Stat3 and its isoforms belong to a family of cytoplasmic transcription factors that affect the synthesis of various proteins. Caspases are cysteinyl-aspartate proteases that function under apoptotic and non-apoptotic conditions. We now report that, in addition to transcriptional splicing, Stat3 fragmentation can be mediated by caspasas. Caspase activation in DU145 cells was achieved by staurosporine (STS) exposure, and Western analysis revealed a reduction in full-length Stat3 (fl-Stat3) expression that was caspase-mediated. This proteolytic relationship was further studied by exposing purified Stat3 protein to a mixture of active caspases under cell-free conditions. This demonstrated that caspases directly cleaved Stat3 and Stat3 cleavage was accompanied by the apparent formation of cleavage fragment(s). Stat3 cleavage fragments, reflecting multiple caspase cleavage sites, also were observed in vitro following STS exposure in DU145 cells and in HEK293T cells transfected to express Stat3 truncation mutants. The impact of cleavage on Stat3 transcriptional activity next was assessed and revealed that cleavage of fl-Stat3 was accompanied by reductions in Stat3-DNA binding, Stat3-driven reporter protein (luciferase) activity, and the expression of selected Stat3-dependent genes. Further, reduced Stat3 expression correlated with increased sensitivity to apoptotic stimuli. In concomitant experiments, reporter activity was assessed in Stat3 truncation mutant-expressing HEK293T cells and revealed that, under non-apoptotic conditions, expression of different Stat3 fragments induced differential effects on Stat3-driven luciferase activity. These findings demonstrate that fl-Stat3 undergoes proteolytic processing by caspases that reduces its expression and leads to the formation of cleavage fragments that may modulate Stat3 transcriptional activity.

Seven distinct gene products make up the STAT family in mammals and are designated Stat1 through Stat6 (with Stat5a and Stat5b). Additionally, Stats 1, 3, 4, and 5 are expressed as two isoforms, designated α and β, that exert different transcriptional activities (1–5). It is conventionally held that, after phosphorylation, most commonly as a consequence of cytokine-receptor binding and JAK activation, STATs combine as hetero- or homo-dimers and translocate to the nucleus where they affect the synthesis of key proteins involved in a wide variety of cellular processes, including differentiation and apoptosis (1, 6, 7). Of interest, the overexpression and constitutive phosphorylation of several Stat family members, for example Stat3, have been associated with the development of a neoplastic phenotype in various cell types (8–11). The variety of proteins and cellular processes impacted upon by STAT signaling reflects, in part, the diverse mechanisms that control STAT activation and deactivation. Presently, more than 40 different ligands of cytokine receptors can activate JAKs and induce STAT tyrosine phosphorylation (2, 7, 8, 12). Furthermore, intrinsic receptor tyrosine kinases may directly induce phosphorylation and circumvent the requirement for JAK participation in STAT activation (8). Recent evidence has even challenged the conventional view that STAT phosphorylation and dimerization are necessary for gene transactivation. For example, we have reported that Stat3 acetylation and deacetylation modulates its transcriptional activity (13). Similarly, Stark and colleagues (14, 15) have found that Stats 1 and 3 can induce the expression of a wide spectrum of target genes independent of their phosphorylation status or participation in dimer formation. In related studies, Azam et al. (16) presented evidence that limited proteolytic processing by serine proteases generated C-terminal truncated STAT proteins that could negatively regulate Stat3-, Stat5-, and Stat6-mediated signaling. These truncated molecules, designated STAT-γ, appear to function as dominant-negative regulators of transcription. In addition to processing by serine proteases(s), Stats 3, 5, and 6 also are converted into their γ moiety by the calcium-dependent cysteine protease calpain (17, 18).

Caspases are cysteinyl aspartate-specific proteases whose activation and function traditionally have been associated with apoptosis, or programmed cell death (19). Apoptosis is carried out by two predominant cellular pathways (20). Intrinsic (type-2, mitochondrial-mediated) apoptosis is often induced in tumor cells in response to chemotherapy. The initiating event usually is associated with DNA damage leading to mitochondrial depolarization and the activation of caspase 9, the apical caspase in this pathway (21, 22). Extrinsic (type-1, receptor-mediated) apoptosis occurs in a variety of cell types under physiological conditions and is triggered by ligand binding to specific receptors, such as CD95. This leads to "death-inducing signaling complex" formation and the activation of the apical caspases 2, 8, or 10 (20, 23, 24). Both pathways result in cell death associated with the activation of additional executioner caspases, such as caspase 3, and the proteolytic demolition of specific target proteins such as PARP (20, 22). Relevant to the present study, Goodbourn and King reported that Stat1α is a specific target for demolition by caspase 3 in HeLa cells after induction of apoptosis following exposure to double-stranded RNA (dsRNA) (25). There also is a
Stat3 Cleavage by Caspases

growing appreciation that certain caspases can be activated under non-apoptotic conditions. As reviewed by Schwerk and Schulze-Osthoff (26), caspase 8 activity appears to be required for normal T-cell activation and function. Similarly, Ishizaki and coworkers (27), as well as others (28-30) have shown that caspases 3, 9, and 14 participate in cellular differentiation. Therefore, although intimately involved in apoptosis, selected caspases may be activated under non-apoptotic conditions and participate in cellular processes not associated with programmed cell death.

During the course of studies to elucidate the mechanism(s) by which antineoplastic agents promote cell death, we observed that Stat3 protein levels were reduced during chemotherapy-induced apoptosis. Consequently, an analysis of the relationship between Stat3 protein expression and caspase activity in human cell lines was initiated. We now report that fl-Stat3 expression was reduced in a caspase-dependent manner following the induction of apoptosis in the DU145 cell line. Experiments conducted under cell-free conditions revealed that the relationship between caspase activity and fl-Stat3 expression was direct and accompanied by the apparent formation of Stat3 cleavage fragments. Preliminary studies to map the location of Stat3 cleavage sites were carried out in HEK293T cells transfected to express defined Stat3 truncation mutants with various domain deletions and revealed that Stat3 contains multiple cleavage sites. Finally, assessment of the functional implications of these findings revealed that Stat3 cleavage and fragment expression are capable of modulating Stat3 gene transactivation under both apoptotic and non-apoptotic conditions. These findings reveal that Stat3 expression and function are directly affected by caspase activities.

MATERIALS AND METHODS

Reagents

Cell culture cell culture flasks and pipettes were obtained from Fisher Scientific. Active recombinant caspases 1 through 10 and an antibody to caspase 9 were purchased from BIOMOL, Z-VAD-fmk was from Promega Corp., and Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk were from Calbiochem/EMD Biosciences. The PARP antibody was obtained from Zymed Laboratories. Antibodies to caspase 8 and TRAIL were from BD Pharmingen. C- and N-terminal antibodies for Stat3 and the Stat3-DNA binding site oligonucleotide-agarose conjugate were purchased from Santa Cruz Biotechnology. The antibody against fl-Stat3 was purchased from Imgenex, and the phospho-(pY705)-Stat3 and c-Myc antibodies were from Santa Cruz Biotechnology. Antibodies against FasL and Bcl-xl were purchased from BD Transduction Laboratories. The Seize® kit and reagents were from Pierce Biotechnology. Oncostatin-M (OSM) and IL-6 were purchased from R&D Systems.

9NC was generously provided by Dr. P. Pantazis at the University of Oklahoma. The β-actin antibody and all other reagents and chemicals were purchased from Sigma Co. unless otherwise noted.

Cell Lines

The relationship between caspase activity and Stat3 expression was evaluated in the DU145 and PC3 human prostate tumor cell lines, the HeLa human cervical carcinoma cell line, and in the HEK293T human embryonic kidney cell line. All cell lines were obtained from the American Type Culture Collection. Selected studies were also conducted in PC3 cells stably transfected to express Stat3 (PC3/Stat3), as previously described (13). DU145 and PC3 cells were carried in Roswell Park Memorial Institute (RPMI) 1640 media plus 10% FBS. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% FBS, and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% FBS. All cell lines were maintained in a humidified incubator at 37 °C under an atmosphere of 5% CO2.

Cytotoxicity

1 × 10^6 DU145, PC3, or PC3/Stat3 cells were plated into each well of a 96-well flat-bottom plate (Costar, Corning Inc.) in 100 µl of media plus serum. After 24 h, STS (0–1000 nM) (31–33) or 9NC (34) was added to each well in a volume of 100 µl. At various times thereafter up to 24 h, 100 µl of a 2 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS was added to each well, and the plate was processed to determine the absorbance of each well using a Bio-Tek Instruments EL800 plate reader, as previously described (35).

Flow Cytometry

Two × 10^6 cells were incubated for 24 h in medium containing serum. 24 h later, STS or 9NC were added in a volume to achieve final concentrations of 1000 nM or 40 nM, respectively. 24 h later, cells were harvested by trypsinization and resuspended in ice-cold PBS. The cells then were stained for 30 min at room temperature in the dark with a solution of 50 µg/ml propidium iodide, 0.6% Igepal, and 0.1% sodium citrate. Flow cytometry was performed by FACSscan (BD Biosciences) using the ModFit LT program (BD Biosciences). Statistical analysis was performed with the Kruskal-Wallis non-parametric analysis of variance test followed by Dunn’s multiple comparisons test using Instat (36).

Analysis of the Relationship between Caspase Activity and Stat3 Protein

In Vitro Studies—Approximately 2 × 10^6 DU145 or HeLa cells were seeded in 75-cm² flasks. On the following day 0–1000 nM STS, 50 nM 9NC (34), 40 µM Z-VAD-fmk, a pan-caspase inhibitor (37, 38), or combinations of these agents, were added. After an additional 12 h (STS) or 24 h (9NC), cells were harvested and lysed (39). Total proteins from cell lysates (60–75 µg/condition) were separated on a 10% polyacrylamide gel containing SDS and transferred to nitrocellulose filters that were processed and probed with antibodies to detect Stat3, caspase 8, caspase 9, actin, or PARP by chemiluminescence (39).

Alternatively, HEK293T cells where transiently transfected with one of several cDNAs encoding specific c-Myc-tagged Stat3 truncation mutants. Construction of these expression vector pcDNAs was as previously described (13). The c-Myc epitope was inserted at the 5′-end of the following Stat3 constructs: Stat3 full-length (1-770), Stat3 1-130, Stat3 1-320, Stat3 1-585, Stat3 1-720, Stat3 466-770, and Stat3 586-770. These Stat3 domain deletion constructs then were transiently transfected with Lipofectamine® (40). Transfected cells were exposed to 1000 nM STS for 12 h, and total proteins from whole cell lysates (25 µg/condition) were separated on a 10% SDS-PAGE and probed with antibodies to detect tagged proteins or β-actin, as described above.

Cell-free Studies—A cell-free assay was employed to assess the direct relationship between caspase activity and Stat3 protein. Stat3 was isolated and purified from DU145 cells by immunoprecipitation methods using the Seize® kit. Briefly, 2–5 × 10^7 cells were lysed, and Stat3 proteins were immunoprecipitated overnight at 4 °C. Immobilized protein then was eluted using a low pH buffer, neutralized, renatured, and concentrated in preparation for analysis or for storage at 4 °C for up to 3 days. To assess Stat3 proteolysis, purified Stat3 protein was incubated at 37 °C for 1 h with a mixture of active caspases (1 through 10) and pre-and postincubation Stat3 protein levels compared by Western analysis. Parallel experiments using equivalent amounts of Stat3 protein incubated in a caspase mixture that was heat-inactivated (100 °C for 10 min) prior to use served as control.
Functional Impact of Caspase Activity on Stat3 Signaling

Phospho-(pY705)-Stat3 Expression—DU145 cells were exposed to STS, Z-VAD-fmk, or their combination as described above. After 12 h, cells were harvested, processed for SDS-PAGE analysis, and probed with appropriate antibodies to detect phospho-(pY705)-Stat3 protein, as described above.

Electrophoretic Mobility Shift Assay—DU145, PC3, or PC3/Stat3 cells were seeded at a density of ~7 × 10^5 cells/flask. On the following day all cells were exposed to IL-6 (20 ng/ml), and DU145 cells also were exposed to STS (1000 nM), Z-VAD-fmk (40 μM), or their combination. After an additional 12 h all cells were harvested, and nuclear lysates were generated by previously reported methods (41). Aliquots of nuclear extract (5 μg of protein in 1 μl) then were combined with 1 μg of poly[d(I-C)], 32P-labeled Stat3-DNA binding oligonucleotide probe (42), and binding buffer in a final volume of 10 μl. Samples then were incubated for 30 min at 0 °C after which the reaction was stopped by adding an equal volume of loading buffer. Sample proteins were separated by SDS-PAGE methods on a 6% gel and binding species were visualized by conventional autoradiography (43).

Stat3-DNA Binding—A standard transcription factor enrichment assay was modified to assess Stat3 dimer binding to DNA. DU145 cells were seeded at a density of 4 × 10^6 in 150-cm² flasks and after 24 h exposed to STS, Z-VAD-fmk, or their combination. After 12 h, cells were harvested, and cell lysates were generated by incubation for 2 h at 4 °C in 1 ml of 20 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% glycerol, 20 mM dithiothreitol, and protease inhibitors. Particulate material was removed by centrifugation, and the supernatant (4 mg of total protein/condition) was incubated overnight at 4 °C in 3 ml of 100 mM HEPES-KOH (pH 7.9), 25 mM MgCl₂, 300 mM KCl, 50 mM dithiothreitol, 0.5% Nonidet P-40, and 50% glycerol, containing 50 μg of the Stat3-DNA binding site oligonucleotide (5'-GATCTCTTCTGG-GAATTCTTAGATC-3') (44)-agarose conjugate. Agarose beads then were removed by centrifugation, washed, and incubated in 50 μl of Laemmli buffer for 10 min at 100 °C. Immediately after, the supernatant was removed by centrifugal elution, and the eluant was stored at −20 °C for subsequent Western analysis of Stat3 content.

Luciferase Reporter Assay—DU145 cells (5 × 10⁵ cells/60-mm dish) were transiently transfected with 0.5 μg of a luciferase plasmid containing the consensus DNA Stat3 binding sequence for the SIE fragment (44) of the promoter region of mouse IRF1 gene using Lipofectamine® (Invitrogen) in serum-free medium (Opti-MEM, Invitrogen) (13). After 4 h the media was replaced with 2 ml of Opti-MEM plus 20% FBS and then replaced 24 h later with 2 ml of RPMI plus 10% FBS containing IL-6 (20 ng/ml) and STS, Z-VAD-fmk, or their combination. Six hours later cells were harvested, washed with PBS, and lysed in 300 μl of passive lysis buffer (Promega), and luciferase activity was evaluated using the Dual Luciferase Reporter Assay (Promega).

Alternatively, HEK293T cells (5 × 10⁵ cells/60-mm dish) were co-transfected with 0.5 μg of a luciferase plasmid containing the consensus DNA-Stat3 binding sequence and with one of several cDNAs encoding c-Myc-tagged Stat3 truncation mutants, as described above. After 12 h the media was removed and replaced with 2 ml of Opti-MEM plus 20% FBS for 42 h after which the media was replaced with 2 ml of RPMI 1640 plus 10% FBS alone or containing either OSM (50 μg/ml) or IL-6 (20 ng/ml). After an additional 6 h, luciferase activity was evaluated as described above.

Expression of Selected Stat3 Target Genes—DU145-, PC3-, or Stat3-expressing PC3 cells were exposed to IL-6 (20 ng/ml). DU145 cells also were exposed to STS (1000 nM), as previously described. After 12 h, cells were harvested, processed for SDS-PAGE analysis, and probed with appropriate antibodies to assess the expression of fl-Stat3, c-Myc, FasL, TRAIL, and Bcl-xl, as described above.

RESULTS

This study assessed the relationship between caspase activity and Stat3 expression. For in vitro experiments STS was used to activate caspasas, because it consistently induces caspase-mediated apoptosis in a variety of cell models (31–33). To confirm STS-induced caspase activation in our model, DU145 cells first were exposed to STS alone (0–1000 nM) for 12 h after which PARP cleavage was assessed (38, 45, 46). In Fig. 1A it is clear that STS efficiently induced apoptosis as evidenced by robust concentration-dependent PARP cleavage and induction of cytotoxicity. Under these conditions an STS-dependent reduction in Stat3 expression also is evident. A parallel study assessed the temporal effect of STS exposure on these parameters. For this experiment, DU145 cells were exposed to 1000 nM STS, and cell lysates were acquired every 90 min for 12 h. This experiment revealed that caspase 8 and caspase 9 activation and PARP cleavage were evident within 3 h of STS exposure and, again, correlated with the cytotoxicity (Fig. 1B). Assessment of Stat3 expression under these conditions revealed that caspase activation preceded the obvious reduction in Stat3 expression and suggested that the reduction in Stat3 expression was caspase-mediated.

To further define this relationship DU145 cells were exposed to STS alone, the caspase inhibitor Z-VAD-fmk alone (40 μM), or STS plus Z-VAD-fmk for 12 h after which Stat3 expression again was assessed. STS exposure again induced a dramatic reduction in Stat3 expression while exposure to STS plus Z-VAD-fmk preserved Stat3 expression (Fig. 2A), thus supporting the notion that this effect was caspase-mediated in these cells. To assess the generality of this effect, parallel experiments were carried out in the HeLa cells. This model was selected for comparison, because Goodbourn and King reported that, in these cells, Stat1 expression was directly demolished by caspase 3 following dsRNA-induced apoptosis (25). The results demonstrate that, in HeLa cells also, a 12-h exposure to STS reduced Stat3 expression (Fig. 2A). Again, co-exposure to STS and Z-VAD-fmk preserved Stat3 expression and supported the notion that the reduced expression was caspase- and not cell line-dependent. Experiments also determined whether the observed reduction in Stat3 expression was STS-dependent. To this end, DU145 cells were exposed to the pro-apoptotic topoisomerase I inhibitor 9NC (50 nM × 24 h) (34), alone or combined with Z-VAD-fmk. Exposure to 9NC also reduced Stat3 expression and induced PARP cleavage. In the presence of 9NC and Z-VAD-fmk, Stat3 and PARP expression were preserved (Fig. 2A).

The above findings support the contention that the reduction in Stat3 expression observed under apoptotic conditions was caspase-mediated. To further assess the relationship between caspase activity and Stat3 cleavage the effect of specific caspase inhibitors on STS-induced reductions in Stat3 expression next was determined. For these studies DU145 cells were exposed to STS alone or combined with Z-VAD-fmk (pan-caspase inhibitor), 20 μM Z-DEVD-fmk (caspase 3 inhibitor), 20 μM Z-IETD-fmk (caspase 8 inhibitor), or 20 μM Z-LEHD-fmk (caspase 9 inhibitor). The results revealed that, as previously observed, exposure to Z-VAD-fmk effectively inhibited the STS-induced reduction in Stat3 expression and effectively prevented STS-induced PARP cleavage (Fig. 2B). In contrast, exposure to the caspase 3, 8, or 9 inhibitors was less effective at preventing both the reduction in Stat3 expression and PARP cleavage following exposure to STS (Fig. 2B), suggesting that, although exposure to STS reduced Stat3 expression in a caspase-mediated fashion, the observed reduction in Stat3 did not specifically reflect cleavage by caspases 3, 8, or 9.
These findings could suggest that cellular processes other than caspase-mediated proteolysis were responsible for Stat3 cleavage under these conditions. Specifically, other proteases (i.e., calpains) (17, 18) or proteolytic processes (i.e., proteasome-mediated degradation) (16, 18, 47) also are activated in a caspase-dependent manner during programmed cell death, and these alternate processes could be responsible for Stat3 proteolysis. Thus, a cell-free assay system was employed to determine if there existed a direct relationship between Stat3 proteolysis and caspase activity. Stat3 was purified from DU145 cells by immunoprecipitation and incubated with a mixture of purified active caspases 1 through 10. These studies revealed that the amount of Stat3 was reduced as a direct consequence of caspase exposure (Fig. 2C). This effect was not a reflection of general proteolysis under these conditions, because the p20 fragment of caspase 9 was unaffected by this incubation.

This cell-free model then was employed to evaluate the temporal relationship between caspase activity and Stat3 expression. Purified Stat3 was exposed to the caspase mixture, and aliquots were removed at 45-min intervals. The results confirmed that exposing Stat3 to this caspase mixture reduced fl-Stat3 expression as a function of time (Fig. 3A). Furthermore, the reduction in Stat3 corresponded with the appearance of a peptide fragment in the 50-kDa range with affinity for the N-terminal antibody. Of interest, when this blot was reprobed with a C-terminal Stat3 antibody the 50-kDa fragment was not evident (data not shown). Although these findings do not rule out the possibility that other processes contribute to the reduction in Stat3 expression observed during apoptosis (15, 17, 18, 47), they do reveal that caspases directly cleave Stat3.

Because, under cell-free conditions, caspase-mediated Stat3 cleavage resulted in an apparent cleavage fragment in the 50-kDa range with affinity for the N-terminal Stat3 antibody, studies next determined if caspase-generated Stat3 cleavage fragments could be detected in whole cells using this antibody. For these studies two model systems were
employed. First, DU145 cells were exposed to STS, Z-VAD-fmk, or their combination. Thereafter, the cells were harvested and assessed by Western blot. This revealed several bands, with apparent molecular masses in the 70- and 50-kDa range, whose appearance corresponded with caspase activation (Fig. 3B). In contrast, employing either the C-terminal and fl-Stat3 antibodies, while revealing changes in total Stat3 expression, did not reveal cleavage fragments whose appearance corresponded with Stat3 demobilization.

These findings suggested that multiple Stat3 fragments were generated as a consequence of caspase activation. Examination of the amino acid sequence of Stat3 (48) revealed that it contained several potential cleavage sites containing either the “D” sequence motifs; at amino acids 169, 184, 371, 563, 625, and 723 (Fig. 3C). To confirm the presence of multiple caspase cleavage sites in Stat3, experiments next were conducted in HEK293T cells transfected to express selected c-Myc-tagged Stat3 truncation mutants. These cells were exposed to STS (1000 nM × 12 h) and assessed by Western analysis using a c-Myc antibody to detect tagged fragments. The results revealed that, with the exception of a small N-terminal Stat3 fragment corresponding to amino acids 1–130, the expression of all Stat3 truncation mutants was reduced as a consequence of STS exposure (Fig. 3D). These findings confirmed the absence of a cleavage site in the extreme N-terminal region (1–130) and support the notion that Stat3 contains multiple caspase cleavage sites bounded by amino acids 130–320 and 586–770.

The above finding also suggested that caspase-mediated Stat3 cleavage could affect Stat3 signaling, and therefore studies next determined the impact of STS-induced caspase activation on selected Stat3-signaling parameters. Because phosphorylated Stat3 participates in cytokine-mediated signal transduction (1, 2, 8, 9, 49), studies first determined whether the expression of tyrosine-phosphorylated (pY705)-Stat3 was reduced following STS exposure. These studies were carried out in cells exposed to STS and/or Z-VAD-fmk, and Western analysis revealed that changes in phospho-(pY705)-Stat3 expression closely mirrored those of total cellular Stat3 under the same exposure conditions (Fig. 4A). Subsequent experiments determined if this reduction in phospho-Stat3 reduced binding to DNA. Extracts were generated from DU145 cells exposed for 12 h to either STS, Z-VAD-fmk, or their combination. In initial experiments Stat3 binding to DNA was assessed by electrophoretic mobility shift assay. The results of this analysis revealed that the consequence of this reduction in the cellular content of phospho-Stat3 was a significant reduction in Stat3 dimer binding to DNA (Fig. 4B). To confirm the specificity of this effect Stat3 binding to DNA also was assessed in total cell extracts by exposure to the Stat3 DNA binding site consensus sequence immobilized on agarose beads. Western analysis of proteins associated with the Stat3 binding sequence revealed a dramatic reduction in Stat3 dimer recovery in cells exposed to STS alone, whereas exposing cells to STS plus Z-VAD-fmk partially prevented this effect (Fig. 4C).

This confirmed that apoptosis-associated caspase activity disrupted Stat3 phosphorylation and interaction with DNA. To determine if this effect impacted upon Stat3 transcriptional activity experiments next assessed if STS-induced caspase activation affected Stat3-driven luciferase reporter protein activity. DU145 cells were transiently transfected with the IRF1 luciferase reporter cDNA construct under the control of the Stat3 binding SIE fragment (13, 44). Cells then were exposed to IL-6 (20 ng/ml) and Z-VAD-fmk and/or STS after which luciferase activity was quantified. This revealed that luciferase activity was reduced over 90% in cells exposed to STS. Exposing cells to Z-VAD-fmk plus STS restored activity to about one-third of controls (Fig. 4D).

FIGURE 2. Stat3 cleavage is caspase-mediated in vitro and under cell-free conditions. A, analysis of the effect of a 12-h exposure to STS (1000 nM), Z-VAD-fmk (40 μM), 9NC (50 nM), or their combination on the expression of Stat3 and PARP in DU145 cells or HeLa human cervical carcinoma cells. 2 × 10⁶ cells were seeded in flasks, and 24 h later STS and/or Z-VAD-fmk or 9NC and/or Z-VAD-fmk was added. After an additional 12 h (STS) or 24 h (9NC), cells were harvested and processed for Western analysis as described in Fig. 1A with the exception that the C-terminal Stat3 antibody was used to visualize Stat3. B, analysis of the effect of a 12-h exposure to STS (1000 nM), alone or combined with the pan-caspase inhibitor Z-VAD-fmk (40 μM), the caspase 3 inhibitor Z-DEVD-fmk (20 μM), the caspase 8 inhibitor Z-IETD-fmk (20 μM), or the caspase 9 inhibitor Z-LEHD-fmk (20 μM), on the expression of Stat3 and PARP in DU145 cells. 2 × 10⁶ cells were seeded in flasks, and 24 h later STS and/or the selected caspase inhibitors were added. After an additional 12 h, cells were harvested and processed for Western analysis as described in A. C, Western blot analysis of the effect of exposure to a mixture of caspases (1–10) on purified Stat3 protein. Stat3 was isolated from DU145 cells by microbatch immunoprecipitation. Purified Stat3 protein was exposed to a mixture of either active or heat-inactivated caspases for 60 min at 37 °C. Immediately thereafter, pre- and postincubation samples were processed for Western blot analysis to detect Stat3 protein using the C-terminal Stat3 antibody.

JUNE 30, 2006•VOLUME 281•NUMBER 26

JOURNAL OF BIOLOGICAL CHEMISTRY 17711
These findings suggest that caspase-induced changes in Stat3 expression, activation, and signaling impact upon Stat3-driven gene expression. Therefore, complementary studies were conducted to compare the impact of altering fl-Stat3 expression on the expression of selected Stat3-dependent genes under both apoptotic and non-apoptotic conditions. For these studies, the expression of selected Stat3-dependent genes under non-apoptotic conditions was assessed in IL-6-exposed PC3 cells (Stat3 null) and in PC3 cells stably transfected to express Stat3. The results revealed that the expression of c-Myc and BclXL, which are involved in the regulation of apoptosis (50, 51), was relatively lower in Stat3 null PC3 cells (Fig. 5A). In parallel studies, the expression of these selected Stat3-dependent genes also was assessed in IL-6-exposed DU145 cells (with or without STS). The results of this study reveal that in STS-treated cells Stat3 expression was reduced, and this again correlated with a reduction specifically in the expression of c-Myc and BclXL (Fig. 5A). These findings reveal that caspase-induced reductions in fl-Stat3 expression can selectively affect the expression of Stat3 target genes involved in apoptosis.

These studies were extended to determine if the proteolytic processing of fl-Stat3 indeed impacted on cellular response to apoptotic agents. For these studies the sensitivity of PC3 and PC3/Stat3 cells to STS- or 9NC-induced cytotoxicity was quantified and revealed that Stat3 null PC3 cells were more sensitive to the cytotoxic effects of both agents (Table 1). Apoptosis-associated DNA fragmentation also was assessed in both cell lines after exposure to these agents and was greater in Stat3 null PC3 cells again suggesting that these cells were more sensitive to the pro-apoptotic activity of STS and 9NC (Table 1). These findings, combined with our observation that reduction in the expression of fl-Stat3 correlates with alterations in the expression of selected Stat3 target genes, suggest that the proteolytic control of Stat3 expression may in fact be physiological relevant in survival and apoptotic processes.

In addition to reducing the expression of the fl-protein, the proteo-
FIGURE 4. Stat3 cleavage inhibits Stat3 signaling and transcriptional activity. A, Western blot analysis of the effect of a 12-h exposure to STS, Z-VAD-fmk, or their combination, on the expression of fl-Stat3 or phospho-(pY705)-Stat3 in DU145 cells. Cell lysates were generated and processed as described in Fig. 2A. The filter then was stripped and reprobed with the (pY705)-Stat3 antibody. Densitometric analysis of Stat3 (solid bars) and phospho-(pY705)-Stat3 (open bars) expression as a function of the above exposure conditions is presented in the contained graph. For this comparison, expression in non-treated (control) samples was normalized to 100% (bars mean ± S.D., n = 4–5). B, electrophoretic mobility shift assay analysis of Stat3 dimer binding to DNA as a function of exposure (12 h) to IL-6, STS, and/or Z-VAD. Following exposure, DU145, PC3, or PC3/Stat3 cells were harvested, and nuclear lysates were generated. Particulate material then was removed by centrifugation, and the resulting supernatant (5 μg of protein/condition) was combined with 1 μg of poly(dI-dC), 32P-labeled DNA binding probe, and binding buffer in a final volume of 10 μl. After 30 min at 0 °C, the reaction was terminated and sample proteins were separated by SDS-PAGE methods on a 6% gel. Binding species were visualized by conventional autoradiography. As indicated, nuclear extract from non-IL-6-treated cells and extract containing anti-Stat3 was included in the DNA binding reaction to identify Stat3 dimers and supershift the Stat3-DNA complex. C, Western blot analysis of Stat3-DNA binding as a function of exposure (12 h) to STS and/or Z-VAD. Following exposure, cells were harvested and cell lysates were generated. Particulate material then was removed by centrifugation, and the resulting supernatant (4 mg of total protein/condition) was incubated overnight at 4 °C in binding buffer containing 50 μg of Stat3-DNA binding site oligonucleotide-agarose conjugate. Adsorbed protein was removed by denaturation in Laemmli buffer for 10 min at 100 °C. The resulting supernatant was analyzed by Western blot methods to assess Stat3 content, using the N-terminal antibody. D, effect of changes in fl-Stat3 expression on Stat3-driven luciferase activity in PC3, PC3/Stat3, and DU145 cells. In DU145 cells, the effect of STS-induced caspase activation on Stat3-driven luciferase activity was assessed. Cells were transiently transfected with a luciferase plasmid containing the consensus DNA Stat3 binding sequence for the SIE fragment of the promoter region of mouse IRF1 gene using Lipofectamine. After 4 h the media was replaced with Opti-MEM plus 20% FBS, and 24 h later cells were exposed to IL-6 (20 ng/ml) and STS, Z-VAD-fmk, or their combination, for 6 h. Therefore, luciferase activity was measured in cytosol extracts and compared with activity observed in non-treated (control) cells, in which the observed activity was set at 100 arbitrary units. The PC3 parental cell line (Stat3 null) was stably transfected to express full-length Stat3, as previously described. Both PC3/Stat3-expressing cells and the PC3 parental line were exposed to IL-6 (20 ng/ml) for 6 h. Luciferase activity then was measured in cytosol extracts. Activity detected in Stat3-expressing PC3 cells was set at 100 arbitrary units (bars mean ± S.D., n = 3–4).
lytic processing of Stat3 generates cleavage fragments. To determine if Stat3 fragments also possessed signaling activity, complementary studies to those described above were carried out in which Stat3-driven luciferase activity was quantified in HEK293T cells transfected to express selected Stat3 truncation mutants under non-apoptotic conditions. Surprisingly, this study revealed that none of the expressed Stat3 fragments inhibited reporter activity below that detected in empty vector-transfected (control) cells. Indeed, the expression of N- or C-terminal fragments corresponding to amino acids 1–320 and 466–770 resulted in nearly the same degree of reporter activity as observed in cells transfected with full-length (1–770) Stat3 (Fig. 5B). Surprisingly, expression of the N-terminal fragment corresponding to amino acids 1–720, an N-terminal fragment lacking the region of Stat3 implicated in transcriptional activation (52, 53) (Fig. 3C), resulted in reporter activity significantly above that obtained following transfection with fl-Stat3 (Fig. 5B). Parallel studies also were carried out to determine if expression of Stat3 truncation mutants affected either OSM- or IL-6-driven luciferase reporter activity. The results of these studies revealed that expression of either fl-Stat3 or the N-terminal fragment corresponding to amino acids 1–720, combined with cytokine exposure, resulted in reporter activity greater than that observed in control (empty vector-transfected) cells, whereas the expression of the other Stat3 fragments slightly reduced luciferase activity under these conditions (Fig. 5C). Thus, under apoptotic conditions, caspase activation presumably results in Stat3 demolition accompanied by the inhibition of Stat3-signaling processes and a reduction in the expression of selected Stat3-de-
**TABLE 1**

**Correlation between fl-Stat3 expression and sensitivity to pro-apoptotic agents**

For IC₅₀ determinations, cells were seeded in 96-well (flat bottom) plates (1 × 10⁴ cells/well) in a volume of 100 µl and incubated at 37 °C. 24 h later 9NC (1–100 nM) or STS (100–1000 nM) was added to each well in a volume of 100 µl. Then, 24 h later, 100 µl of a 2 mg/ml solution of MTT in PBS was added to each well, and the plate was processed to determine the absorbance of each well using a Bio-Tek Instruments EL800 plate reader. Cells not exposed to drug, or medium containing no cells, were used as positive or negative controls, respectively. An absorbance of 2× the negative control was considered positive for viable cells. To assess DNA fragmentation, cells were seeded into 25-cm² flasks at a density of 3 × 10⁴ cells/flask. After another 24 h, 9NC (50 nM) or STS (1000 nM) was added. 12 (STS) or 24 (9NC) h later cells were harvested and stained for 30 min at room temperature in the dark with a solution of 0.05 mg/ml propidium iodide (BD Biosciences) and 0.1% sodium citrate. Flow cytometry was performed by FACScan (BD Biosciences) using the ModFit LT program (BD Biosciences). Statistical analysis was performed with the Kruskal-Wallis non-parametric analysis of variance test followed by Dunn’s multiple comparisons test using Instat. (IC₅₀, values are mean ± S.D., n = 4–8, sub-G₀/G₁, values are the average of two determinations.)

| Cell line | Agent | IC₅₀ | Sub-G₀/G₁ |
|-----------|-------|-----|---------|
| PC3       | 9NC   | 28 ± 4 | 40.8 |
| PC3/Stat3  | 9NC   | 40 ± 7 | 22.0 |
| PC3       | STS   | 213 ± 30 | 31.4 |
| PC3/Stat3  | STS   | 513 ± 86 | 19.4 |

**DISCUSSION**

Many processes influence Stat3 signaling, and it is now recognized that limited proteolysis can modulate Stat3 transcriptional activity by controlling the size and/or composition of the cytoplasmic pools of this protein (2, 8, 10, 16, 18). Here we report that cysteiny1 aspartate-specific proteases, caspases, also are capable of directly cleaving Stat3 and generating multiple Stat3 cleavage fragments. Reflecting the close association between caspases and programmed cell death, it is interesting to speculate that an early consequence of apoptosis is the down-regulation, via the caspase-mediated demolition, of Stat3-driven anti-apoptotic protein synthesis (54–57). Indeed, we found that caspase-mediated Stat3 demolition correlated with the progression of prostate tumor cells through apoptosis to cell death and corresponded with a reduced expression of c-Myc and BclXL, both regulators of apoptosis (50, 51). Support for the concept that Stat3 is demolished during apoptosis also is derived from analysis of its amino acid sequence that reveals several caspase cleavage sites (Fig. 3C) that, in the face of multiple caspase activities, should result in the generation of numerous polypeptide fragments. That Stat3 contains multiple caspase cleavage sites also was confirmed by our observation that several caspase-generated Stat3 fragments were generated under cell-free conditions and under apoptotic conditions in both DU145 cells and Stat3 fragment expressing HEK293T cells (Fig. 3). Indeed, the studies in HEK293T cells revealed that, with the exception of the small N-terminal fragment (1–130), every expressed Stat3 truncation mutant was cleaved as a consequence of STS-induced caspase activation.

Another implication of these findings reflects the dual functionality of caspases. Without question, caspases play an important role in apoptosis. However, there is a growing appreciation for the roles caspases play in proliferation, differentiation, and cell cycle regulation (26). For example, considerable evidence suggests that caspase 8 activity is required for the activation and proliferation of both T- and B-cells (26, 58–60). Further, Zhou et al. (61), studying T-cells, found that this caspase can be activated independent of CD95 and preferentially cleaves proteins involved in cell cycle regulation, such as WEE1. In addition to this non-apoptotic role for caspase 8, Ishizaka et al. (27) reported that caspase 3 is required for the terminal differentiation of rodent eye-lens epithelial cells. Similarly, caspase 3 and 9 activities appear to be required for normal erythroid progenitor cell differentiation and platelet formation from megakaryocytes (62, 63). Our present finding, that caspase activation reduces Stat3 expression while generating specific Stat3 cleavage fragments, may represent a novel mechanism that links the activity of specific caspases to non-apoptotic cell functions. In support of this possibility we observed that the expression of selected Stat3 domain deletion fragments under non-apoptotic conditions can modulate Stat3 transcriptional activity. This suggests that the limited proteolytic processing of Stat3, as could be predicted to occur under limited, non-apoptotic caspase activation, also may modulate Stat3 signaling. This notion is supported by the location of the putative caspase cleavage sites of Stat3. Stat3 has six functional domains (52, 53) (Fig. 3C). The first 130 amino acids in the N-terminal region (Fig. 3C, N-term) functions to stabilize Stat3 dimer interactions with DNA, whereas the region between amino acids 138 and 319 is implicated in dimer formation and other protein-protein interactions (coiled-coil domain; Fig. 3C, Coiled-coil). The DNA binding domain for Stat3 (DNA binding) is bracketed by amino acids 321 and 465. Stat3 contains an SH2 domain between amino acids 584 and 674 that is linked to the N-terminal region by a linker domain between amino acids 466 and 583. Tyrosine and serine phosphorylation occurs at amino acids 705 and 727, respectively, and transcriptional activation appears to require the C-terminal region bounded by amino acids 721 and 770. Thus, large N-terminal or C-terminal intact Stat3 fragments, generated by limited caspase-mediated proteolysis, will possess intact functional domains that may allow modified Stat3-DNA binding interactions, Stat3 dimer formation, and/or transcriptional activity.

To aid in defining the sequence and function of naturally generated fragments, we have initiated a study to assess the relationship between individual caspase activities and the generation of discrete Stat3 fragments under cell-free conditions. In preliminary studies we have exposed purified Stat3 to individual caspases under cell-free conditions and have observed that Stat3 is not demolished with equal efficiency by all caspases (64). Interestingly, only caspases 2, 4, 5, and 10 appear capable of cleaving Stat3 and generating specific cleavage fragments, a finding consistent with data presented here (Fig. 28). Functionally, caspases 8, 9, 10, and possibly 2 are the ‘initiator’ caspases responsible for interpreting cellular or environmental signals and initiating an apoptotic cascade, whereas caspases 3, 6, and 7 are ‘executioner’ caspases that amplify an apoptotic response. Caspases 1, 4, and 5 are the interleukin converting enzyme-like caspases that participate in cytokine processing, post-translational modifications, and apoptosis (65–69). Thus, our preliminary findings support the position that caspase-mediated Stat3 cleavage is not confined to late stage apoptosis (i.e. not mediated by executioner caspases) and that caspase-generated Stat3 fragments may be generated under non-apoptotic conditions.

Our observation that STS-induced apoptosis reduces Stat3 expression in HeLa cells also extends the findings of King and Goodburn (25) who reported that, in this model, apoptosis induced by exposure to either dsRNA or etoposide resulted only in the cleavage of Stat1. The difference in the spectrum of STAT family members targeted by caspases in their report as compared with our findings could reflect differences in the apoptotic pathways induced by exposure to dsRNA, etoposide, and STS. For example, exposure to dsRNA mimics a viral challenge (70, 71), whereas etoposide is a specific inhibitor of topoisomerase II that causes the accumulation of double-stranded DNA breaks (72). One could speculate that the particular caspases activated...
by exposure to either dsRNA or etoposide could result in the targeting of a specific subset of STAT molecules in response (25). In contrast, STS inhibits protein kinases A, G, and C, as well as a variety of other tyrosine kinases (31–33). The myriad of pathways thus affected, and the resulting multifaceted induction of apoptosis, could activate multiple caspases that consequently result in the targeting of different STAT family members.

Finally, the present results also suggest that there may be a link between caspase activities and the oncogenic properties associated with the overexpression and/or constitutive phosphorylation of STAT proteins. For example, in prostate tumor cells, STAT activation and overexpression are oncogenic and associated with the overexpression of anti-apoptotic proteins and the down-regulation of pro-apoptotic proteins (9, 11). Indeed, recent studies by Barton and coworkers (73, 74) support this notion and reveal that STAT overexpression is closely linked to prostate cell survival and that modulating the expression, either positively or negatively, of STAT3 alone can alter the balance between cell survival and apoptosis. Clearly, the up-regulation of Stat3 in prostate cancer cells can reflect multiple factors, including alterations in cytokine receptor expression and JAK activity. Our present findings suggest that modulating caspase activities may also increase Stat3 expression by disrupting its proteolytic processing and thus affect the expression of Stat3-targeted genes (Fig. 5A). This potential relationship between disrupted caspase activities and Stat3 oncosogenesis may be particular relevant in neoplastic cells where apoptotic signaling, and presumably caspase activation, clearly are defective. Derived studies to correlate differences in caspase expression and activation with differences in constitutive Stat3 expression in both normal human prostate epithelial cells and DU145 human prostate tumor cells are planned.

In conclusion, STAT3 undergoes proteolytic processing by caspases. This processing is associated with a reduction in Stat3 expression, the formation of discrete cleavage fragments, and alternations in Stat3 transcriptional activity. As such, this caspase-mediated processing may represent another mechanism to modulate Stat3 signaling under apoptotic and non-apoptotic conditions.

REFERENCES
1. Ihle, J. N., and Kerr, I. M. (1995) Trends Genet. 11, 69–74
2. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
3. Wang, D., Straropodis, D., Teglund, S., Kriazawa, J., and Ihle, J. N. (1996) Mol. Cell. Biol. 16, 6141–6148
4. Maritano, D., Sugrue, M. L., Tininini, S., Dewilde, S., Strobl, B., Fu, X., Murry-Tait, V., Chiarle, R., and Poli, V. (2004) Nat. Immunol. 5, 401–409
5. Hoey, T., Zhang, S., Schmidt, N., Yu, Q., Ramchandian, S., Xu, X., Naeger, L. K., Sun, Y. L., and Kaplan, M. H. (2003) EMBO J. 22, 4327–4348
6. Heim, M. H. (1999) J. Recept. Signal. Transduct. Res. 19, 75–120
7. Pfeffer, L. M., Dinarello, C. A., Herberman, R. B., Williams, B. R., Borden, E. C., King, P., and Goodbourn, S. (1998) J. Biol. Chem. 273, 8699–8704
8. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Poli, V. (2004) Nat. Immunol. 5, 401–409
9. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
10. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2349
11. Nam, S., Buettner, R., Turkson, J., Kim, D., Cheng, J. Q., Muehlbeyer, S., Hippe, F., Vatter, S., Meier, K.-H., Eisenbrand, G., and Jove, R. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 5908–6003
12. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
13. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
14. Zhang, S., Schmidt, N., Yu, Q., Ramchandian, S., Xu, X., Naeger, L. K., Sun, Y. L., and Kaplan, M. H. (2003) EMBO J. 22, 4327–4348
15. Heim, M. H. (1999) J. Recept. Signal. Transduct. Res. 19, 75–120
16. Pfeffer, L. M., Dinarello, C. A., Herberman, R. B., Williams, B. R., Borden, E. C., King, P., and Goodbourn, S. (1998) J. Biol. Chem. 273, 8699–8704
17. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Poli, V. (2004) Nat. Immunol. 5, 401–409
18. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
19. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2349
20. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
21. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
22. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
23. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
24. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
25. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
26. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
27. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
28. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
29. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) Mol. Cell. Biol. 24, 9390–9400
6785–6790
62. DeBotton, S., Sabri, S., Daugas, E., Zermati, Y., Guidotti, J. E., Hermine, O., Kroemer, G., Vainchenker, W., and Debili, N. (2002) Blood 100, 1310–1317
63. Sordet, O., Rebe, C., Plenchette, S., Zermati, Y., Hermine, O., Vainchenker, W., Garrido, C., Solary, E., and Dubrez-Daloz, L. (2002) Blood 100, 4446–4453
64. Darnowski, J. W., Goulette, F. A., and Cousens, L. P. (2004) Proc. Am. Assoc. Cancer Res. 45, 1195
65. Shi, Y. (2000) Mol. Cell. 9, 459–470
66. Ashkenazi, A., and Dixit, V. M. (1999) Curr. Opin. Cell Biol. 11, 255–260
67. Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., and Salvesen, G. S. (1999) J. Biol. Chem. 274, 8359–8362
68. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
69. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) J. Biol. Chem. 272, 17909–17911
70. Takizawa, T., Fukuda, R., Miyawaki, T., Ohashi, K., and Nakanishi, Y. (1995) Virology 209, 288–296
71. Takizawa, T., Ohashi, K., and Nakanishi, Y. (1996) J. Virol. 70, 8128–8132
72. Walker, P. R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J. F., and Sikorska, M. (1991) Cancer Res. 51, 1078–1085
73. Barton, B. E., Murphy, T. F., Shu, P., Huang, H. F., Meyerhofen, M., and Barton, A. (2004) Mol. Cancer Ther. 3, 1183–1191
74. Huang, H. F., Murphy, T. F., Shu, P., Barton, A. B., and Barton, B. E. (2005) Mol. Cancer 12, 2