The 14-3-3 proteins are a family of highly conserved eukaryotic regulatory molecules that play important roles in many biological processes including cell cycle control and regulation of cell death. They are able to carry out these effects through binding and modulating the activity of a host of signaling proteins. The ability of 14-3-3 to inhibit Bad and other proapoptotic proteins argues that 14-3-3 can support cell survival. To examine this issue in a global sense, a specific inhibitor of 14-3-3/ligand interactions, difopein, was used. Difopein expression led to induction of apoptosis. Studies using various components of survival and death signaling pathways were consistent with a vital role for 14-3-3/ligand interactions in signal transduction from upstream pro-survival kinases to the core apoptotic machinery. Because these kinases often become activated during oncogenesis, the effect of difopein on cell death induced by antineoplastic drugs was examined. It was found that difopein enhances the ability of cisplatin to kill cells. These data support the model that 14-3-3, through binding to Bad and other ligands, is critical for cell survival signaling. Inhibition of 14-3-3 may represent a useful therapeutic target for treatment of cancer and other diseases involving inappropriate cell survival.

The 14-3-3 proteins are a family of conserved dimeric eukaryotic regulatory molecules. 14-3-3 proteins are known for their ability to bind multiple cellular protein ligands, with more than 70 binding partners identified to date. 14-3-3 binding proteins are very diverse and include kinases, phosphatases, receptors, structural proteins, and transcription factors (see Ref. 1 for a review). Most 14-3-3 ligands require phosphorylation before 14-3-3 can interact with them, and consensus phosphoserine containing 14-3-3 binding motifs have been defined (2, 3). The large number and diversity of 14-3-3 ligands has led to the suggestion that 14-3-3 is involved in many different cellular processes, including mitogenesis, cell cycle control, and apoptosis.

Although 14-3-3 has some targets, such as Bad (4), that are proximal components of the apoptotic machinery, the number of 14-3-3 ligands that feed into apoptosis signaling pathways in a more indirect manner is staggering. 14-3-3 binding proteins such as Raf-1 (5), protein kinase C (6), phosphatidylinositol 3-kinase (7), and mitogen-activated protein kinase kinase kinase 1 (8) have been shown to have significant involvement in signaling cell survival and death. Because of this, determination of the role of 14-3-3 in survival signaling demands a global approach to controlling 14-3-3/ligand interactions. The first steps in this direction have been taken, using 14-3-3 ligand binding-defective mutants. Such mutants can have a dominant negative effect on some 14-3-3-mediated processes (see Ref. 9 for an example), possibly by forming inactive heterodimers with endogenous wild type 14-3-3 proteins. It was found that mutant 14-3-3 proteins could sensitize cells to apoptosis induced by UV irradiation or serum withdrawal and that this effect was mediated by p38 mitogen-activated protein kinase (10). Such a result may be explained by the ability of 14-3-3 to inhibit the death-inducing mitogen-activated protein kinase kinase kinase homolog ASK1 (9), which is upstream of p38. Although this work provides valuable insights into the function of 14-3-3 in p38-regulated apoptosis, technical limitations related to the use of stable cell lines place restrictions on its applicability to other 14-3-3-mediated processes.

The peptide R18 is a 20-mer isolated from a phage display screen for its ability to bind 14-3-3 (11). This peptide was found to interact with 14-3-3 very specifically; however, it does not possess selectivity among the 14-3-3 isoforms. Notably, the R18/14-3-3 interaction does not require phosphorylation of R18, and R18 was one of the first documented 14-3-3 ligands to possess this trait. However, this novelty also raised the possibility that R18 bound 14-3-3 in a manner unlike natural ligands. This issue was resolved when the co-crystal structures of 14-3-3 with R18, a Raf-1-derived phosphopeptide (pS-Raf-259); Ref. 12), and a phosphopeptide taken from the 14-3-3 binding epitope of polyoma virus middle T antigen (3) were determined. The phosphate groups of pS-Raf-259 and middle T antigen were found to contact a cluster of residues, including Lys49, Arg56, Tyr128, and Arg127, in the charged side of the conserved amphipathic ligand binding groove of 14-3-3. A core motif in R18, 12WLDLE14, was found in a position similar to that of the phosphopeptides, with negatively charged Asp12 and Glu14 making contacts similar to those of phosphoserine. The hydrophobic residues in the R18 core make extensive contacts with the hydrophobic side of the amphipathic groove. Thus, although R18 lacks phosphoserine, it interacts with 14-3-3 in a manner very similar to phosphorylated ligands.

Because R18 shares a common binding site on 14-3-3 with other ligands, it is expected that R18 could competitively interfere with 14-3-3/ligand interactions. Indeed, this has been shown for several 14-3-3 target proteins in vitro, including Raf-1 (11), ASK1 (9), and exoenzyme S (13). This phenomenon is not restricted to R18, because phosphopeptides derived from 14-3-3 ligands have been similarly used on several occasions (see Ref. 14 for an example). However, because R18 does not require phosphorylation, we hypothesized that it could be ex-
pressed in cells from a DNA construct to inhibit 14-3-3/ligand interactions without activation by a kinase and without sensitivity to phosphatases. Expression of R18 should globally inhibit 14-3-3/ligand interactions. By using 14-3-3 inhibitor peptides, we have found that 14-3-3/ligand interactions are critical mediators of an antiapoptotic signal in multiple cell types. This signal is in part delivered through maintenance of mitochondrial integrity. Disruption of 14-3-3 survival signaling may provide new opportunities for therapeutic intervention in diseases involving insufficient apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids—The insert for pSCM110, coding for N-terminally Myc-tagged R18, was made by polymerase chain reaction against R18-pGEX-2T (11) using the primers 5′-A AAG CTG ATC AGC GAG GAC GAT GAC GAT AAG ATG GGA GTG ACC CGG GCC GAC GGG-3′ and subcloned into the vector pCR3.1-Uni using the Uni-directed Eukaryotic TA cloning kit (Invitrogen). A similar construct, pSCM121, containing difopein in place of R18 was made by subcloning the 100-base pair SalI/HindIII fragment of pSCM110 into the 5-kilobase BamHI/EcoRV fragment of the same vector. The predicted protein sequence for C54 is GVTRR sequence of difopein without tag is SADGA (PHAES, pSCM184. DNA coding for the C-terminal 54 amino acids of exoenzyme S (CS4) was obtained by polymerase chain reaction against pL2X200 using the primers 5′-GGAC GAC GTA TGC TGT GTC GAC ACC CCA AAA GGG CAA GCA TGG GCC CAC GGG-3′ and subcloned into the vector pCR3.1-Uni, using the Uni-directional Eukaryotic TA cloning kit (Invitrogen). A similar construct, pSCM136, respectively, into pEYFP-C1 (CLONTECH) cut with BglII/Apal to create pSCM136 and pSCM158, pSCM174, containing the mutant R18/Lys was made using the QuikChange site-directed mutagenesis kit (Stratagene) with pSCM136 as a template and 5′-GGG ATC GGACTT TCTGGTT TCTGGTT TCTGGTT-3′ as a primer. This vector was adapted for use with the Gateway system vector pDONR201 (Invitrogen) by insertion of the RF cassette after digestion with EcoRI and treatment with Klenow fragment, creating pSCM158. DNA coding for the C-terminal 54 amino acids of exoenzyme S (CS4) was obtained by polymerase chain reaction against pL2X200 using the primers 5′-GGG ACT TTT GTA CAA GGA AGC TGG GTA TCA GCC GAC GCC CCA-3′ and subcloned into the vector pCR3.1-Uni using the Uni-directed Eukaryotic TA cloning kit (Invitrogen).

Cell Culture—HEK 293, HeLa, and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech) containing 10% fetal calf serum. All cell lines were obtained from the American Type Culture Collection. Transfection was performed in 35-mm dishes at a density of 3×104/well in 35-mm dishes at a density of 3×104/well in 35-mm dishes and transfected as for the attachment-based assay. Twenty-four hours after transfection, the cells were fixed with 5% glutaraldehyde + 2% formaldehyde and stained for β galactosidase using 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal) (15). At least 500 stained cells in each sample were counted in a blind fashion, with dead cells distinguished by their rounded, blebbled appearance.

RESULTS

Difopein Binds 14-3-3 in Cells and Inhibits 14-3-3/Ligand Interactions—Before starting our study of the effect of 14-3-3/ligand interaction disruption on survival, we first wanted to develop a high affinity 14-3-3 antagonist. It was shown that dimerization of a 14-3-3 binding phosphopeptide led to increased affinity, presumably through bidentate interaction with dimeric 14-3-3 (3). Thus, we placed two R18 coding sequences separated by a sequence coding for a short peptide linker in a mammalian expression vector to create difopein (dimeric fourteen-three-three peptide inhibitor; Fig. 1). Difopein was readily expressed in HEK293 cells as an EYFP fusion (Fig. 2). The ability of difopein to bind 14-3-3 in cells was determined using an immunoprecipitation assay. 14-3-3 precipitates contained EYFP-difopein but not EYFP (Fig. 2). It was not necessary to专门 treat the cells to induce difopein binding, as might be expected if phosphorylation-dependent 14-3-3 ligands were used.

The 14-3-3/Raf-1 interaction was used as a model system to determine whether expressed difopein is capable of disrupting 14-3-3/ligand binding. Immunoprecipitated 14-3-3 was examined for the presence of endogenous Raf-1 (Fig. 2). In the presence of EYFP, a significant amount of Raf-1 was found in

1 The abbreviations used are: EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; β-gal, β-galactosidase; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline.
the 14-3-3 complex; however, expression of EYFP-difopein reduced Raf-1 to nearly undetectable levels. These data indicate that difopein is an effective competitor of the 14-3-3/Raf-1 interaction in cells. Based on this result and on the finding that the conserved amphipathic groove of 14-3-3 is the primary binding site for R18 (12) as well as physiological ligands (17, 18), it is reasonable to propose that difopein can disrupt many, if not all, of the 14-3-3/ligand interactions that occur in cells.

**Difopein Expression Induces Apoptotic Cell Death**

The ability of 14-3-3 to bind and inhibit several proapoptotic proteins supports the idea that 14-3-3 acts to promote cell survival. However, 14-3-3 also binds many proteins not known to be involved in cell death, and the overall effect of 14-3-3 on survival is not known. We used the difopein 14-3-3 inhibitor in several cell death assays to address this issue. For the first method, COS-7 cells were co-transfected with Myc-difopein and a β-galactosidase (β-gal) marker. Later, floating, dead cells were gently washed away, and the attached cells were assayed for β-gal. Cell death is detected as a decrease in the β-gal activity relative to the vector control. Transfection with Myc-difopein led to a decrease in β-gal activity and thus viability (Fig. 3A). This supports the hypothesis that 14-3-3/ligand interactions are required for critical cellular processes, possibly including pro-survival signaling. Similar results were seen in a cell morphology based assay (Fig. 3B).

Because disruption of survival signaling is expected to induce apoptosis rather than necrosis, it is essential to characterize the nature of the cell death caused by R18. Caspase 3 is a downstream effector protease that is commonly activated during apoptosis. Upon expression of EYFP-difopein, but not EYFP, the levels of activated caspase 3 increase in COS-7 cells (Fig. 3C), supporting the hypothesis that difopein induces apoptosis. We also used a flow cytometry based DNA content assay to determine the population of cells containing cleaved DNA.
Internucleosomal DNA cleavage, which is a commonly used marker of apoptosis, produces small fragments that can diffuse out of cells during ethanol fixation, resulting in decreased signals being seen on staining for DNA (sub-G₀ DNA content). In COS-7 cells, EYFP-difopein caused a dramatic increase in the fraction of sub-G₀ cells (Fig. 3D), whereas EYFP behaved similarly to empty vector (data not shown). Thus, difopein-induced disruption of 14-3-3/ligand interactions can cause apoptosis in COS-7 cells, supporting a role of 14-3-3 in survival signaling.

It is possible that the effects of 14-3-3 inhibition on apoptosis are restricted to COS-7 cells. Three additional cell lines were used to examine this issue: A549 lung cancer cells, DU145 prostate cancer cells, and HeLa cervical carcinoma cells. In the attachment-based viability assay, Myc-difopein was found to kill all three of these cell lines to varying degrees (Fig. 4), supporting the generality of the difopein effect. Because 14-3-3 proteins are ubiquitously expressed and because 14-3-3 can potentially target many different apoptosis regulating molecules, we anticipate that most cells will show some degree of sensitivity to 14-3-3 inhibitors.

**Difopein Causes Cell Death through 14-3-3 Inhibition**—Although the parent molecule of difopein, R18, is highly specific for 14-3-3, binding no other proteins in radiolabeled cell lysates

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**Fig. 3. Disruption of 14-3-3/ligand interactions by difopein induces apoptosis.** A, cell attachment assay. COS-7 cells were co-transfected with difopein or a control vector (80% of DNA) along with a lacZ reporter gene (20% of DNA). After 45 h the floating cells were gently washed away, and the attached cells were lysed and assayed for β-galactosidase. Decreased β-gal activity indicates a loss of viability. The results are normalized to the control vector β-gal activity (mean ± S.E.; n = 3). B, cell morphology assay. COS-7 cells were transfected as in A. Twenty-four hours later, the cells were fixed and stained for β-gal. Transfected (β-gal-positive) cells in each sample were examined microscopically for a rounded, blebbed morphology indicative of death. The ratios above the bars are live cells/total cells counted. C, caspase-3 activity assay. Twelve hours after transfection, COS-7 cells were lysed and assayed for the ability to cleave acetyl-DEVD-p-nitroaniline using the caspase 3 assay kit. D, DNA content distribution. COS-7 cells were transfected with plasmids coding for EYFP-difopein or EYFP along with a farnesylated EGFP marker. Twenty-four hours later, the cells were fixed in ethanol overnight, stained with 7-aminoactinomycin D, and run on a flow cytometer. Histograms for the 7-aminoactinomycin D signal, representing DNA content, are shown (left panel) for transfected cells (EGFP-F-positive) only. Histograms are normalized to the total number of transfected cells such that the area under each curve is 100. The right panel shows the fraction of cells containing sub-G₀ DNA content, which represent apoptotic cells with fragmented DNA. The results shown are representative of at least three independent experiments.
The 14-3-3 proteins are essential regulators of apoptosis induced by many different stimuli. This family, which contains both pro- and anti-apoptotic members, acts in part by controlling the status of the mitochondria. Two antiapoptotic Bcl-2 homologs, Bcl-2 and Bcl-X<sub>L</sub>, were transfected into COS-7 cells to determine their ability to block EYFP-difopein-induced death. Both Bcl-X<sub>L</sub> (Fig. 6B) and Bcl-2 (data not shown) were able to strongly inhibit difopein-induced apoptosis, even under conditions where they had relatively small effects on death caused by the proapoptotic Bcl-2 homolog Bad (data not shown). These results suggest that the 14-3-3 survival signal acts upstream of the Bcl-2 proteins, possibly aiding in the maintenance of mitochondrial integrity.

Protein phosphorylation is a common mechanism for regulating cellular processes, including apoptosis, and many kinases are known to promote (i.e. ASK1; Ref. 21) or inhibit (i.e. Akt/ PKB; Refs. 22-24) cell death. Because phosphorylation of serine or threonine is usually required to induce 14-3-3-ligand interaction, it is possible that the effects of difopein on cellular processes would be insensitive to kinase activity. This was tested for a key survival signaling kinase, Akt. As predicted, constitutively active Akt had little effect on EYFP-difopein-induced apoptosis (Fig. 6C), despite its robust expression (data not shown) and its ability to decrease the basal level of cell death. Together, the data presented in this section suggest that 14-3-3-ligand interactions are important for signal transmission from upstream survival signaling kinases to the mitochondria, although it is likely that other roles for 14-3-3 in regulating apoptosis await discovery.

**Disruption of 14-3-3/Ligand Interactions Can Enhance Sensitivity to the Antineoplastic Agent Cisplatin**—During oncogenesis, most cancers develop defects in apoptosis regulation (25, 26), often because of activation of upstream survival signaling pathways. Because conventional antineoplastic agents rely on induction of apoptosis for their efficacy (27), agents that can blunt this overactivation of survival mechanisms may enhance the utility of current anticancer therapies. Treatment of COS-7 cells with the antitumor drug cisplatin caused a dose-dependent decrease in viability as measured by an attachment-based assay (Fig. 7 and data not shown). Transfection of the cells with Myc-R18 prior to cisplatin treatment enhanced the ability of cisplatin to kill cells (Fig. 7). It was possible to see a decrease in viability caused by the combination of R18 and cisplatin at doses where each alone produced no detectable effect, and at higher treatment levels the combination was still more active than either agent alone. In addition, it was possible to see similar effects in the HeLa cervical cancer cell line (data not shown). These results are encouraging and show that in principle inhibition of 14-3-3-ligand interactions could be a useful therapeutic strategy for the treatment of cancer.

**DISCUSSION**

The 14-3-3 proteins are biochemically well characterized as phosphoserine-binding proteins (2). However, the large number and diversity of 14-3-3 ligands has made 14-3-3 difficult to classify in terms of function. The data presented here strongly support a role for 14-3-3 in prevention of apoptosis through transduction of survival signals. Expression of 14-3-3-ligand interaction inhibitors in cells led to the induction of apoptosis. These results indicate that 14-3-3 proteins are essential in mammalian cells, consistent with results of 14-3-3 knockout in yeast (28-30). To some degree our findings contrast with a previous study using ligand binding-defective mutants of 14-3-3 (10). These mutants have dominant negative effects in some systems, presumably via heterodimerization with wild
type 14-3-3. It was reported that dominant negative 14-3-3 can enhance the death caused by some stimuli, such as UV irradiation, but by itself cannot induce apoptosis (10). It is likely that part of this difference is due to the use of stable cell lines. The 14-3-3 mutant proteins were continuously expressed during the generation of these lines, so that only clones that developed resistance to the proapoptotic effects of 14-3-3 inhibition could be isolated. An additional difficulty is that the mutants used may have heterogenous effects on ligand binding affinity with respect to different ligands (17), making it possible that only a small subset of 14-3-3/ligand interactions were disrupted. Thus, the use of a global inhibitor of 14-3-3 in transient transfection systems is expected to provide a more complete view of the role of 14-3-3 in apoptosis.

One of the most important issues related to the use of 14-3-3/ligand interaction inhibitors is that of their specificity. The R18 peptide is highly specific for binding to 14-3-3 proteins (11). In addition, the abilities of R18 to bind 14-3-3 and induce apoptosis were both abolished by mutation of two residues shown to be critical for contacting 14-3-3 (12). This argues strongly that the phenotypes seen upon R18 expression require 14-3-3 binding. These results also support the hypothesis that difopein is acting through specific binding and inhibition of 14-3-3. However, it is impossible to completely rule out the presence of 14-3-3-independent effects of R18 or difopein. Thus, we tested the structurally unrelated 14-3-3 inhibitor C54 and found that it could induce apoptosis. Because of its lack of homology to difopein, it is unlikely that difopein and C54 share common non-14-3-3-specific effects. Together our results support the interpretation that the phenotypes induced by difopein reflect a loss of 14-3-3/ligand interactions.

A multitude of proteins have been described that positively or negatively regulate the induction of apoptosis. 14-3-3 can be placed in this network as acting upstream of the Bcl-2 proteins...
and effector caspases but downstream or independent of the pro-survival kinase Akt. This is consistent with the finding that Akt inhibition of the proapoptotic Bcl-2 homolog Bad requires 14-3-3 (32). It is possible that difopein acts to disrupt the 14-3-3/Bad complex and liberate free, active Bad. Although this simple model may account for a portion of the 14-3-3 effect on cell survival, the large number of 14-3-3 ligands directly or indirectly involved in cell death regulation makes it unlikely that it can completely explain the ability of difopein to induce apoptosis.

Studies of oncogenesis have shown that transforming mutations in preneoplastic cells often predispose them to apoptosis, either intrinsic to the cell or mediated by tumor surveillance systems in the host (27, 31). To compensate for this, many cancers develop enhanced antiapoptotic survival signaling networks (26). This provides a compelling rationale to support the targeting of apoptosis in general and survival signaling in particular for the treatment of cancer. Additionally, many currently available antineoplastic therapies act via induction of apoptosis (27), further supporting such a strategy. The ability of difopein to inhibit the antiapoptotic activity of the survival signaling kinase Akt and the additive effect of difopein on cisplatin-induced cell death suggest that blockade of 14-3-3/ligand interactions could be a therapeutically relevant target for cancer. It is interesting to note that the tumor suppressor protein p53 is not required for difopein-induced cell death, because difopein is active in COS-7 cells, which express the p53 inactivating SV40 T antigen, and in DU145 cells, which are mutated in the p53 gene. This is an important point because many antineoplastic agents are relatively ineffective against cells

**FIG. 6. Placement of the 14-3-3 survival signal in the apoptosis signaling network.** A, COS-7 cells were treated with the pan-caspase inhibitor zVAD-fmk at 50 μM or with vehicle (Me2SO) prior to transfection with EYFP or EYFP-difopein. DNA fragmentation was used to determine apoptosis as in Fig. 3D. B and C, the effects of Bcl-XL (B) and constitutively active Akt (C) on difopein-induced COS-7 cell death were measured as in Fig. 3D.

**FIG. 7. Cisplatin and R18 decrease the viability of COS-7 cells in an additive fashion.** COS-7 cells were co-transfected with R18 DNA (amount used shown as the fraction of total DNA) and a lacZ marker gene. 12 h after transfection, the indicated concentrations of cisplatin were added. Approximately 36 h later the viability of the cells (mean ± S.E., n = 3) was determined as in Fig. 3A.
that lack p53 and because p53 mutation is extremely common in cancer. Mechanistically, the additive effects of 14-3-3 inhibitors and cisplatin are consistent with the hypothesis that disruption of the 14-3-3 survival signal sensitizes cells to cisplatin-induced apoptosis.

Although the work described here focused on apoptotic cell death, 14-3-3 proteins are thought to be involved in many other cellular processes. The ability of 14-3-3 inhibitors to induce apoptosis does not imply that they produce only this phenotype. For example, expression of difopein can blunt the accumulation of HeLa cells in the S phase of the cell cycle caused by treatment with low concentrations of doxorubicin (data not shown). It may be possible to use low levels of 14-3-3 inhibitors, cells partially resistant to difopein-induced cell death, or treatment with apoptosis inhibitors such as Bcl-2 to examine the role of 14-3-3/ligand interactions in modulating other actions of the cell.

In summary, through the use of inhibitors of 14-3-3/ligand interactions, it has been shown that 14-3-3 proteins are important mediators of survival signals. This suggests that the 14-3-3 proteins are essential in mammalian cells, as has been previously shown in yeast. This work may have practical applications in treatment of diseases involving disregulated apoptosis and could also aid in the dissection of the function of 14-3-3 in other cellular processes.

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