAK-2p34 (Figure 3*A*). In all three cell lines, levels of caspase-activated PAK-2p34 sustained up to 32 hours but decreased at 48 hours. In Hs578T cells, immunoreactive bands appeared above and below PAK-2p34 at 48 hours, but it is unknown if these bands correspond to modified forms of PAK-2p34.

In Hs578T cells, cisplatin also caused high levels of activated caspases 3 and 7 and of cleaved PARP, a substrate of caspases 3 and 7. MDA-MB435 cells showed lower levels of activated caspases 3 and 7 and cleaved PARP, and MCF-7 cells also showed lower levels of activated caspase 7 and cleaved PARP but, as expected, no activated caspase 3 (Figure 3*A*). Cisplatin is believed to activate the intrinsic apoptotic pathway resulting in the formation of the apoptosome and autocleavage of caspase 9 at Asp-315 [25,26]. Consequently, we observed autocleaved caspase 9 at the earliest time points and the highest levels in Hs578T cells, which were most sensitive to cisplatin. In MDA-MB435 and MCF-7 cells, which were less sensitive to cisplatin, autocleaved caspase 9 appeared at later time points and at lower levels (Figure 3*A*). Caspase 9 is also cleaved at Asp-330 by caspase 3, which has been interpreted as a positive feedback amplification loop to accelerate apoptosis. However, newer data have shown that cleavage at Asp-330 does not activate caspase 9 directly but prevents inhibition of autocleaved caspase 9 by X-linked inhibitor of apoptosis (XIAP) [26]. Cisplatin induced the highest levels of caspase 9 cleavage at Asp-330 in Hs578T cells, which also showed high levels of activated caspase 3 (Figure 3*A*). In MDA-MB435 cells, which showed lower levels of caspase 3, levels caspase 9 cleavage at Asp-330 were also lower. However, MCF-7 cells, which lack caspase 3, showed low but detectable levels of caspase 9 cleavage at Asp-330, indicating that another caspase can cleave caspase 9 at Asp-330 at least to some degree.

In comparison to cisplatin, taxol treatment resulted in less caspase activation of PAK-2, activation of caspases 3 and 7, cleavage of PARP, autocleavage of caspase 9 at Asp-315, and cleavage of caspase 9 at Asp-330 (Figure 3*B*). Levels of caspase-activated PAK-2p34, activated caspases 3 and 7, PARP cleavage, and cleavage of caspase 9 at Asp-330 were more similar in Hs578T and MDA-MB435 cells in response to taxol than they were in response to cisplatin. However, the kinetics of the response was later and more sustained in MDA-MB435 cells than in Hs578T cells. Treatment with taxol resulted in activated caspase 7 and PARP cleavage but no caspase-activated PAK-2p34 and cleavage of caspase 9 at Asp-330 in caspase 3–deficient MCF-7 cells (Figure 3*B*). Autocleavage of caspase 9 at Asp-315 was not detected in Hs578T

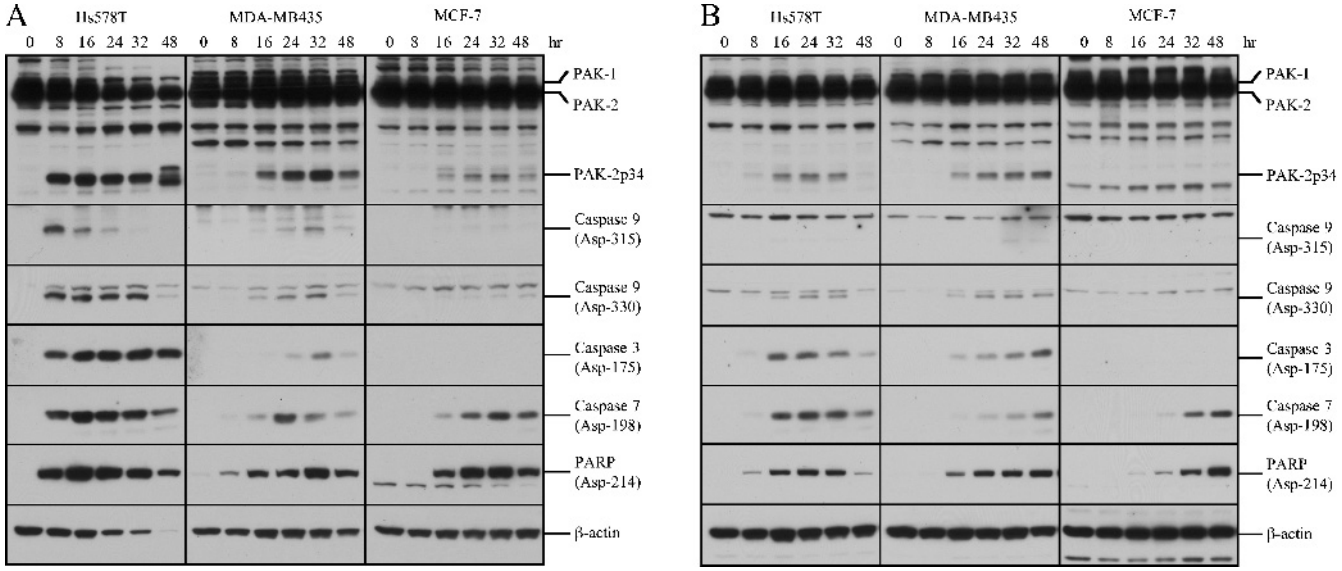

Figure 3. Activation of caspases and caspase activation of PAK-2 in human breast cancer cells in response to the anticancer drugs cisplatin and taxol. Hs578T human breast cancer cells were treated with (A) 50 μM cisplatin or (B) 50 nM taxol for 0, 8, 16, 24, 32, and 48 hours. Both detached and adherent cells were harvested and combined. Caspase activation of PAK-2 and activation of caspases were analyzed by Western blot. Caspase activation of PAK-2 was determined with the C-terminal anti-PAK antibody. Activation of caspases 3, 7, and 9 and cleavage of the caspase substrate PARP were determined with antibodies specific for cleaved fragments of these caspases and PARP. Positions of PAK-1, PAK-2, caspase-activated PAK-2p34, and cleaved caspases or PARP are indicated at the right. Even protein loading was verified by Western blot with an anti–β-actin antibody, but late apoptotic cell lysates such as of Hs578T cells at 24, 32, and 48 hours of cisplatin treatment showed decreased β-actin levels.

and MCF-7 cells and was barely detectable in MDA-MB435 cells in response to taxol, suggesting that alternate mechanisms mediate taxol-induced activation of caspases 3 and 7 and cell death. Therefore, activation of caspases correlates with caspase activation of PAK-2 and cell death in response to cisplatin or taxol, and treatment of Hs578T cells with cisplatin resulted in the highest levels of activated caspases, caspase activation of PAK-2, and cell death.

Elevated Activity Levels of Full-length PAK-2 Are Present in Breast Cancer

Elevated protein or activity levels of PAKs have been associated with the malignant phenotype of cancer cells, and elevated PAK signaling seems to contribute to malignant growth [14–16,27]. Therefore, we analyzed full-length PAK-2 activity and protein levels in Hs578Bst normal breast epithelial cells and Hs578T, MCF-7, and MDA-MB435 breast cancer cells as well as in matched surgical samples of cancer and normal breast tissue. Activity levels of PAK-2 were analyzed by in-gel assays using myelin basic protein as a substrate. PAK-2 protein levels were determined with a specific PAK-2 antibody (Figure 4). PAK-2 activity migrated as two bands with approximately 58 and 62 kDa, which were previously shown to represent PAK-2 using mouse brain and BALB3T3 fibroblasts [8]. Most of the PAK-2 protein levels correspond to the 62-kDa band, whereas the 58 kDa contains low or undetectable protein levels of PAK-2 (Figure 4). Therefore, the 58-kDa band seems to correspond to highly active PAK-2. This is in agreement with an earlier report that endogenously active PAK-2 (the article uses the older nomenclature PAK I) purified from rabbit reticulocytes migrates at 58 kDa [28]. PAK-2 activity was markedly elevated in MCF-7 and MDA-MB435 breast cancer cells compared with Hs578T breast cancer cells and Hs578Bst normal breast epithelial cells, whereas PAK-2 protein levels were only slightly increased. Most of the surgical breast cancer samples (2-8) had markedly higher PAK-2 activity levels than the matched normal breast tissue samples (Figure 4). PAK-2 protein levels were not significantly increased in the cancer samples. Therefore, elevated full-length PAK-2 activity levels are present in the breast cancer cell lines MCF-7 and MDA-MB435 as well as surgical breast cancer suggesting a role of hyperactive PAK-2 in breast cancer development and/or progression.

Elevated Full-length PAK-2 Activity Induces Loss of Contact Inhibition and Anchorage-Independent Growth

To analyze the role of hyperactive PAK-2 in breast cancer, we constructed a conditionally active PAK-2 (Figure 5*A*). The L106F mutation was introduced into the regulatory domain of PAK-2 to release the autoinhibitory interaction with the catalytic domain and create the constitutively active PAK-2L106F [13]. The EGFP domain was added to the N-terminus to facilitate detection. For conditional activation with 4-HT the hormone-binding domain of the mouse ER [20] was added to the C-terminus to generate EGFP-PAK-2L106F-ER. Stable Hs578T cells for expression of EGFP-PAK-2L106F-ER (Hs578T-EGFP-PAK-2L106F-ER) were generated and the conditional activation of EGFP-PAK-2L106F-ER was tested after incubation of Hs578T-EGFP-PAK-2L106F-ER cells in the absence or presence of 100 nM 4-HT for 16 hours. Cell lysates were analyzed by Western blot with three different anti–phospho-PAK antibodies that specifically detect autophosphorylated (active) PAK-2 (Figure 5*B*). Little basal activity of EGFP-PAK-2L106F-ER was observed in the absence of 4-HT, whereas addition of 4-HT resulted in greatly enhanced activity. Total levels of EGFP-PAK-2L106F-ER were only slightly increased in the presence of 4-HT.

Addition of 4-HT to confluent Hs578T-EGFP-PAK-2L106F-ER cells resulted in three-dimensional growth and the formation of foci (Figure 5*C*). In the absence of 4-HT, Hs578T-EGFP-PAK-2L106F-ER cells continued growing as a monolayer. Parental Hs578T cells in absence or presence of 4-HT did not form foci and continued growing as a monolayer. Therefore, elevating activity levels of full-length PAK-2 by activation of EGFP-PAK-2L106F-ER resulted in loss of contact inhibition. Furthermore, in the presence of 4-HT, Hs578T-EGFP-PAK-2L106F-ER cells grew colonies in soft agar within 96 hours, whereas no colonies were visible in the absence of 4-HT (Figure 5*D*). Parental Hs578T cells showed no colony growth in the absence or presence of 4-HT. Therefore, elevating activity levels of full-length PAK-2 by activation of EGFP-PAK-2L106F-ER resulted in anchorage-independent growth. Loss of contact inhibition and anchorage-independent growth typically stimulate the metastatic potential of cancer cells.

Elevated Full-length PAK-2 Activity Stimulates Cell Survival and Protects from Cell Death in Response to the Anticancer Drug Cisplatin

To determine whether elevated full-length PAK-2 activity affects cisplatin-induced cell death, Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were treated with cisplatin in the absence or presence of 4-HT. Treatment with cisplatin resulted in the stimulation of cell death in parental Hs578T cells in the absence and presence of 4-HT and in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT (Figure 6*A*). However, activation of EGFP-PAK-2L106F-ER with 4-HT dramatically reduced levels of cell death. To verify this effect of activation of EGFP-PAK-2L106F-ER, we also determined cell viability of parental Hs578T and Hs578T-EGFP-PAK-2L106F-ER

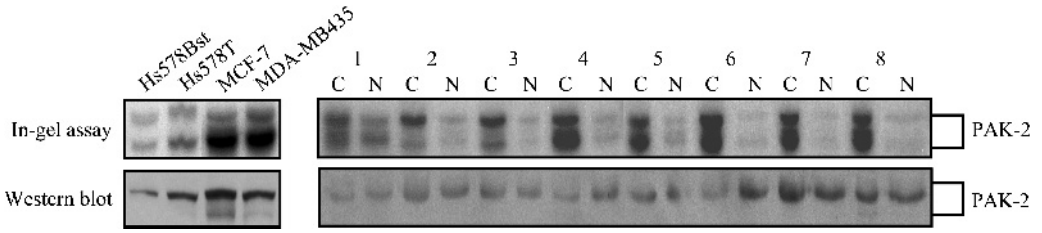

Figure 4. Elevated activity levels of full-length PAK-2 in breast cancer. Activity levels of PAK-2 in human breast cancer cell lines and surgical human breast cancer samples (C) and matched normal breast tissue samples (N) were analyzed by in-gel assays using 11% SDS–polyacrylamide gels containing myelin basic protein. Total levels of PAK-2 were analyzed by Western blot with an anti–PAK-2 antibody. The positions of activity and protein bands corresponding to full-length PAK-2 (62 and 58 kDa) are indicated at the right.

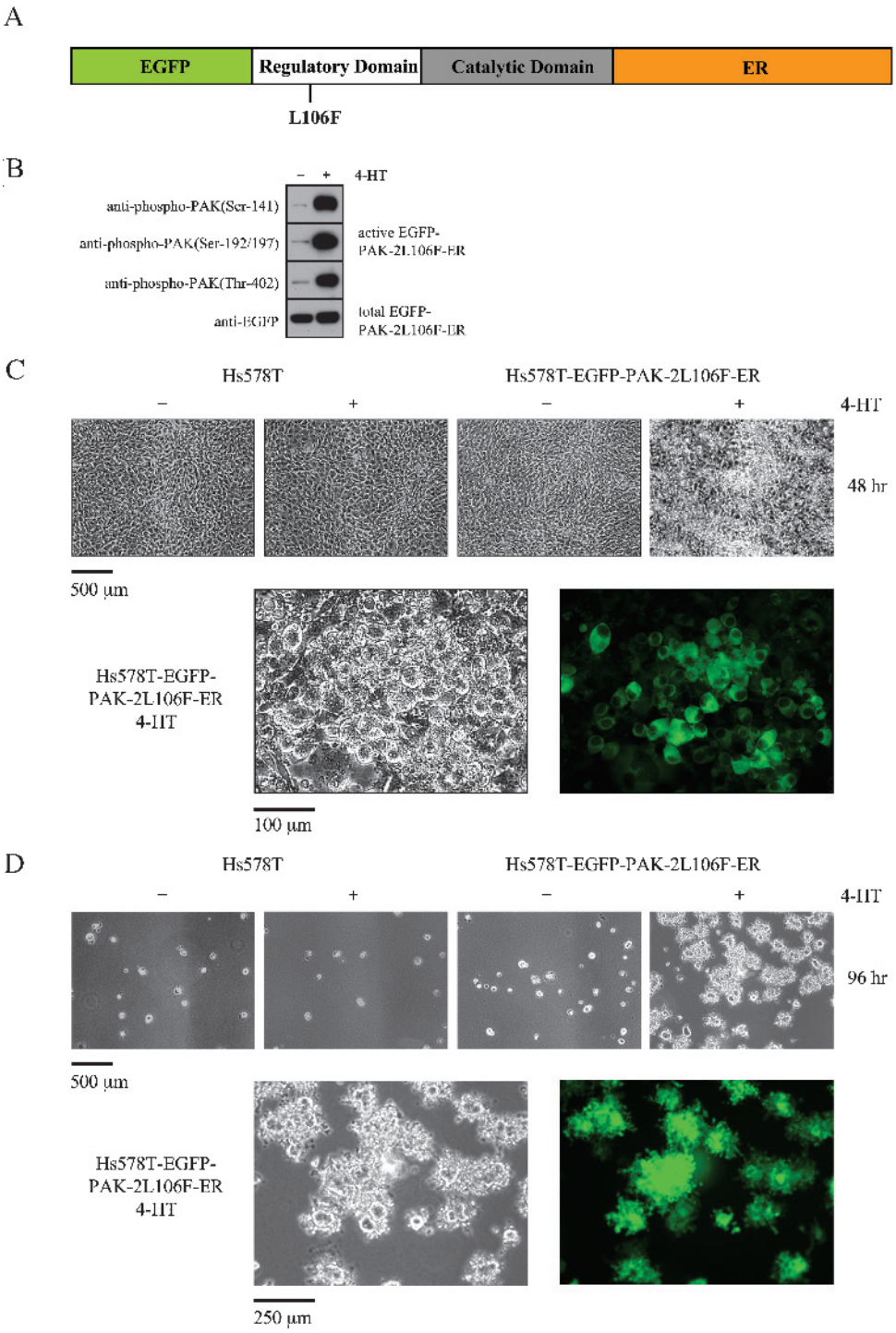

Figure 5. Activation of EGFP-PAK-2L106F-ER induces loss of contact inhibition and anchorage-independent growth. (A) The L106F mutation was introduced into the regulatory domain of PAK-2 to release the autoinhibitory interaction with the catalytic domain. The EGFP domain was added to the N-terminus to facilitate detection and the hormone-binding domain of the mouse estrogen receptor to the C-terminus for conditional activation with the ligand 4-HT. (B) Stable Hs578T-EGFP-PAK-2L106F-ER cells were incubated in the absence or presence of 100 nM 4-HT for 16 hours. Cell lysates were analyzed by Western blot with three anti–phospho-PAK antibodies that specifically detect autophosphorylated (active) EGFP-PAK-2L106F-ER or an anti-EGFP antibody to detect total EGFP-PAK-2L106F-ER. (C) Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were grown to confluency and then further incubated for 48 hours in the absence or presence of 100 nM 4-HT. Phase contrast pictures are shown for each of the four samples. Higher-magnification phase contrast and fluorescent pictures are shown for Hs578T-EGFP-PAK-2L106F-ER cells in the presence of 4-HT. (D) Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were seeded in soft agar in the absence or presence of 100 nM 4-HT and incubated for 96 hours. Phase contrast pictures are shown for each of the four samples. Higher-magnification phase contrast and fluorescent pictures are shown for Hs578T-EGFP-PAK-2L106F-ER cells in the presence of 4-HT.

cells after treatment with cisplatin for 24 hours in the absence or presence of 4-HT with a colorimetric viability assay. Cisplatin decreased cell viability in parental Hs578T cells in the absence and presence of 4-HT and in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT (Figure 6*B*). However, activation of EGFP-PAK-2L106F-ER with 4-HT dramatically stimulated cell viability. Therefore, elevating activity levels of full-length PAK-2 by activation of EGFP-PAK-2L106F-ER protects Hs578T cells from cisplatin-induced cell death.

Elevated Full-length PAK-2 Activity Negatively Regulates Caspase Activation of PAK-2, Activation of Caspase 3, and Apoptotic Histone 2B Phosphorylation

Cisplatin treatment causes high levels of cell death and caspase activation of PAK-2 in Hs578T cells (Figures 1–3), and stabilization of caspase-activated PAK-2p34 results in stimulation of cell death in Hs578T cells [12]. Therefore, we examined whether elevated full-length PAK-2 activity affects caspase activation of PAK-2 in response to cisplatin. Cell lysates were analyzed by Western blot with a C-terminal anti-PAK antibody that detects endogenous PAK-1, PAK-2, and caspase-activated PAK-2p34 as well as recombinant EGFP-PAK-2L106F-ER and its caspase-cleaved PAK-2p34-ER fragment (Figure 7*A*). As observed previously, treatment with cisplatin resulted in caspase activation of PAK-2 in parental Hs578T cells. The kinetics of the response in these experiments were slower than in previous experiments (Figure 3*A*), which is likely because of the varying efficacies of different batches of cisplatin. Caspase cleavage of PAK-2 was evident by decreased levels of full-length PAK-2 and generation of the PAK-2p34 fragment starting at 16 hours of cisplatin treatment (Figure 7*A*). Only slightly less caspase activation of PAK-2 was observed with Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT. However, activation of EGFP-PAK-2L106F-ER with 4-HT greatly reduced caspase activation of PAK-2. In Hs578T-EGFP-PAK-2L106F-ER cells in the presence of 4-HT, levels of full-length PAK-2 decreased only slightly, and levels of the PAK-2p34 fragment were markedly decreased compared with parental cells and Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT. Interestingly, levels of EGFP-PAK-2L106F-ER also decreased significantly in response to cisplatin in the absence of 4-HT but not in the presence of 4-HT (Figure 7*A*). Unexpectedly, levels of the PAK-2p34-ER fragment (indicated by the *arrowhead* in Figure 7*A*) did not correlate with the decrease of EGFP-PAK-2L106F-ER because they were higher in the presence than in the absence of 4-HT. This indicates that the PAK-2p34-ER fragment is rapidly degraded in the absence of 4-HT but not in the presence of 4-HT. Similar results were obtained with an antibody for the ER domain (data not shown). Our results suggest that elevating activity levels of full-length PAK-2 by activation of EGFP-PAK-2L106F-ER downregulates caspase activation of PAK-2.

To determine whether activation of EGFP-PAK-2L106F-ER downregulates caspase activation of PAK-2 by affecting the activation of caspases, we analyzed the activation of the initiator caspase 9 and the effector caspases 3 and 7 using antibodies specific for cleaved fragments of these caspases and the caspase substrate PARP (Figure 7*B*). In Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT, levels of activated caspase 3 were slightly decreased compared with parental cells, whereas in the presence of 4-HT, activated caspase 3 was undetectable (Figure 7*B*). Unexpectedly, levels of activated caspase 7 were increased in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT compared with parental Hs578T cells. In the presence of 4-HT, levels of activated caspase 7 decreased but were still at higher levels than in parental Hs578T cells. Levels of cleaved PARP were increased in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT compared with parental Hs578T cells but decreased to similar levels as in parental Hs578T cells in the presence of 4-HT (Figure 7*B*). Because PARP is a substrate of caspases 3 and 7, the results are consistent with the differential up- and down-regulation of the activation of these caspases in Hs578T-EGFP-PAK-2L106F-ER cells. Treatment of Hs578T cells with cisplatin resulted in activation and autocleavage of caspase 9 at Asp-315. Levels of autocleaved caspase 9 were increased

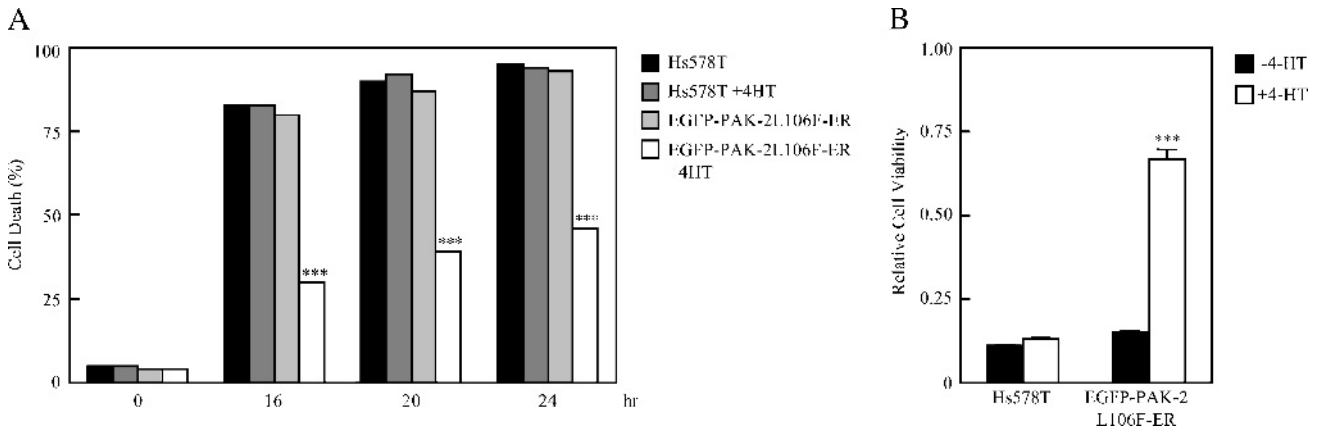

Figure 6. Activation of EGFP-PAK-2L106F-ER reduces levels of cell death and stimulates cell survival in response to cisplatin. (A) Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were treated for the indicated times with 50 μM cisplatin in the absence or presence of 100 nM 4-HT. Levels of cell death were determined by staining with Hoechst 33342 and analyzing apoptotic chromatin condensation by fluorescence microscopy. At least 500 cells were counted and categorized as normal or apoptotic by absence or presence of chromatin condensation and/or fragmentation. Levels of cell death are shown as percentages of total cells. *P* values for differences to parental Hs578T cells were calculated by Fisher's exact test. ***$P < .001$. (B) Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were grown in 96-well plates and treated for 24 hours with 50 μM cisplatin in the absence or presence of 100 nM 4-HT. MTS assays were performed with quadruplicate samples, and relative cell viability is shown as mean values and SDs in comparison to viability of cells before treatment, which was set arbitrarily at 1. *P* values for differences to parental Hs578T cells were calculated by Fisher's exact test. ***$P < .001$.

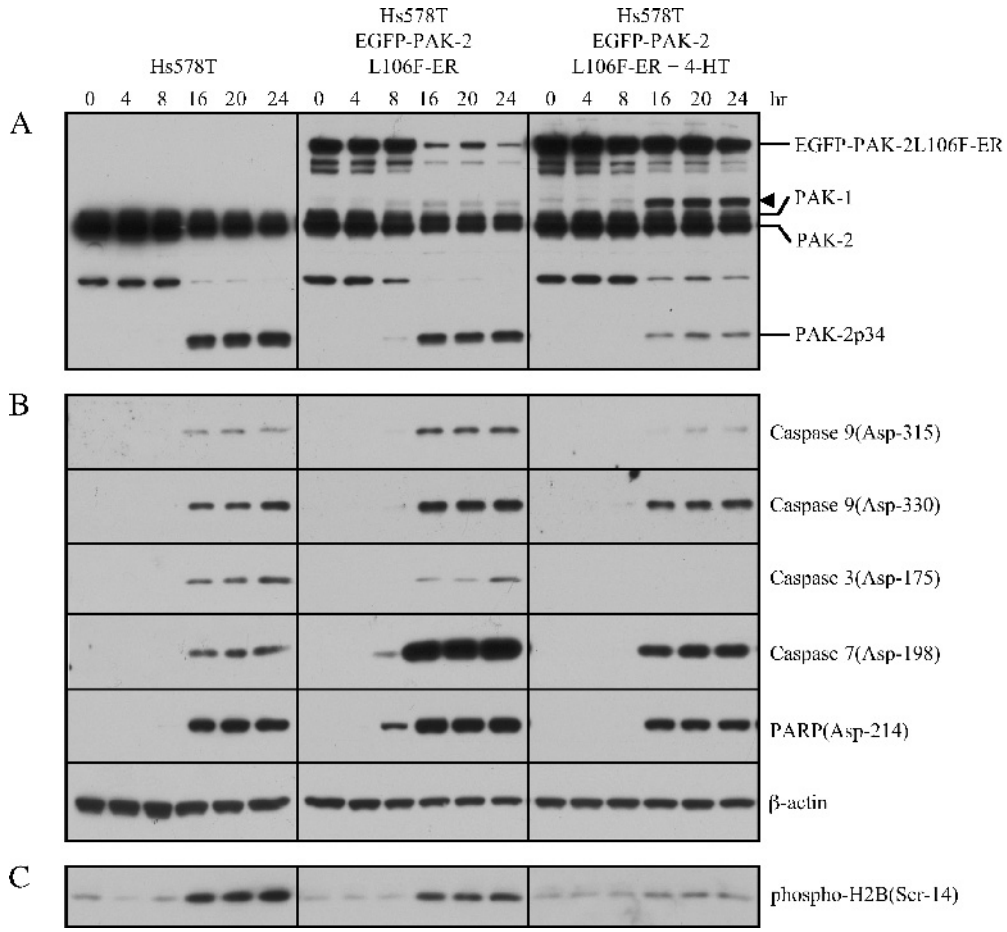

Figure 7. Activation of EGFP-PAK-2L106F-ER regulates caspase activation of PAK-2, activation of caspases, and proapoptotic histone 2B phosphorylation. Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells were treated with 50 μM cisplatin at indicated times in the absence or presence of 100 nM 4-HT. Adherent and detached cells were harvested and lysed. (A) Cell lysates were analyzed by Western blot with a C-terminal anti-PAK antibody. Positions of EGFP-PAK-2L106F-ER, PAK-1, PAK-2, and caspase-activated PAK-2p34 are indicated at the right; position of the caspase-cleaved PAK-2p34-ER fragment is indicated by the arrowhead. (B) Activation of caspases 3, 7, and 9 and cleavage of the caspase substrate PARP were determined with antibodies specific for cleaved fragments of these caspases and PARP. Even protein loading was verified by Western blot with an anti–β-actin antibody, but late apoptotic cell lysates such as those of Hs578T and Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT at 24 hours of cisplatin treatment showed decreased β-actin levels. (C) Histones were acid-extracted from cell lysates. Phosphorylation of histone 2B (H2B) at Ser-14 was analyzed by Western blot with an anti–phospho(S14)H2B antibody.

in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT compared with parental Hs578T cells. In the presence of 4-HT, levels of autocleaved caspase 9 decreased to levels slightly lower than those in parental Hs578T cells (Figure 7*B*). Caspase 9 cleavage at Asp-330 by caspase 3 also increased in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT compared with parental Hs578T cells. In the presence of 4-HT, caspase 9 cleavage at Asp-330 decreased to levels similar to those in parental Hs578T cells. The elevated levels of activated caspases 7 and 9 in Hs578T-EGFP-PAK-2L106F-ER cells could be due to a compensation mechanism caused by the basal activity levels of EGFP-PAK-2L106F-ER in the absence of 4-HT (Figure 5*B*). Interestingly, this increase in caspases 7 and 9 activation does not seem to contribute to cell death because Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT showed similar levels of cisplatin-induced cell death as parental Hs578T cells and addition of 4-HT decreased levels of cell death (Figure 6). Our results show that elevated activity of full-length PAK-2 downregulates activation of caspase 3. Because PAK-2 is cleaved by caspase 3, it seems that activated full-length PAK-2 negatively regulates its own cleavage to the proapoptotic PAK-2p34 fragment.

We have shown that apoptotic histone 2B phosphorylation at Ser-14 correlates with caspase activation of PAK-2 in response to treatment of breast cancer cells with taxol or cisplatin and that PAK-2p34 phosphorylates histone 2B at Ser-14 *in vitro*. Because activation of EGFP-PAK-2L106F-ER reduces levels of PAK-2p34, we analyzed if it also affects apoptotic histone 2B phosphorylation (Figure 7*C*). Apoptotic histone 2B phosphorylation was slightly reduced in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT and was dramatically reduced in the presence of 4-HT compared with that in parental cells. The results provide further evidence that PAK-2p34 is likely the protein kinase that phosphorylates histone 2B at Ser-14 in Hs578T cells in response to cisplatin.

Discussion

PAK family protein kinases seem to be involved in the development or progression of human cancer. Elevated PAK-1 and PAK-2 activity

have been associated with human breast cancer and seem to promote a more invasive and malignant phenotype [14–16]. Interestingly, *PAK-2* is among a panel of "invasiveness" genes that have been strongly associated with breast cancer metastasis and mortality [18]. Metastatic cancer is typically treated by chemotherapy with drugs that kill circulating cancer cells, but a subset of circulating cancer cells often escape cell death and become resistant to anticancer drug treatment. Full-length PAK-2 has been shown to stimulate cell survival and protect cells from stress-induced cell death, whereas caspase-activated PAK-2p34 is involved in the cell death response [8,12,13]. Here, we show that elevated PAK-2 activity causes anchorage-independent survival and growth as well as resistance to anticancer drug–induced cell death of human breast cancer cells. It is suggested that active full-length PAK-2 downregulates levels of active caspase 3 and its own cleavage to the proapoptotic PAK-2p34 fragment, and thereby restrains cell death.

Caspase activation of PAK-2 occurs during anticancer drug–induced cell death of human breast cancer cells and correlates with cell death and apoptotic histone 2B phosphorylation. In Hs578T cells, caspase activation of PAK-2 and apoptotic histone 2B phosphorylation correlates with cell death and activation of caspases. Levels of cell death of Hs578T cells, activation of caspases 3, 7, and 9, and caspase activation of PAK-2 are higher in response to cisplatin than taxol. Because levels of activated caspases 3, 7, and 9 are low in response to taxol, it is suggested that taxol-induced cell death involves other caspases or is, at least in part, caspase-independent. During apoptosis, histone 2B is phosphorylated at Ser-14, but the extent of phosphorylation varies depending on the cell type and apoptotic stimulus [24]. Caspase-cleaved Mst1 has been shown to mediate apoptotic histone 2B phosphorylation, but we have not detected caspase-cleaved Mst1 in Hs578T, MDA-MB435, and MCF-7 cells treated with cisplatin or taxol (data not shown). However, apoptotic histone 2B phosphorylation correlates with levels of caspase-activated PAK-2p34 in Hs578T, MDA-MB435, and MCF-7 cells treated with cisplatin or taxol, and conditional activation of EGFP-PAK-2L106F-ER greatly reduces levels of caspase-activated PAK-2p34 and apoptotic histone 2B phosphorylation in response to cisplatin. Because PAK-2p34 phosphorylates histone 2B at Ser-14 *in vitro*, it is possible that caspase-activated PAK-2p34 directly mediates apoptotic histone 2B phosphorylation in breast cancer cells. Alternatively, caspase-activated PAK-2p34 could mediate apoptotic histone 2B phosphorylation indirectly by stimulation of another protein kinase.

Cisplatin seems to induce apoptosis through the intrinsic apoptotic pathway resulting in the formation of the apoptosome and autocleavage of caspase 9 at Asp-315 [25,26]. Activated caspase 9 then cleaves and activates the effector caspases 3 and 7, which cleave other cellular proteins including PAK-2 during the execution of apoptosis [29,30]. Hs578T human breast cancer cells are highly sensitive to cisplatin, showing rapid and high levels of activation and autocleavage of caspase 9, activation of caspases 3 and 7, and caspase activation of PAK-2 and high levels of cell death within 24 hours. Caspase 9 is also cleaved at Asp-330 in a feedback loop by activated caspase 3. However, this does not seem to directly activate caspase 9 but rather abrogate inhibition by XIAP [26,31]. MDA-MB435 and MCF-7 cells are less sensitive to cisplatin than Hs578T cells and show less activation of caspases, caspase activation of PAK-2, and a later onset and lower levels of cell death.

Treatment of Hs578T, MDA-MB435, and MCF-7 human breast cancer cells with taxol induced lower levels of activated caspases, caspase-activated PAK-2p34, and cell death than treatment with cisplatin. However, in MDA-MB435 cells, differences between cisplatin and taxol treatments were smaller and less pronounced than in Hs578T and MCF-7 cells. Within the three breast cancer cell lines, Hs578T is highly sensitive to cisplatin and moderately sensitive to taxol, MDA-MB435 is moderately sensitive to cisplatin and taxol, and MCF-7 is moderately sensitive to cisplatin but resistant to taxol. The lack of caspase 3 does not seem to be the only reason for the higher resistance of MCF-7 cells against anticancer drug–induced apoptosis. MCF-7 cells also show very little activation and autocleavage of caspase 9, which is upstream of caspase 3. Introduction of the caspase 3 gene into MCF-7 cells restored morphologic nuclear and DNA fragmentation in response to Bax overexpression but did not change the rate of cell death [32].

Importantly, elevated PAK activity has been reported in human breast cancer samples and several human breast cancer cell lines. We have detected elevated PAK-2 activity in seven of eight surgical human breast cancer samples. In addition, we have detected elevated PAK-2 activity in MDA-MB435 and MCF-7 human breast cells but not in Hs578T human breast cancer cells or Hs578Bst human mammary epithelial cells. To examine the role of elevated PAK activity in cancer development or progression, we have generated a conditionally active form of PAK-2 and introduced it into Hs578T human breast cancer cells with low endogenous PAK activity levels. Conditional activation of EGFP-PAK-2L106F-ER resulted in loss of contact inhibition and anchorage-independent growth. This indicates a more malignant phenotype because metastatic cancer cells need to disseminate from the mammary epithelium, survive in circulation, and grow at distant sites [33–35]. Normal epithelial cells require contacts of integrins with extracellular matrix proteins for survival and die by anoikis (detachment-induced apoptosis) when they leave the epithelial layer and become detached from the extracellular matrix. In contrast, metastatic cancer cells have the ability to survive outside the epithelial layer independent of these integrin–extracellular matrix contacts [36–39]. PAKs have been shown to mediate survival signals downstream from integrin–extracellular matrix contacts [40,41]. Therefore, elevated PAK-2 activity may act as a constitutive survival signal and thereby allow anchorage-independent survival and growth. Interestingly, conditional activation of EGFP-PAK-2L106F-ER in Hs578T human breast cancer cells also suppresses cell death induced by the anticancer drug cisplatin suggesting that stimulated PAK-2 survival signaling blocks anoikis and anticancer drug–induced apoptosis. Recent findings support such an antiapoptotic role of elevated PAK signaling in mammary epithelial cells. Expression of a constitutively active PAK-1 mutant suppresses activation of caspase 3 in detached MCF-10A mammary epithelial cells [42]. In contrast, dominant-negative PAK-1 mutants reduce the malignant morphology of three-dimensionally cultured MCF-10A, NeoT, and AT1 cells [43]. Furthermore, elevated integrin-dependent Rac-PAK-1 signaling supports resistance to taxol-induced apoptosis of three-dimensional acini of mammary epithelial cells by allowing stress-induced activation of NF-κB [44]. Inhibition of PAK activity with a dominant-negative Rac or the protein kinase inhibitor domain of PAK renders mammary acini sensitive to cell death.

The balance of antiapoptotic full-length PAK-2 and proapoptotic PAK-2p34 activity seems to regulate cell survival and cell death. Cisplatin treatment causes caspase activation of PAK-2 and generation of the proapoptotic PAK-2p34 fragment, but conditional activation of EGFP-PAK-2L106F-ER greatly reduces levels of caspase-activated

PAK-2p34. This seems to be the result of suppression of caspase 3 activation because levels of activated caspase 3 are also dramatically reduced. Interestingly, levels of activated caspase 7 and, to a lesser degree, caspase 9 autocleavage at Asp-315 and caspase 3–mediated cleavage at Asp-330 are upregulated in Hs578T-EGFP-PAK-2L106F-ER cells in the absence of 4-HT. Because EGFP-PAK-2L106F-ER has basal activity levels, which are higher than those of endogenous PAK-2 in Hs578T cells, the increase of activated caspases 7 and 9 could be a compensatory effect caused by the continuous presence of moderately elevated PAK-2 activity. However, increased levels of activated caspases 7 and 9 do not increase levels of cisplatin-induced cell death or apoptotic histone 2B phosphorylation. These results indicate that elevated PAK-2 activity suppresses the apoptotic response by diminishing levels of activated caspase 3 independent of activation of caspase 7 or 9.

In summary, our results show that elevated PAK-2 activity suppresses apoptosis and causes anchorage-independent survival and growth of human breast cancer cells (Figure 8). Apoptotic stimuli such as anticancer drugs or detachment from the extracellular matrix initiate the intrinsic or extrinsic apoptosis pathways resulting in activation of initiator caspases. Elevated PAK-2 activity seems to suppress the apoptotic response downstream of activation of initiator caspases by down-regulating activation of caspase 3 and thereby preventing its own caspase cleavage to the proapoptotic PAK-2p34 fragment. At this point, it remains unknown whether PAK-2 affects caspase 3 activation directly or indirectly through other antiapoptotic factors such as NF-κB and/or inhibitor of apoptosis (IAP) proteins [45,46]. NF-κB has been shown to mediate elevated Rac–PAK-1 signaling and to induce expression of IAPs [44,45]. Inhibition of detachment-induced apoptosis results in anchorage-independent survival, which, in the presence of growth factors, may be sufficient for anchorage-independent growth. However, elevated PAK-2 activity could stimulate anchorage-independent growth directly by affecting the growth factor pathway. Group 1 PAKs have been shown to stimulate ERK-1/2 activation through phosphorylation of the upstream protein kinases Raf-1 and MEK-1 [1]. In addition, stimulation of anchorage-independent growth of MCF-7 human breast cancer cells by overexpression of a constitutively active PAK-1 mutant seems to be mediated by ERK-1/2 [16]. Elevated PAK-2 activity may promote malignant progression through multiple targets and pathways and, therefore, represents a potential therapeutic target to prevent metastasis and restore sensitivity to chemotherapeutic drugs.

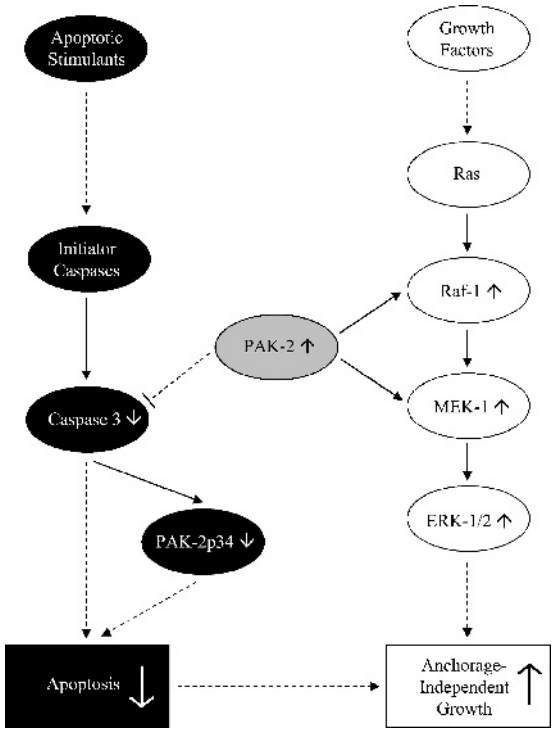

Figure 8. Proposed molecular mechanisms by which elevated PAK-2 activity suppresses apoptosis and causes anchorage-independent growth. Apoptotic stimuli result in activation of initiator caspases through the extrinsic or intrinsic pathway. Activated initiator caspases cleave and activate caspase 3. Caspase 3 executes apoptosis by cleavage of many intracellular proteins including PAK-2, which leads to generation of the proapoptotic PAK-2p34 fragment. Elevated activity of PAK-2 results in decreased caspase 3 activation and thereby prevents its own caspase cleavage and the execution of apoptosis. Growth factors stimulate cell growth through activation of the GTPase Ras, which activates the protein kinase cascade consisting of Raf-1, MEK-1, and ERK-1/2. Elevated PAK-2 activity could stimulate the growth factor pathway through phosphorylation of Raf-1 or MEK-1 and thereby cause anchorage-independent growth. Elevated activity of PAK-2 is indicated by an upward arrow, and effects of elevated PAK-2 activity on protein/activity levels of downstream mediators, or apoptosis and anchorage-independent growth are indicated by up- or downward arrows, respectively.

Acknowledgments

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