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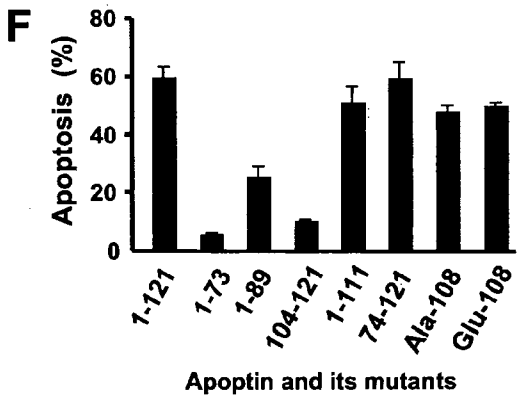
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(54) **Title:** METHODS OF INDUCING APOPTOSIS OF CANCEROUS CELLS USING APOPTIN DERIVATIVES



(57) **Abstract:** This application relates to methods of inducing apoptosis in a cancerous cell, which comprise administering isolated or purified apoptin- derived peptides comprising the amino acids sequence PKPPSK.

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Molecular Dissection of the Role of the PI3-Kinase/Akt Pathway; Redirection from Pro-Survival Signaling to a Death Pathway through Nuclear Relocation of Akt.

PRIOR APPLICATION INFORMATION

This application claims the benefit of US Provisional Patent Application 60/793,654, filed April 21, 2006.

BACKGROUND OF THE INVENTION

The Phosphatidylinositol 3'-kinase (PI3-K) is a lipid kinase that catalyses phosphorylation of the Inositol ring of phosphoinositides [PI, PI(4)P and PI(4,5) P_2] at the D3 position [1, 2]. Three classes of PI3-K have been identified which differ in their primary structure, regulation and substrate specificity (reviewed in [3]). Class I PI3-Ks have been the major focus of PI3-K studies and are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (p85). Four isoforms of the p110 subunit have been described (α, β, γ and δ), and three mammalian genes encode the adapter subunits p85α, p85β and p55γ. The p85 subunit contains an N-terminal SH3 domain followed by a proline-rich domain, a breakpoint cluster region homology domain, a second proline-rich domain and two SH2 domains. It also has a putative coiled coil domain between the two SH2 domains (iSH2 domains) that mediates the stable dimerization with the p110 catalytic subunit. The p110 subunit contains a closely-spaced N-terminal binding site for p85 and a c-terminal kinase domain [4, 5]. Regulation of activity of p85/p110 PI3-K is a complex process. In general, p85 inhibits p110-associated catalytic activity. The binding of the N-terminal SH2 domain of p85 with the phosphorylated tyrosine residues within the specific docking sites (YxxM) in the cellular domain of receptor tyrosine kinases [6, 7] or in other intracellular non-receptor tyrosine kinase adaptors, leads to a conformational change in p85, which alleviates p110 inhibition and further activates PI3-K [8-11]. Interaction of the nSH2 domain with the tyrosine-phosphorylated proteins is universally accepted to be responsible for PI3-K activation, but there are other studies reported the activation of PI3-K via alternate mechanisms. For example, the conformational switch within the p85-p110 holoenzyme can also occur via the interactions of SH3 domain/proline rich sequences, BCR-homology domain/GTP loaded adaptor proteins and others [12-15]. Akt/PKB, a serine/threonine kinase, is a crucial kinase downstream of the PDK1 recruitment to the PtdIns(3,4,5)P3 lipid messenger produced via the PI3-K activation [16]. Activated Akt modulates the function of numerous substrates related to the regulation of cell proliferation, such as glycogen syntase kinase-3 (GSK-3), Cyclin dependent kinase inhibitors, $p21^{Cip1/Waf1}$, $p27^{kip1}$, and mammalian target of rapamycin (mTOR) [17, 18].

Another important function of activated PI3-K/Akt in cells is maintaining the cell survival via inhibition of apoptosis. The targets for Akt during this process include Bad phosphorylation (a pro-apoptotic Bcl-2 family member), FKHRL1 inactivation (a transcription factor for FasL and Bim) and NF-κB activation [19, 20].

Several molecules in the literature have been assigned a dual role in both cell survival and cell death mechanisms. For instance the oncogenes Myc [21, 22], NF-κB [23], molecules of Ras/Map kinase pathway [24, 25], Bcl-2 [26, 27], caspases [28], and even an orphan nuclear receptor Nur77 [29] are all involved in promoting either cell proliferation or cell death, depending on the context and the stimulus. So far, the PI3-K/Akt pathway has been implicated only in the promotion of cell survival, proliferation, growth, transcription and translation (reviewed in [30]). The specific role of the PI3-K/Akt pathway in a pro-cell death pathway has thus far not been clarified.

Apoptin is a 14 kDa protein derived from the chicken anemia virus. Apoptin selectively induces apoptosis in cancer cells but not in primary cells (reviewed in: [31, 32]). In primary cells, apoptin remains in the cytoplasm, whereas in transformed cells it migrates into the nucleus and ultimately kills the cell by activation of the mitochondrial death pathway, in a Nur77-dependent manner independently of death receptors [33-36]. However, targeted translocation of apoptin into the nuclei of primary cells is not sufficient for apoptin's toxicity. Thus, additional interaction partners or specific activation of other signaling pathways in the cancer cells preceding nuclear accumulation might be necessary for apoptin's tumor specific toxicity. Nuclear accumulation is strongly linked to phosphorylation of apoptin at the threonine-108 (Thr-18) residue by an unknown kinase [37, 38] specifically in cancer cells but not normal cells. Thus, identifying the pathways responsible for the phosphorylation of apoptin might provide clues about its tumor specific toxicity. These pathways may also represent attractive targets for the development of new highly selective anticancer drugs.

In this study, we have investigated the role of the PI3-K/Akt pathway in apoptin-induced cell death. Our results intriguingly demonstrate that apoptin interacts with the regulatory $p85^{PI3-K}$ subunit, which leads to the constitutive activation of PI3-K; inhibition of this activation process severely impairs apoptin-induced cell death. We also demonstrate nuclear translocation of Akt during apoptin-induced cell death, apparently through an apoptin-mediated piggy-back effect, and further, show the cell cycle regulators $p27^{kip1}$ and CDK2 to be nuclear Akt targets during the apoptotic process. Nuclear translocation of Akt alone does not have a toxic effect, but it strongly potentiates the toxicity of apoptin and also some anticancer drugs. Finally, we have identified CDK2 as the previously

unidentified kinase phosphorylating apoptin, which regulates its tumor cell-specific nuclear localization.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method of inducing apoptosis in a cancerous cell comprising administering an effective amount of an isolated or purified peptide comprising PKPPSK (SEQ ID NO:3).

According to a second aspect of the invention, there is provided a method of manufacturing a pharmaceutical composition comprising mixing an effective amount of an isolated or purified peptide comprising PKPPSK (SEQ ID NO:3) with a suitable excipient.

According to a third aspect of the invention, there is provided a purified or isolated peptide comprising amino acids PKPPSK (SEQ ID NO:3).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Apoptin selectively kills cancer cells via the interaction conferred by the SH3 domain of the PI3-Kinase p85 subunit and apoptin's proline rich motif. (A) B-cells from either normal Peripheral Blood Lymphocytes (PBLs) or the CLL PBLs were double stained for CD5/CD19 surface markers using FITC-conjugated CD5 and Per CP-conjugated CD19 antibodies at different time points after either TAT-GFP or TAT-Apoptin treatment. The samples were then analyzed by flow cytometry and the number of CD5/CD19 double-positive cells were plotted. (**B**) GST-pull down assay performed with MCF-7 lysate using either GST control or GST-Apoptin and the proteins ($p85^{PI3\text{-}K}$ and Akt) specific for apoptin interaction identified by mass spectrometry are indicated. (**C**) GST pull down assay performed with PC-3, MCF-7, 293 and L929 cell lysates with either GST or GST-apoptin. The presence of the p85 subunit of PI3-K and Akt in apoptin complexes was determined by immunoblotting with the respective antibodies. (**D**) Co-immuno-precipitation indicates the interaction of apoptin with p85 and Akt. PC-3 cell lysates were immuno-precipitated with anti-GFP antibody (GFP-apoptin) at different time-points upon transfection with GFP-apoptin. Co-immuno-precipitated p85 and Akt were detected by Western blot. Reciprocal immuno-precipitation with either anti-p85- or anti-Akt antibodies and detection of apoptin by immunoblotting is shown at the bottom. (**E**) Schematic representation of apoptin deletion mutants tagged with N-terminal GFP. The deletion mutants were transfected into PC-3 cells, immuno-precipitated with anti-GFP antibody after 18 hours post transfection and the presence (association) of p85 detected by immunoblotting. (**F**) The percentage of cell death induced by different apoptin mutants

assessed by flow cytometry (Nicoletti method) 24 hours post-transfection. (**G**) Schematic representation of the PI3-K p85 deletion mutants highlighting the structural domains. The wild type and p85ΔiSH2 were not tagged, with the remaining four mutants possessing a HA tag. The p85 deletion mutants were co-transfected together with apoptin into PC-3 cells. 28 hours post-transfection, cells were lysed, and p85 immuno-precipitated using either anti-p85 antibody (the wt, and ΔiSH2 mutants) or anti-HA antibody for other mutants. Apoptin interaction with the mutants was detected by immunoblotting using anti-apoptin antibodies and the expression of deletion mutants detected by anti-p85 or anti-HA antibodies.

Figure 2: PI3-K is constitutively activated during apoptin-induced apoptosis. (**A**) The PI3-K activity was measured by an ELISA-based assay after immuno-precipitating PI3-K from the lysates of PC-3 and MCF-7 cells transfected to express apoptin (time points indicate time post-transfection), as described in the methods section, and the fold induction was calculated. PI3-K activity in non-transfected cells was considered as a basal level (1x). (**B**) PC-3 cells were either transfected with apoptin alone, pretreated with wortmannin, LY294002 followed by apoptin transfection, or transfected to co-express apoptin and a PI3-K dominant negative vector. The PI3-K activity was measured 24 hours post-transfection as described in 2A. (**C**) PI3-K activity was measured 24 hours after transfecting the cells to express the different apoptin deletion mutants. (**D**) PI3-K-mutants described in Fig. 1G were expressed after transfection in PC-3 cells (see main text for details). Cells were lysed 24 hours later, and PI3-K forms immuno-precipitated as described in the legend to figure 1G. Kinase activity was then measured by ELISA as per (**A**) above. Similar results were obtained with MCF7 cells. (**E**) The effect of PI3-K inhibition on apoptin-induced cell death was assessed by flow cytometry (Nicoletti). The cells were either transfected to express GFP (control) or GFP-apoptin in the absence of presence of treatment with wortmannin or LY294002, or transfection to coexpress the dominant negative PI3-K PI3-K-DN. Apoptosis was then measured 24 and 48 hours post transfection, with results compared to those for control treatments of wortmannin or LY294002 alone, or PI3-K-DN. Expression alone. (**F**) Cells were either transfected with p85 siRNA plasmid (Upstate) or control siRNA plasmid for 48 hours, and p85 expression in the presence or absence of siRNA detected by immunoblotting; tubulin was used a loading control (top panel). PC-3 cells were transfected to express apoptin alone, or transfected with or without p85-inhibitory siRNA, followed by transfection to after 48 hours, to express apoptin. After a further 24- or 48 hours, cell death was measured by flow cytometry (Nicoletti). (**G**) The localization of GFP-apoptin in both MCF-7 and PC-3 cells was detected by confocal microscopy after transfection in the absence or presence of

wortmannin. (**H**) The effect of p85 inhibition on apoptin localization was determined by confocal microscopy, 24 hours upon transfection of cells to express GFP-apoptin either without or with co-transfected p85-targetting siRNA plasmid. The expression of p85 was detected by anti-p85 antibody followed by Cy-3 conjugated secondary antibody. (**I**) The nuclear/cytoplasmic distribution of apoptin in the presence of wortmannin or p85-inhibitory siRNA in both MCF-7 and PC-3 cells was investigated by sub-cellular fractionation followed by Western blotting.

Figure 3: Akt translocates to the nucleus during apoptin-induced cell death. (**A**) The activation of Akt by apoptin was detected in PC-3 cells by immunoblotting using an antibody against Akt-phosphorylated at Ser-473 at different time points after transfection to express apoptin. Total Akt was detected by immunoblotting. (**B**) PC-3 cells were transfected to express apoptin in the absence or presence of treatment with wortmannin or LY294002, and phosphorylated Akt, then detected by immunoblotting. Akt activation was also determined by immunoblotting in lysates from cells transfected to express apoptin alone or apoptin together with either dominant negative PI3-K (DN), PDK1-DN, wild type PTEN, or phosphatase deficient C124S-PTEN mutant. (**C**) The effect of Akt inhibition on apoptin toxicity was assessed in PC-3 cells by flow cytometry (Nicoletti method), either 24 hours or 48 hours after transfection to express apoptin either alone or together with PDK1-DN, Akt-DN (adenoviral vector), wild type PTEN, or C124S-PTEN. (**D**) The localization of Akt either in the absence or presence of apoptin in MCF-7 and PC-3 cells was detected by confocal microscopy, after immuno-staining with anti-Akt antibody followed by Cy3 conjugated secondary antibody. DAPI was used as counter-stain for the nuclei, and the images overlaid (right panels). (**E**) The MCF-7 cells were transfected to express the indicated apoptin derivatives (see Fig. 1G for details), and Akt immuno-detected as per **D**. (**F**) The cells were infected with either adenoviral Myc-tagged NLS-Akt alone or co-transfected to express apoptin and the localization of NLS-Akt was detected using anti-Myc-tag antibody followed by Cy3-marked secondary antibody. The arrows indicate representative cells undergoing apoptosis that show chromatin condensation. (**G**) Quantitative assessment of apoptin-triggered apoptosis in NLS-Akt expressing cells as compared to controls. Cells were infected with adenovirus encoding NLS-Akt, and/or transfected to express GFP-apoptin. Apoptotic cell death was detected by flow cytometry (Nicoletti) 24 or 48 hours after transfection. (**H**) MCF-7 and PC-3 cells were infected with adenovirus coding for NLS-Akt, 2 hours later, cells were treated with anticancer drugs (methotrexate 10 μM, docetaxel 50 nM, doxorubicin 50 μg/ml, cisplatin 75 μg/ml, staurosporine 2.5 μM, anti-CD95 50 ng/ml), and 24 hours later apoptosis was assessed by flow cytometry (Nicoletti). Data represent the average of three independent experiments.

Figure 4: Akt phosphorylates- and mediates $p27^{kip1}$ downregulation in the nucleus. (**A**) Phosphorylation of $p27^{kip1}$ at serine and threonine residues either in the presence of GFP control plasmid or GFP-apoptin was detected by specific phospho-serine or phospho-Thr-specific antibodies after immuno-precipitation using a anti-$p27^{kip1}$ antibody. Threonine phosphorylation was also detected using antibody against $p27^{kip1}$ phosphorylated at Thr-157. (**B**) $p27^{kip1}$ phosphorylation after transfection to express apoptin at different time points after transfection was detected by immunoblotting using anti-phospho-$p27^{kip1}$-Thr-157 antibody, with tubulin as a loading control. (**C**) The effect of PI3-K inhibition on $p27^{kip1}$ phosphorylation at Thr-157 was detected by immunoblotting 18 hours after transfecting the cells to express apoptin with or without treatment with wortmannin, or PD98059 (upper panel). The effect of PI3-K, PDK1, Akt and PKC on $p27^{kip1}$ phosphorylation was also determined by immunoblotting. Cells were transfected to express apoptin alone or together with either 'wild type' PI3-K, constitutively active PDK1 (CA), dominant-negative PI3-K (DN), PDK1-DN, Akt-DN and PKC-DN. After 16 hours, cells were lysed and phosphorylated $p27^{kip1}$ detected by Western blotting. (**D**) Co-immuno-precipitation of Akt and $p27^{kip1}$. PC-3 cells were transfected with apoptin and 12 hours later Akt was immuno-precipitated. The precipitates were then resolved by SDS-PAGE, and $p27^{kip1}$ detected using a specific antibody. The association between Akt and $p27^{kip1}$ could be detected over 12-24 hours after transfection to express apoptin. Similar results were observed in MCF-7 cells. (**E**) The levels of $p27^{kip1}$ in GFP-apoptin transfected PC-3 cells were followed within 24 hours post-transfection by Western blotting, with tubulin as a loading control. A control experiment with cells transfected to express GFP showed no decrease of $p27^{kip1}$ in the cells (not shown). (**F**) The effect of selected kinases on $p27^{kip1}$ protein levels, in the presence of apoptin. $p27^{kip1}$ was detected by immunoblotting in lysates of PC-3 cells 24 hours after transfection to express GFP (Control), or GFP-apoptin either alone or together with PI3-K-DN, PDK1-DN, Akt-DN and PKC-DN. Where indicated, cells were treated with the kinase inhibitors PD98059, wortmannin, or MG-115. Similar results were also obtained in MCF-7 cells. (**G**) The effect of the inhibition of $p27^{kip1}$ expression on apoptin-triggered apoptosis. PC-3 cells were transfected with either $p27^{kip1}$ siRNA or scrambled (control) siRNA for 36 hours and the $p27^{kip1}$ expression was controlled by immunoblotting. PC-3 cells were then transfected to express GFP (control) or GFP-apoptin (AP), or transfected with $p27^{kip1}$-siRNA alone, with or without GFP-apoptin encoding plasmid. Apoptosis was measured by flow cytometry (Nicoletti) at the indicated time points. The data represent results from three independent experiments, with similar experimental outcome obtained using MCF-7 cells.

Figure 5: CDK2 is activated during apoptin-induced cell death. (**A**) CDK2, CDK1, Cyclin E and Cyclin A immuno-precipitated from the cells transfected to express GFP or GFP-apoptin 24 hours post-transfection, were used in an *in vitro* kinase assay on Histone H1 substrate. The levels of Histone H1 phosphorylation were detected by immunoblotting with an antibody against the phosphorylated Histone. (**B**) The kinase activity of CDK1 and CDK2 at different times of post-transfection to express GFP or GFP-apoptin as indicated was measured as per (**A**) and the kinase activity, determined by quantifying the immuno-blot signals against the non-transfected control, plotted. (**C**) PC-3 cells were transfected to express apoptin either alone or together with the indicated dominant negative (DN) kinases, treated without or with PI3-K inhibitors as indicated, and CDK2 activity measured as in (**A**). Total CDK2 levels (bottom) are indicated by immunoblotting. (**D**) The effect of CDK2 inhibitor on apoptin toxicity was determined by transfecting cells to express apoptin with or without treatment with CDK2 inhibitor. Cell death was measured by flow cytometry (Nicoletti method) after 24 and 48 hours respectively, with results compared to those for cells treated with CDK2 inhibitor alone. (**E**) PC-3 cells were transfected with either CDK2 siRNA plasmid or control siRNA for 48 hours and CDK2 expression determined by immunoblotting. The level of apoptosis in cells transfected to express GFP (control) or GFP-apoptin in the absence or presence of CDK2-targetting siRNA at 24 and 48 hours was determined by flow cytometry. (**F**) CDK2 kinase activity was measured 24 hours after transfection to express apoptin either without or with treatment with CDK2 inhibitor, caspase inhibitor zVAD-fmk or Bcl2-over-expression prior to transfection. (**G**) The levels of cytosolic cytochrome c were detected by cellular fractionation followed by immunoblotting with cells transfected to express apoptin without or with treatment with CDK2 inhibitor or Bcl2 over-expression. Indicated samples were lipofected with Bcl2-expression vector 24 hours before apoptin transfection. (**H**) The nuclear or cytoplasmic localization of CDK2 was detected by cellular fractionation followed by Western blotting at different time-points after apoptin transfection. (**I**) PC-3 cells were transfected with apoptin, and 20 hours later CDK2 was immuno-precipitated. The precipitates were then resolved on SDS-PAGE, and Bcl2 was detected in the immune complexes a by specific antibody. (**J**) The phosphorylation levels of Bcl2 were detected, at indicated time-points after transfection with apoptin, by immunoblotting of Bcl2-preacipitates, with anti-phospho-Ser, anti-phospho-Thr, and anti-Bcl2-phopho-Thr-56 specific antibodies. The total Bcl2 levels were detected by immunoblotting with anti-Bcl2 antibody. (**K**) PC-3 cells were either transfected with apoptin alone, co-transfected with apoptin, PI3-K siRNA, and/or CDK2 siRNA or pre-treated with a proteosome inhibitor MG-115. 20 hours later, the level of Bcl2 phosphorylation at Thr-56 residue and the total Bcl2

were detected by immunoblotting with anti-Bcl2-phospho-Thr-56 antibody and anti-Bcl2 antibody respectively.

Figure 6: CDK2 is the tumor specific apoptin kinase. (**A**) *In vitro* kinase assay performed with GST-Apoptin and TAT-Apoptin as substrates using active CDK1/Cyclin B, CDK2/Cyclin E or CDK2/Cyclin A. Apoptin phosphorylation was detected by immunoblotting using an antibody against phosphor-threonine-proline. Total apoptin levels were detected by anti-apoptin antibody. Histone was used as a positive control. (**B**) Active CDK2, CDK1, Cyclin B, Cyclin E, Cyclin A were immuno-precipitated using their respective antibodies and the CDK2 T160A mutant was immuno-precipitated using anti-HA antibody, and used in a kinase assay with TAT-GFP, TAT-apoptin or H1 as substrates. Phosphorylation was monitored as in (**A**). (**C**) PC-3 cells were transfected to express GFP-apoptin, in the presence or absence of CDK2 inhibitor or CDK2-targetting siRNA, and GFP-apoptin was immuno-precipitated 24 hours later using anti-GFP antibodies. Apoptin phosphorylation in the immuno-precipitates was detected by anti-phospho-Thr-Pro antibodies and immunoblotting. Total apoptin and CDK2 levels are indicated. (**D**) The effect of CDK2 inhibition on apoptin localization in PC-3 and MCF-7 cells. Cells were transfected to express GFP-apoptin in the presence or absence of CDK2-targetting siRNA plasmid followed by confocal imaging. CDK2 expression was detected using anti-CDK2 antibody and Cy-3 conjugated secondary antibodies. (**E**) The localization of apoptin in the presence of co-expressed active PDK1 (PDK1-CA), PDK1-DN, wild type Akt or Akt-DN in PC-3 cells. Confocal imaging was performed 24 hours after transfection. (**F**) The level of phosphorylation of apoptin in PC3 cells in the presence of the indicated co-expressed dominant negative (DN) kinases was determined as per as described in figure 6C. Total apoptin was detected with anti-apoptin antibodies. Similar results were observed in MCF7 cells.

Figure 7: Model for the role of PI3-K/Akt pathway activation during cell death induced by apoptin, and other cell death stimuli. During apoptin-induced cell death, PI3-K is constitutively activated, which leads to PDK1-dependent Akt activation and its nuclear translocation, probably through a piggy-back transport mechanism. Nuclear Akt phosphorylates and downregulates $p27^{kip1}$, which leads to aberrant CDK2 activation and propagation of the cell death signal. Activated CDK2 phosphorylates, among other substrates, apoptin at Thr-108 site and regulates its nuclear accumulation in cancer cells. Phosphorylated CDK2 translocates to the cytoplasm and phosphorylates Bcl2, which is then targeted for proteosome-dependent degradation. The resulting imbalance in the levels of the cell's anti-apoptotic factors leads to apoptosis.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

Apoptin is a chicken anemia virus derived protein that kills cancer cells specifically without affecting normal cells. Our experiments were designed to study the mechanisms of tumor specific apoptosis induced by Apoptin. This knowledge of mechanism is subsequently utilized in designing the shorter Apoptin peptides that will be much more effective in inducing apoptosis and at the same time retaining the cancer specific toxicity.

The amino acid sequence of apoptin is:

MNALQEDTPP GPSTVFRPPT SSRPLETPHC REIRIGIAGI TITLSLCGCA NARAPTLRSA TADNSESTGF KNVPDLRTDQ PKPPSKKRSC DPSEYRVSEL KESLITTTPS RPRTAKRRIR L (SEQ ID NO:1)

Described herein is an isolated or purified peptide comprising an amino acid sequence of $PX_1X_2PX_3(R/K)$ (SEQ ID NO:2) wherein X_1 X_2 and X_3 are any amino acid. In alternative embodiments, comprises an isolated or purified peptide consisting of or consisting essentially of an amino acid sequence of $PX_1X_2PX_3(R/K)$ wherein X_1 X_2 and X_3 are any amino acid. As will be appreciated by one of skill in the art, as used herein, 'consisting essentially of' means that the small peptide may be fused to a carrier or may be otherwise presented to the cell as part of a larger molecule which retains the anti-cancer activity of the small peptide.

In an alternative embodiment, the peptide comprises an amino acid sequence of PKPPSK (amino acids 81-86 of apoptin (SEQ ID NO:1), SEQ ID NO:3). In alternative embodiments, comprises an isolated or purified peptide consisting of or consisting essentially of an amino acid sequence of PKPPSK. As will be appreciated by one of skill in the art, as used herein, 'consisting essentially of' means that the small peptide may be fused to a carrier or may be otherwise presented to the cell as part of a larger molecule which retains the anti-cancer activity of the small peptide.

In an alternative embodiment, the peptide comprises an amino acid sequence of PKPPSKKR (amino acids 81-88 of apoptin (SEQ ID NO:1), SEQ ID NO:4). In alternative embodiments, comprises an isolated or purified peptide consisting of or consisting essentially of an amino acid sequence of PKPPSKKR. As will be appreciated by one of skill in the art, as used herein, 'consisting essentially of' means that the small peptide may

be fused to a carrier or may be otherwise presented to the cell as part of a larger molecule which retains the anti-cancer activity of the small peptide.

In a yet further embodiment, the peptide may comprise or may consist of or may consist essentially of amino acids 1-111 (SEQ ID NO:5), 74-121 (SEQ ID NO:6), 74-111 (SEQ ID NO:7) or 74-104 (SEQ ID NO:8) of apoptin.

In some embodiments, the apoptin-derived peptide is a recombinant peptide and comprises an amino acid sequence of at least one of SEQ ID NO:2-8. As will be appreciated by one of skill in the art, as used herein, 'recombinant' is distinct from 'isolated or purified' and refers to the amino acid sequence in question being presented in a non-native context. That is, the recombinant peptide comprises an amino acid sequence of at least one of SEQ ID NO:2-8 and the recombinant peptide is not full-length apoptin (SEQ ID NO:1). That is, the recombinant peptide may comprise a truncated apoptin peptide that includes at least one of SEQ ID NO:2-8 or may comprise SEQ ID NO:2-8 inserted in a non-native peptide for example a carrier peptide.

In some embodiments, the apoptin-derived peptide consists of or consists essentially of a peptide of approximately 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids comprising SEQ ID NO:2 or SEQ ID NO:3, as discussed herein. As discussed herein, in some embodiments, there is provided the proviso that the apoptin-derived peptide is not native apoptin (SEQ ID NO:1).

In some embodiments, the apoptin-derived peptide consists of or consists essentially of a peptide of approximately 6-100, 7-100, 8-100, 9-100, 10-100, 15-100, 20-100, 25-100, 30-100, 35-100, 40-100, 45-100, 50-100, 55-100, 60-100, 65-100, 70-100, 75-100, 80-100, 85-100, 90-100, 95-100, 100 or more amino acids comprising SEQ ID NO:2 or SEQ ID NO:3, as discussed herein. As discussed herein, in some embodiments, there is provided the proviso that the apoptin-derived peptide is not native apoptin (SEQ ID NO:1).

In a further embodiment of the invention, an apoptin-derived peptide as described above is used to induce or trigger cell death in a cancerous cell, to induce or trigger apoptosis in a cancerous cell, to direct the PI3-K pathway from cell survival to cell death or to activate PI3-K, thereby inducing apoptosis. As will be appreciated by one of skill in the art, these methods involve administering an effective amount of an apoptin-derived peptide as described above to an individual in need of such treatment. As will be appreciated by one of skill in the art, an 'effective amount' is an amount sufficient to achieve the desired result and will of course depend on many factors including but by no means limited to the age, weight and condition of the patient. In some embodiments, the

desired result is an amount sufficient to direct the PI3-K pathway from cell survival to cell death or to trigger or induce apoptosis in a cancerous cell as discussed herein.

In a further embodiment of the invention, there is provided a method for manufacturing a pharmaceutical composition comprising mixing a purified or isolated apoptin-derived peptide as described above with a suitable excipient. As discussed herein, the pharmaceutical composition may be for treating cancer, for inducing apoptosis or cell death in a cancerous cell, for directing the PI3-K pathway from cell survival to cell death or for activating PI3-K, as discussed herein.

We have shown that Apoptin interacts with the SH3 domain of the p85 regulatory subunit of PI3-Kinase which is normally a survival molecule and directs the PI3-kinase pathway towards cell death away from the cell survival signaling. The interaction of Apoptin with the SH3 domain of PI3-Kinase occurs via the amino acids 80-90 in apoptin, which we later identified as a proline rich motif. Further based on the knowledge of this Apoptin and PI3-kinase interaction, we designed the shorter version of the Apoptin peptides which has the intact proline rich motif and thus can kills the cancer cells specifically via interacting with the PI3-Kinase and possibly other SH3 domain containing proteins. The novelty in the project lies in the fact that the Apoptin derived peptides, which are significantly smaller (as small as 6-10 amino acids) compared to full length Apoptin (121 amino acids) effectively induces cell death in a cancer specific manner and the mechanism utilized by both full Apoptin and its peptide derivates is based on this novel pathway, where the apoptosis inducing molecules redirect the survival/proliferation pathways towards cell death pathways. Apoptin induced redirection of PI3-K pathway is based on the translocation of Akt, a downstream molecule of PI3-kinase, to the nucleus where it phosphorylates different substrates like CDK2 and p27, which might be different from the survival signaling that occurs normally in the cytoplasm.

In this study, we have investigated for the first time the role of several signaling molecules involved in cancer-selective cell death triggered by apoptin. Interestingly, our results show that the PI3-K/Akt signaling pathway, universally accepted to promote cell survival and proliferation, can also have a critical role in promoting cell death in response to certain stimuli. Moreover, we demonstrate that the inhibition of PI3-K activation by either pharmacological inhibitors or by genetic methods can abrogate cell death induced by the viral protein apoptin. It is of note that the treatment of cancers using PI3-kinase inhibitors has been largely unsuccessful. Our strategy is novel because it causes the nuclear transfer of Akt, and subsequently accesses a new set of substrates, and the possibility to use the same pro-survival pathways towards killing the cancer cells, as described herein. Although PI3-K is known to be involved in cell survival, several publications have hinted at

the fact that active PI3-K may contribute to apoptosis under certain conditions [43-45]. Intriguingly in this context, Akt-inhibitors have proved to be only moderately successful in experimental cancer therapy [46, 47]. Here, we provide, for the first time, the molecular basis for the role of the PI3-K/Akt pathway in the induction of apoptosis and its propagation (summarized in Fig. 7). The PI3-K/Akt pathway, under normal conditions, targets several substrates either in the cytosol or in the nucleus, and promotes cell survival and proliferation. This is achieved by various mechanisms, including the control of the abundance, activity and stability of certain regulators of the cell cycle (reviewed in: [16, 30]). We document here for the first time that activated Akt, if translocated to the nucleus, can stimulate rather than protect against apoptosis induced by certain cytotoxic stimuli, including several anticancer drugs. We hypothesize that in the presence of apoptin, activated Akt targets substrates and/or pathways that lead to the aberrant activation of CDK2, resulting in perturbation of cell cycle progression, and ultimately cell death. Thus, the net-outcome of CDK2-activation could vary according to the "signaling context", the type of stimuli and the temporal characteristic of signals that they trigger (e.g. transient versus constitutive signaling). There are well-established examples of such context-dependent, dramatic changes in the final outcome of activation of certain signaling pathways. For example, the proto-oncogene c-myc stimulates cell proliferation in the presence of the appropriate survival stimuli (including an activated PI3-K/Akt-pathway) and triggers apoptosis in their absence [48]. This dual capacity ensures that cell growth is restricted to the correct paracrine environment, co-activation of a pro-survival signaling pathway, and/or co-expression of anti-apoptotic molecules, and is thereby strictly controlled by multiple mechanisms [49-51].

We have demonstrated that the interaction of apoptin with the p85 regulatory subunit constitutively activates PI3-K. The interaction of different molecules with the SH3 domain of p85 is known to change the conformation and activate its p110 catalytic subunit, thus leading to the constitutive PI3-K activation. Interestingly, a detailed analysis of the sequence of apoptin revealed a proline-rich motif between amino acids 80 and 90. The PKPPSK (SEQ ID NO:3) sequence (amino acids 81-86) in fact constitutes a perfect consensus motif (PxxPxR/K)(SEQ ID NO:2) recognized by SH3 domains. Derivatives of apoptin lacking these residues are severely impaired in inducing apoptosis, implying that apoptin, by interacting with the SH3 domain of the p85 regulatory subunit, activates PI3-K constitutively.

In addition to apoptin's interaction with PI3-K, we observed transient interaction with Akt. Furthermore, interaction of Akt with apoptin appears to facilitate Akt nuclear localization. Recent studies have indicated that under certain conditions, activated Akt can

be translocated to the nucleus with the help of certain cytosolic proteins, since Akt itself does not possess an intrinsic nuclear localization signal sequence [52, 53]. Here, we show that Akt nuclear translocation occurs early during apoptin-induced apoptosis. Furthermore, interaction of activated Akt with apoptin is crucial for its nuclear translocation, as demonstrated by the fact that Akt is translocated to the nucleus only in the presence of apoptin mutants which have the ability to activate PI3-K, and that Akt is observed in the nucleus only in the presence of apoptin derivatives with the ability to accumulate in the nucleus. This implies that apoptin interaction with Akt contributes to its nuclear import, possibly through a piggy-back mechanism and the nuclear transporter importin beta 1 [54]. Apoptin thus effects Akt's nuclear translocation, presumably enabling access to pro-apoptotic phosphorylation targets that do not normally come into contact with cytoplasmic Akt. Nuclear access of Akt alone is not sufficient to induce apoptosis, since NLS-Akt alone does not induce cell death, but can potentiate apoptin-induced cell death. Thus, NLS-Akt potentiated classical cell death stimuli, such as methotrexate, docetaxel, doxorubicin, and cispratin, but not others such as staurosporine or CD95-triggering. In fact, NLS-Akt even demonstrated some protective effects with respect to staurosporine- or CD95-induced cell death, implying that nuclear Akt may have pro-survival and proliferation-promoting function, depending on the experimental conditions. For example, Trotman and colleagues reported that the PML-tumor suppressor prevents cancer by dephosphorylating and inactivating Akt inside the nucleus [55] (see also below). Other researchers have reported that phosphorylated-, nuclear-, but not cytoplasmic- Akt interacts with Ebp1, (an inhibitor of CAD-dependent apoptotic DNA-fragmentation), and enhances its anti-apoptotic action independently of Akt-kinase activity in a cell-free experimental system [56]. Thus, nuclear Akt contributes to cell death pathways only in the presence of certain apoptotic stimuli.

There are several reported nuclear targets of Akt, including FOXO3a [57], Nur77 [58] and $p21^{cip/waf}$ [59]. We have previously shown that Nur77 translocates from the nucleus to mitochondria during apoptin-induced cell death [33]. We still have to define the connection between nuclear Akt and translocation of Nur77 to the mitochondria. We have also observed the downregulation of $p21^{cip/waf}$ 24 hours after cell death induction by apoptin, although this occurs independently of Akt activation, since Akt inhibition by wortmannin did not affect p21 levels. Downregulation of p21 may be the result of cleavage by caspases, as a secondary event, downstream of mitochondrial death pathway activation. Here, we report $p27^{kip1}$ as a nuclear target of Akt-phosphorylation and demonstrate the importance of this signaling event in apoptin-induced cell death. $p27^{kip1}$, a cyclin dependent kinase inhibitor and a negative regulator of the cell cycle, plays a role both in proliferation and apoptosis, in a stimulus-dependent manner [60, 61]. $p27^{kip1}$ is

downregulated during apoptin-induced cell death dependent on Akt activation. Akt phosphorylates $p27^{kip1}$ at Thr-157 in the nucleus and targets it for proteosomal degradation. Previously, it was shown that $p27^{kip1}$-phosphorylation at Thr-187 by CDKs triggers the ubiquitination and degradation of $p27^{kip1}$ by SCF/Skp2 ubiquitin ligase complexes [62]. We found no change in the levels of phospho-$p27^{kip1}$-Thr-187 during apoptin treatment, although CDK2 was activated during apoptin-induced cell death. Also, it was reported that $p27^{kip1}$ phosphorylation at Ser-10, Thr-157 and Thr-198 results from activation of the PI3-K/Akt pathway. The phospohorylation of $p27^{kip1}$ at these sites affects its nuclear localization, resulting in the cytoplasmic accumulation with the help of the 14-3-3 scaffold protein, thus blocking $p27^{kip1}$'s ability to inhibit CDKs [63, 64]. However, our data (not shown) indicate that there is no change in the nuclear localization of $p27^{kip1}$ during apoptin-induced cell death. Akt is also known to control expression of $p27^{kip1}$ at the mRNA level via inactivation of a FOXO3a transcription factor [65]. Though, we have observed the phosphorylation and inhibition of FOXO3a by Akt during apoptin-induced cell death, we have not observed changes in $p27^{kip1}$ mRNA levels. Thus, taken together, Akt mediated phosphorylation of $p27^{kip1}$ at Thr-157 only affects the stability of the protein through the proteosome pathway, thereby relieving downstream effector molecules from $p27^{kip1}$-mediated inhibition.

As a consequence of $p27^{kip1}$ downregulation, cyclin A-associated CDK2 is constitutively activated by apoptin. Though CDK2 is a crucial player in the cell cycle during the progression from G1 to S phase [66], it is also known to regulate apoptosis [67, 68]. We show here that CDK2 inactivation by either pharmacological inhibitors or by siRNA, severely impairs apoptin-induced cell death. Also, we observed that the levels of CDK2 protein did not change with apoptin expression (Fig. 5C), whereas the levels of cyclin A increased; this did not appear to occur at the transcriptional level, suggesting that it is rather due to the prevention of protein degradation. This may be partially due to the inhibition of the C-subunit of the Anaphase-Promoting-Complex (APC/C) by apoptin as reported earlier [39]. Furthermore, we demonstrated that cyclin A/CDK2 also regulates apoptin's nuclear localization by direct phosphorylation of Thr-108 (Fig. 6A-D). Phosphorylation at Thr-108 has previously been reported to be specific for tumor cells, and required for apoptin induced cell death [37, 38]. Interestingly, it has been shown previously that, the phosphorylation level of apoptin is severely impaired by the apoptin mutant lacking residues 80-90, even if residues around the phosphorylation site (Thr-108) are intact [38]. The results here explain this interesting observation in that we show that apoptin interacts with PI3-K via amino acids 80-90, and that this interaction is required for downstream CDK2 activation. In contrast to previous results, the Ala-108 apoptin mutant

is still toxic for cancer cells under our experimental conditions, suggesting that the Thr-108 phosphorylation is of a secondary importance, and a consequence of CDK2 activation during apoptin-induced cell death, rather than a triggering event directly involved in apoptin's toxicity. Our results, however, still confirm that the Thr-108 phosphorylation is critical for apoptin nuclear accumulation. Nevertheless, aberrant CDK2 activation is clearly crucial and absolutely necessary for apoptin-induced cell death. The targets for CDK2 during apoptosis are still unknown; although p53 has been reported to be activated downstream of CDK2 [67], we and others, have ruled out the role of p53 in apoptin-induced cell death [33, 39, 69]. We do show here, however, that upon cytoplasmic translocation, CDK2 phosphorylates Bcl2, and targets it for proteosome-dependent degradation. This, in turn, affects the balance between anti- and pro-apoptotic Bcl2-family members to promote apoptosis. Thus, although CDK2 activation downstream of Akt is important, it may not be the sole mechanism for the pro-apoptotic role of the PI3-K/Akt pathway in apoptin-induced cell death. We predict that some additional Akt-targets, either in the nucleus or in the cytoplasm, exist that are phosphorylated during the course of apoptin-triggered cell death. Thus, Thr108 phosphorylation is important only for keeping the translocated protein in the nucleus but not necessary for nuclear import itself. The nuclear import is mediated by the nuclear localization sequence (NLS) located around the amino acids 80-90. Thus the smaller peptide still can go to the nucleus via its NLS and doesn't require 108-p as it can't be exported back because it lacks the nuclear export sequence (NES) reported in the full length apoptin.

Different components of the PI3-K/Akt pathway are involved in tumorigenesis and are highly active in various types of cancers compared to normal cells (reviewed in: [70, 71]). Furthermore, PTEN, a phosphatase that counteracts PI3-K's action, is the second most commonly mutated tumor suppressor gene after p53 [71]. Both CDK2 and Cyclin A are reported to be highly over-expressed in several tumors compared to the normal tissues (reviewed in: [72, 73]). Hyper-activation of the above pathways leads to a poor clinical prognosis and also contributes to drug resistance during cancer treatment. Thus, apoptin's targeting of these very pathways may explain its unique properties of tumor specific toxicity. Our data strongly indicate that apoptin "hijacks" these survival pathways and redirects them from their normal survival/proliferatory action towards the activation of the cell death. Importantly, the discovery here of the novel mechanism of the redirection of survival signaling into death pathways, will very likely lead to the development of a novel class of anti-tumor drugs.

The invention will now be described by way of examples. However, the examples do not necessarily limit the invention.

Apoptin selectively kills cancer cells relaying on the PI3-K pathway

We firstly reassessed the cancer cell-selective toxicity of apoptin, a controversial question in apoptin biology, using peripheral blood lymphocytes (PBLs) from Chronic Lymphocytic Leukemia (CLL) patients compared to the peripheral blood lymphocytes from a normal healthy individual. B-cells in CLL and normal PBLs were detected by double staining for the specific surface markers CD5 and CD19. In normal PBLs, there was no significant difference in the B-cell population before (6.1%) or after (5.2%) 48 hours of treatment with TAT-apoptin. In contrast, there was a significant decrease in the CLL PBLs upon TAT-apoptin treatment (11.2%) compared to the control (40.1%) (Fig. 1A). Thus, the data indicates that apoptin effectively kills the malignant B-cells from CLL patients, but not the normal counterparts, therefore confirming apoptin's selective toxicity (Fig. 1A). TAT-GFP, used as a negative control for the toxicity, had no significant effect on the survival of both normal and malignant cells.

As a first step to identify cellular targets of apoptin action in the cell, we generated an expression construct encoding glutathione-S-transferase (GST) fused to the apoptin N-terminus. GST-apoptin and control GST were used in a pull-down assay with cell extracts derived from MCF-7 breast cancer cells (Fig. 1B). Mass spectrometric analysis of proteins specifically bound to apoptin identified the two major components of the PI3-K/Akt pathway: the p85 regulatory subunit of PI3-K, and the serine/threonine kinase Akt, a kinase downstream of PI3-K. We also found that apoptin interacts with proteins such as chaperones, actin and tubulin family members as observed previously [39]. We confirmed the mass spectrometric data by detecting p85 and Akt by Western blot ir. the samples from the GST-apoptin pull-down assay (Fig. 1C), as well as using total cell extracts from PC-3 prostate cancer cells, L929 mouse fibrosarcoma cells, and 293 transformed human embryonic kidney cells. The interaction of apoptin with PI3-K and Akt was also examined *in vivo*, whereby PC-3 cells were transfected with GFP-apoptin, and total cell extracts immuno-precipitated with anti-GFP antibody at different time points post transfection, with the composition of the immune complexes was analyzed for PI3-K and Akt by immunoblotting. In a separate series of experiments, PI3-K (p85), and Akt were immuno-precipitated, and apoptin was detected by Western blot. Figure 1D shows that PI3-K and Akt both interact with apoptin confirming the data from Figure 1B. The interaction of apoptin with PI3-K could be detected 4-6 hours after apoptin transfection, thus preceding apoptin induced cell death, which is initiated at least 18-24 hours later. The above experiment indicates that PI3-K interaction with apoptin is a very early event in apoptin induced cell death. We consistently observed strong interaction of PI3-K with apoptin

within 6-48 hours post-transfection, whereas interaction of apoptin with Akt seems weaker and is seen only at certain time-points upon apoptin treatment.

Apoptin interacts with the SH3 domain of the p85 regulatory subunit via its proline-rich sequence

To identify and map the sites on apoptin responsible for interaction with PI3-K, PC-3 cells were transfected to express either full-length GFP-apoptin, or various deletion mutant derivatives of apoptin tagged with an N-terminal GFP as listed in Figure 1E. Apoptin was immuno-precipitated with anti-GFP antibodies 24 hours post transfection and the immune complexes were analyzed for the interaction with PI3-K by immunoblotting using anti-p85 antibodies. PI3-K was found in the immuno-precipitates of full-length apoptin and apoptin derivatives that harbored amino acids from 74-100 (a proline-rich region, see above), implying that this region of apoptin is important for interaction with PI3-K (Fig. 1E). We then tested if interaction of PI3-K with apoptin is essential for apoptin-induced cell death by assessing the percentage of apoptosis induced by the different mutants. In agreement with the interaction data, only those mutants that had the intact interaction site for PI3-K were able to induce apoptosis (Fig. 1F). Interestingly, in our model system the mutant Ala-108, which is a non-phosphorylable apoptin stated to be non-toxic to the cells in previous reports [38], was still able to induce significant apoptosis as long as its interaction site with the PI3-K was intact.

Next, we investigated the apoptin interaction site on PI3-K by co-transfecting with a vector encoding full-length apoptin, together with full length PI3-K or various deletion derivatives thereof. Some PI3-K deletion mutants were tagged with haemagglutinin tag (HA) at their N-terminus (Fig. 1G). Both full length PI3-K and the mutant lacking the iSH2 domain were immuno-precipitated by anti-p85 antibody, while other mutants were immuno-precipitated using anti-HA antibodies. Immuno-detection of apoptin in the immune complexes of PI3-K and its deletion mutant derivatives implied that apoptin interacts with the intact SH3 domain of PI3-K (Fig. 1G).

PI3-K is constitutively activated during apoptin-induced apoptosis

To determine the functional significance of apoptin's interaction with the p85 regulatory subunit, we measured PI3-K activity using a non-radioactive ELISA-based method. Surprisingly, MCF-7 and PC-3 cells transfected to express GFP-Apoptin revealed constitutive activation of PI3-K in apoptin-transfected cells (Fig. 2A). PI3-K activity was increased nearly four-fold in apoptin treated MCF-7 cells and up to six-fold in PC-3 cells, compared to the GFP-transfected control. PI3-K activation was seen around six hours

after transfection, consistent with the interaction data (Fig. 1FG), with activation retained at a similar level for up to ~40 hours. In a control experiment, various PI3-K inhibitors prevented apoptin-triggered generation of PIP_3 (Fig. 2B), whilst co-transfection of apoptin with a dominant negative PI3-K vector reduced apoptin-induced PI3-K activation to basal levels (Fig. 2B). We then tested if the activation of PI3-K is a direct consequence of apoptin's interaction by monitoring PI3-K activity in cells transfected to express different apoptin deletion derivatives. As expected, the full-length apoptin induced six-fold PI3-K activation, while the apoptin 1-89 derivative that harbors only the partial PI3-K interaction site, was much less active. The apoptin derivatives 1-111, 74-121, Ala-108 and Glu-108 were all able to activate PI3-K to an extent comparable to full-length apoptin, whereas the 1-73 and 104-121 derivatives were unable to activate PI3-K. This observation implies that the intact p85 interaction site on apoptin is crucial for the activation of PI3-K (Fig. 2C). On the other hand, PI3-K activity in cells co-transfected to express full-length apoptin and p85 deletion mutants confirmed that the interaction of apoptin with the SH3-domain of p85 is essential for its activity. The p85 derivatives that retain both SH3 and iSH2 domains have constitutive PI3-K activity in the presence of apoptin, whereas others lacking these domains are severely impaired in their activity (Fig. 1D). Interestingly, the mutant N+I+C SH2 having only the SH2 domains but lacking the SH3 domain have relatively higher PI3-K activity compared to other deletion mutants and to the control, indicating that the intact SH3 domain in the wild-type p85 protein might have an inhibitory action on its activity. Thus, the interaction of apoptin with the SH3 domain appears to elicit a conformational change in the p85 protein, converting it from an inhibitory- to an active state.

In order to investigate the direct effect of PI3-K inhibition on apoptin induced cell death, we pre-treated the cells with the PI3-K inhibitors, wortmannin and LY294002, 30 minutes before transfection to express apoptin and cell death assayed 24 and 48 hours later. To our surprise, both inhibitors afforded significant protection against apoptin-induced cell death, despite generally being known to enhance the cell death process through inhibition of PI3-K activity. This effect was further confirmed by co-transfection of cells to express apoptin together with a dominant-negative derivative of PI3-K, which significantly protected cells from apoptin-induced cell death (Fig. 2E).

To support the data from the inhibitor and transfection experiments, we used siRNA to test whether knocking down endogenous PI3-K would affect resistance to apoptin-induced cell death. In PC-3 cells, the expression of p85 was completely abrogated by p85-specific siRNA but not by the control siRNA (Fig. 2F), as assayed approximately 30 hours post-transfection. The assessment of cell death revealed that cells that lack p85 expression were strongly resistant towards apoptin induced cell death compared to control

siRNA-expressing cells (Fig. 2F). Similar observations were also made in the MCF-7 and 293 cell types, consistent with the idea that the effect is not cell type specific.

Apart from the negative effect of PI3-K inhibition on apoptin induced cell death, we also tested if PI3-K inhibition affects apoptin sub-cellular localization. Apoptin is mainly localized in the nucleus of PC-3 and MCF-7 cells, but in the presence of wortmannin, apoptin was found mainly distributed in the cytoplasm as shown either by the immuno-staining followed by confocal laser microscopic imaging (Fig. 2G) or by sub-cellular fractionation followed by Western blotting (Fig. 2I). The effect of PI3-K inhibition on apoptin's localization was further studied using p85 siRNA. Upon the inhibition of p85 expression, nuclear localization of apoptin was almost completely abrogated (Fig. 2H) indicating that PI3-K activity is not only necessary for apoptin-induced cell death but also for the localization of the protein during its apoptotic action in cells. Previous reports indicated that the tumor-specific phosphorylation of the Thr-108 residue in apoptin is required for the differential localization of the protein. PI3-K is mainly a lipid kinase, though some protein kinase activity has been reported; the possibility that PI3-K might directly phosphorylate apoptin was firstly excluded in *in vitro* kinase assays (data not shown). It thus seems likely that one of the downstream effectors of the PI3-K pathway phosphorylates apoptin and controls its localization, but in a PI3-K dependent manner (see below).

Akt translocates to the nucleus during apoptin induced cell death

We next investigated the effect of apoptin on downstream targets of the PI3-K pathway by transfecting the PC-3 cells to express apoptin, and assessing Akt activation by Western blotting at different time-points post-transfection. In agreement with the results for PI3-K activation, increased levels of activated Akt were seen around 6-12 hours post-transfection, with pronounced levels of phosphorylated Akt detectable even 24 hours post-transfection (Fig. 3A). The activation of Akt is downstream of-, and dependent on PI3-K activation; pretreatment of cells with the inhibitors wortmannin or LY294002, or co-transfection of apoptin-expressing cells with constructs encoding PI3-K- or PDK1-dominant-negative mutants (PI3-K-DN, PDK1-DN) impaired Akt activation. Also, over-expression of wild type PTEN, a negative regulator of Akt activation, severely reduced apoptin-induced Akt activation, whereas over-expression of a phophatase deficient PTEN mutant had no effect (Fig. 3B). To further confirm the role of PI3-K in apoptin-induced cell death, and to validate the role of Akt in this process, we transiently over-expressed a PDK1-DN, and/or the dominant negative Akt (Akt-DN) using adenoviral vectors. Both dominant-negative kinase mutants as well as over-expression of wild type PTEN

significantly protected against apoptin-induced cell death, thus confirming the key role of the PI3-K/Akt pathway in this process (Fig. 3C). Akt is generally regarded as a survival-, or proliferation-promoting kinase and not at all a pro-apoptotic molecule. However, in the presence of apoptin, Akt is clearly acting as a pro-cell death molecule as its inhibition severely inhibits the cell death pathways triggered by apoptin. Next, we asked if apoptin redirects Akt to different cellular targets by modulating its sub-cellular localization. As shown in Figure 3D, Akt is mainly localized in the cytoplasm of both PC-3 and MCF-7 cells in the absence of apoptin expression, but is clearly nuclear in its presence. Analysis of the kinetics of Akt nuclear translocation revealed that most of the cytoplasmic Akt translocates to the nucleus within 12 hours of transfection to express apoptin (data not shown). Furthermore, to determine the effect of different apoptin mutants on Akt translocation and test if Akt nuclear translocation depends on apoptin/PI3-K interaction, we investigated the localization of Akt in cells transfected to express different apoptin deletion mutants. As shown in figure 3E, Akt translocates to the nucleus only in cells expressing apoptin mutants 1-121, 1-89, 1-111, 74-121 and the Glu-108 mutant, all which are nuclear localizing, and have an intact PI3-K interaction site. Cells expressing the apoptin mutants 1-73 and 104-121 did not exhibit Akt nuclear translocation. This is in perfect accordance with their ability both to interact with PI3-K and induce apoptosis (Fig. 2C). In the presence of the Ala-108 mutant, Akt translocates to the nucleus together with apoptin around 18 hours post transfection, but later at 24 hours traces of Akt were observed in the cytoplasm along with apoptin. This is in accordance with previously-published data [37]. Apoptin mutant T108A is still able to enter the nucleus, and facilitate the nuclear translocation of Akt, but it is deficient in nuclear accumulation. This clearly implies that apoptin might be acting as a carrier molecule for Akt, directly mediating its nuclear transport via a "piggy back" interaction.

To determine if nuclear Akt alone is sufficient to induce cell death even in the absence of an apoptotic stimulus, we infected PC-3 cells with an NLS-Akt adenoviral vector in the absence and presence of apoptin expression. As shown in figure 3F, nuclear Akt alone was unable to induce apoptosis, whereas in the presence of apoptin, it significantly enhanced the cell death. Quantitative analysis, using flow cytometry, to determine the extent of cell death (Fig. 3G) indicated that NLS-Akt exhibits some residual toxicity (13%) compared to the negative (wild type) control (7%). Co-expression of NLS-Akt together with apoptin sensitized the cells towards apoptin-induced cell death. Importantly, NLS-Akt also sensitized the cells towards several other clinically relevant apoptotic stimuli, such as methotrexate, docetaxel, cisplatin, and doxorubicin, but not staurosporine or CD95-triggering (Fig. 3H). Together, the data indicates that nuclear Akt

alone is not sufficient for cell death induction, but can clearly facilitate cell death in the presence of certain apoptotic stimuli.

Akt phosphorylates and downregulates $p27^{kip1}$ in the nucleus

As most of the Akt is localized to the nucleus during apoptin induced cell death, we next investigated the role of $p27^{kip1}$, a cell cycle inhibitor, as a potential nuclear target of Akt activity. $p27^{kip1}$ was immuno-precipitated from the lysates of untransfected cells and cells transfected to express apoptin, and phosphorylation was monitored using anti-phospho-serine or anti-phospho-threonine antibodies. $p27^{kip1}$ threonine phosphorylation levels increased in the presence of apoptin, but apoptin had no effect on serine phosphorylation (Fig. 4A). We confirmed the phosphorylation of $p27^{kip1}$ at threonine residues by using an antibody against phosphorylated Thr-157 (a potential Akt consensus site). Analysis of the kinetics of phosphorylation of $p27^{kip1}$ at Thr-157 revealed that it occurs soon after Akt nuclear translocation. A significant increase in the phophorylation at Thr-157 of $p27^{kip1}$ is seen 12 hours after transfection to express apoptin and diminishes after 24-30 hours (Fig. 4B). The increase in threonine phosphorylation is dependent on Akt activation, as treatment of cells with wortmannin but not with the MAP kinase inhibitor, PD98059, abolished $p27^{kip1}$ phosphorylation even in the presence of apoptin (Fig. 4C). The decreased $p27^{kip1}$ phosphorylation levels after 24 hours may be due to the decrease in the total $p27^{kip1}$ protein levels (see below). Phosphorylation of $p27^{kip1}$ at Thr-157 was revealed to be strongly dependent on Akt activation, which is further dependent on PI3-K and PDK1 upstream activation. The co-transfection of cells to express apoptin together with dominant negative PI3-K, PDK1, or Akt mutant derivatives significantly decreased the phosphorylation at Thr-157 of $p27^{kip1}$. In contrast, phosphorylation of the Thr-157 residue was not affected by co-transfection to express a dominant negative form of the unrelated kinase PKC (Fig. 4C). Furthermore, we determined that phosphorylation of $p27^{kip1}$ at Thr-157 is directly mediated by Akt, by examining $p27^{kip1}$/Akt interaction. Co-immuno-precipitation experiments revealed that significant levels of $p27^{kip1}$ could be found in the immune complexes, together with Akt, during apoptin-induced cell death (Fig. 4D).

$p27^{kip1}$ phosphorylation targets it into the proteosome-dependent degradation pathway. To determine the functional significance of $p27^{kip1}$ phosphorylation by Akt, we checked the protein levels of $p27^{kip1}$ before and after transfection to express apoptin. Strikingly, we have found a dramatic decrease in the levels of $p27^{kip1}$ in apoptin-expressing cells compared to the GFP control expressing cells (Fig. 4E). The kinetics of $p27^{kip1}$ downregulation revealed that about 24 hours post-transfection, $p27^{kip1}$ is completely downregulated in PC-3 cells. While the kinetics were observed to vary somewhat in

different cell lines, a similar trend of transient phosphorylation followed by depletion was clearly evident. The decrease in the levels of $p27^{kip1}$ protein is entirely dependent on $p27^{kip1}$ phosphorylation by Akt, as the protein levels are restored to the control levels in apoptin expressing cells that have been pre-treated with wortmannin but not with PD98059 (Fig. 4F). The restoration of $p27^{kip1}$ levels was also seen upon co-expression of PI3-K-DN, PDK1-DN or Akt-DN but not with PKC-DN. That the decrease in the protein levels of $p27^{kip1}$ was due to proteosome-dependent degradation was indicated by the fact that treatment of cells with the proteosome inhibitor MG-115 restored the $p27^{kip1}$ levels even in the presence of apoptin expression (Fig. 4F). The levels of $p27^{kip1}$ mRNA did not change upon apoptin treatment, thus indicating that the decrease in the $p27^{kip1}$ protein levels was entirely dependent on Akt-mediated phosphorylation enhancing degradation of the protein via the proteosome pathway.

To investigate the role of $p27^{kip1}$ downregulation during apoptin-induced cell death, we used a siRNA-based approach. $p27^{kip1}$ expression was depleted upon transfection to express $p27^{kip1}$-specific but not control siRNA (Fig. 4G). Furthermore, transfection to express apoptin of the $p27^{kip1}$-deficient cells revealed that $p27^{kip1}$ downregulation sensitizes the cells to apoptin-induced cell death; as shown in Figure 4G, the percentage of apoptin induced cell death in PC-3 cells, determined by flow cytometry after propidium iodide staining, clearly showed a significant increase upon $p27^{kip1}$-inhibition as compared to apoptin alone.

CDK2/cyclin A activity is elevated and is required during apoptin-induced cell death

To investigate the role of CDKs downstream of $p27^{kip1}$ downregulation during apoptin's pro-apoptotic signaling, we measured the activation of two major cyclin-dependent kinases, CDK1 and CDK2, using an *in vitro* kinase assay and the substrate histone H1. PC-3 cells were transfected to express either GFP or GFP-apoptin and the CDK activity was measured 24 hours post transfection by immuno-precipitating using CDK1 or CDK2 specific antibodies. In the presence of apoptin expression, CDK2 but not CDK1 activity was elevated as indicated by the H1 phosphorylation activity (Fig. 5A). To determine if the increased CDK2 activity during apoptin-induced cell death was associated with cyclin E or A, immuno-precipitation was performed using specific antibodies, either in the presence of over-expressed GFP or GFP-apoptin. Figure 5A, shows that only cyclin A- but not cyclin E-associated CDK2 phosphorylated histone H1, suggesting that only cyclin A/CDK2 has a role during apoptin-induced cell death. CDK2 activity was detectable at 16 hours and peaked at 24 hours post transfection (Fig. 5B). We also tested if this increase in CDK2 activity during apoptin-induced apoptosis is dependent on the upstream PI3-K/Akt

activation. Figure 5C shows that CDK2 is activated only when the PI3-K/Akt pathway is active; inhibition of Akt activation by wortmannin, Ly294002, PI3-K-DN, PDK1-DN or Akt-DN significantly decreased apoptin-induced CDK2 activation.

To test if CDK2 activity is required for apoptin-induced cell death, we used two different approaches. The first was to inhibit CDK2 activity using a CDK2-specific inhibitor Roscovitine. Figure 5D shows that in the presence-, as compared to the absence of CDK2 inhibitor, PC-3 cells were significantly resistant to apoptin-induced cell death. The background levels of cell death seen with the combination of CDK2 inhibitor and apoptin expression may be attributed to the CDK2 inhibitor itself, as the inhibitor itself is slightly toxic. The second approach was to knock down the expression of CDK2 by transfection with a plasmid encoding a CDK2 specific siRNA, and test its effect on apoptin induced cell death. As shown in Figure 5E, CDK2 expression in PC-3 cells was downregulated by CDK2-inhibitory siRNA 48 hours after transfection, with the CDK2-negative cells concomitantly highly resistant to apoptin-induced cell death.

We have previously shown that apoptin's death signaling converges at the mitochondrial death pathway [33]. Here, we further investigated whether CDK2 activation is upstream, or if it is a consequence of mitochondrial death pathway activation. As shown in Figure 5F, CDK2 activation is unaffected either by Bcl-2 over-expression or by caspase inhibition using the inhibitor zVAD-fmk, but the prevention of CDK2 activation by CDK2 inhibitor completely blocked release of cytochrome c from the mitochondria to the cytosol (Fig. 5G), indicating that CDK2 activation is upstream of the mitochondrial death pathway.

Sub-cellular localization of CDK2 determines whether it drives either cell proliferation or cell death [40]. CDK2 is localized mainly in the nucleus during the execution of its normal cell cycle regulatory function, but in the presence of apoptin, CDK2 was observed predominantly in the cytoplasm (Fig. 5H, see also Fig. 6D). We tested the functional significance of the cytoplasmic CDK2 by investigating its substrates in the cytoplasm by co-immuno-precipitation, whereby Bcl2 was detected in the CDK2 immune complexes in the presence of apoptin (Fig. 5I). Bcl2-phosphorylation is known to facilitate its degradation via the proteosome pathway [41, 42]; to determine the effect of CDK2 on Bcl2, we tested the levels of Bcl2 phosphorylated either at serine- or threonine residues after immuno-precipitation from lysates from cells transfected to express apoptin. Increased Bcl2 phosphorylation at threonine but not serine residues was observed. The increased phosphorylation of Bcl2 appears to occur specifically at Thr-56 (Fig. 5J); significant changes in phosphorylation of Bcl2 at Ser-70, Thr-74 and Ser-87 were not observed either in the presence or absence of apoptin expression. Bcl2 phosphorylation results in a dramatic decrease of Bcl2 protein (Fig. 5J), apparently due to proteosome-

mediated degradation, since treatment with the proteosome inhibitor MG-115 restored Bcl2 levels (Fig. 5K). Both Bcl2 phosphorylation and the decrease in total protein was found to be dependent on CDK2 activity, since downregulation of CDK2-expression by siRNA prior to transfection to express apoptin resulted in a significant decrease in Bcl2 phosphorylation and little change to protein levels observed. That Bcl2 phosphorylation by CDK2 is dependent on upstream activation of the PI3-K/Akt pathway, was indicated by the fact that depletion of PI3-K-expression using a specific siRNA showed an effect on Bcl2 similar to that of the downregulation of CDK2-expression. Thus, PI3-K/Akt mediated activation of CDK2 leads to the Bcl2-degradation, with the low Bcl2 level altering the balance between the pro- and anti-apoptotic Bcl2 family members in favor of pro-apoptotic molecules, and ultimately activating the mitochondrial death pathway.

Activated cyclin A-associated CDK2 is the apoptin kinase that regulates its nuclear localization in cancer cells

Apoptin phosphorylation at Thr-108 has been previously reported to be critical for its activity and tumor cell-specific nuclear localization [37, 38]. To test whether CDK2 may directly phosphorylate apoptin, we performed an *in vitro* kinase assay using recombinant GST-apoptin and TAT-apoptin as substrates. As shown in Figure 6A, detection of phosphorylated apoptin using a phospho-threonine-proline specific antibody revealed that apoptin can be phosphorylated by active, recombinant CDK2/cyclin A. Interestingly, active, recombinant CDK2/Cyclin E was not able to phosphorylate apoptin *in vitro*, nor was CDK1/Cyclin B, although both were able to phosphorylate Histone H1 *in vitro*. We further confirmed the CDK2 phosphorylation of apoptin using immuno-precipitated CDK2, CDK1, cyclin A, cyclin E, and cyclin B, with inactive CDK2 (CDK2 T160A) as a negative control. Apoptin was phosphorylated only by the immune complex of active CDK2 and cyclin A, but not by other combinations of CDKs and cyclins (Fig. 6B). This data correlates with the activation of cyclin A-associated CDK2, and not other CDKs, in the presence of apoptin (see above).

We next investigated, if activated CDK2 phosphorylates apoptin *in vivo* at Thr-108 by two different approaches. First, we inhibited CDK2 activity in PC-3 cells using a CDK2-specific inhibitor and transfected the cells to express apoptin. 24 hours later, apoptin was immuno-precipitated and its phosphorylation-status assessed using the phospho-specific Thr-Pro antibody. Figure 6C shows that apoptin is phosphorylated in the presence of active CDK2, with the level of phosphorylation significantly reduced in the presence of the CDK2 inhibitor Roscovitine. Secondly, we tested the effect on apoptin phosphorylation of inhibiting the expression of CDK2 using siRNA. Figure 6C shows that apoptin

phosphorylation was severely abrogated by reduced CDK2 expression in the cells, implying that CDK2 is the apoptin kinase. We next investigated the role of CDK2 in controlling apoptin's nuclear localization by inhibiting CDK2 expression in both PC-3 and MCF-7 cells using siRNA. Figure 6D shows that in the presence of CDK2, apoptin is exclusively in the nucleus, but knock down of CDK2 by siRNA severely impaired apoptin nuclear accumulation in both cell lines. This suggests strongly that apoptin-activated CDK2 is the apoptin kinase controlling apoptin nuclear accumulation in cancer cells. Furthermore, upstream activation of PI3-K/Akt is necessary for CDK2 kinase activity towards apoptin, as co-transfection of a PDK1 dominant negative mutant, or Akt-DN, severely reduces apoptin nuclear localization (Fig. 6E). Co-expression of apoptin with active PDK1 or Akt facilitated rather than prevented apoptin nuclear accumulation. Also, phosphorylation of apoptin by CDK2 is reduced in the presence of PDK1-DN or Akt-DN, but not upon co-expression of PKC-DN (Fig. 6F).

Materials and Methods

Cell culture and reagents

MCF-7, PC-3, 293 and L929 cells were grown in RPMI-1640 medium supplemented with 10% FBS (Hyclone), 100μg/ml penicillin and 0.1μg/ml streptomycin (Gibco BRL). The cells were grown at 37°C with 5% CO_2 in a humidified incubator. The peripheral blood lymphocytes were isolated from Chronic Lymphocytic Leukemia (CLL) patients or normal healthy individuals by ficoll gradient fractionation, as described previously [74] and maintained in RPMI medium. The following antibodies were used: murine anti-PI3-K (p85), anti-mouse IgG-HRP, anti-rabbit IgG-HRP (all from Upstate Cell Signaling), goat anti-Akt, rabbit anti-$p27^{kip1}$, murine anti-tubulin, rabbit anti-GFP, rabbit anti-phospho-$p27^{kip1}$-Thr-187, rabbit anti-CDK2, anti-CDK1, anti-Cyclin E, anti-Cyclin A, murine anti-cytochrome c, anti-goat IgG-HRP (all from Santacruz Biotechnologies), murine anti-phospho-Akt Ser-473, Mouse anti-phoshpho-Thr antibody, murine anti-phospho-Thr-Pro antibody (all from Cell Signaling), anti-human CD5-FITC, CD19-PerCP (BD biosciences), murine anti-HA, murine anti-phospho-Ser antibody, anti-goat Cy3, anti-rabbit Cy3, anti-murine Cy3 (all from Sigma), anti-Myc antibody (Invitrogen), and anti-phospho-$p27^{kip1}$-Thr-157 (R&D systems). The following inhibitors were used: wortmannin (IC_{50} 5nM), LY294002 (IC_{50} 1.4μM), PD98059 (IC_{50} 2μM), CDK2 specific inhibitor, Roscovitine (Kd 700nM) (all from Calbiochem), and zVAD-fmk Enzyme System Products (Aurora, OH, USA).

B-cell staining and FACS analysis

Peripheral blood lymphocytes from normal individuals and CLL patients either left untreated or TAT-Apoptin treated for the indicated times were washed twice with ice cold PBS, and then incubated with both CD5-FITC and CD19-PerCP antibodies (each 0.5μg per 10^6 cells) for 30 minutes at 4°C in dark. The cells were then washed twice with cold PBS and resuspended in 300μl of PBS. Samples were analysed by flow cytometry by using both FL1 (FITC) and FL2 (PerCP) channels and percentage of B-cells obtained by gating the double positive cells compared to unstained control.

Protein purification, GST-pull down assay and protein identification

The TAT-GFP and TAT-Apoptin proteins were purified as previously [75]. GST and GST-apoptin were purified by using glutathione sepharose high performance beads (Amersham Biosciences) according to the manufacturer's protocol. The GST-pull down assay was performed to detect apoptin's interacting partners. Briefly, either purified GST or GST-apoptin along with total PC-3 cell lysate was immobilized on glutathione sepharose beads overnight at 4°C. The beads were washed thrice with ice-cold lysis buffer and the bound proteins were isolated on SDS-PAGE. The proteins specific for apoptin were subjected to in-gel digestion and further identified by MALDI-TOF mass spectometry at the proteomics centre at the University of Manitoba. Finally, proteins from the GST-pull down assay were identified by immunoblotting.

Plasmids, transfections and adenoviral infections

The following plasmid were used: GFP-apoptin (apoptin cloned into pEGFP-C1 vector, clonetech), GST-apoptin (apoptin cloned into PGEX-2T vector, Amersham biosciences), PI3-K dominant negative vector, Akt wild type vector (J. Downward, UK, [49]), Apoptin mutant plasmids (Fig. 1EF) [37]), p85 deletion mutants (Fig. 1G) (T. Mustelin, [76]), PDK1 dominant negative and constitutively active vectors (A. Halayko, Winnipeg), PTEN wt and PTEN C124S phosphatase dead mutant (D.R. Anderson, Saskatchewan Cancer Agency, Saskatchewan), PKC dominant negative vector (E. Kardami, Winnipeg), CDK2 T160A mutant (D.O. Morgan, San Franscisco, [77], Ad-NLS-Akt (M.A Sussman, San Diego, [78], and Ad-Akt-dominant negative vector (K.Walsh, [79]). Transfection was performed using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. Adenoviral transfections with Akt-dominant-negative mutant vector and nuclear targeted Akt were performed as previously [80].

PI3-kinase ELISA

A non-radioactive competitive ELISA based assay was used to assess the PI3-kinase activity under different conditions (Fig 2A-D) according to the manufacturers protocol (Echleon Biosciences). Briefly, equal amounts of PI3-K from the PC-3 cell lysates were immuno-precipitated with anti-p85 antibodies overnight at 4°C and then incubated with protein A-Sepharose beads for 1 hour at 4°C. The bead-bound enzymes were incubated with 100 pM of phosphatidylinositol (4, 5) bisphosphate (PI(4, 5)P2) substrate in kinase reaction buffer (4 mM $MgCl^2$, 20 mM Tris, pH 7.4, 10 mM NaCl, and 25 µM ATP) for 2 h at room temperature. The mixtures were then incubated with phosphatidylinositol (3,4,5) trisphosphate (PI(3,4,5)P3) detector for 1 h at room temperature in the dark, and subsequently added to PI(3,4,5)P3-coated microplate wells, and incubated for 30 min at room temperature in the dark. After thorough washing, peroxidase-linked secondary detection reagent was added, and PI(3,4,5)P3 detector protein binding to the plate was assessed by measuring absorbance at 450nm. The data for the kinase activity are expressed as fold induction in transfected cells compared with the activity in untreated cells.

In-vitro kinase assays

The in-vitro kinase assays using Histone H1 (Upstate), GST-apoptin and TAT-apoptin as substrates was performed as described earlier [57], but in a non-radioactive assay. Briefly, 5 ng of the recombinant CDK2/Cyclin A (New England Biolabs), CDK2/Cyclin E, CDK1/Cyclin B (Upstate) or the immuno-precipitated CDK2, CDK1, Cyclin A, Cyclin B, Cyclin E were used in a kinase reaction with 5µg of Histone H1, GST-Apoptin or TAT-Apoptin as substrates in the presence of 200µM ATP in a kinase assay buffer (25mM Tris pH 7.5, 5mM β-Glycerophosphate, 2mM DTT, 0.1mM Na_3VO_4 and 10mM $MgCl_2$). The kinase reaction was performed at 30°C for 30 minutes and the end-products were resolved by SDS-PAGE (12-15%) and detected by immunoblotting using their respective phospho-specific antibodies.

Immuno-precipitation and immunoblotting

Cells were washed twice with cold PBS, lysed with ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, Protease inhibitor cocktail), incubated for 10 min on ice, and centrifuged for 10 min at 4°C. Immunoprecipitations were performed with the indicated antibodies (Figs. 1, 2, 4 and 5) and the immuno-complexes captured with protein A-agarose beads (Amersham

Pharmacia Biotech). After three washing steps with cell lysis buffer, bead-bound proteins were subjected to Western blot analysis as described previously [33].

RNA interference

The plasmids coding for PI3-K siRNA (pKD-PI3 Kinase, p85-V3), CDK2 siRNA (pKD-CDK2-v6) and the negative control siRNA were purchased from Upstate cell signaling. The $p27^{kip1}$ siRNA was obtained commercially from Santacruz Biotechnologies. The described plasmids or the siRNA sequences were transfected into the cells grown to 70% confluency using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The expression of the proteins was analysed by Western blotting after 48 hours of transfection.

Immunolocalization studies

MCF-7 and PC-3 cells were transfected to express apoptin in the absence or presence of various treatments and fixed 24 h later in 4% Paraformaldehyde in PBS, permeabilized in 0.2% Triton X-100 and stained with either anti-p85-, anti-Akt-, or anti-CDK2 antibodies followed by their respective secondary antibodies conjugated to Cy3. The fluorescent images were then analysed by a confocal microscopy.

Cell fractionation

The cytoplasmic and nuclear fractions were separated by using differential centrifugation exactly as described previously [33].

Apoptotic assays

The measurement of apoptosis was performed by the Nicoletti method followed by flow cytometry as previously [33].

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.

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CLAIMS

1. A method of inducing apoptosis in a cancerous cell comprising administering an effective amount of an isolated or purified peptide comprising PKPPSK (SEQ ID NO:3).

2. The method according to claim 1 wherein the peptide comprises PKPPSKKR (SEQ ID NO:4).

3. The method according to claim 1 wherein the peptide comprises amino acids 1-111 of SEQ ID NO: 5.

4. The method according to claim 1 wherein the peptide comprises amino acids 1 to 48 of SEQ ID NO:6.

5. The method according to claim 1 wherein the peptide comprises amino acids 1 to 38 of SEQ ID NO:7.

6. The method according to claim 1 wherein the peptide comprises amino acids 1 to 31 of SEQ ID NO:8.

7. The method according to claim 1 with the proviso that the peptide is not native apoptin (SEQ ID NO:1).

8. A method of manufacturing a pharmaceutical composition comprising mixing an effective amount of an isolated or purified peptide comprising PKPPSK (SEQ ID NO:3) with a suitable excipient.

9. The method according to claim 8 wherein the peptide comprises PKPPSKKR (SEQ ID NO:4).

10. The method according to claim 8 wherein the peptide comprises amino acids 1-111 of SEQ ID NO: 5.

11. The method according to claim 8 wherein the peptide comprises amino acids 1 to 48 of SEQ ID NO:6.

12. The method according to claim 8 wherein the peptide comprises amino acids 1 to 38 of SEQ ID NO:7.

13. The method according to claim 8 wherein the peptide comprises amino acids 1 to 31 of SEQ ID NO:8.

14. The method according to claim 8 with the proviso that the peptide is not native apoptin (SEQ ID NO:1).

15. A purified or isolated peptide comprising amino acids PKPPSK (SEQ ID NO:3).

16. The peptide according to claim 15 wherein the peptide comprises PKPPSKKR (SEQ ID NO:4).

17. The peptide according to claim 15 wherein the peptide comprises

amino acids 1-111 of SEQ ID NO: 5.

18. The peptide according to claim 15 wherein the peptide comprises amino acids 1 to 48 of SEQ ID NO:6.

19. The peptide according to claim 15 wherein the peptide comprises amino acids 1 to 38 of SEQ ID NO:7.

20. The peptide according to claim 15 wherein the peptide comprises amino acids 1 to 31 of SEQ ID NO:8.

21. The peptide according to claim 15 with the proviso that the peptide is not native apoptin (SEQ ID NO:1).

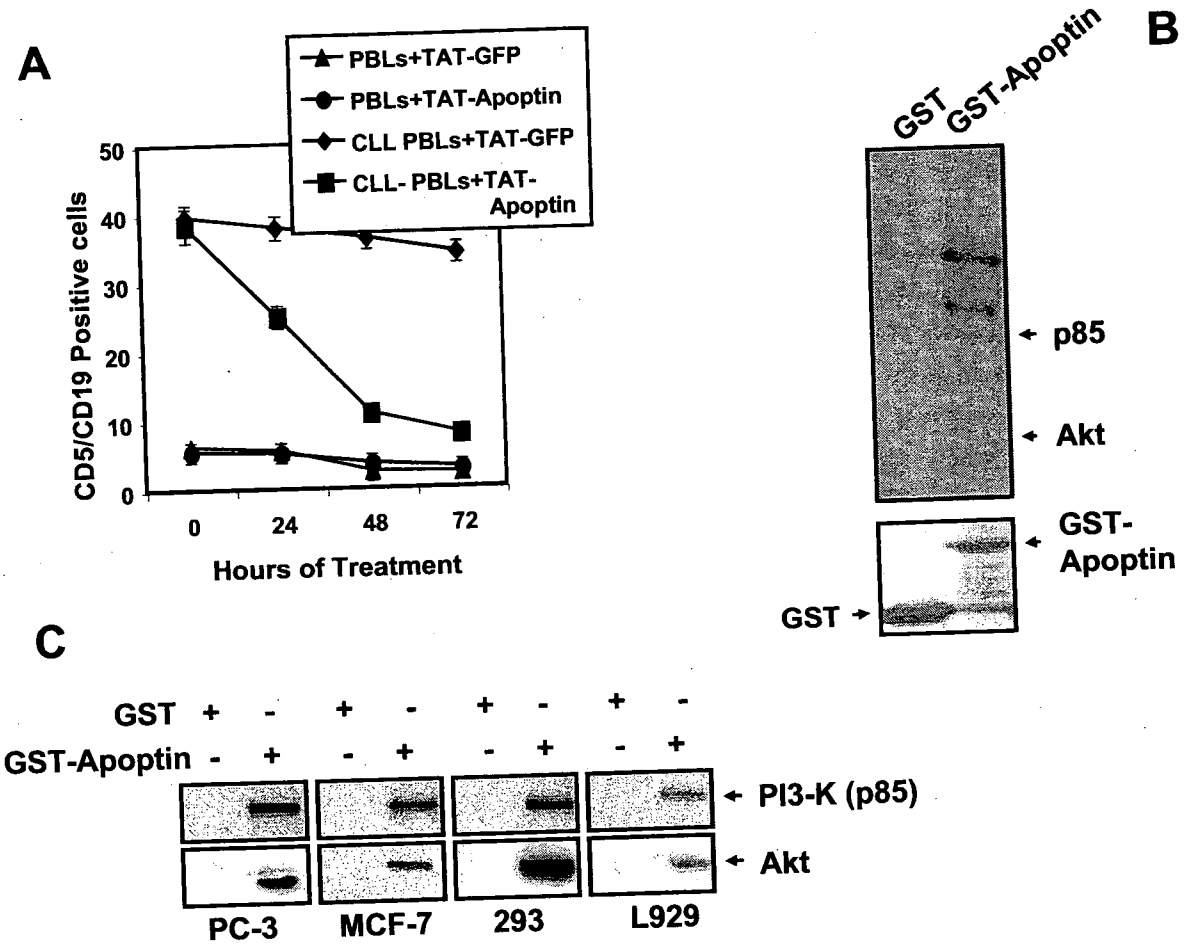

A
PBLs+TAT-GFP
PBLs+TAT-Apoptin
CLL PBLs+TAT-GFP
CLL- PBLs+TAT-Apoptin
CD5/CD19 Positive cells
50
40
30
20
10
0
0
24
48
72
Hours of Treatment
B
GST
GST-Apoptin
p85
Akt
GST-Apoptin
GST
C
GST + - + - + - + -
GST-Apoptin - + - + - + - +
PI3-K (p85)
Akt
PC-3
MCF-7
293
L929

Figure 1-1

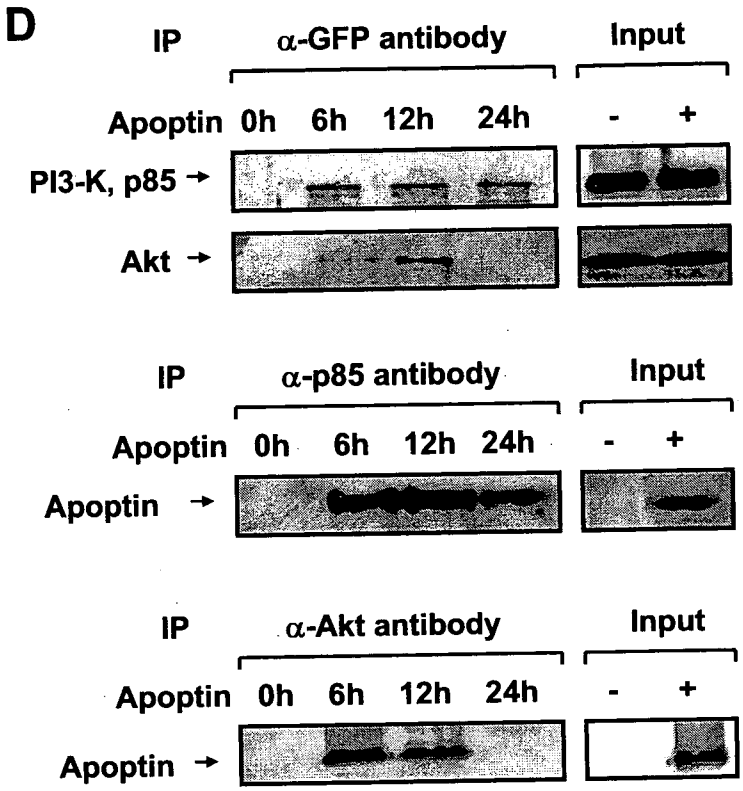

D
IP
α-GFP antibody
Input
Apoptin 0h 6h 12h 24h - +
PI3-K, p85 →
Akt →
IP
α-p85 antibody
Input
Apoptin 0h 6h 12h 24h - +
Apoptin →
IP
α-Akt antibody
Input
Apoptin 0h 6h 12h 24h - +
Apoptin →

Figure 1-2

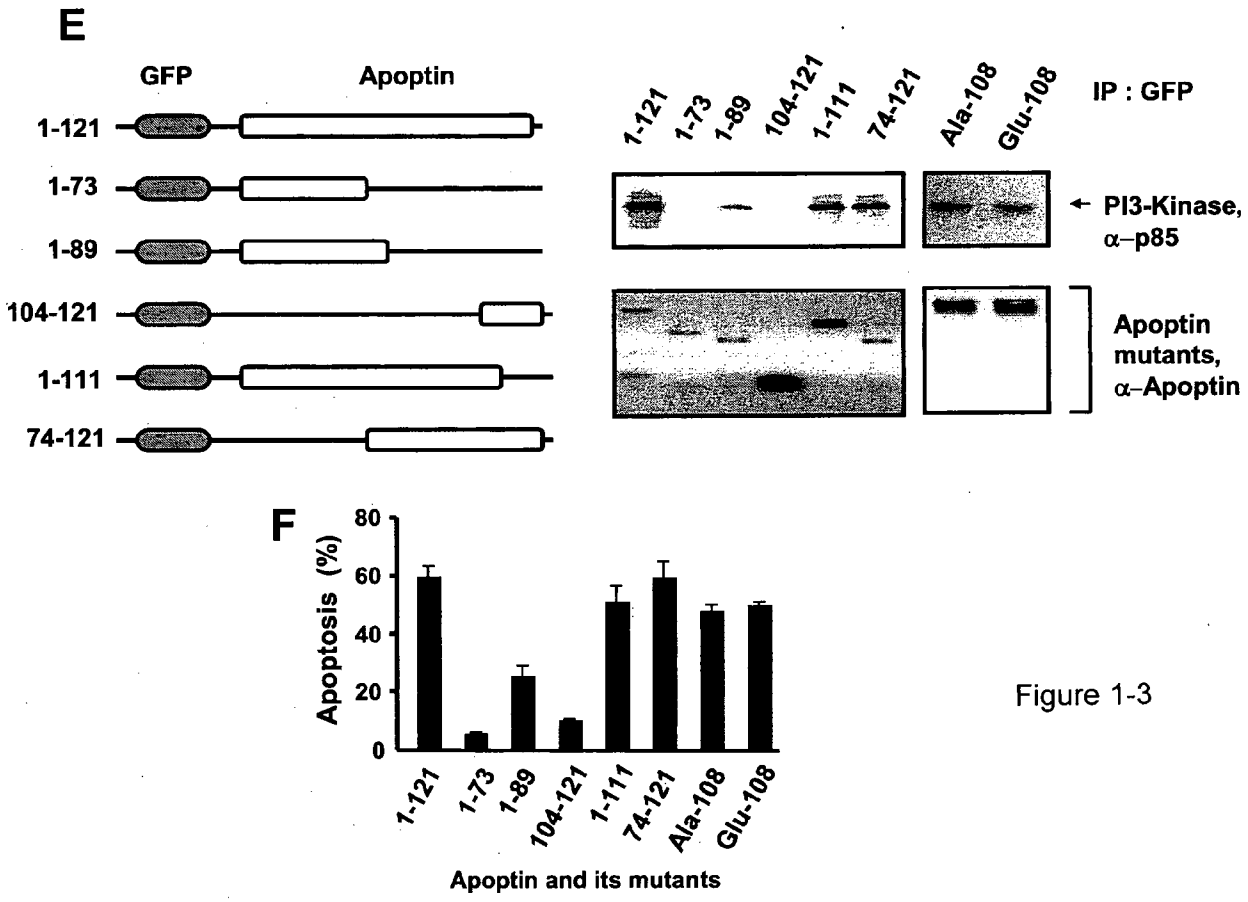

E
GFP
Apoptin
1-121
1-73
1-89
104-121
1-111
74-121
1-121
1-73
1-89
104-121
1-111
74-121
Ala-108
Glu-108
IP : GFP
PI3-Kinase, α–p85
Apoptin mutants, α–Apoptin
F
Apoptosis (%)
0
20
40
60
80
1-121
1-73
1-89
104-121
1-111
74-121
Ala-108
Glu-108
Apoptin and its mutants

Figure 1-3

G

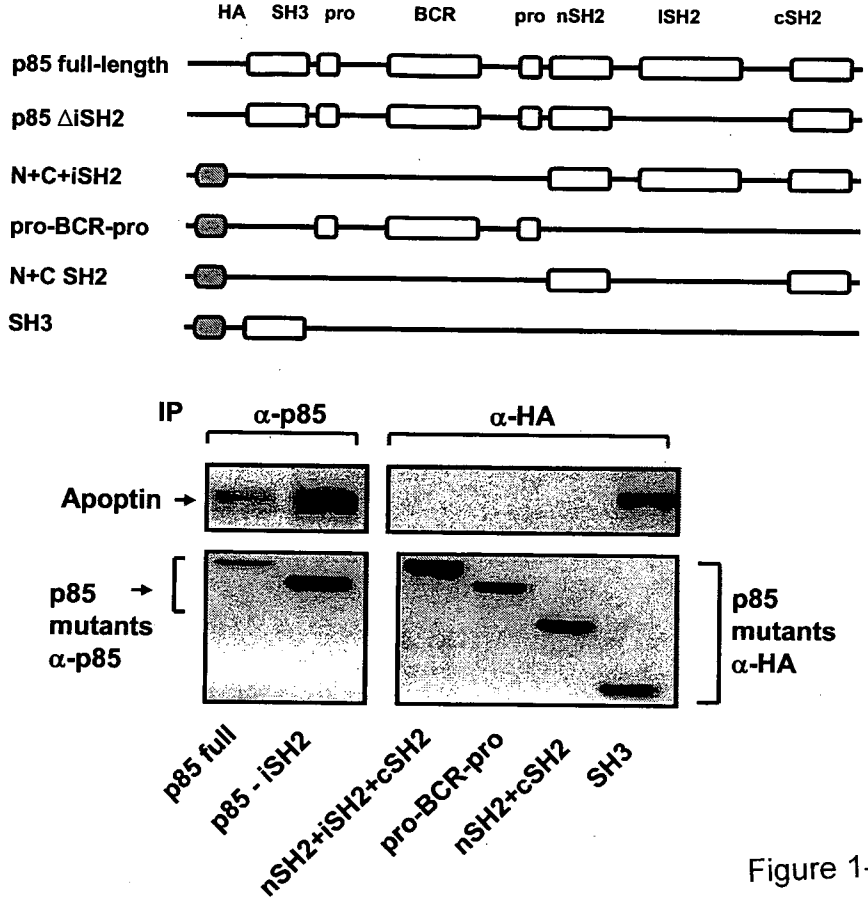

HA
SH3
pro
BCR
pro
nSH2
ISH2
cSH2
p85 full-length
p85 ΔiSH2
N+C+iSH2
pro-BCR-pro
N+C SH2
SH3
IP
α-p85
α-HA
Apoptin
p85 mutants α-p85
p85 mutants α-HA
p85 full
p85 - iSH2
nSH2+iSH2+cSH2
pro-BCR-pro
nSH2+cSH2
SH3

Figure 1-4

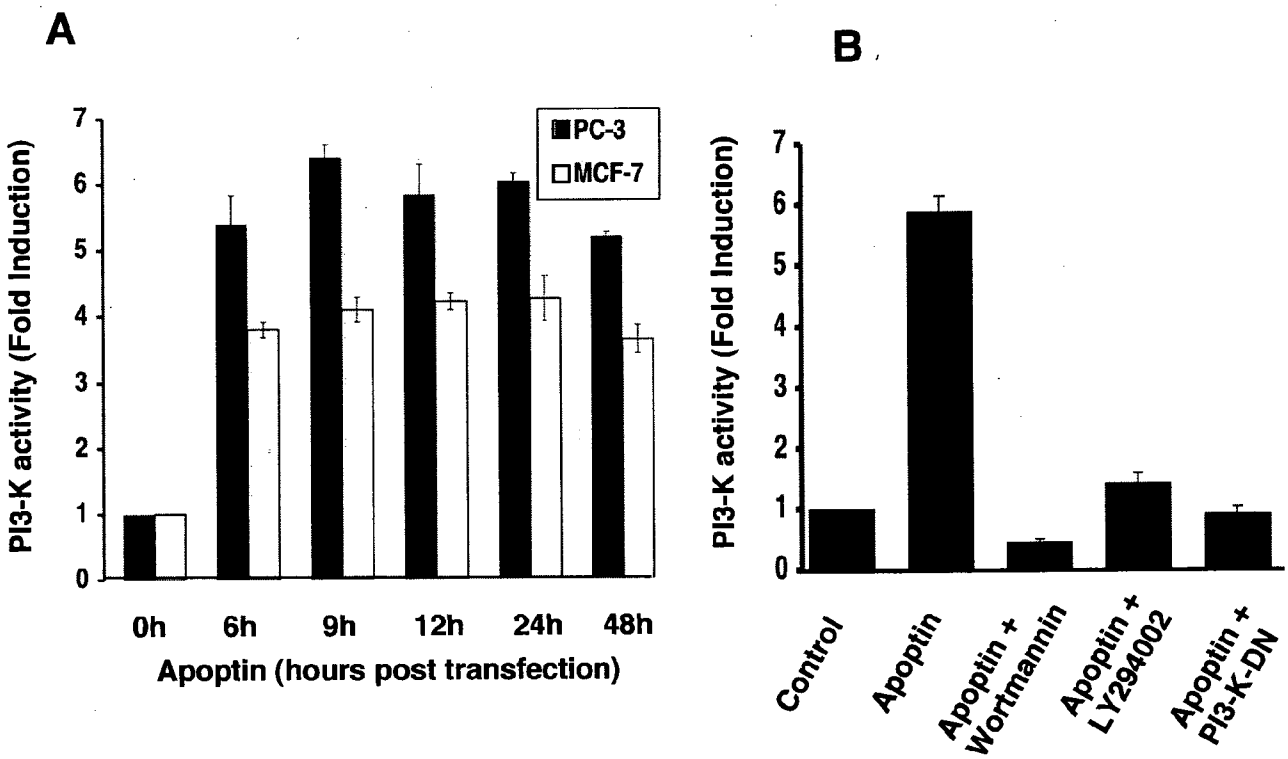

A
PI3-K activity (Fold Induction)
PC-3
MCF-7
0h
6h
9h
12h
24h
48h
Apoptin (hours post transfection)
B
PI3-K activity (Fold Induction)
Control
Apoptin
Apoptin + Wortmannin
Apoptin + LY294002
Apoptin + PI3-K-DN

Figure 2-1

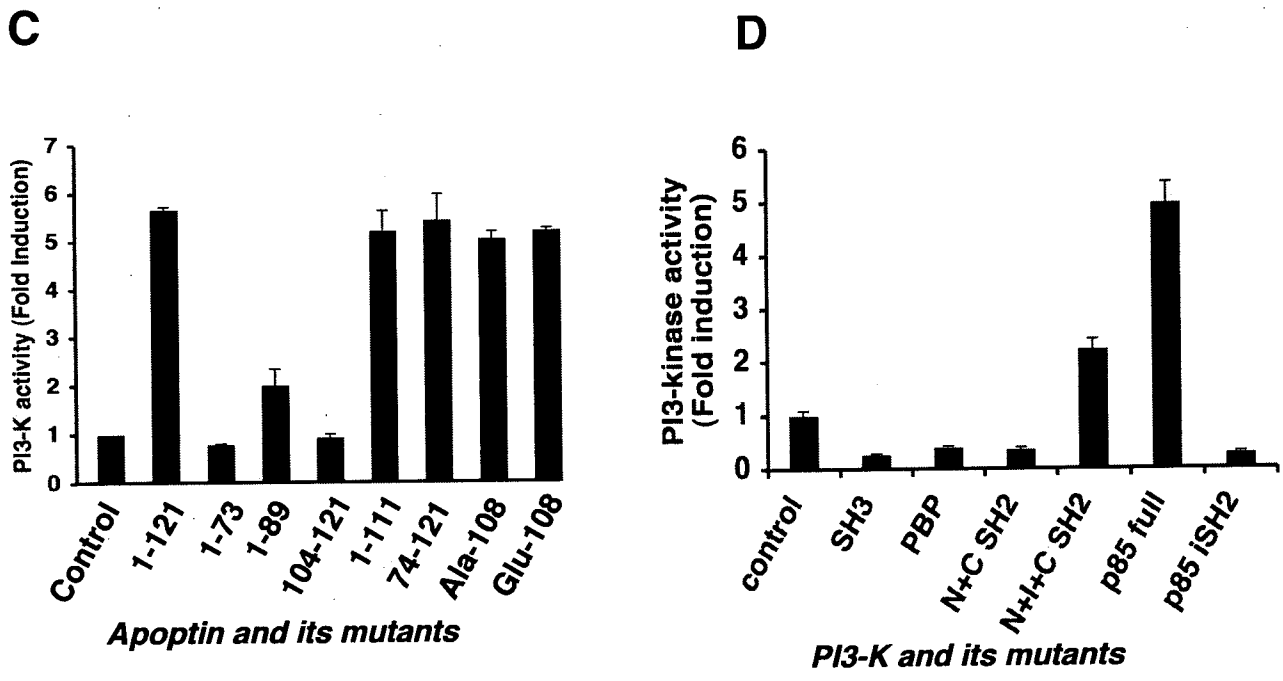

C
PI3-K activity (Fold Induction)
0
1
2
3
4
5
6
7
Control
1-121
1-73
1-89
104-121
1-111
74-121
Ala-108
Glu-108
Apoptin and its mutants
D
PI3-kinase activity (Fold induction)
0
1
2
3
4
5
6
control
SH3
PBP
N+C SH2
N+I+C SH2
p85 full
p85 iSH2
PI3-K and its mutants

Figure 2-2

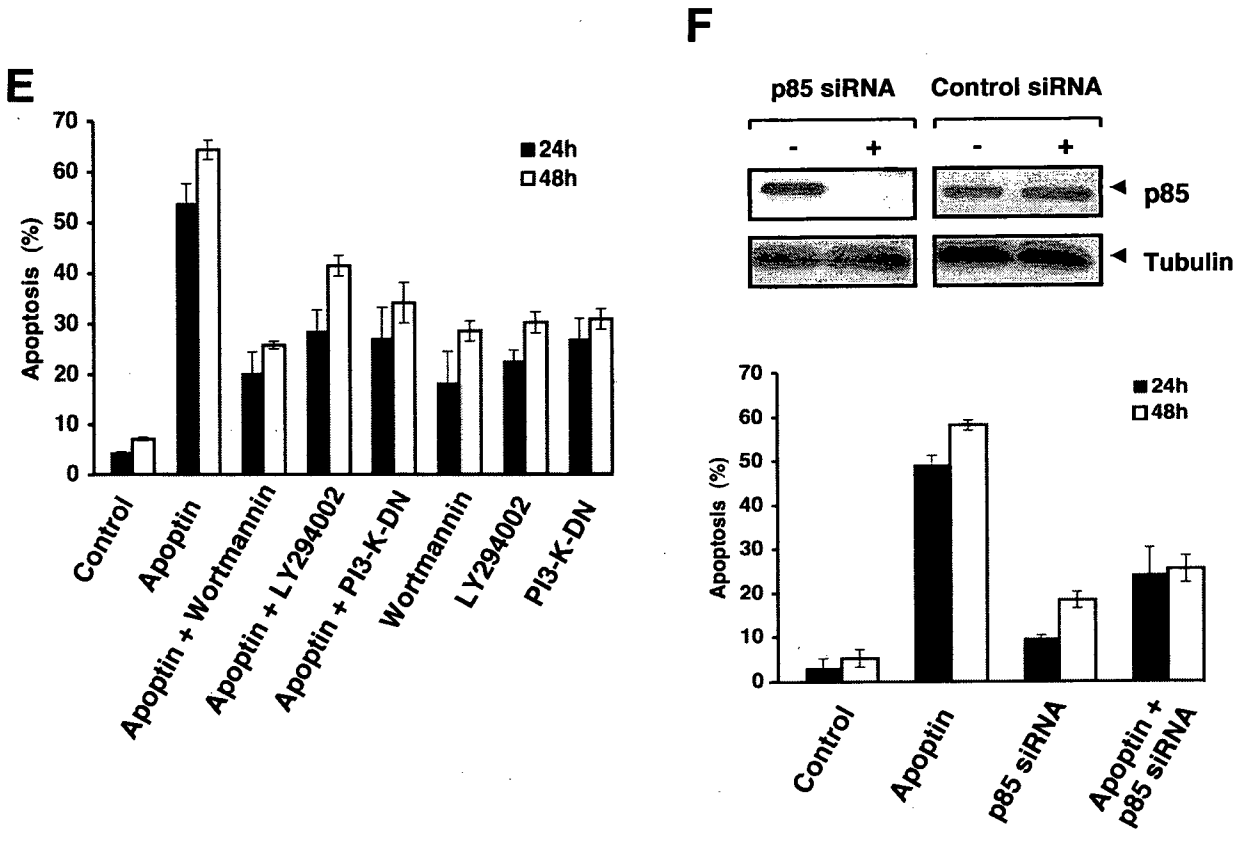

E
Apoptosis (%)
70
60
50
40
30
20
10
0
24h
48h
Control
Apoptin
Apoptin + Wortmannin
Apoptin + LY294002
Apoptin + PI3-K-DN
Wortmannin
LY294002
PI3-K-DN
F
p85 siRNA
Control siRNA
-
+
-
+
p85
Tubulin
Apoptosis (%)
70
60
50
40
30
20
10
0
24h
48h
Control
Apoptin
p85 siRNA
Apoptin + p85 siRNA

Figure 2-3

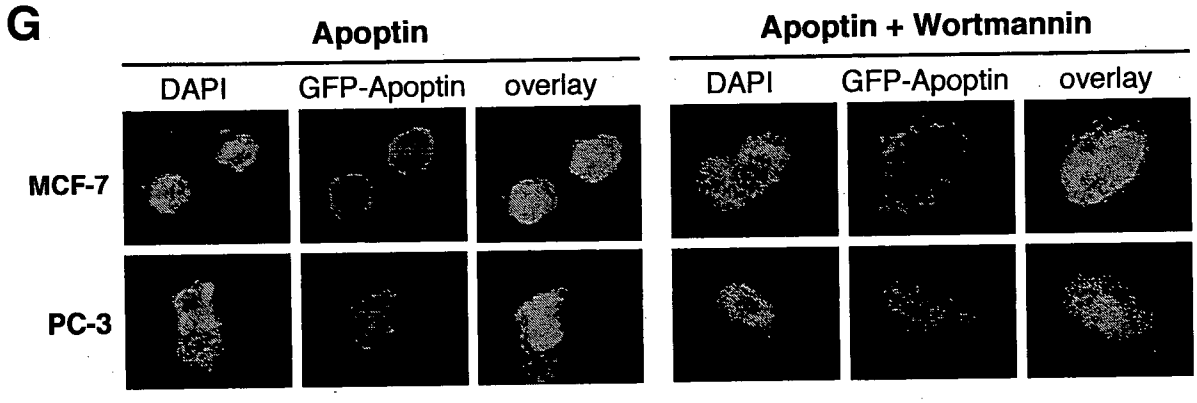

G
Apoptin
Apoptin + Wortmannin
DAPI
GFP-Apoptin
overlay
DAPI
GFP-Apoptin
overlay
MCF-7
PC-3

Figure 2-4

H

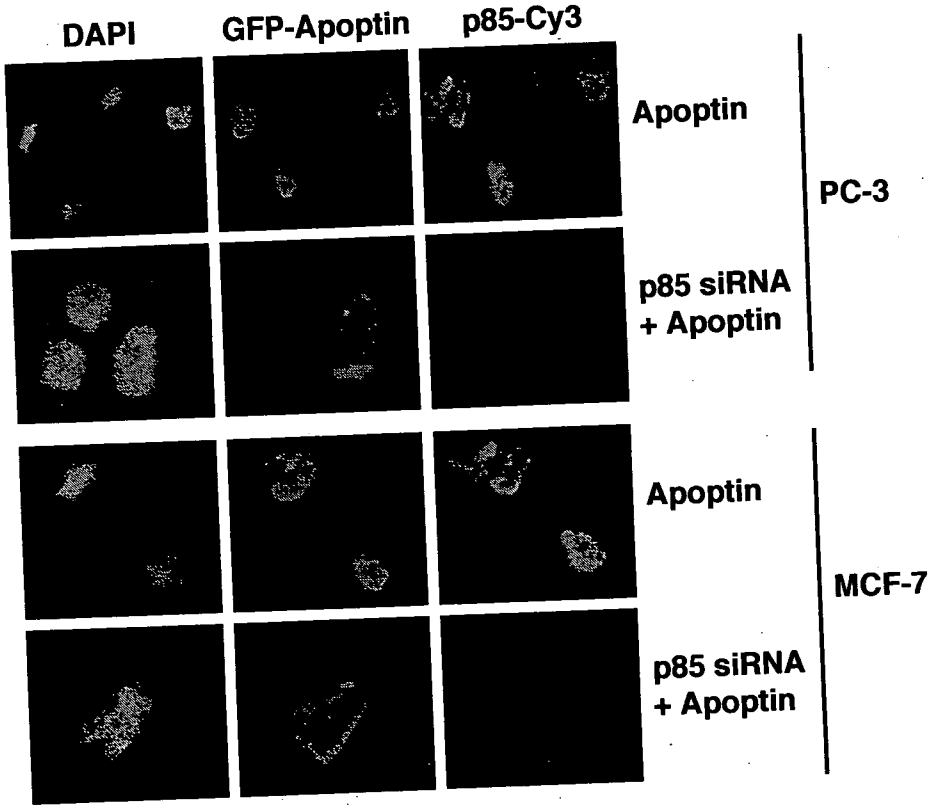

DAPI
GFP-Apoptin
p85-Cy3
Apoptin
PC-3
p85 siRNA
+ Apoptin
Apoptin
MCF-7
p85 siRNA
+ Apoptin

Figure 2-5

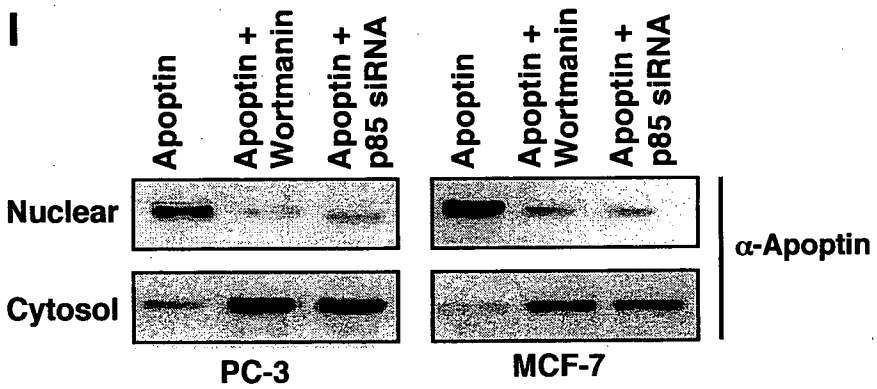

Apoptin
Apoptin + Wortmanin
Apoptin + p85 siRNA
Apoptin
Apoptin + Wortmanin
Apoptin + p85 siRNA
Nuclear
Cytosol
α-Apoptin
PC-3
MCF-7

Figure 2-6

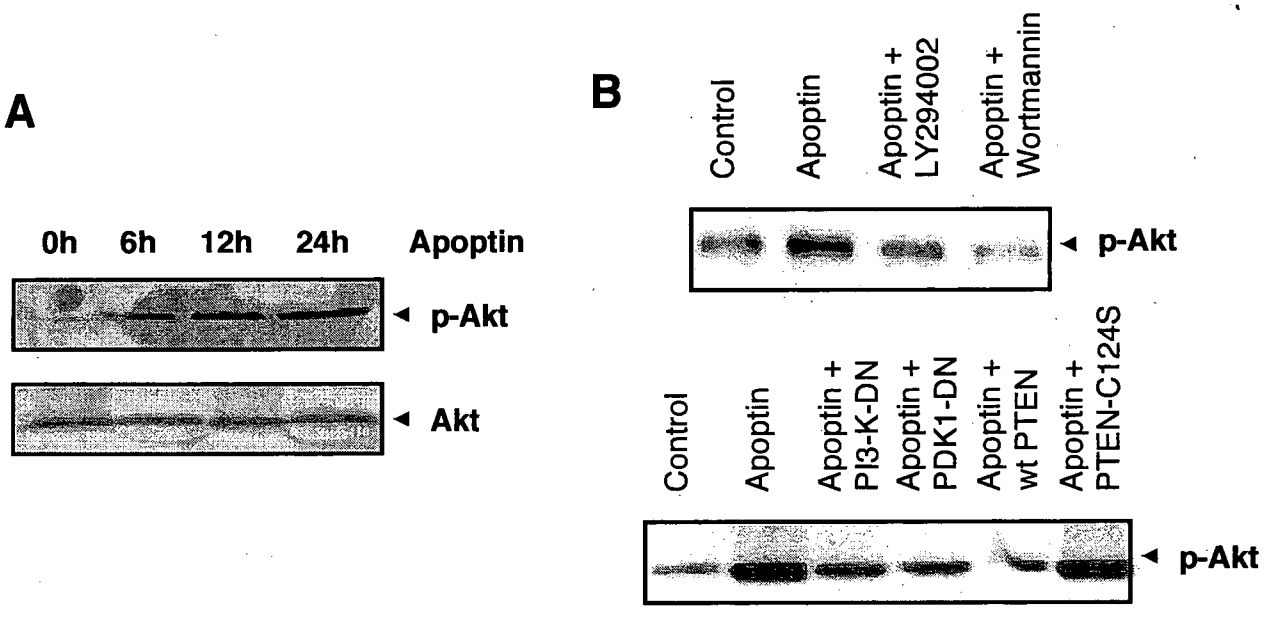
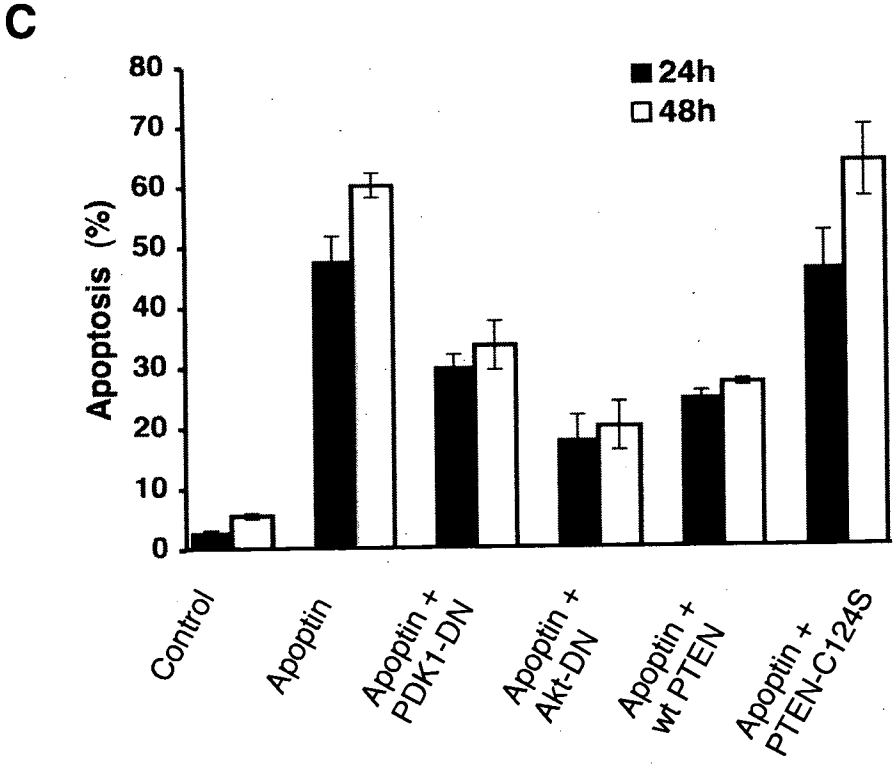

A
0h
6h
12h
24h
Apoptin
p-Akt
Akt
B
Control
Apoptin
Apoptin + LY294002
Apoptin + Wortmannin
p-Akt
Control
Apoptin
Apoptin + PI3-K-DN
Apoptin + PDK1-DN
Apoptin + wt PTEN
Apoptin + PTEN-C124S
p-Akt

Figure 3-1


C
24h
48h
Apoptosis (%)
80
70
60
50
40
30
20
10
0
Control
Apoptin
Apoptin + PDK1-DN
Apoptin + Akt-DN
Apoptin + wt PTEN
Apoptin + PTEN-C124S

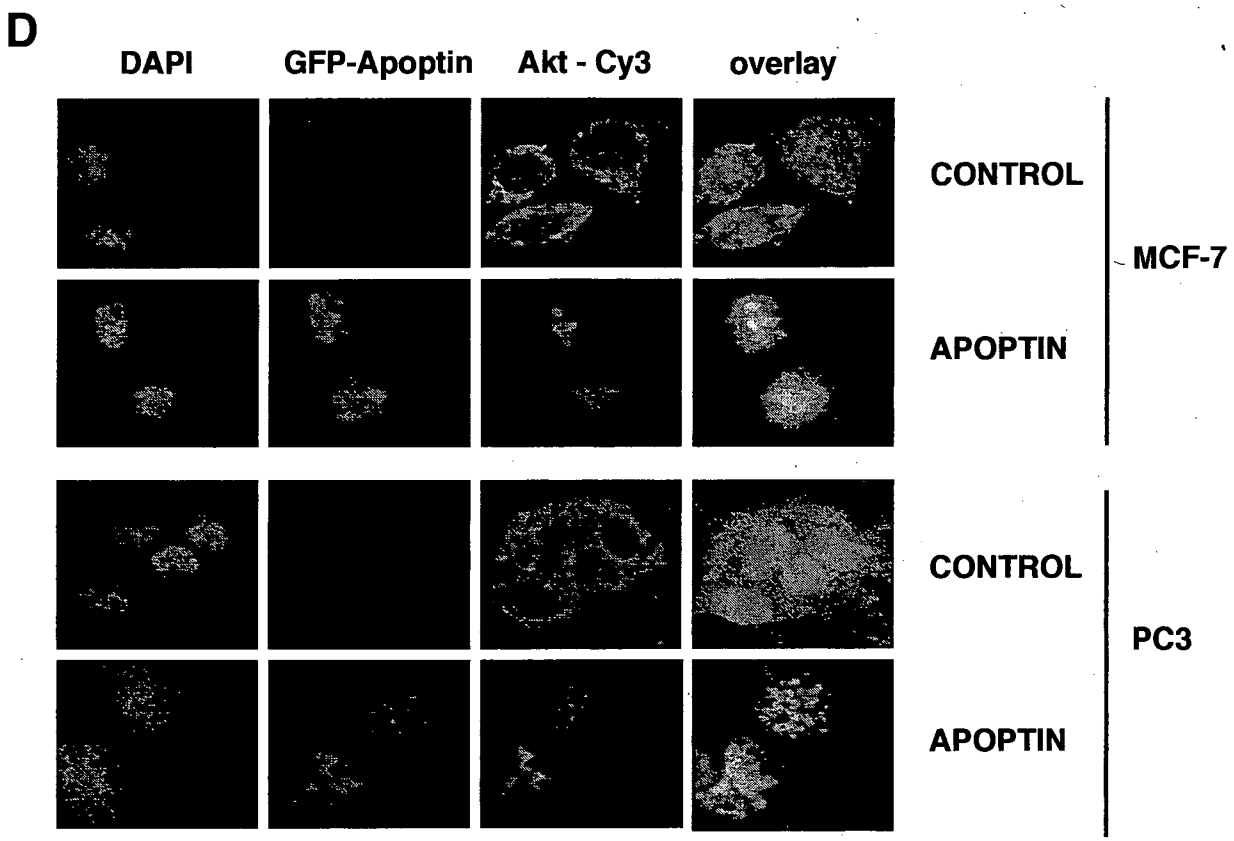
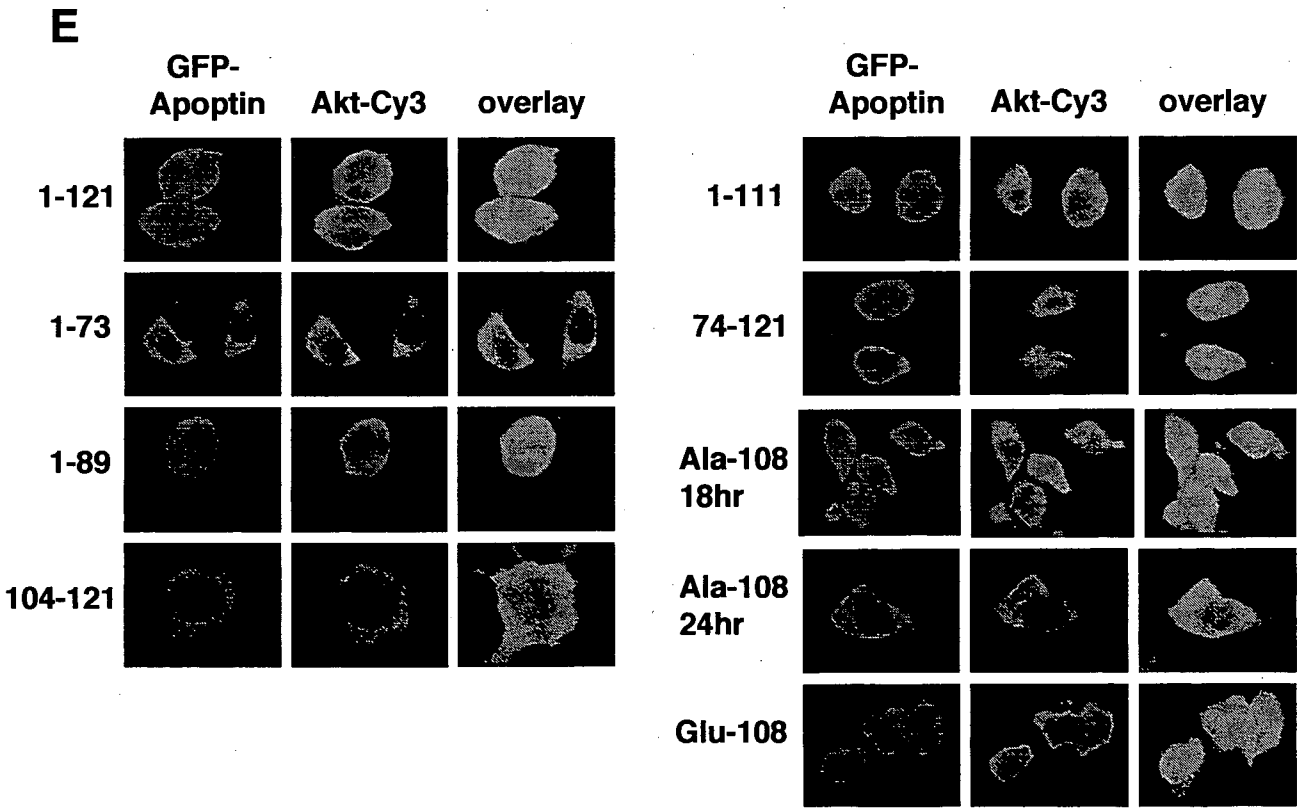

D
DAPI
GFP-Apoptin
Akt - Cy3
overlay
CONTROL
APOPTIN
MCF-7
CONTROL
APOPTIN
PC3

Figure 3-2


E
GFP-Apoptin
Akt-Cy3
overlay
1-121
1-73
1-89
104-121
GFP-Apoptin
Akt-Cy3
overlay
1-111
74-121
Ala-108 18hr
Ala-108 24hr
Glu-108

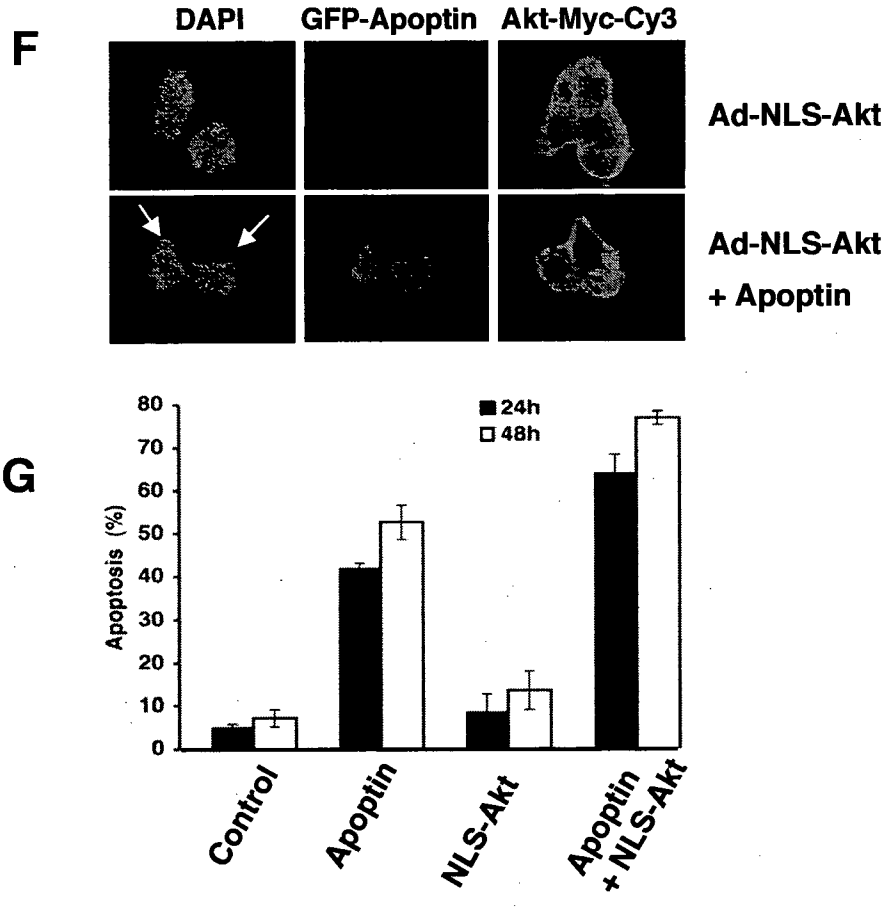

F
DAPI
GFP-Apoptin
Akt-Myc-Cy3
Ad-NLS-Akt
Ad-NLS-Akt
+ Apoptin
G
24h
48h
Apoptosis (%)
80
70
60
50
40
30
20
10
0
Control
Apoptin
NLS-Akt
Apoptin
+ NLS-Akt

Figure 3-3

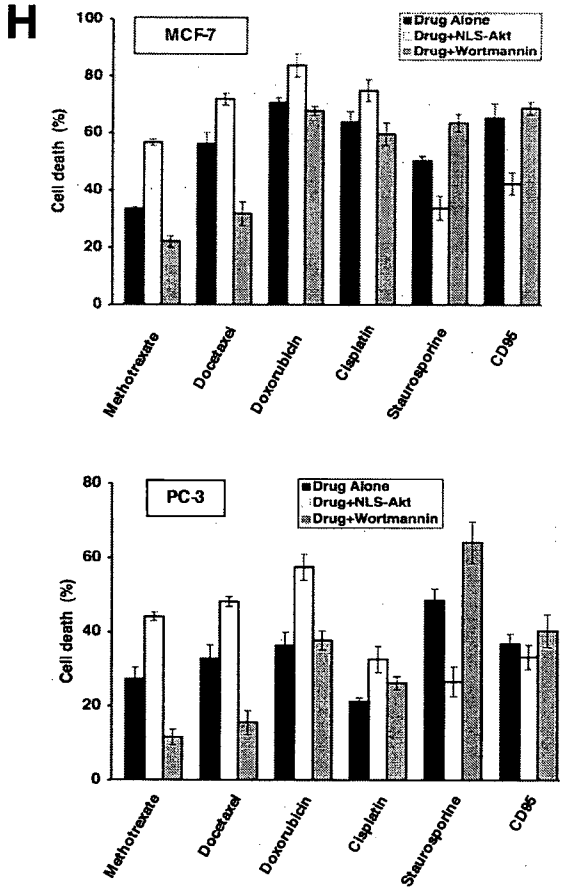

H
MCF-7
Drug Alone
Drug+NLS-Akt
Drug+Wortmannin
Cell death (%)
100
80
60
40
20
0
Methotrexate
Docetaxel
Doxorubicin
Cisplatin
Staurosporine
CD95
PC-3
Drug Alone
Drug+NLS-Akt
Drug+Wortmannin
Cell death (%)
80
60
40
20
0
Methotrexate
Docetaxel
Doxorubicin
Cisplatin
Staurosporine
CD95

Figure 3-4

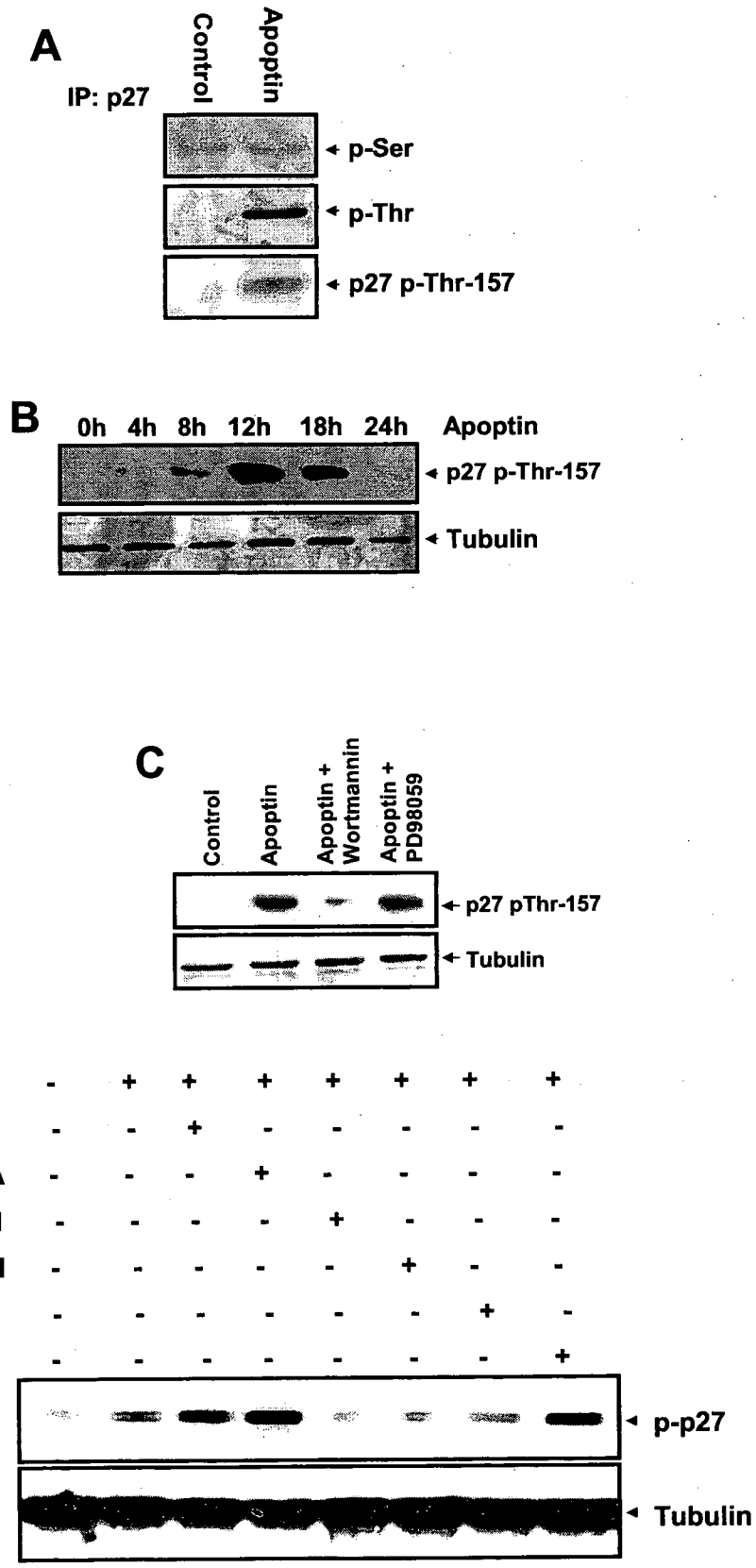
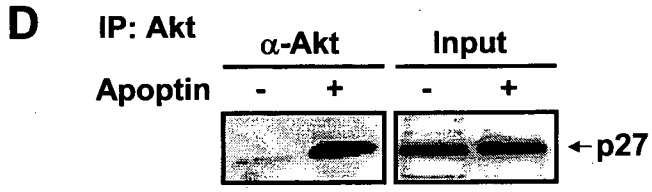
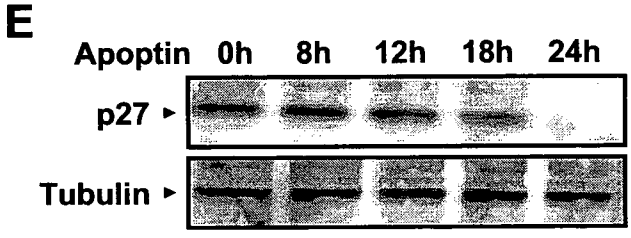

A
IP: p27
Control
Apoptin
p-Ser
p-Thr
p27 p-Thr-157
B
0h 4h 8h 12h 18h 24h
Apoptin
p27 p-Thr-157
Tubulin
C
Control
Apoptin
Apoptin + Wortmannin
Apoptin + PD98059
p27 pThr-157
Tubulin
Apoptin - + + + + + + +
PI3-K wt - - + - - - - -
PDK1 CA - - - + - - - -
PI3-K-DN - - - - + - - -
PDK1-DN - - - - - + - -
Akt-DN - - - - - - + -
PKC-DN - - - - - - - +
p-p27
Tubulin

Figure 4-1


D
IP: Akt
α-Akt
Input
Apoptin
-
+
-
+
←p27


E
Apoptin 0h 8h 12h 18h 24h
p27 ▸
Tubulin ▸

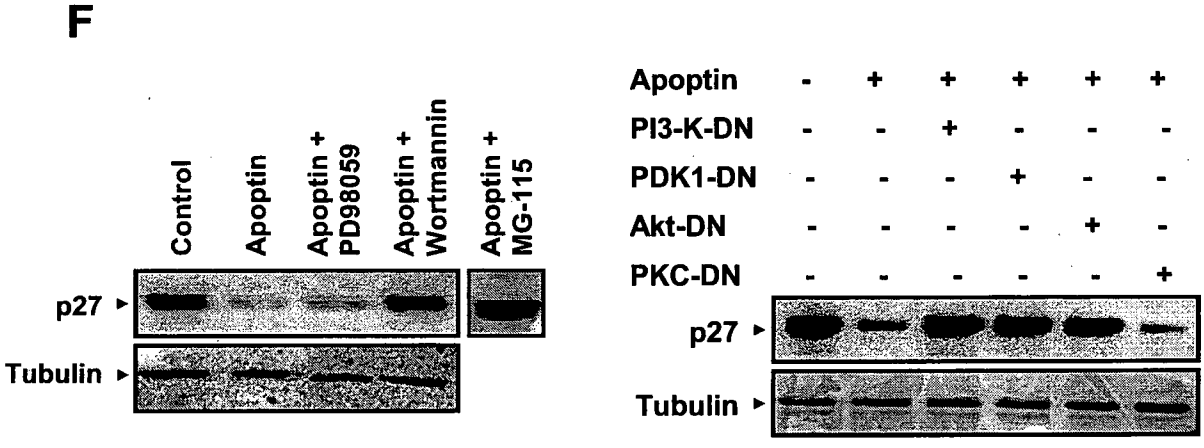

F
Control
Apoptin
Apoptin + PD98059
Apoptin + Wortmannin
Apoptin + MG-115
p27 ▸
Tubulin ▸
Apoptin - + + + + +
PI3-K-DN - - + - - -
PDK1-DN - - - + - -
Akt-DN - - - - + -
PKC-DN - - - - - +
p27 ▸
Tubulin ▸

Figure 4-2

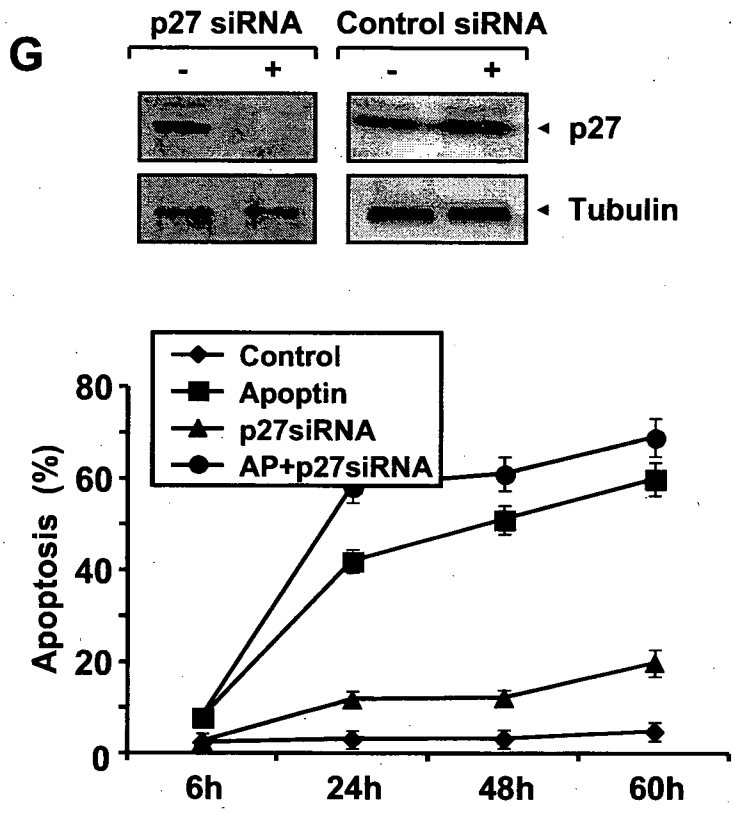

G
p27 siRNA
Control siRNA
-
+
-
+
p27
Tubulin
Control
Apoptin
p27siRNA
AP+p27siRNA
Apoptosis (%)
80
60
40
20
0
6h
24h
48h
60h

Figure 4-3

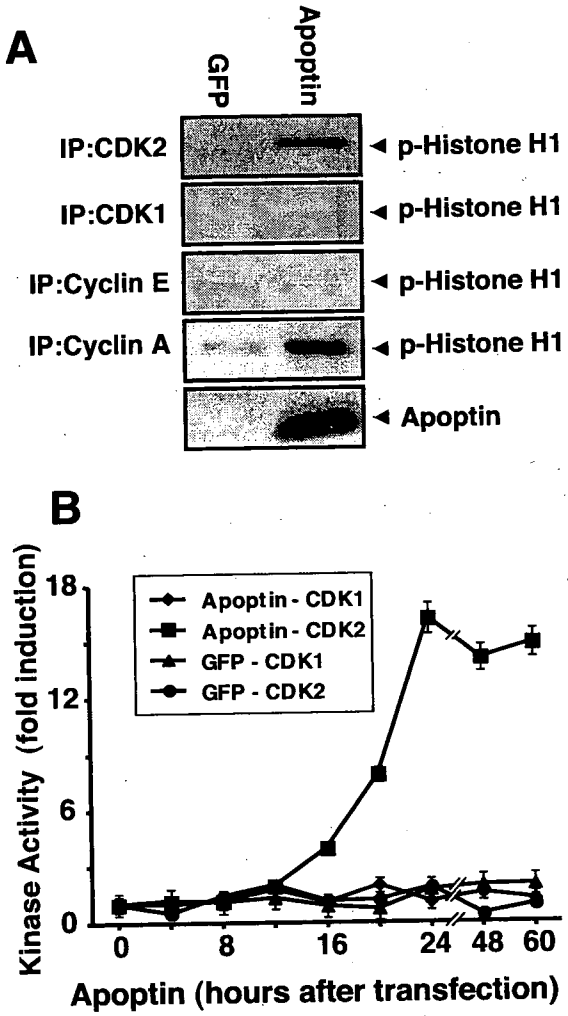
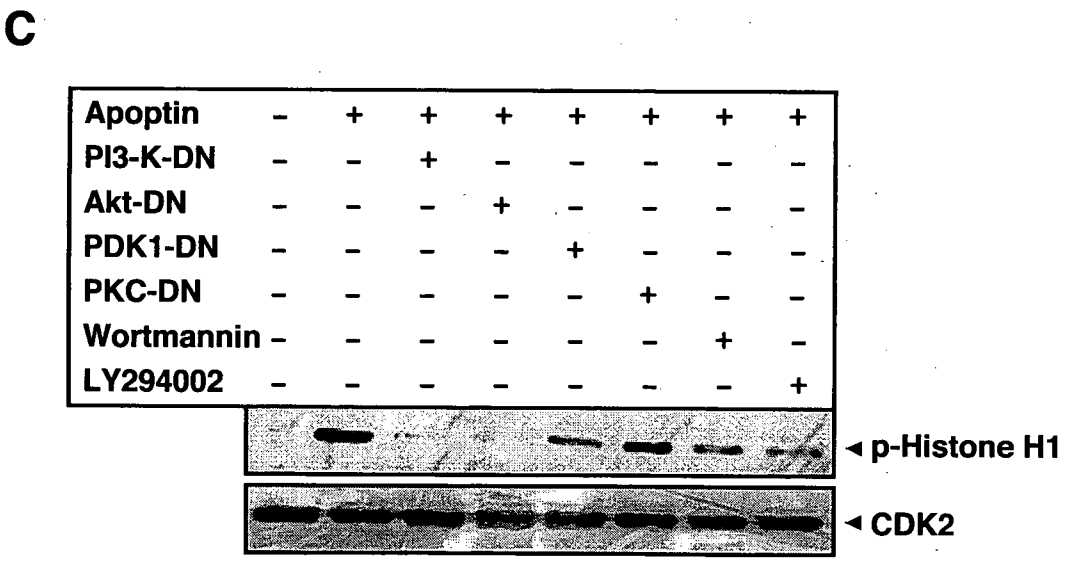
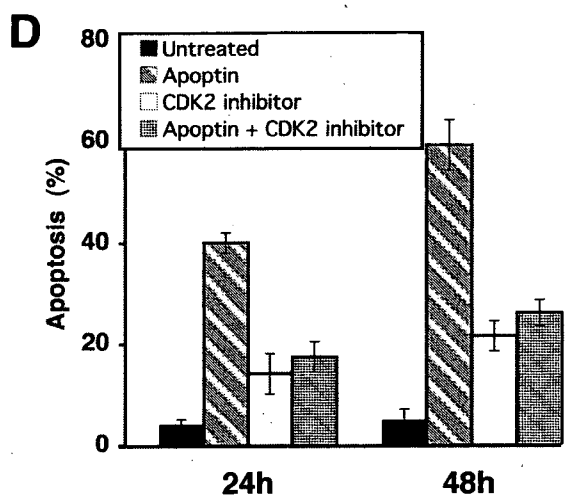

A
GFP
Apoptin
IP:CDK2
IP:CDK1
IP:Cyclin E
IP:Cyclin A
p-Histone H1
p-Histone H1
p-Histone H1
p-Histone H1
Apoptin
B
Kinase Activity (fold induction)
18
12
6
0
Apoptin - CDK1
Apoptin - CDK2
GFP - CDK1
GFP - CDK2
0
8
16
24
48
60
Apoptin (hours after transfection)

Figure 5-1


C
Apoptin - + + + + + + +
PI3-K-DN - - + - - - - -
Akt-DN - - - + - - - -
PDK1-DN - - - - + - - -
PKC-DN - - - - - + - -
Wortmannin - - - - - - + -
LY294002 - - - - - - - +
p-Histone H1
CDK2


D
Untreated
Apoptin
CDK2 inhibitor
Apoptin + CDK2 inhibitor
Apoptosis (%)
0
20
40
60
80
24h
48h

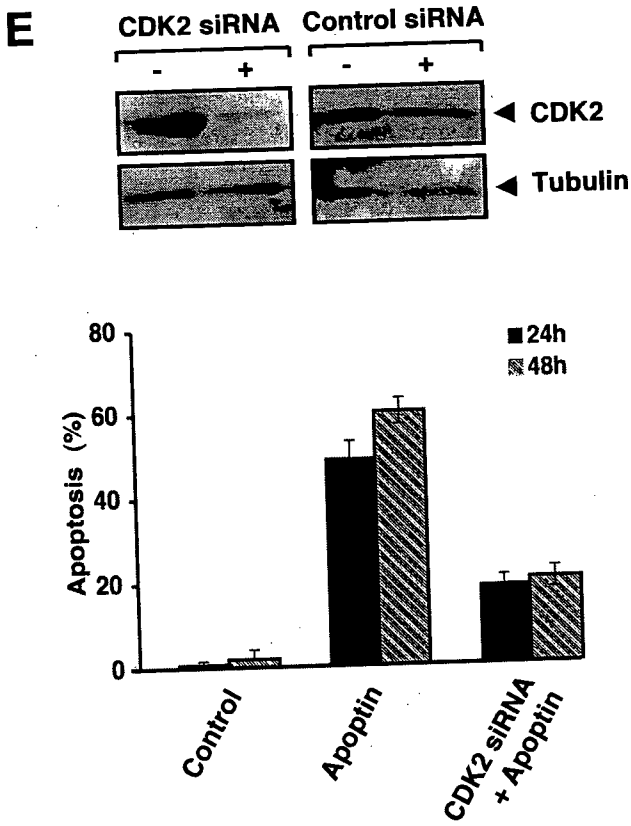

E
CDK2 siRNA
Control siRNA
-
+
-
+
CDK2
Tubulin
24h
48h
Apoptosis (%)
0
20
40
60
80
Control
Apoptin
CDK2 siRNA + Apoptin

Figure 5-2

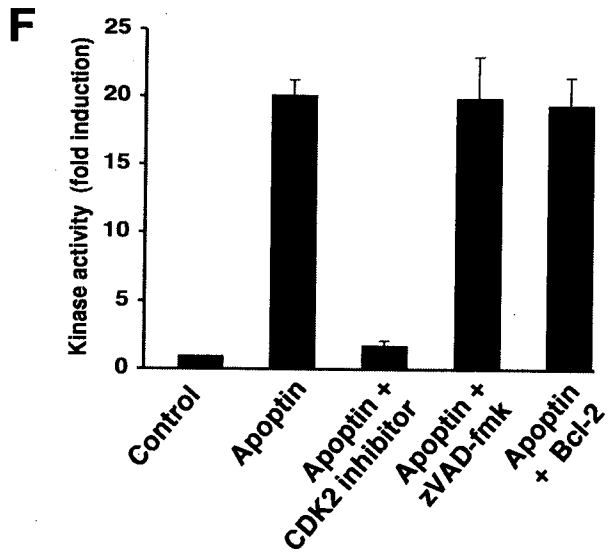
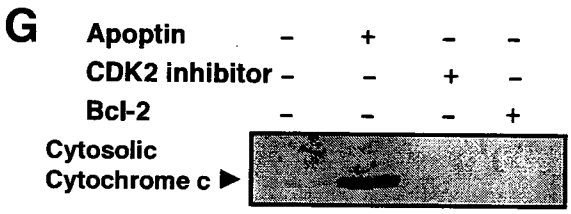
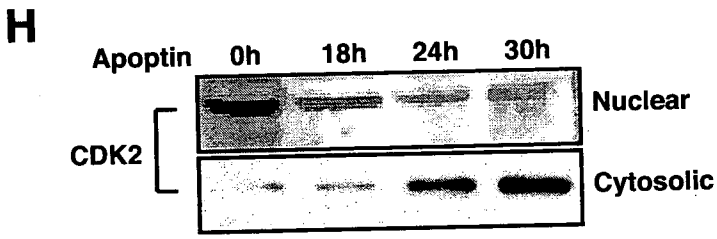
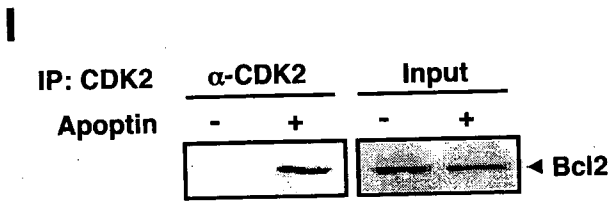

F
Kinase activity (fold induction)
25
20
15
10
5
0
Control
Apoptin
Apoptin + CDK2 inhibitor
Apoptin + zVAD-fmk
Apoptin + Bcl-2

Figure 5-3


G
Apoptin - + - -
CDK2 inhibitor - - + -
Bcl-2 - - - +
Cytosolic Cytochrome c


H
Apoptin 0h 18h 24h 30h
CDK2
Nuclear
Cytosolic


I
IP: CDK2
α-CDK2
Input
Apoptin
- + - +
Bcl2

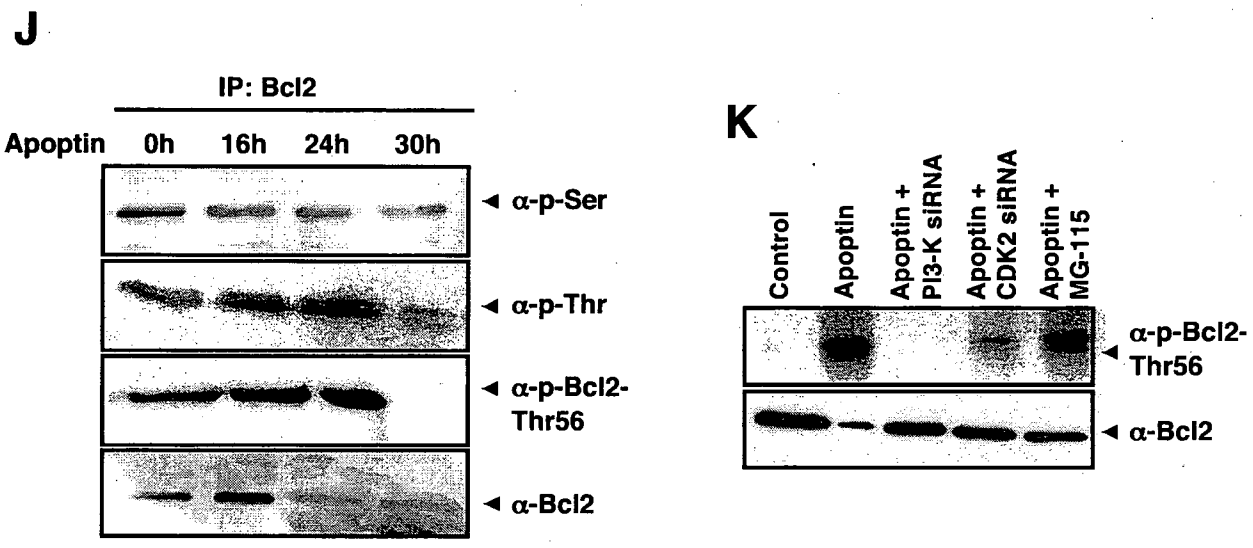

J
IP: Bcl2
Apoptin
0h
16h
24h
30h
α-p-Ser
α-p-Thr
α-p-Bcl2-Thr56
α-Bcl2
K
Control
Apoptin
Apoptin + PI3-K siRNA
Apoptin + CDK2 siRNA
Apoptin + MG-115
α-p-Bcl2-Thr56
α-Bcl2

Figure 5-4

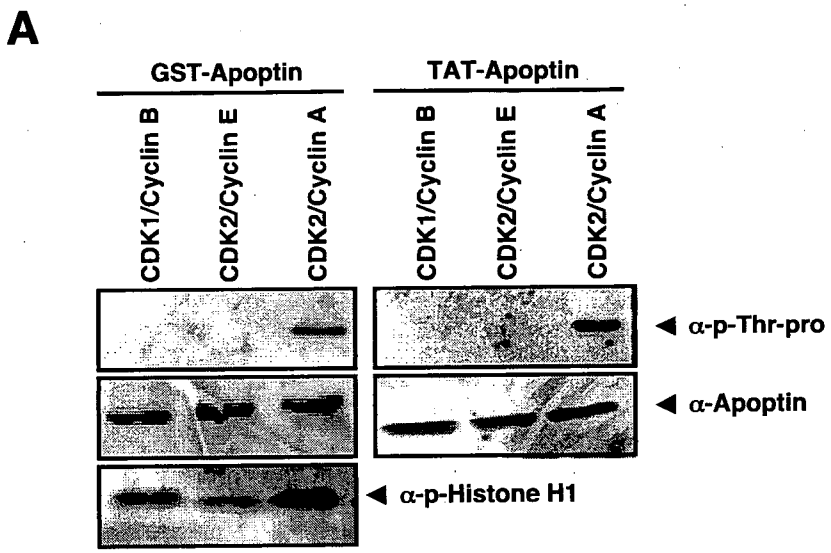
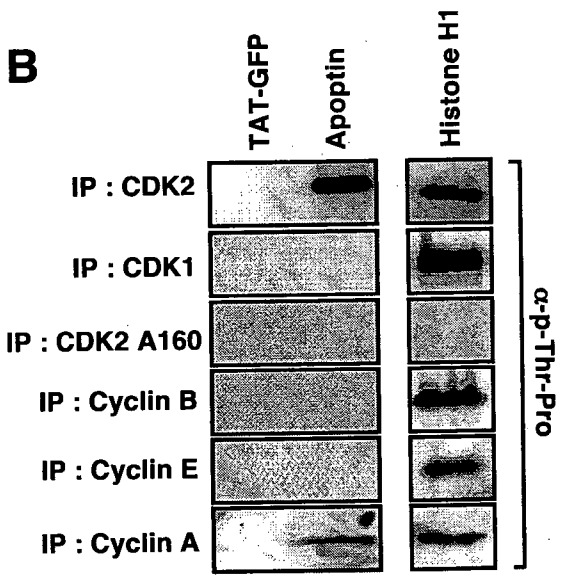
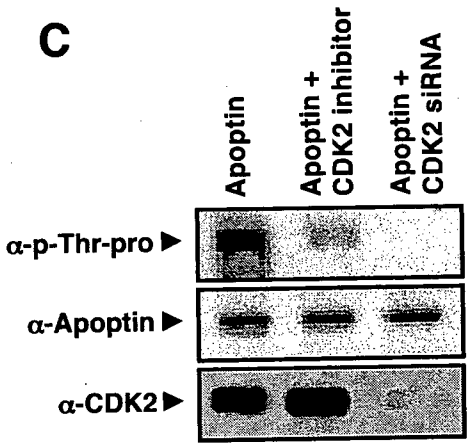

A
GST-Apoptin
TAT-Apoptin
CDK1/Cyclin B
CDK2/Cyclin E
CDK2/Cyclin A
CDK1/Cyclin B
CDK2/Cyclin E
CDK2/Cyclin A
α-p-Thr-pro
α-Apoptin
α-p-Histone H1

Figure 6-1


B
TAT-GFP
Apoptin
Histone H1
IP : CDK2
IP : CDK1
IP : CDK2 A160
IP : Cyclin B
IP : Cyclin E
IP : Cyclin A
α-p-Thr-pro


C
Apoptin
Apoptin + CDK2 inhibitor
Apoptin + CDK2 siRNA
α-p-Thr-pro
α-Apoptin
α-CDK2

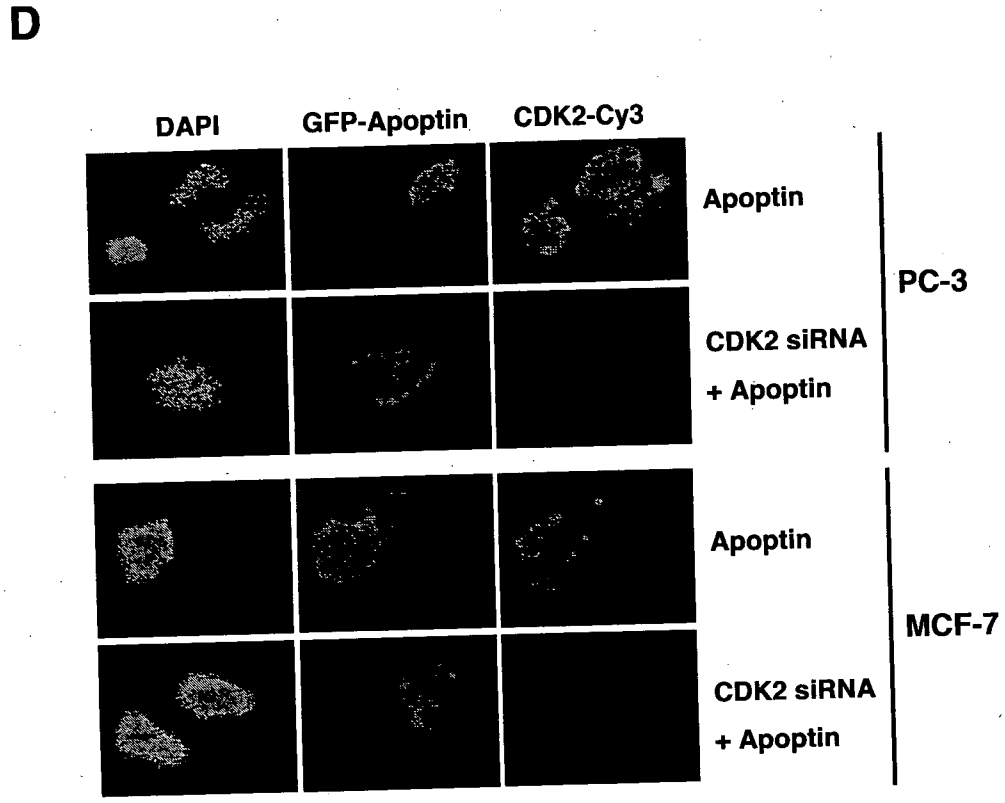
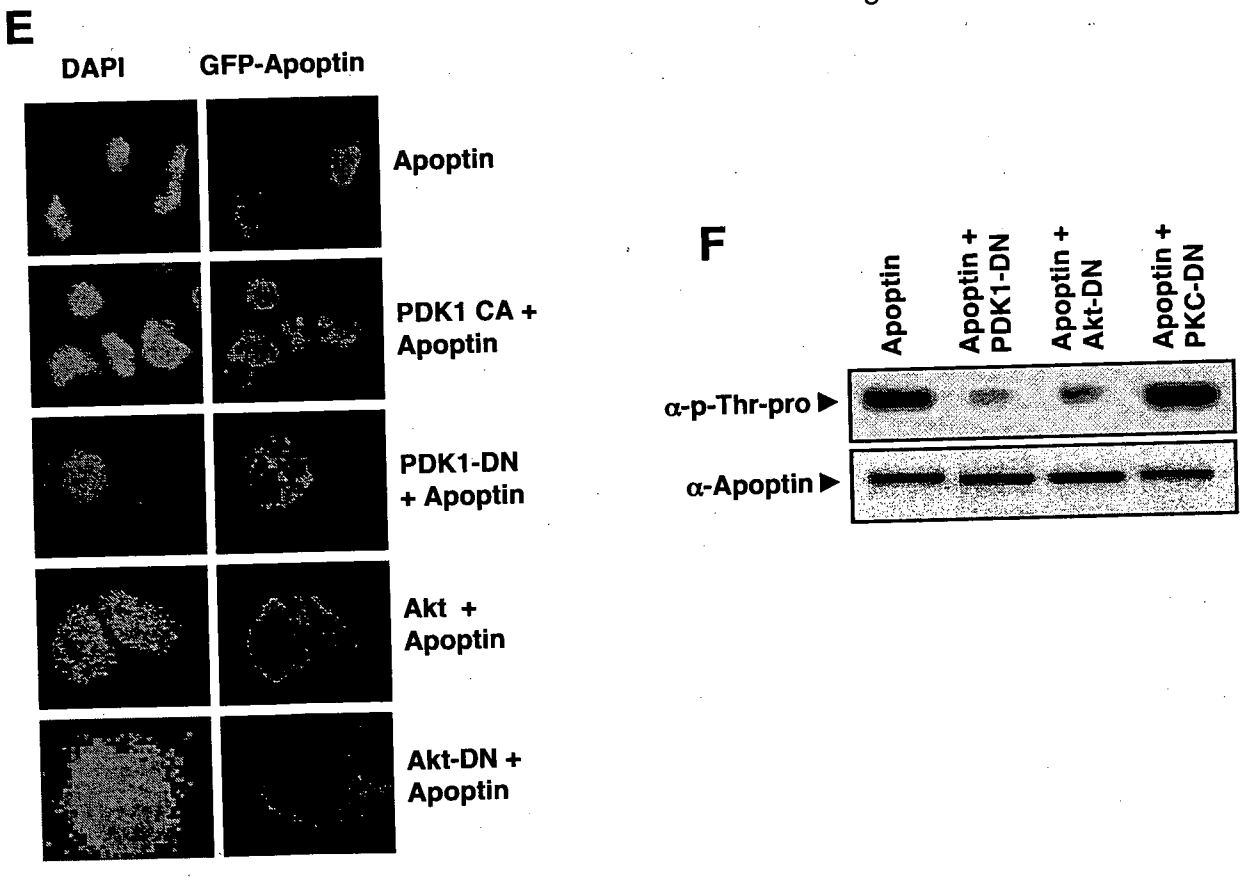

D
DAPI
GFP-Apoptin
CDK2-Cy3
Apoptin
CDK2 siRNA
+ Apoptin
PC-3
Apoptin
CDK2 siRNA
+ Apoptin
MCF-7

Figure 6-2


E
DAPI
GFP-Apoptin
Apoptin
PDK1 CA +
Apoptin
PDK1-DN
+ Apoptin
Akt +
Apoptin
Akt-DN +
Apoptin
F
Apoptin
Apoptin +
PDK1-DN
Apoptin +
Akt-DN
Apoptin +
PKC-DN
α-p-Thr-pro
α-Apoptin

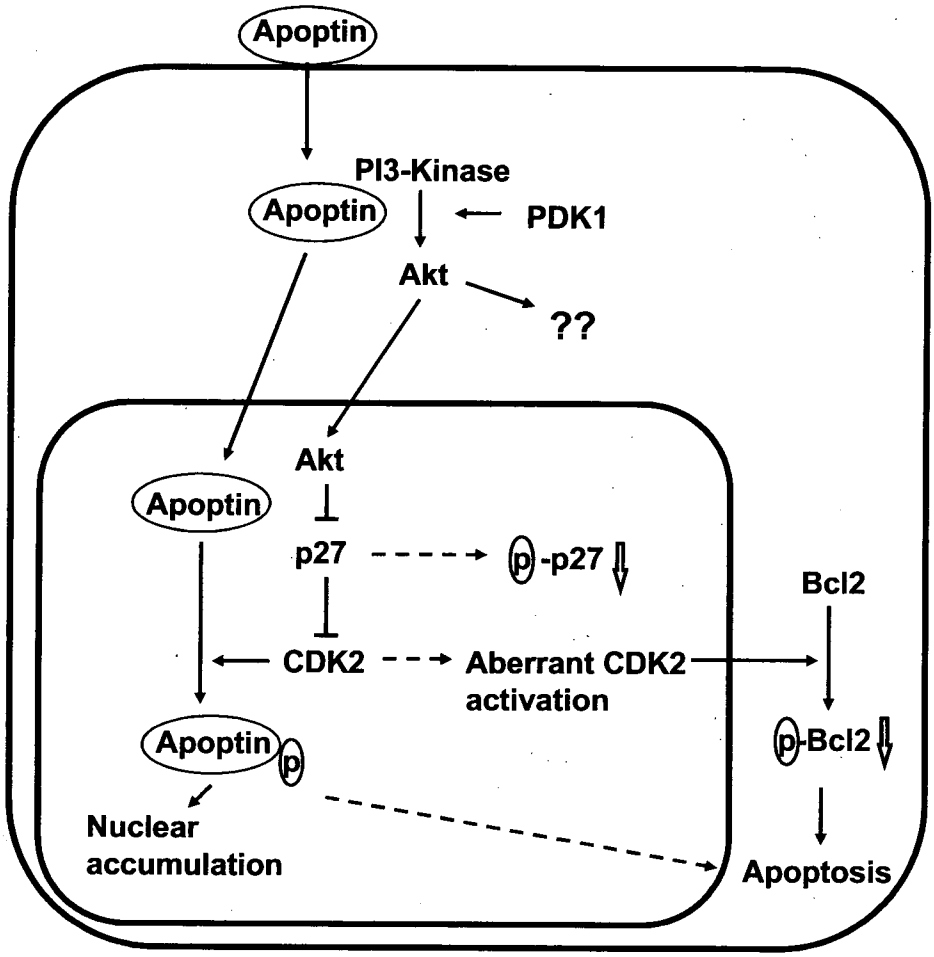

Apoptin
PI3-Kinase
Apoptin
PDK1
Akt
??
Akt
Apoptin
p27
p-p27
Bcl2
CDK2
Aberrant CDK2 activation
Apoptin p
p-Bcl2
Nuclear accumulation
Apoptosis

Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000681

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 14/01*** (2006.01) , ***A61K 38/08*** (2006.01) , ***A61K 38/16*** (2006.01) , ***A61P 35/00*** (2006.01) , ***C07K 7/06*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 14/01 (2006.01) , ***A61K 38/08*** (2006.01) , ***A61K 38/16*** (2006.01) , ***A61P 35/00*** (2006.01) , ***C07K 7/06*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
GenomeQuestLive, Pubmed, Delphion, Canadian Patent Database, Espacenet, Scopus, USPTO, Keywords: apoptin, apoptosis, tumour, cancer, peptide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US2006/0057652 A1 (GREEN, M. et al.), 16 March 2006 whole document	1-21
X	WO03089467 A1 (NOTEBORN, M. H. M. and DANEN-VAN OORSCHOT, A. A. A. M.), 30 October 2003 whole document	1-21
X	WO0232954 A2 (NOTEBORN, M. H. M. et al.), 25 April 2002 whole document	1-21

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2007 (14-06-2007)	6 August 2007 (06-08-2007)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Yolanda Romsicki 819- 997-1044

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DANEN-VAN OORSCHOT, A. A. A. M. et al. "The chicken anemia virus-derived protein apoptin requires activation of caspases for induction of apoptosis in human tumor cells", Journal of Virology, August 2000 Vol. 74, pages 7072-7078 ISSN: 0022-538X	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/000681

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 1-7

because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 1-7 encompass methods of treatment of a human or animal which this Authority is not obliged to search under Rule 39.1(iv) of the PCT, the search has been carried out based on the alleged purpose/use of the peptides referred to therein.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2007/000681

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US2006057652 A1	16-03-2006	NONE	
WO03089467 A1	30-10-2003	AU2003214721 A1	03-11-2003
WO0232954 A2	25-04-2002	AU1283502 A	29-04-2002
		EP1199363 A1	24-04-2002
		EP1373514 A2	02-01-2004
		US2003199009 A1	23-10-2003