14-3-3 Proteins Mediate an Essential Anti-apoptotic Signal*

Received for publication, June 27, 2001, and in revised form, September 17, 2001 Published, JBC Papers in Press, September 27, 2001, DOI 10.1074/jbc.M105971200

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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 τ (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 14-3-3ζ 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, ¹⁰WLDLE¹⁴, 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-

^{*} This work was supported by National Institutes of Health Grants GM53165 and GM60033 (to H. F.) and by a Predoctoral Fellowship in Pharmacology/Toxicology from the Pharmaceutical Research and Manufacturers of America Foundation (to S. C. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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 in an isoform-independent manner, making it useful for determination of the cellular roles of 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 Myctagged R18, was made by polymerase chain reaction against R18pGEX-2T (11) using the primers 5'-A AAG CTT ATG GAG CAG AAG CTG ATC AGC GAG GAG GAC CTG GGA TCC GAA TTC GTC GAC TCG GCC GAC GGG-3' and 5'-A TCT AGA TCA CTC GAG CCC AGC GGC CCC-3' and subcloned into the vector pCR3.1-Uni using the Unidirectional Eukaryotic TA cloning kit (Invitrogen). A similar construct, pSCM121, containing difopein in place of R18 was made by subcloning the ~100-base pair Sall/XbaI fragment of pSCM110 into the ~5-kilobase pair XhoI/XbaI fragment of the same vector. The predicted protein sequence of difopein without tag is SADGA (PHCVP RDLSW LDLEA NMCLP) GAAGL DSADG A(PHCV PRDLS WLDLE ANMCL P)GAAG LE, with the individual R18 monomers in parentheses. R18 and difopein were fused to an N-terminal enhanced yellow fluorescent protein (EYFP)¹ tag by subcloning the BamHI/ApaI fragments of pSCM110 and pSCM121, respectively, into pEYFP-C1 (CLONTECH) cut with BglII/ ApaI to create pSCM136 and pSCM138. 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 TGT CGT GGC TTA AGC TTA AGG CGA ATA TG-3' and its reverse complement as primers. pSCM182 is a mammalian expression vector for enhanced cyan fluorescent protein (ECFP), created by inserting the NdeI/SmaI fragment from pECFP-C1 (CLONTECH) into NdeI/EcoRV cut pcDNA3 (Invitrogen). This vector was adapted for use with the Gateway system (Invitrogen) by insertion of the RfC cassette after digestion with EcoRI and treatment with Klenow fragment, creating pSCM184. DNA coding for the C-terminal 54 amino acids of exoenzyme S (C54) was obtained by polymerase chain reaction against pLXZ200 using the primers 5'-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TCC GAC GAT GAC GAT AAG ATG GGA GTG ACC CG-3' and 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GGC CAG ATC-3'. This was placed in the Gateway system vector pDONR201 (Invitrogen) using BP clonase according to the manufacturer's protocol to create pSCM186. A vector coding for C54 with an N-terminal ECFP tag (pSCM187) was made using the Gateway LR reaction with pSCM186 and pSCM184. The predicted protein sequence for C54 is GVTRR VLEEA ALGEQ SGHSQ GLLDA LDLAS KPERS GEVQE QDVRL RMRGL DLA. Mammalian expression plasmids for human Bcl-2 and Bcl-X_L were generous gifts from Dr. Hong-Gang Wang.

Cell Culture—HEK 293, HeLa, and COS-7 cells were grown in Dulbecco's modified Eagle's medium (Mediatech) containing 10% fetal calf serum (Atlanta Biologicals). A549 and DU145 cells were grown in RPMI 1640 (Mediatech) containing 10% fetal calf serum. All cell lines were obtained from the American Type Culture Collection. Transfection was accomplished using FuGENE 6 cationic lipid reagent (Roche Molecular Biochemicals).

Immunoprecipitation—48 h post transfection, 8×10^5 HEK 293 cells were resuspended in lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% Nonidet P-40, 5 mM NaF, 5 mM Na₄P₂O₇, 2 mM Na₃VO₄, 10 mg/liter aprotinin, 10 mg/liter leupeptin, 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 1 h, and centrifuged to remove insoluble materials. The resulting lysates were precleared with 50 μ l of 50% protein G-Sepharose 4FF (Amersham Pharmacia Biotech). 1 μ l of 4 g/liter mouse anti-FLAG M2 antibody (Sigma-Aldrich) was added to the cleared lysates, and 1.5 h later 50 μ l of 50% protein G-Sepharose 4FF was added. This mixture was rocked gently for 1.5 h before the beads were washed three times with ice-cold lysis buffer and once with 50 mM HEPES, pH 7.5. Bound proteins were eluted by boiling and detected by Western blot.

Attachment-based Viability Assay—Cells seeded at 5 \times 10⁴/well were grown in 24-well plates and transfected with a lacZ marker and various test plasmids, using at least three wells for each condition. Forty-five hours later the samples were washed twice with PBS to remove floating and loosely attached cells and then lysed with 200 μ l/well of Z buffer (100 mM Na_2HPO_4, pH 6.95, 10 mM KCl, 1 mM MgSO_4, 50 mM β -mercaptoethanol, 0.2% Nonidet P-40). After addition of 50 μ l of 1 g/liter chlorophenolred- β -D-galactopyranoside (Roche Molecular Biochemicals) in Z buffer, the solutions were transferred to a microtiter plate, and $A_{550~\rm nm}$ was determined using a kinetic plate reader (Molecular Devicees). β -Galactosidase activity was determined as the slope of the best fit line to the observed $A_{550~\rm nm}$ versus time curve. This corresponds to the viability of the transfected cells.

Morphology-based Assay for Cell Death—COS-7 cells were seeded at 3×10^5 in 35-mm dishes and transfected as for the attachment-based assay. Twenty-four hours after transfection, the cells were fixed with 0.5% glutaraldehyde + 2% formaldehyde and stained for β -galactosif ase using 5-bromo-4-chloro-3-indolyl- 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, blebbed appearance.

Caspase 3 Activity Assay—The caspase 3 assay kit, Colorimetric from Sigma-Aldrich, which contains the substrate acetyl-DEVD-*p*-nitroaniline, was used according to the manufacturer's protocol.

Flow Cytometric Measurement of DNA Content-Cells were seeded in 35-mm dishes at a density of 3×10^5 1 day prior to transfection with various test DNAs in combination with pEGFP-F (20% of total DNA; CLONTECH). This marker vector codes for an enhanced green fluorescent protein containing a farnesylation signal sequence at the C terminus. Twenty-four hours after transfection, the cells were trypsinized and washed with PBS (130 mM NaCl, 20 mM phosphate, pH 7.5). Samples of the washed cells were taken for analysis of protein expression by Western blot. The remaining cells were fixed by adding 3 ml of ice-cold 95% ethanol and were stored at 4 °C overnight to allow small fragments of DNA to diffuse out of the cells (16). The next day cells were washed twice with PBS containing 1% bovine serum albumin (Sigma-Aldrich) and then resuspended in 0.8 ml of PBS, 1% bovine serum albumin. To the cells was added 100 µl of 10 g/liter RNase A (Sigma-Aldrich) in PBS, 1% bovine serum albumin, and 100 μ l of a DNA binding dye (Sigma-Aldrich): either 0.1 g/liter 7-aminoactinomycin D or 0.5 g/liter propidium iodide. Both of these dyes behaved equivalently and were used interchangeably. The samples were then stored in the dark at room temperature for at least 30 min prior to running them on a FACSort flow cytometer (Becton Dickinson). 1 to 3×10^5 events were counted for each sample. The data were analyzed using WinMDI v2.8 (J. Trotter; Scripps Research Institute) to exclude debris, clumps, and nontransfected cells.

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 specially 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.



Difopein: Dimeric Fourteen-three-three Peptide Inhibitor



FIG. 2. Difopein expressed in cells can bind 14-3-3 and inhibit 14-3-3/ligand interactions. HEK293 cells were transfected with DNA coding for FLAGtagged 14-3-3ζ, EYFP, or EYFP-difopein as indicated. 14-3-3 was immunoprecipitated using anti-FLAG (M2; Sigma-Aldrich). The presence of various species in the lysates and precipitated complexes was determined by Western blot using anti-Raf-1 (sc-133; Santa Cruz Biotechnology; top panels), anti-GFP (8367; CLON-TECH; middle panels), and anti-14-3-3 (sc-629; Santa Cruz Biotechnology; bot*tom panel*). The anti-GFP antibody recog-nizes GFP, EGFP, ECFP, and EYFP.

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.

Lysate

FLAG IP

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 Mycdifopein 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.



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 \pm 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, Colorimetric (Sigma-Aldrich). The results shown represent the mean caspase activity \pm S.E. (n = 3). *D*, DNA content distribution. COS-7 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)ositive) 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.

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- G_0 DNA content). In COS-7 cells, EYFP-difopein caused a dramatic increase in the fraction of sub- G_0 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



FIG. 4. Difopein is active in multiple cell lines. A549, DU145, and HeLa cells were transfected and assayed for β -galactosidase as in Fig. 3A. The β -gal activity for each cell line is relative to the vector control activity for that line and represents the mean \pm S.E. (n = 3).

(11), this type of approach is limited by the possibility that critical proteins of low abundance will not be detected. We have developed two lines of evidence that strongly support the hypothesis that the effects of difopein on cell death are caused by inhibition of 14-3-3. First, we changed two acidic residues of R18 (Asp¹² and Glu¹⁴), which are involved in coordinating the basic cluster of 14-3-3 (12), to lysines, creating R18(Lys). This peptide is not expected to bind 14-3-3, but it could retain interaction with other cellular partners if they exist. Indeed, EYFP-R18(Lys) was unable to be co-precipitated with $14-3-3\zeta$ from HEK293 cells even under conditions where EYFP-R18 could be (Fig. 5A). When tested for its ability to induce DNA fragmentation, EYFP-R18(Lys) behaved essentially identically to EYFP and did not induce apoptosis (Fig. 5B), despite its robust expression (data not shown). The second approach used a 14-3-3 binding molecule that is structurally unrelated to difopein, which should therefore have a different spectrum of non-14-3-3-specific protein interactions. We have characterized the phosphorylation-independent interaction of 14-3-3 with Pseudomonas aeruginosa exoenzyme S(13, 18, 19), finding that it binds in the amphipathic groove of 14-3-3 with high affinity. Others later determined that the 14-3-3 binding epitope of exoenzyme S is localized to its C-terminal 54 residues (20). This domain, termed C54, was able to bind 14-3-3 (Fig. 5A) and induce apoptosis in COS-7 cells as determined by DNA content (Fig. 5C). Thus, a strong correlation exists between the abilities of these molecules to bind 14-3-3 and to cause cell death. This argues that the apoptosis caused by difopein is due to inhibition of 14-3-3/ligand interactions and that 14-3-3, by binding other cellular proteins, mediates a critical prosurvival signal.

The 14-3-3 Survival Signal Acts Upstream of Mitochondria— Because apoptosis is irreversible once a critical commitment step is reached, the initiation of this process is subject to multiple levels of regulation. To place the 14-3-3 survival signal among other known death regulators, the effect of overexpression or inhibition of several regulators on EYFP-difopein-induced apoptosis was examined. First, the pan-caspase inhibitor zVAD-fmk was used to disable both upstream signaling caspases as well as downstream effector caspases. Treatment of COS-7 cells with zVAD-fmk at 50 μ M abolished the ability of EYFP-difopein to kill cells (Fig. 6A). This result is most likely explained by a requirement for effector caspases in the apoptosis caused by disruption of 14-3-3/ligand interactions and is consistent with the ability of difopein to activate caspase 3 (Fig. 3*C*).

The Bcl-2 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_L, were transfected into COS-7 cells to determine their ability to block EYFP-difopein-induced death. Both Bcl-X_L (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-difopeininduced 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



FIG. 5. The ability of peptides to bind 14-3-3 is correlated with their ability to induce apoptosis. A, various peptides were assayed for FLAG-14-3-3 ζ binding by immunoprecipitation (IP) as in Fig. 2. The peptides in lanes 3-5 and 8 were expressed as EYFP fusions, whereas those in lanes 7 and 9 were ECFP fusions. The top and bottom panels were blotted (8367; CLONTECH) with anti-GFP whereas the middle panel was blotted with anti-FLAG (M2; Sigma-Aldrich). B and C, the ability of R18(Lys) (B) or C54 (C) to induce COS-7 cell death was determined as in Fig. 3D. Western blot (not shown) was used to verify the expression of all peptides.

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),² 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

² H. Wang and H. Fu, unpublished results.

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 μ M 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-X_L (B) and constitutively active Akt (C) on difopeininduced 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 \pm S.E., n = 3) was determined as in Fig. 3A.

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 re-



EYFP-difopein

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 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



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EYFP

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.

Acknowledgments-We thank Drs. Bingcheng Wang and Hong-Gang Wang for the generous gifts of DNA constructs and the members of the Fu laboratory for helpful discussions.

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Shane C. Masters and Haian Fu J. Biol. Chem. 2001, 276:45193-45200. doi: 10.1074/jbc.M105971200 originally published online September 27, 2001

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