**Simac Is Required for Cytochrome c-induced Apoptosis in Prostate Cancer LNCaP Cells**

Jonathan P. Carson, Marcelina Behnam, Jennifer N. Sutton, Chunying Du, Xiaodong Wang, Donald F. Hunt, Michael J. Weber, and George Kulik

Departments of Microbiology [J. P. C., M. B., M. J. W., G. K.] and Chemistry [J. N. S., D. F. H.] at the University of Virginia, Charlottesville, Virginia 22908, and Howard Hughes Medical Institute, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235 [C. D., X. W.]

**Abstract**

Release of cytochrome c from mitochondria to cytosol has been identified as one of the central events of apoptosis. Direct injection of cytochrome c induces apoptosis in some but not in all cell types. We observed that LNCaP prostate cancer cells failed to undergo apoptosis induced by cytochrome c microinjections. Microinjection of cytochrome c with another mitochondrial protein, Smac, was sufficient to activate caspases, however. Smac is believed to function as a neutralizer of caspase inhibitors, and mass spectrometry analysis identified XIAP as a predominant Smac binding protein in LNCaP cells. These findings are consistent with a requirement for a release of Smac from mitochondria to enable caspase activation in prostate cells. Indeed, translocation of Smac from mitochondria to cytosol was observed in LNCaP cells that underwent apoptosis and was inhibited by epidermal growth factor, which is a survival factor for these cells. These results further emphasize the central role of mitochondria in the regulation of apoptosis in prostate cancer cells.

**Introduction**

The high failure rate for castration or chemotherapy to bring about permanent cures for metastatic prostate cancer can be partly explained by antiapoptotic adaptations derived from mutation and natural selection within the neoplasia (1–3). Accordingly, we and others have observed that the Akt survival pathway is constitutively active in LNCaP cells (4–6). In addition, Akt-independent survival signaling pathways can be activated when these cells are treated with EGF,4 androgen, or serum (5–8). However, it is not clear how signaling cascades are required to elicit survival or which proapoptotic molecules are ultimately targeted.

It has been suggested that release of cytochrome c from mitochondria is sufficient to induce the nucleation of “apoptosomes,” multi-protein complexes that contain the zymogen forms of caspase 3 and Apaf-1, and perhaps other factors (9–11). When recruited to the apoptosome, caspase 9 is activated by autocatalytic cleavage and in turn cleaves and activates caspase 3 (12). Consistent with this mechanism, direct injection of cytochrome c into the cell cytosol is reported to induce apoptosis in several cell types (13, 14).

We were intrigued by the question of whether cytochrome c microinjection is sufficient to induce apoptosis in LNCaP prostate cancer cells, knowing this would provide a convenient model to assess if survival signals operate upstream or downstream from mitochondria.

**Materials and Methods**

**Antibodies and Other Reagents.** Antibodies directed against the active form of caspase 3 (CM-1) were from Idun Pharmaceuticals (La Jolla, CA). Monoclonal antibody to cytochrome c was from Pharmingen (San Diego, CA). Anti-Smac rabbit antisera and recombinant Smac protein were described previously (15). Anti-poly(ADP-ribose) polymerase goat antiserum was from R&D Systems, Inc. (Minneapolis, MA); monoclonal antibody to Erk2 (1B3B9) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibody to XIAP was from BD Transduction Labs (Lexington, KY). Antibody to caspase 7 was a gift from Dr. Junying Yuan (Harvard University). Antibody to caspase 9 was from Pharmingen. Secondary fluorophore-conjugated goat antimouse or goat anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Secondary horseradish-peroxidase-conjugated antibodies used for Western blots were from Amersham Pharmacia Biotech (Piscataway, NJ). Cytochrome c protein, LY294002, DAPI, Texas Red, cycloheximide, and other chemicals were from Sigma Chemical Co. (St. Louis, MO). BSA was from Boehringer Mannheim (Indianapolis, IN). Recombinant EGF and TNF-α were from Upstate Biotechnology, Inc., zVAD-fmk was from Bachem (King of Prussia, PA), and DEVD-AMC was from Calbiochem (San Diego, CA). Tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD). Microinjection equipment was from Eppendorf (Hamburg, Germany).

**Cell Lines.** PrE normal prostate epithelial cells were purchased from Clonetics-Cambrex (Rutherford, NJ) and maintained in media supplied by the company. LNCaP cells were a gift from the laboratory of Dr. Leland Chang (University of Virginia) and maintained in T Medium with 5% FCS. Generation and maintenance of RIG cells was described previously (16). All cells were kept in 5% CO₂ at 37°C.

**Microinjections and Apoptosis Detection.** Before analyses, cells were plated 1.5 × 10⁶/3-cm dish with microinjection coverslip. The next day, medium was changed to serum-free, phenol red-free RPMI for an additional 24 h. Then cells were injected into the cytoplasm with BSA, cytochrome c, or recombinant Smac (4 mg/ml). To mark injected cells, 2 mg/ml Dextran 10,000 conjugated with Texas Red were added to injection mixtures. Cells with active caspases were revealed by indirect immunofluorescence with CM-1 antibodies that recognize active caspase 3 followed by goat antirabbit antibodies conjugated with Cy-5 fluorochrome. Nuclei were visualized by staining with DAPI. Details of immunofluorescent procedures were described previously (8). Percentage of injected apoptotic cells was determined by examining the superimpositions of green, red, and blue visual fields using a Nikon microscope equipped with a digital camera and software from Inovision (Durham, NC).

**Hypotonic Lysis for Anti-Cytochrome c and Anti-Smac Western Blots.** LNCaP cells were plated on 15-cm dishes and grown to 80% confluence. Then, medium was changed to serum-free, phenol red-free RPMI for an additional 24 h. After administering treatments for 24 h, cytosolic extracts were obtained as previously described (5), and 25 µg of protein/lane were used for Western blots. The integrated pixel densities of immunoreactive electrophoretic bands of Smac, cytochrome c, and Erk2 were calculated using software from Scion (Frederick, MD), normalized to the corresponding density of the Erk2 bands, and calculated as a factor of the negative control.

1 Supported by Grants CA39076 and CA76465 from NIH, a gift from CapCure, and Grant 01-09 from Virginia’s Commonwealth Health Research Board.

2 To whom requests for reprints should be addressed, at Department of Microbiology, University of Virginia Cancer Center, JPA 1300, Jordan Hall, room 216, Charlottesville, VA 22908. Phone: (804) 924-8710; Fax: (804) 982-0689; E-mail: gak5g@virginia.edu.

3 The abbreviations used are: EGF, epidermal growth factor; DAPI, 4’,6-diamidino-2-phenylindole; TNF, tumor necrosis factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; IAP, inhibitor of apoptosis protein.
**Immunoprecipitation of Smac for Mass Spectroscopy and Western Blotting.** Ten 15-cm dishes of LNCaP cells were grown to near confluency and washed with phenol red-free, serum-free RPMI. Then, cells were lysed in CHAPS lysis buffer [0.5% CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, and protease inhibitors] and transferred to separate tubes for centrifugation at 15,000 rpm (Sorvall S34 rotor) for 30 min. After centrifugation, supernatants and protease inhibitors were added and incubated with either 40 μl of anti-Smac rabbit antiserum or 40 μl of preimmune serum as a negative control. After cell lysis preparation and antibody immunoprecipitation, the beads were washed three times with 500 μl of CHAPS lysis buffer. Next, 10 ml of the cell lysate was divided evenly into two tubes, into which beads with either anti-Smac antibodies or preimmune antibodies were added and incubated (3 h, 4°C) under rotation. After immunoprecipitation, beads were pelleted, and 1 ml of each cell lysate was preserved for subsequent Western blotting. After the removal of supernatants, beads were washed three times with CHAPS lysis buffer, and bound proteins were eluted with 100 mM Glycine (pH 2.6, 30 min, 4°C). Subsequently eluted proteins were analyzed by mass spectrometry and Western blotting.

**Mass Spectrometry.** Glycine-eluted proteins were pH adjusted with 100 mM NH₄HCO₃, pH 8.0. Reduction of proteins was with a solution of 10 mM DTT in 100 mM NH₄HCO₃, 1 h at 56°C. Carboxymethylation was achieved by adding 20 mM iodoacetamide in 100 mM NH₄HCO₃, 45 min at room temperature in the dark. Trypsin (0.5 μg) was added and allowed to digest at 37°C for 12 h. Tryptic peptides from the solution digest were introduced into a Thermofinnigan LCQ Deca mass spectrometer using nanoflow high-performance liquid chromatography-microelectrospray ionization. Peptides were eluted over a 4-h linear gradient using solvent A 0.1 M acetic acid in water and solvent B 60% Acetonitrile in 0.1 M acetic acid in water. Data-dependent MS/MS analysis was performed. The MS/MS data were then searched using SEQUEST algorithm against the nonredundant database that is maintained by National Center for Biotechnology Information.5

**Results**

**Microinjection of Cytochrome c Does Not Induce Apoptosis in LNCaP Cells.** It has been reported previously that microinjection of cytochrome c induces apoptosis in several cell lines, including 293 HEK, HeLa, and Rat-1 fibroblasts (13, 14, 17). To determine whether cytosolic cytochrome c was sufficient to induce apoptosis in LNCaP prostate cancer cells, we first used RIG cells and 293 cells as positive controls for cytochrome c-induced apoptosis. Injection of horse cytochrome c at a concentration of 2 mg/ml was sufficient to induce apoptosis in almost all injected 293 (data not shown) and RIG cells (Fig. 1A). Within 1 h after injection, cells showed specific hallmarks of apoptosis: intensive membrane blebbing, activation of caspase 3, and nuclear condensation. To our surprise, none of these features was observed in LNCaP cells injected with 5 mg/ml cytochrome c (Fig. 1B). No evidence of apoptosis was observed in LNCaP cells even 16 h after cytochrome c was injected at a concentration of 10 mg/ml.

Inability of LNCaP cells to respond to cytochrome c microinjection by caspase activation was surprising because these cells can readily activate caspases 3 and 7 after being subjected to various proapoptotic stimuli (8, 18). In fact, we were able to find cells that undergo spontaneous apoptosis and were stained positively with antibodies to active caspase 3 outside of the area with microinjected cells (Fig. 1C).

**Inhibition of ConstitutivelyActive Akt Does Not Sensitize LNCaP Cells to Cytochrome c-induced Apoptosis.** It has been shown that in prostate cells expressing constitutively active V12Ras, Akt becomes activated and may phosphorylate and inhibit caspase 9 (19). Because LNCaP cells have constitutively active Akt (5, 6), we suspected that it can inhibit apoptosis downstream of cytochrome c release. We demonstrated previously that LY294002 inhibits Akt activity and induces apoptosis in LNCaP cells deprived of survival factors. To test the hypothesis that the elevated Akt activity found in LNCaP cells obviates the proapoptotic effect of cytosolic cytochrome c, we compared apoptosis in LNCaP cells treated with LY294002 for 12 h and injected with 4 mg/ml cytochrome c or BSA. Although the percentage of apoptosis in LY294002-treated cells varied between experiments, no increase in the percentage of apoptosis was observed in cells injected with cytochrome c versus cells injected with BSA (Fig. 1D). Because the percentage of apoptosis is no higher in cells injected with cytochrome c, the apparent resistance of LNCaP cells to cytochrome c-induced apoptosis does not depend on antiapoptotic signaling by Akt.

Results from microinjection experiments seemingly contradict data that show a strong correlation between cytochrome c release and apoptosis in LNCaP cells (5, 8, 20). The absence of apoptotic response to cytochrome c microinjection prompted us to speculate that coordinate release of additional factors into the cytosol together with cytochrome c may be required for apoptosis induction.

---

Smac and Cytochrome c in LNCaP Cells. Recently, the proapoptotic human protein Smac was identified. This protein is released from mitochondria in HeLa cells undergoing apoptosis. Smac (and its mouse homologue DIABLO) can bind to IAP proteins and prevent them from inhibiting caspase activation, thus sensitizing cells to the cytochrome c–induced apoptosis (15, 21).

We assessed if Smac is expressed in LNCaP cells and released into the cytosol together with cytochrome c in cells undergoing apoptosis. Smac shows a distribution similar to cytochrome c when followed by indirect immunofluorescence on the single cell level (Fig. 2A) or by cell fractionation (Fig. 2B).

When LNCaP cells are treated with LY294002 or TNF-α, cytochrome c and Smac are released from mitochondria into the cytosol. Inhibitor of caspases z-VAD prevents apoptosis induced by either LY294002 or TNF-α; however, only in cells treated with TNF-α, release of apoptogenic proteins is inhibited. Thus, TNF-α requires and LY294002 does not require caspase activity to induce mitochondrial events. Treatment of LNCaP cells with EGF inhibits release of both Smac and cytochrome c from mitochondria and also inhibits caspase activity induced by LY294002. This is consistent with our previous observation that the survival mechanism activated by EGF targets stress-activated apoptosis upstream of mitochondria (8).

Simultaneous Injection of Cytochrome c and Smac Activates Caspases in LNCaP Cells. We injected purified Smac protein together with cytochrome c to test if the presence of Smac in the cytosol will make LNCaP cells sensitive to cytochrome c–induced apoptosis. After injection (4 h) with a mixture of Smac and cytochrome c, 15% of cells showed increased caspase 3 activation. At the same time, Smac alone led to activation of caspase 3 in <2% of injected cells (Fig. 3). Thus, when released into the cytosol together, these proteins are sufficient to induce activation of effector caspases. Although the original discovery of Smac/DIABLO emphasized its role in activation of caspase 9 and 3, it was not clear whether Smac and cytochrome c are sufficient for activation of the caspase cascade downstream from mitochondria in LNCaP cells or whether additional factors are required. Microinjection experiments support the former hypothesis.

Smac Interacts with XIAP in LNCaP Cells. The proapoptotic effect of Smac has been originally attributed to its ability to bind IAPs (15, 21); however, a recent report demonstrated a proapoptotic effect of Smac β in the absence of IAP binding (22). To obtain unbiased information on Smac interactions in LNCaP cells, we analyzed proteins present in Smac immunoprecipitates by mass spectrometry. Five tryptic peptides were identified from Smac, allowing 34% coverage of the protein; also, eight tryptic peptides were identified from XIAP,
providing 18% protein coverage (Fig. 4A). Thus, mass spectrometry indicates that XIAP is a predominant Smac-binding partner in LNCaP cells. The high stoichiometry of interaction between these proteins was further confirmed by analysis of XIAP in Smac immunoprecipitates by Western blotting that show depletion of XIAP from cell lysates (Fig. 4B).

XIAP binds and inhibits caspases 3, 7, and 9 (23, 24). Comparison of XIAP and caspase levels in LNCaP and human cell line HeLa, where cytochrome c injection induces apoptosis, did not show significant differences in XIAP expression; however, levels of caspases 7 and 9 in LNCaP were ~2-fold lower than in HeLa (Fig. 4C). Thus, it is possible that the ratio between caspases and XIAP determines whether Smac is needed to allow activation of caspases in response to proapoptotic stimuli in a given cell type.

**Discussion**

The concept that resistance to apoptosis in prostate cancer contributes to a poor response to chemotherapy or androgen ablation is becoming increasingly popular. Therefore, understanding the molecular mechanisms that underlie apoptotic resistance may provide information that can be used to improve the efficiency of existing therapies or develop novel treatment modalities.

It has been suggested that cytochrome c release from mitochondria...
is a “point of no return” where a cell commits itself to apoptosis. This idea prompted the search for cytotoxic agents that can mimic the effect of proapoptotic proteins of the Bcl family and induce apoptosis by attacking mitochondria (25). Consistent with this hypothesis, survival mechanisms observed in prostate cells can prevent cytochrome c release from mitochondria (5). On the other hand, evidence is accumulating that the role of mitochondria in apoptosis may be more subtle, e.g., growth factor-mediated survival signals have been reported to operate downstream of cytochrome c release (26), and proteins of the IAP family may inhibit caspases directly, even in the presence of cytosolic cytochrome c (27). Because protective Bcl-2 family members are known to be targets of caspases (28), it is likely that apoptosis inhibition through IAPs may serve to dampen an activation loop where caspase activity and mitochondrial disintegration both precipitate one another (29).

Smac released from mitochondria would bind XIAP and allow further activation of initiator caspase 9, as well as effector caspases 3 and 7. Interaction between XIAP and caspases shared by the death receptor and mitochondrial apoptotic pathways can explain synergistic effects of proapoptotic agents that operate via death receptors and mitochondrial pathway reported in LNCaP cells (30). Smac, when released from mitochondria, would titrate off XIAP and result in more effective activation of effector caspases by caspase 8. Additionally, decrease of XIAP levels in response to cycloheximide is in line with higher sensitivity of LNCaP cells to TNF-α and LY294002-induced apoptosis in the presence of inhibitors of protein synthesis. However, because there are several binding partners for XIAP and other IAP proteins can bind caspases, predictions based on analysis of limited set of proteins should be treated with caution.

Results presented in this communication further emphasize the role of mitochondria in the regulation of apoptosis in prostate cancer cells. Our observation that cytochrome c microinjections do not induce apoptosis in normal (data not shown) and malignant prostate cells without Smac may imply a predominant role for proteins of the IAP family in that tissue. One possible reason to maintain a high threshold of antiapoptotic defense in prostate cells is to protect these cells from Fas-induced apoptosis. Indeed, both Fas and FasL are reportedly expressed in the normal prostate gland, perhaps serving to maintain a site of immune privilege (31–33) or ensuring sperm with a defense against maternal immunosurveillance during conception. In turn, activation of nuclear factor κB signaling via death receptors was reported to contribute to increased IAP levels (34). During the development of neoplasia, prostate cells can be expected to further exploit IAP expression level, which is increased in a broad spectrum of cancers, including prostate adenocarcinoma (35), and therefore may be linked to oncogenic progression and decreased patient survival (36). Our finding that XIAP is the predominant IAP protein found in Smac immunoprecipitates may provide a rationale for drug design that seeks to neutralize IAP proteins by “Smac mimetics” in the hopes that such an approach would sensitize prostate cancer cells to conventional chemotherapy.

Acknowledgments

We thank Jeffrey Shabanowitz for the help with mass spectrometry; Joshua Rovin for intellectual contributions; Karen Martin, Michelle Haskell, Cliff Martin, and Karissa Lofton for technical help; Junying Yuan for antibodies to caspase 7; Peter Krammer for antibodies to caspase 8; and Idun Pharmaceuticals for providing CM-1 antibodies to active caspase 3.

References


J. P. Carson, unpublished data.


Smac Is Required for Cytochrome c-induced Apoptosis in Prostate Cancer LNCaP Cells


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/1/18

Cited articles  This article cites 36 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/1/18.full.html#ref-list-1

Citing articles  This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/62/1/18.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.