Survival-promoting functions of 14-3-3 proteins

Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322, U.S.A.

Abstract

The 14-3-3 proteins are a family of phosphoserine/phosphothreonine-binding molecules that control the function of a wide array of cellular proteins. We suggest that one function of 14-3-3 is to support cell survival. 14-3-3 proteins promote survival in part by antagonizing the activity of associated proapoptotic proteins, including Bad and apoptosis signal-regulating kinase 1 (ASK1). Indeed, expression of 14-3-3 inhibitor peptides in cells is sufficient to induce apoptosis. Interestingly, these 14-3-3 antagonist peptides can sensitize cells for effective killing by anticancer agents such as cisplatin. Thus, 14-3-3 may be part of the cellular machinery that maintains cell survival, and targeting 14-3-3-ligand interactions may be a useful strategy to enhance the efficacy of conventional anticancer agents.

Introduction

14-3-3 proteins bind a variety of proteins that are critical mediators of intracellular signalling

Key words: apoptosis, ASK1, Bad, difopein.

Abbreviations used: PKB, protein kinase B; ASK1, apoptosis signal-regulating kinase 1; EYFP, enhanced yellow fluorescent protein.

To whom correspondence should be addressed (e-mail hfu@emory.edu).
pathways [1,2]. Through protein–protein interactions, 14-3-3 participates in the regulation of diverse cellular processes, including cell proliferation and cell-cycle checkpoint control. Recent work on the interaction of 14-3-3 with death-promoting proteins illustrates an important role of 14-3-3 in promoting cell survival signalling and preventing cell death. Because anti-neoplastic therapies ultimately eliminate tumour cells by the induction of apoptosis, understanding how 14-3-3-mediated survival pathways inhibit apoptosis may allow the use of 14-3-3 antagonists to sensitize tumour cells for effective therapy.

**14-3-3 supports cell survival by inhibiting multiple death-promoting pathways**

Apoptosis is a conserved, active process of cell death that plays a critical role in normal development, maintenance of tissue homeostasis and elimination of damaged or unwanted cells [3–5]. It is clear that cell death pathways are tightly associated with cell survival pathways to ensure that cell death occurs only when needed [5]. For example, the proapoptotic function of Bad, a Bcl-2 family member, can be inhibited by multiple kinases, including Akt/protein kinase B (PKB) and ribosomal S6 kinase (RSK), in response to a variety of survival signals.

The first indication that 14-3-3 is involved in regulation of cell death came when 14-3-3 was found to be associated with Bad in response to the survival factor interleukin-3 [6], resulting in the inhibition of Bad proapoptotic activity. Greenberg and colleagues later discovered that 14-3-3–Bad association is regulated by Akt/PKB through phosphorylation of Bad, providing a direct link between a survival signalling kinase and a death promoter [7]. Bad contains three known phosphorylation sites, Ser-112, Ser-136 and Ser-155, and a host of kinases, including PKA, p21-activated kinase (‘PAK’) and RSK, in response to various survival signals.

The inhibition of both Bad- and ASK1-induced cell death in the presence of transiently expressed 14-3-3 ζ. Indeed, overexpression of 14-3-3 blocked ASK1WT, but not ASK1ΔΨ, -induced apoptosis. Interestingly, co-expression of a ligand-binding-defective mutant of 14-3-3 ζ, K49E, did not inhibit ASK1-induced cell death, but instead promoted it. These data imply that this 14-3-3 mutant interferes with the function of endogenous 14-3-3 proteins, exhibiting a dominant negative effect. Another 14-3-3 mutant protein, R56/60E, has been shown to accelerate apoptosis induced by UV irradiation or serum withdrawal [22]. These data confirm a critical role of 14-3-3 in controlling the death-promoting activity of ASK1. This suggests that association with 14-3-3 inhibits the death-promoting activity of ASK1. To directly test whether 14-3-3 can inhibit the proapoptotic activity of ASK1, we monitored ASK1-induced cell death in the presence of transiently expressed 14-3-3 ζ. Indeed, overexpression of 14-3-3 blocked ASK1WT, but not ASK1ΔΨ, -induced apoptosis. Interestingly, co-expression of a ligand-binding-defective mutant of 14-3-3 ζ, K49E, did not inhibit ASK1-induced cell death, but instead promoted it. These data imply that this 14-3-3 mutant interferes with the function of endogenous 14-3-3 proteins, exhibiting a dominant negative effect. Another 14-3-3 mutant protein, R56/60E, has been shown to accelerate apoptosis induced by UV irradiation or serum withdrawal [22]. These data confirm a critical role of 14-3-3 in controlling the death-promoting activity of ASK1. This suggests another mechanism by which prosurvival kinases can engage 14-3-3 to block apoptosis. Thus regulation of ASK1 by both prosurvival and anti-apoptotic signals may provide a critical point of control for cell death and cell survival.

Inhibition of both Bad- and ASK1-induced apoptosis by 14-3-3 lends weight to the possibility
that 14-3-3 may have a general anti-apoptotic function. In support of this notion, 14-3-3 proteins also target many other proteins that are intimately involved in the regulation of apoptosis, including FKHRL1 (a member of the forkhead family of transcription factors), insulin-like growth factor-I receptor (‘IGFIR’), phosphoinositide 3-kinase and A20 [23–28].

**14-3-3 employs a conserved groove for ligand binding, providing a target for pharmacological intervention**

Using a combination of structural biology and genetic approaches, we have identified the primary protein-interaction site of 14-3-3 involved in ligand binding. In collaboration with Robert Liddington’s laboratory, we solved the structure of 14-3-3 ζ and postulated that an amphipathic groove in each 14-3-3 monomer, which is conserved among all 14-3-3 proteins, is the primary binding site for diverse ligands [29]. Mutational analysis of residues in this groove provided strong support for this model [30-32]. Crystal structures of 14-3-3 in complex with phosphorylated peptides derived from middle T antigen (‘MT’) and Raf-1 [33,34], with arylalkylamine N-acetyltransferase [35], and with a non-phosphorylated peptide, R18 [33], confirmed that the amphipathic groove of 14-3-3 is its main ligand-binding domain. Therefore, this conserved groove of 14-3-3 provides a specific target for disruption of 14-3-3-ligand interactions.

**14-3-3 antagonists can disrupt 14-3-3-ligand interactions in vitro and in vivo**

To control 14-3-3-ligand interactions, we desired a peptide antagonist of 14-3-3 proteins. In collaboration with B. Wang, we isolated R18 (PHC-VPDLSWLDLEANCLP) from a phage display library based on its affinity for 14-3-3 proteins [36]. This peptide binds different isoforms of 14-3-3 with similar affinity ($K_d \approx 80$ nM) and high specificity, thus it may serve as a general 14-3-3 inhibitor. To provide a structural understanding of the binding of R18 to 14-3-3, we co-crystallized R18 in complex with 14-3-3 ζ (Figure 1). As expected, our structure unambiguously demonstrated that R18 binds 14-3-3 ζ via its conserved amphipathic ligand-binding groove. Competition between R18 and natural ligands for the same residues in the binding groove of 14-3-3 should cause inhibition of 14-3-3-ligand interactions. Indeed, we found that R18 effectively inhibited the interaction of 14-3-3 ζ with multiple ligands, such as ASK1, Bad and Raf-1 [21,35-37]. Because R18 binds 14-3-3 without prior phosphorylation, it can be expressed in cells to inhibit 14-3-3-ligand interactions, a significant advantage over most other 14-3-3-binding peptides. To increase the affinity of this peptide, we generated a dimeric R18 connected by a peptide linker, termed difopein (dimeric fourteen-three-three peptide inhibitor) [38]. To determine whether difopein can bind 14-3-3 in cells, an immunoprecipitation assay was used. 14-3-3 precipitates contained enhanced yellow fluorescent protein (EYFP)-difopein, but not EYFP (Figure 2). Significantly, expression of EYFP-difopein effectively dissociated 14-3-3 from its ligands, such as Raf-1 (Figure 2). Thus it is likely that difopein can disrupt many, if not all, of the 14-3-3-ligand interactions that occur in cells, making it a valuable reagent to probe the function of 14-3-3 in many physiological processes.

**Disruption of 14-3-3-ligand interactions leads to apoptotic cell death**

The ability of 14-3-3 to inhibit several proapoptotic proteins while enhancing the function of prosurvival proteins supports the idea that 14-3-3 acts to promote cell survival. If this hypothesis is correct, blockade of 14-3-3–ligand binding would be expected to induce apoptosis. We have examined multiple markers of viability and apoptosis in COS-7 cells expressing R18 or difopein to address this issue. Upon expression of EYFP-difopein, the levels of activated caspase 3 increase, as determined using the substrate acetyl-DEVD-$\rho$-nitroanilide. This effect is not mediated by
**Figure 2**

**Difopein can bind 14-3-3 and inhibit endogenous 14-3-3-ligand interactions**

HEK-293 cells were transfected with DNA coding for FLAG-14-3-3ζ and either EYFP or EYFP-difopein as indicated. 14-3-3 was immunoprecipitated using anti-FLAG (M2). The presence of various species in the lysates and precipitated complexes was determined by Western blot using anti-Raf-1 (top), anti-green fluorescent protein (GFP, middle) and anti-14-3-3 (bottom) antibodies. The anti-GFP antibody recognizes GFP, enhanced GFP, enhanced cyan fluorescent protein ('ECFP') and EYFP equally well.

**Figure 3**

**Difopein-mediated disruption of 14-3-3-ligand interactions induces apoptosis**

COS-7 cells were transfected with plasmids coding for EYFP-difopein or EYFP along with a famesylated enhanced green fluorescent protein (EGFP) marker. 24 h later, cells were fixed in ethanol, stained with 7-aminoactinomycin D (7AAD) and measured by flow cytometry. Histograms for the 7AAD 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, which represents apoptotic cells with fragmented DNA. EYFP, as EYFP alone does not cause detectable caspase 3 activation. Another hallmark of apoptosis is the occurrence of DNA fragmentation. EYFP-difopein caused a dramatic increase in the fraction of cells containing fragmented DNA (sub-G₀), while EYFP itself behaved similarly to empty vector (Figure 3). In addition, difopein also caused the mitochondrial transmembrane potential, ΔΨₘ, to decline. Thus we conclude that difopein-induced disruption of 14-3-3-ligand interactions can indeed cause apoptosis in COS-7 cells.

A concern with the interpretation of these results is the possibility that R18 or difopein may have effects on proteins other than 14-3-3. To address this issue, we tested the 14-3-3-binding-defective mutant peptide R18(D12K, E14K) and found that it could not induce apoptosis. Thus the epitope of R18 responsible for induction of apoptosis overlaps the 14-3-3-binding domain, supporting the idea that R18 kills cells by inhibiting 14-3-3. We have also tested another 14-3-3 inhibitor peptide, C54, derived from *Pseudomonas aeruginosa* exoenzyme S [39], which shares no sequence homology with R18, and have shown that it can cause apoptosis. These data support an essential role of 14-3-3 in transmission of survival signals.

**14-3-3-mediated survival signalling acts upstream of the mitochondrial death machinery**

It is believed that apoptosis signalling uses two major pathways, mitochondria-dependent and mitochondria-independent, that converge on the downstream effector caspases [3,4]. To place 14-3-3 among other known death regulators, we
tested the effect of overexpression or inhibition of several regulators on EYFP-difopein-induced apoptosis. We found that the pan-caspase inhibitor zVAD-fmk (benzoyloxycarbonyl-Val-Ala-DEpfluoromethylketone) abolished the ability of EYFP-difopein to kill cells, which is consistent with the ability of difopein to activate caspase 3. This suggests that disruption of 14-3-3–ligand interactions causes cell death in an effector-caspase-dependent manner. The Bcl-2 family proteins are essential regulators of apoptosis induced by multiple stimuli that act in part by controlling the status of the mitochondria. We found that two anti-apoptotic Bcl-2 homologues, Bcl-2 and Bcl-XL, were both able to dramatically inhibit difopein. These results argue that the 14-3-3 survival signal acts upstream of Bcl-2 and Bcl-XL. It is likely that difopein can disrupt the 14-3-3–Bad interaction to liberate Bad, which then acts directly on Bcl-2 and Bcl-XL. However, given the large number of 14-3-3 ligands involved in apoptosis, it seems unreasonable that such a simple model can completely account for the cellular effects of 14-3-3. Because phosphorylation of serine or threonine often serves as a signal to induce 14-3-3–ligand interaction, it is possible that 14-3-3–ligand interaction inhibitors would be insensitive to kinase activity. This was tested for a key survival signalling kinase, Akt/PKB, which promotes survival in part through phosphorylation of the 14-3-3 ligands Bad and FKHR11. Constitutively active Akt/PKB had little effect on difopein-induced apoptosis, despite its robust expression and its ability to decrease the basal level of cell death. Taken together, these data argue that 14-3-3–ligand interactions are important for signal transmission from upstream survival signalling kinases to the core apoptotic mechanisms.

The 14-3-3 antagonist R18 sensitizes tumour cells to cisplatin-mediated cell death

Insufficient induction of apoptosis is now recognized as a hallmark of cancer [40]. We examined the effects of 14-3-3 antagonists in A549 (lung), DU145 (prostate) and HeLa (cervix) cancer cell lines to determine whether they could be effectively killed. Difopein decreased viability in all three lines to varying degrees. Given the ubiquitous nature of 14-3-3, it is likely that difopein may have a broad anti-tumour spectrum. Because conventional anti-neoplastic agents rely on induction of apoptosis for their efficacy, agents that can blunt the overactivation of survival mechanisms may enhance the utility of current anticancer therapies. To test the possibility that inhibition of 14-3-3–ligand interactions facilitates induction of apoptosis by cytotoxic agents, we examined the effect of R18 on cisplatin-induced cell death. Transfection of COS-7 cells with Myc-R18 prior to cisplatin treatment enhanced the ability of cisplatin to kill cells. When used independently at low levels neither cisplatin nor R18 had a significant effect on viability. However, low-dose cisplatin and R18 in combination were toxic to the cells. This result indicates that R18 may sensitize COS-7 cells to cisplatin-induced death. In addition, it was possible to see similar effects in cisplatin-treated HeLa cells. Inhibition of 14-3-3–ligand interactions could be a useful therapeutic strategy with the potential to improve conventional cancer therapy.

In conclusion, 14-3-3 proteins appear to mediate an essential anti-apoptotic signal in cells. 14-3-3 may promote cell survival in part by binding and inhibiting proapoptotic proteins, such as Bad and ASK1, and in part by enhancing the function of prosurvival proteins. Because many diseases, including cancer, have up-regulated survival machinery, inhibition of 14-3-3–ligand interactions may offer therapeutic opportunities. Our 14-3-3 antagonists that target the same binding site as natural ligands will not only allow us to dissect the function of 14-3-3 in many cellular processes, but may also lead us to novel strategies for the treatment of diseases involving disregulated cell survival.

References

14-3-3 Proteins and photoneuroendocrine transduction: role in controlling the daily rhythm in melatonin

D. C. Klein*, S. Ganguly*, S. Coon*, J. L. Weller*, T. Obsil†, A. Hickman† and F. Dyda†

*Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4480, U.S.A., and †Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892-4480, U.S.A.

Abstract

This paper describes the role 14-3-3 proteins play in vertebrate photoneuroendocrine transduction. 14-3-3 proteins form a complex with arylalkylamine N-acetyltransferase (AANAT), the enzyme which turns melatonin production on during the day and off at night. Complex formation is triggered at night by cAMP-dependent phosphorylation of the enzyme, and results in activation and protection against proteolysis. This enhances melatonin production > 10-fold. Light exposure results in dephosphorylation of the enzyme and disassociation from 14-3-3, leading to destruction and a rapid drop in melatonin production and release and circulating levels.

Key words: cAMP-dependent protein kinase, N-acetyltransferase, pineal.

Abbreviations used: AANAT, arylalkylamine N-acetyltransferase; PKA, cAMP-dependent protein kinase.

*e-mail klein@helix.nih.gov

Received 20 March 2002