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MINIREVIEW

Targeting Protein Serine/Threonine Phosphatases for Drug Development

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ABSTRACT

With the recent clinical success of drugs targeting protein kinase activity, drug discovery efforts are focusing on the role of reversible protein phosphorylation in disease states. The activity of protein phosphatases, enzymes that oppose protein kinases, can also be manipulated to alter cellular signaling for therapeutic benefits. In this review, we present protein serine/threonine phosphatases as viable therapeutic targets, discussing past successes, current challenges, and future strategies for modulating phosphatase activity.

Numerous cellular processes, including metabolism, immune response, synaptic plasticity, cell growth and proliferation, and apoptosis, are controlled by intricate signal transduction networks composed of molecules and macromolecular protein complexes that are responsive to biological or chemical stimuli in the cell's immediate environment. A common mechanism used by cells to either propagate or terminate intracellular signal transduction pathways is reversible protein phosphorylation, whereby the addition or removal of a negatively charged phosphate can alter the conformation of a target protein and/or its interactions with other proteins. Ultimately, phosphorylation/dephosphorylation reactions affect the activity, function, half-life, or subcellular localization of the substrate; hence, the underlying molecular mechanisms controlling this reversible post-translational modification are of great physiological importance.

Careful study has been afforded to the structure, function, and regulation of the enzymes that catalyze phosphorylation or dephosphorylation reactions—protein kinases and phosphatases, respectively. It is widely accepted that proper spatial and temporal regulation of both protein kinases and phosphatases is crucial for maintaining the appropriate balance of phosphorylation required for cellular homeostasis (Bauman and Scott, 2002). Because deregulation of these enzymes has been implicated in a variety of diseases (e.g., cancer, diabetes, cardiac hypertrophy, and neurodegeneration), emerging therapeutic strategies have focused on the design of drugs that affect the biological actions of kinases and phosphatases.

Protein kinases have become increasingly popular drug targets, constituting ~30% of several pharmaceutical manufacturers' drug discovery programs (Cohen, 2002a). The approval of rapamycin (Sirolimus) for immunosuppression,

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ABBREVIATIONS: CnA, calcineurin A subunit; CnB, calcineurin B subunit; CsA, cyclosporin A; Cyp, cyclophilin; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FCP, transcription factor II F-interacting carboxyl terminal domain phosphatase; FK506, tacrolimus; FKBP12, FK506 binding protein of 12 kDa; GADD34, growth arrest and DNA damage gene 34; HDAC, histone deacetylase; HSV, herpes simplex virus; IκB, inhibitor of κB; IKK, IκB kinase; INCA, inhibitors of NFAT-calcineurin association; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T cells; Plk1, polo-like kinase 1; PP1, protein serine/threonine phosphatase 1; PP1c, catalytic subunit of PP1; PP2, protein serine/threonine phosphatase 2; PPM, magnesium/manganese-dependent protein phosphatase; PPP, phosphoprotein phosphatase; PSTP, protein serine/threonine phosphatase; PTP, protein tyrosine phosphatase; sal, salubrinal; SAR, structure-activity relationship; SV40, simian virus 40; TNF, tumor necrosis factor-α; TSA, trichostatin A.

imatinib (Gleevec) for chronic myelogenous leukemia and gastrointestinal tumors, and gefitinib (Iressa) for non–small-cell lung cancer has paved the way for development of additional kinase-targeted drugs that are currently under evaluation in clinical trials. The tremendous efforts put forth by the pharmaceutical industry and academic research institutions to modulate kinase activity for the treatment of various disease states have also invigorated discussions regarding phosphatases as plausible drug targets. Several protein tyrosine phosphatases, dual-specificity phosphatases, and lipid phosphatases have recently become attractive candidates for the treatment of a variety of diseases (e.g., diabetes, obesity, and cancer) (Lyon et al., 2002; Ducruet et al., 2005; Lazar and Saltiel, 2006; Tonks, 2006). To date, however, the only U.S. Food and Drug Administration-approved drugs targeting a phosphatase are the immunosuppressants cyclosporin A and FK506, which inhibit protein serine/threonine phosphatase 2B (PP2B or calcineurin). Both compounds are naturally occurring products that, like rapamycin, were not specifically designed for their molecular target but approved for clinical use based upon the merit of their immunosuppressive activity. In this review, we examine the case for protein serine/threonine phosphatase-targeted drug design, presenting past successes, current challenges, and future possibilities.

Overview of Protein Serine/Threonine Phosphatases

Protein phosphatases are classified according to their substrate specificity and are generally divided into two major categories—protein serine/threonine phosphatases (PSTPs) and protein tyrosine phosphatases (PTPs), with dual-specificity phosphatases (DSPs) existing as a subclass of the tyrosine phosphatases. PTPs catalyze dephosphorylation reactions on phospho-tyrosine residues, PSTPs on phospho-serine and phospho-threonine residues, and DSPs on phospho-tyrosine, phospho-serine, and phospho-threonine residues. PSTPs and PTPs have distinct structural characteristics, which give rise to very different catalytic and regulatory mechanisms. Although both types of phosphatases perform nucleophilic attacks on the targeted phosphate group, they do so via different nucleophiles. PTPs use a catalytic cysteine residue as a nucleophile, whereas the PSTP nucleophilic attack is facilitated via a metal-activated water molecule positioned in the catalytic groove.

Studies of the structure, function, and regulation of PSTP catalytic subunits have imparted a systematic classification of these enzymes that is based upon their biochemical properties and amino acid sequence homology. Three distinct PSTP gene families have been described: PPM, FCP, and PPP. The PPM family is composed of Mg^{2+}-dependent phosphatases that include PP2C, pyruvate dehydrogenase phosphatase, and PP2C-"like" phosphatases (Barford et al., 1998). The newly emerging FCP family includes FCP1 and small C-terminal domain phosphatases (SCPs) 1 to 3, which share a common substrate—the C-terminal domain of RNA polymerase II (Yeo et al., 2003, 2005; Kamenski et al., 2004; Gallego and Virshup, 2005). The PPP family consists of the PP1, PP2A, PP2B, PP5, and PP7 subfamilies, which exhibit high amino acid sequence homology within their catalytic domains and probably share similar catalytic mechanisms (Barford et al., 1998; Honkanen and Golden, 2002; Swingle et al., 2004).

No sequence homology exists between members of the PPP and PPM families; however, comparison of their crystal structures suggests that an analogous mechanism evolved for single-step dephosphorylation reactions (Barford et al., 1998). Perhaps the greatest difference between the PPP and PPM families is that members of the PPM family are generally thought to function as monomers, whereas PPP family members form multimeric holoenzyme complexes with a wide variety of regulatory, scaffolding, targeting, and inhibitory proteins that assign substrate selectivity and direct subcellular localization of the catalytic subunit (Janssens and Goris, 2001; Schweighofer et al., 2004). Thus, the activities of distinct phosphatase holoenzymes, each composed of a catalytic subunit and an associated regulatory subunit(s), are indeed highly regulated and specific. Moreover, various post-translational modifications of phosphatase catalytic and regulatory subunits provide additional layers of regulation for some PPP family members (Chen et al., 1992; Bryant et al., 1999; Trockenbacher et al., 2001). The existence of multiple regulatory subunits that have been described for PPP family members suggest that complexity in dephosphorylation reactions lies well beyond the number of genes encoding phosphatase catalytic subunits.

Aberrant serine/threonine phosphatase activity has been linked with several pathological states, including diabetes, cardiovascular disorders, cancer, and Alzheimer's disease (Tian and Wang, 2002; Arroyo and Hahn, 2005; Fiedler and Wollert, 2005; Parameswara et al., 2005). Therefore, the pharmacological manipulation of phosphatase activity is an attractive strategy for the treatment of such conditions. This review examines two different approaches for PSTP drug development: 1) targeting phosphatase catalytic subunits and 2) targeting specific phosphatase complexes (i.e., phosphatase oligomeric holoenzymes and phosphatase-substrate complexes). Both strategies are being actively pursued and have produced multiple compounds that are worthy of clinical testing.

Targeting Serine/Threonine Phosphatase Catalytic Subunits for Drug Development

Molecular targeted drug design requires a thorough knowledge of the enzyme's structure and catalytic mechanism. The crystal structures of PPP family members (PP1, PP2B, PP5, and PP2A) are quite similar, and reveal the N- and C-terminal domains of the catalytic subunits as two distinct domains linked together by a central β-sandwich, which comprises the catalytic core. A narrow channel formed by the interface of the β-sheets creates the catalytic cleft, which contains two metal ions positioned by invariant amino acid residues within the loops of the β-sheets. Although PPM and PPP family members do not share any obvious sequence homologies, the tertiary structures of phosphatases from both families are remarkably similar. The crystal structure of PP2Cα shows a similar catalytic fold as PPP family members—a central β-sandwich flanked by α-helices in the N- and C-terminal domains (Barford et al., 1998). Given the high similarities in tertiary structures, PPP and PPM phosphatases are likely to share the same proposed catalytic mechanism (Barford et al., 1998).

A common structural region within PPP family members—the β12-β13 loop in PP1, PP2A, PP4, PP6, and PP5 and the L7 loop in PP2B—is critical for the binding selectivity of protein inhibitors and small-molecule inhibitors. Deletion of the C terminus of the PP1 catalytic subunit, which includes the β12-β13 loop, abrogates PP1 inhibition by I-1, I-2, NIPP-1, and microcystin (Connor et al., 1999). This loop is adjacent to the catalytic cleft and, when bound to microcystin-LR, obstructs substrate entrance to the catalytic cleft. It is unclear whether protein inhibitors induce a similar conformational change to prevent substrate access, but the general importance of the β12-β13 loop for regulating PP1 activity indicates that this region is an attractive target for the design of novel, selective inhibitors for PPP family members.

Can serine/threonine phosphatase catalytic subunits be good drug targets? Indeed, serine/threonine phosphatase catalytic subunits are already established drug targets. Early drug discovery programs, which focused on the ability of natural compounds to induce phenotypic changes in native systems (i.e., Forward Pharmacology) have led to the identification of numerous naturally occurring compounds that possess immunosuppressive and/or anticancer properties. It was later determined that several of these compounds (e.g., cyclosporin A, FK506, cantharidin, and fostriecin) specifically bind and inhibit serine/threonine phosphatase catalytic subunits (Liu et al., 1991; Li and Casida, 1992; Honkanen, 1993; Walsh et al., 1997). The most successful examples of such compounds are the immunosuppressants cyclosporin A (CsA) and FK506, which potently inhibit PP2B when complexed with their cognate immunophilins (Liu et al., 1991). CsA, a metabolite from the fungus *Tolypocladium inflatum*, was approved for clinical use 8 years before its mechanism of action was elucidated (Cohen, 2002a). Another example of a naturally occurring compound that inhibits serine/threonine phosphatase activity is the blister beetle toxin cantharidin. This compound is the active component of mylabris (an extract derived from the dried body of the Chinese blister beetle), which has been used in China since ~180 CE (Han Dynasty) for the removal of dead tissue and the treatment of external skin conditions (Wang, 1989); cantharidin was used in the United States for topical wart treatment before 1960 (Sakoff and McCluskey, 2004). However, it was not until the 1990s that cantharidin was shown to bind and inhibit both PP1 and PP2A catalytic subunits (Li and Casida, 1992; Honkanen, 1993). Although some of these compounds (e.g., microcystin-LR, nodularin, calyculin A, and cantharidin) have similar IC_{50} values for both PP1 and PP2A, structure-activity relationship (SAR) studies have facilitated the design and synthesis of inhibitor analogs with preferential selectivity for PP1 or PP2A (Aggen et al., 1999; Sakoff and McCluskey, 2004). SAR studies have also been used to create pharmacophores (basic structural features of a molecule that are essential for its biological activity) for the rational design of phosphatase-selective drugs (Sakoff and McCluskey, 2004).

PP2B Catalytic Subunit as a Drug Target

PP2B plays a role in diverse biological processes, including axon guidance, learning and memory, heart valve morphogenesis, and cardiac function (Rusnak and Mertz, 2000); however, its most storied role is regulation of the transcription factor nuclear factor of activated T cells (NFAT) in lymphocytes (Macian, 2005). Activation of the T-cell receptor causes an increase in intracellular calcium levels, which triggers PP2B dephosphorylation of cytosolic NFAT. Removal of NFAT's phosphate groups exposes two nuclear localization signals and induces translocation of NFAT to the nucleus, where it drives the transcription of target genes that include a variety of cytokines. The immunosuppressants CsA and FK506 prevent T cell activation by binding to cellular immunophilin proteins (cyclophilin and FKBP12, respectively), resulting in a drug-immunophilin complex that associates with and inhibits PP2B activity toward NFAT.

Knowledge of the structure of PP2B is crucial to understand the shared mechanism of action of FK506 and CsA. PP2B exists in the cell as an obligate heterodimer, composed of a catalytic subunit (CnA) and a regulatory subunit (CnB). CnA consists of four domains: a catalytic domain, a CnB-binding domain, a calmodulin-binding domain, and an autoinhibitory domain (Rusnak and Mertz, 2000). CnB is a calmodulin-like protein containing two pairs of calcium-binding EF hand motifs separated by a flexible helix linker (Kissinger et al., 1995). Current thinking holds that calcium/calmodulin activates PP2B upon binding to the calmodulin-binding domain of CnA and inducing the dissociation of the autoinhibitory domain from the catalytic domain. Neither FKBP12-FK506 nor CyP-CsA binds directly to the phosphatase catalytic site, but both complexes block substrate access in a noncompetitive manner (Jia, 1997; Huai et al., 2002). Although FK506 and CsA are structurally unrelated, the FKBP12-FK506 and CyP-CsA complexes bind PP2B at the same surface—an interface between the CnA (catalytic subunit) and CnB (regulatory) subunits near the active site (Jia, 1997; Huai et al., 2002).

Despite the great success of CsA and FK506 in treatment of acute organ rejection, long-term usage of these drugs can lead to undesirable side effects such as nephrotoxicity and hepatotoxicity (Martínez-Martínez and Redondo, 2004). These side effects are attributed, in part, to the inhibition of immunophilins, which do not bind PP2B in the absence of drugs but perform distinct cellular functions that include the modulation of an intracellular calcium ion channel (Chelu et al., 2004; Barik, 2006). Therefore, efforts to improve on CsA and FK506 as drugs have included the pursuit of direct, selective PP2B inhibitors. One attractive strategy (that may be applicable as a broad approach for designing selective phosphatase inhibitors) integrates available structural data for PP1 and PP2B catalytic subunits and SAR studies of cantharidin analogs for PP1, PP2A, and PP2B (Baba et al., 2003). Analysis of the SARs has led to the identification of a "phosphatase-selective core" molecule that is able to bind the conserved catalytic site. Computational binding models constructed using the "core" molecule and the PP2B catalytic subunit allowed for an accurate prediction of the appropriate chemical substituents that bind to residues unique to PP2B. The resulting cantharidin analog (1,5-dibenzoyloxymethyl-substituted norcantharidin) is the first selective PP2B inhibitor (IC_{50} = 7 μM) that directly targets the catalytic site. It is noteworthy that additional studies with this compound have demonstrated the importance of stereochemistry at the C1 position for PP2B selectivity, reflecting key differences in the shape of the catalytic grooves of PP2B, PP1, and PP2A (Baba et al., 2005). Current efforts are under way to synthesize pure enantiomers of additional phosphatase inhibitors to allow further testing of how compound chirality influences

phosphatase selectivity. Although their therapeutic value remains to be determined, it is clear that the redesign of nature's compounds can deliver novel and selective phosphatase inhibitors.

PP2A/PP1 Catalytic Subunits as Drug Targets

PP2A and PP1 are involved in diverse cellular processes, including cell growth and proliferation, development, DNA replication and repair, metabolism, neural signaling, and apoptosis. These two phosphatases also account for the majority of serine/threonine phosphatase activity in mammalian cells. Because dysregulation of PP2A and PP1 has been implicated in numerous disease states, they have become attractive pharmacological targets. The specific oligomeric composition of the PP2A and PP1 holoenzymes is crucial for the control of phosphatase activity; thus, it is necessary to understand the role of phosphatase regulatory and targeting subunits.

Native PP2A enzymes primarily exist in two forms—a core dimer and a heterotrimeric holoenzyme. The PP2A core dimer (AC), composed of the scaffolding A subunit and the catalytic C subunit, associates with a regulatory B subunit to generate the heterotrimeric holoenzyme (ABC), which is the predominant form of PP2A in the cell (Janssens and Goris, 2001). PP2A regulatory B subunits are categorized into four different families (B or PR55, B′ or PR56/61, B″ or PR72/130, and B‴ or PR93/110) originating from 14 distinct genes (Janssens and Goris, 2001). The regulatory B subunit plays a crucial role directing the subcellular localization of PP2A; it can also alter the overall shape of the catalytic subunit as well as enzyme kinetics (Strack et al., 1998; Price and Mumby, 2000; Janssens and Goris, 2001; Cho and Xu, 2007).

The substrate specificity and subcellular targeting of the PP1 catalytic subunit (PP1c) is dictated by its association with more than 50 regulatory proteins (Cohen, 2002b; Ceulemans and Bollen, 2004). These subunits exhibit very little homology to one another, but most possess a common PP1c binding motif, [RK]x_{0-1}[VI](P)[FW] (commonly referred to as an RVxF motif, where x is any residue and (P) is any residue but proline), that interacts with a hydrophobic pocket away from the catalytic site (Egloff et al., 1997). This initial common binding event becomes the platform used by many PP1 regulatory subunits to establish secondary, lower affinity interactions that modulate PP1c isoform selectivity. For example, after the binding of PP1c to the RVxF motif of either neurabin or spinophilin (two PP1c regulatory subunits), secondary interactions occur within a region that is C-terminal to the RVxF motif to promote preferential binding of PP1γ1 over PP1β/δ (Carmody et al., 2004, 2008). The interaction of PP1 catalytic subunit with its regulatory subunit can also influence substrate specificity, as is the case for the myosin phosphatase targeting subunit MYPT1. Recent crystallographic data have revealed that upon binding MYPT1, the PP1c catalytic cleft is adapted to create a more suitable binding pocket for phosphomyosin that is remarkably less suitable for other substrates (Terrak et al., 2004).

Inhibition of Phosphatase Activity. The wide range of action of PP2A is precisely the reason that the catalytic subunit of this enzyme was thought to be an unlikely drug target. Nevertheless, fostriecin, a PP2A-selective inhibitor, entered a phase I clinical study after showing promise as a cancer-killing agent (Lewy et al., 2002). Fostriecin is an antibiotic produced by *Streptomyces pulveraceus* and, like many other natural phosphatase-inhibiting compounds, was discovered based on its antitumor properties without any knowledge of its mechanism of action. At first, fostriecin's tumor cytotoxic activity was attributed to the inhibition of DNA topoisomerase II, but its effective concentration (IC_{50} = 40 μM) and cell cycle effects were subsequently found to be inconsistent with classic topoisomerase II inhibitors (Jackson et al., 1985; Chen and Beck, 1993; Honkanen and Golden, 2002; Lewy et al., 2002). Further studies revealed that fostriecin inhibits both PP2A and PP4 (a PP2A family member), triggering premature entry into mitosis and ultimately resulting in apoptosis (Roberge et al., 1994; Walsh et al., 1997; Lewy et al., 2002). In addition, SAR studies supported a strong correlation between inhibition of phosphatases by fostriecin and its cytotoxicity to tumor cells in culture (Buck et al., 2003). Both PP2A and PP4 are actively involved in cell cycle regulation (Wada et al., 2001; Bennin et al., 2002). In fact, addition of the PP2A/PP4 inhibitors okadaic acid and calyculin A (at concentrations that also inhibit PP5 and PP6) to cells in culture produces the same result as treatment with fostriecin—premature entry into mitosis and cell death (Lewy et al., 2002). Thus, the revelation of the antitumor effects and mechanism of action of fostriecin validates PP2A as a viable drug target.

The properties unique to fostriecin over other PP2A inhibitors (e.g., microcystin-LR and calyculin A) that give it a clear advantage for development as an anticancer drug include both low toxicity and high specificity profiles. Fostriecin is 10,000 times more selective for PP2A/PP4 than for PP1/PP5 (Buck et al., 2003) and is much less toxic to normal cells compared with okadaic acid (Walsh et al., 1997). Unfortunately, fostriecin was found to be highly susceptible to oxidation, and the phase I clinical trials were suspended because of drug instability and inadequate purity in the clinical supply of the naturally produced compound (Lewy et al., 2002). However, investigators have employed various complementary strategies to combat these issues, including the development of several novel syntheses for fostriecin analogs (Lewy et al., 2002) and the manipulation of *S. pulveraceus* polyketide synthase gene clusters (a process known as combinatorial biosynthesis) to selectively generate single analogs of fostriecin and fostriecin-like molecules. The latter strategy has been used to engineer bacterial strains that produce single analogs of phoslactomycin B, a compound structurally related to fostriecin (Palaniappan et al., 2003). Phoslactomycins are potent and selective inhibitors of PP2A, and phoslactomycin A has been shown to inhibit lung metastasis in mice through activation of natural killer cells (Kawada et al., 2003). The ability to manufacture a pure supply of phoslactomycins provides a proof of principle for the production of pure supplies of fostriecin analogs that can be tested for their clinical efficacy.

Another natural product known for its antitumor properties is cantharidin. Beneficial therapeutic results have driven its use as a medicinal agent in China for the treatment of skin conditions (see above) as well as for various cancers such as hepatoma and esophageal carcinomas (Wang, 1989). Cantharidin is an inhibitor of PP1, PP2A, PP4, and PP5 [IC_{50} = 1.1, 0.194, 0.05, and 0.60 μM, respectively (Swingle et al., 2007)]; PP2B inhibition is achieved only at relatively high concentrations (IC_{50} = >10 μM). It is currently unknown

whether the antitumor effect of this compound is due to the inhibition of one or more phosphatases. Cantharidin possesses several characteristics that make it ideal for development as an anticancer drug. It is a lipophilic compound that can easily traverse biological membranes and, importantly, is not a substrate for the drug transporter P-glycoprotein (Sieder et al., 1999). Furthermore, one report claims that cantharidin does not induce myelosuppression but instead stimulates hematopoiesis in animals and humans (Wang, 1989).

Western medicine has largely ignored the therapeutic potential of cantharidin because of toxicity issues; however, a demethylated cantharidin analog (norcantharidin) has been shown to have significantly less toxic effects while stimulating hematopoiesis and retaining its anticancer activity and potency for PP1 and PP2A inhibition (IC_{50} = 9 and 3 μM, respectively). Significant effort has been devoted to synthesizing norcantharidin analogs with low toxicity for development as selective phosphatase inhibitor drugs (McCluskey et al., 2000; Hill et al., 2007a,b, 2008). These studies have generated numerous compounds, including the most potent PP2A- and PP1-selective analogs described to date, which have 5- to 15-fold greater potency for growth inhibition (GI_{50} ~9.6 and ~3.3 μM, respectively) in several human cancer cell lines compared with norcantharidin (GI_{50} ~45 μM) (Hill et al., 2007b). Although further studies are required to explore the anticancer properties of these compounds in vivo, it is clear that the development of analogs more potent than the lead compound (norcantharidin) has been successful.

Activation of Phosphatase Activity. Because PP2A/PP1 activity governs several pathways important for cell growth and proliferation, it is difficult to decipher the exact substrate(s) in which abnormal phospho-state (as a result of dysfunctional PP2A/PP1 activity) leads to tumor progression. Perhaps the deregulation of different phosphatase holoenzymes may affect more than one transduction pathway that is required for cell transformation. This could explain why some cancers respond to small-molecule phosphatase inhibitors, whereas others respond to small-molecule phosphatase activators. Preclinical and clinical research will be critical to distinguish the appropriate treatment for specific types of cancer characterized by abnormal protein phosphorylation. For example, cancers that are slowed by kinase inhibitors may also be responsive to treatment with certain phosphatase activators.

PP2A activation. PP2A is a known tumor suppressor—a role first assigned when the catalytic activity of PP2A was shown to be inhibited by the tumor-promoting shellfish toxin okadaic acid (Bialojan and Takai, 1988). Disruption of PP2A activity by a variety of other mechanisms also results in cell transformation. Spontaneous mutations in both α and β isoforms of the A subunit occur at low frequency in several human cancers (e.g., cervical, lung, breast, and colon) (Ruediger et al., 2001a; Arroyo and Hahn, 2005; Cho and Xu, 2007; Westermarck and Hahn, 2008). These mutations, many of which interrupt specific structural contacts between A and C subunits, prevent the formation of the PP2A heterotrimeric holoenzyme and result in decreased phosphatase activity (Ruediger et al., 2001a,b; Westermarck and Hahn, 2008). It is noteworthy that cellular transformation via the SV40 small t antigen is brought about by a similar mechanism—disruption of the PP2A heterotrimeric holoenzyme. Small t antigen disrupts ABC and AB′C holoenzymes by directly binding to the A subunit, thereby competitively displacing the B and B′ subunits (Chen et al., 2004; Arroyo and Hahn, 2005). Targeted disruption of the AB′αC PP2A heterotrimer by SV40 small t promotes increased phosphorylation and activation of the oncogenic protein kinase Akt (Yuan et al., 2002; Zhao et al., 2003). Sustained activation of Akt, and its downstream effectors, by small t is sufficient to transform human mammary epithelial cells (Zhao et al., 2003).

Pharmacological manipulation of PP2A tumor suppressor activity has long been a topic of discussion in the field, and the phosphatase-activating effects of the sphingolipid metabolite ceramide on both PP2A and PP1 have been studied extensively in pursuit of an antiproliferative agent (Dobrowsky et al., 1993; Law and Rossie, 1995; Chalfant et al., 1999, 2004). Yet, only recently has there been any substantial clinical advancement of this concept. In another example of forward pharmacology, activation of PP2A activity was discovered as the underlying mechanism by which the small molecule immunosuppressant FTY720 induces apoptosis (Matsuoka et al., 2003). FTY720 is currently in phase III clinical trials for organ transplantation and multiple sclerosis (Neviani et al., 2007). This synthetic analog of the natural compound myriocin decreases peripheral circulation of T-cell lymphocytes by preventing egress from secondary lymphoid tissues, thereby decreasing T-cell movement into transplanted organs. These physiological effects have been attributed to FTY720's agonism of lymphocyte sphingosine receptors, which ultimately leads to receptor down-regulation and altered lymphocyte trafficking (Brinkmann et al., 2002; Mandala et al., 2002; Chiba, 2005). In addition to its actions at the sphingosine receptor, FTY720 also triggers apoptosis by altering PP2A activity in certain human B- and T-cell leukemia cell lines, including BCR/ABL-transformed myeloid and lymphoid cells and CML-BC (blast crisis chronic myelogenous leukemia) and Ph1-ALL (Philadelphia chromosome-positive acute lymphocytic leukemia) progenitor cells, but does not induce apoptosis in nontransformed cells and normal bone marrow cells (Neviani et al., 2007). FTY720-treated leukemia cells exhibit increased PP2A activity, leading to the dephosphorylation of BCR/ABL, Akt, and ERK1/2 ultimately resulting in cell death (Neviani et al., 2007; Liu et al., 2008). Although the precise mechanism underlying induction by FTY720 of PP2A activation is unclear, evidence suggests that FTY720 has a direct effect on the PP2A heterotrimer (Matsuoka et al., 2003). Ceramide has also been reported to directly activate the PP2A heterotrimer (Dobrowsky et al., 1993; Chalfant et al., 2004); however, a recent study suggests that ceramide directly interacts with the PP2A inhibitor 2 protein (I2PP2A or SET), resulting in decreased PP2A-SET interactions and increased PP2A activity (Mukhopadhyay et al., 2009). Increased SET levels have been reported in BCR/ABL-transformed cells and CML-BC progenitors that undergo FTY720-induced apoptosis (Neviani et al., 2005). Perhaps the effects of FTY720 are also a result of restraining the inhibitory function of SET.

It is noteworthy that FTY720 acts on two of the same signaling pathways (Akt and ERK1/2) as small t to manipulate cell growth. Inhibition of PP2A activity via treatment with okadaic acid or small t reverses the apoptotic effects of FTY720 by preventing Akt and ERK1/2 dephosphorylation (Matsuoka et al., 2003; Neviani et al., 2007; Liu et al., 2008).

Perhaps the best indicator that FTY720 will be an effective clinical weapon against certain leukemias is its ability to restrict leukemogenesis in mouse models of B-cell lymphoma, CML-BC, and Ph1 ALL (Neviani et al., 2007; Liu et al., 2008). In this regard, FTY720 may be a novel therapy for patients with leukemias resistant to imatinib and other kinase inhibitors.

PP1 activation. A recent study has demonstrated close ties between PP1 and Plk1 (polo-like kinase 1), a key kinase regulator of mitotic progression (Yamashiro et al., 2008). Because Plk1 is overexpressed in numerous human tumors (Strebhardt and Ullrich, 2006), it has become a target for small-molecule kinase inhibitors. In fact, one Plk1-selective ATP-competitor, BI 2536 (Steegmaier et al., 2007), is currently in phase I clinical trials. The loss of Plk1 activity in cells leads to metaphase arrest but does not induce arrest in G_2 phase (Strebhardt and Ullrich, 2006). However, overexpression of Plk1 leads to transformation of NIH 3T3 fibroblasts, and overactive Plk1 can override the DNA damage-induced G_2 checkpoint. Yamashiro et al. (2008) reported that Plk1 activity is antagonized by the MYPT1-PP1c complex (myosin phosphatase targeting subunit-PP1 catalytic subunit). Thus, it is possible that activation of a phosphatase, specifically MYPT1-associated PP1c, provides another strategy for combating dysfunctional Plk1.

The interaction of the PP1c regulatory domain (i.e., the region of PP1c that binds RVxF-containing proteins) with proteins/peptides can lead to allosteric changes that modify enzyme activity as well as substrate selectivity. Synthetic RVxF-containing peptides of various lengths (4–13mers) have been shown to activate PP1 in either the absence or presence of the PP1 inhibitor DARPP32, thus indicating that the binding of these peptides to the regulatory domain of PP1c can directly influence phosphatase activity as well as its interaction with PP1c-binding proteins (Tappan and Chamberlin, 2008). A search for small molecules that could mimic the activating effect of these RVxF-containing peptides was recently undertaken; this screen was based on molecular models of the Gm peptide/PP1c crystal and analogs of the "Adda" side chain of microcystins, which are weak activators of PP1 activity (Tappan and Chamberlin, 2008). The resulting "RVXF mimic" compound activated PP1 by 2-fold in in vitro assays (EC_{50} = 2 μM). Although the therapeutic potential of such compounds has not yet been examined, the ability to selectively activate PP1c through an allosteric, regulatory domain represents an attractive complimentary approach to drugs designed to inhibit kinase activities.

PP5 Catalytic Subunit as a Drug Target

Comparison of the three existing crystal structures of the PP5 catalytic subunit with the crystal structures of other PPP family members has revealed both similar and unique properties of PP5. The N-terminal TPR domains and a short C-terminal α-helical subdomain are two features that distinguish PP5 from the other PPP family members. These two regions, acting in concert, provide an important autoregulatory mechanism for PP5 activity. Hydrogen bonds stabilize interactions between the catalytic domain and both the TPR domain and the C-terminal subdomain, thereby physically blocking substrate access to the catalytic fold (Yang et al., 2005). The control of PP5 activity is mediated through its TPR interactions with various proteins and lipids (Chen et al., 1994; Skinner et al., 1997; Ramsey and Chinkers, 2002). Direct binding of Hsp90 or $G\alpha_{12}/G\alpha_{13}$ activates PP5, presumably by disrupting the autoinhibitory interaction between the TPR domain and the catalytic site (Chen et al., 1996; Yamaguchi et al., 2002). In addition, lipid molecules, such as arachidonic acid and unsaturated and saturated fatty acyl coenzyme A esters, directly bind the TPR domain and stimulate PP5 by promoting a structural change within the TPR helices to relieve autoinhibition (Skinner et al., 1997; Yang et al., 2005).

There are no known specific small-molecule inhibitors of the PP5 catalytic subunit, but recent studies have given researchers a preview of the potential clinical benefit of decreasing cellular PP5 expression and/or activity. The PP5 promoter contains an estrogen response element, and estradiol has been reported to increase PP5 expression by ~50% in estrogen-dependent breast carcinoma cells (MCF-7) (Urban et al., 2003). Moreover, overexpression of PP5c in MCF-7 cells promotes cell proliferation in the absence of estrogen, whereas cellular depletion of PP5 (through transfection of antisense oligonucleotides) attenuates estrogen-dependent cell growth (Honkanen and Golden, 2002). Thus, altered PP5 expression directly affects estrogen-dependent cell growth, and increased levels of this phosphatase may lead to metastases. Because decreased PP5 activity restricts estrogen-dependent cell growth, small-molecule inhibitors of PP5 could be useful for treating breast cancers resistant to selective estrogen receptor modulators. The PP5 catalytic subunit also seems to be a desirable target for antitumor therapy, given that depletion of PP5 has been shown to induce G_1 arrest and interfere with the ATR-mediated DNA damage checkpoint signaling pathway in A549 lung carcinoma cells (Zhang et al., 2005). Furthermore, hypoxia-induced elevation of cellular PP5 may aid tumor growth by negatively regulating an ASK1/MKK4/JNK pathway that facilitates an apoptotic response in cells experiencing low-oxygen environments (Zhou et al., 2004). The extant literature on PP5 suggests that a strategy similar to the one used for designing a PP2B selective inhibitor from a phosphatase selective core molecule (see *PP2B Catalytic Subunit as a Drug Target*) may also allow the rational design of a drug that selectively targets the PP5 catalytic site. Given that PP5, like PP2B, has restricted substrate selectivity in the cell, the inhibition of its activity may allow specific manipulation of cell cycle progression without perturbation of other cellular processes.

PPM Catalytic Subunits as Drug Targets

PP2C and PP2C-like phosphatases largely function as negative regulators of stress-activated pathways (Tamura et al., 2006). The expression of one PP2C isoform, PPM1D or Wip1, is induced by p53 as part of a negative feedback loop (Fiscella et al., 1997). Wip1 dephosphorylates and inactivates p38 MAPK, resulting in the abrogation of p53 function after cellular stress (Bulavin et al., 2004). As p38 MAPK functions to activate cell cycle checkpoints through phosphorylation of various targets (p53, cdc25A, cdc25B), a Wip1-mediated decrease in p38 activity promotes cell cycle progression (Yoda et al., 2006). Aberrant Wip1 activity has been implicated in tumorigenesis, and increased levels of Wip1 have been reported in several types of cancers, including neuroblastoma, medulloblastoma, primary breast cancer, and ovarian clear

cell adenocarcinoma (Bulavin et al., 2004; Castellino et al., 2008). Conversely, mice lacking the *wip1* gene display increased apoptosis in breast tumors and reduced tumorigenesis (Bulavin et al., 2004). Together, these results indicate that Wip1 is an attractive target for development of small molecule inhibitors to treat certain cancers.

Nonphosphate inhibitors of any PPM family member did not exist until recent investigations identified several compounds from the National Cancer Institute (NCI) Diversity Set library that selectively inhibit Wip1 and PP2Cα (Belova et al., 2005; Rogers et al., 2006). Recent work has also produced substrate-based phosphopeptide inhibitors of Wip1 (Yamaguchi et al., 2006). Although the cellular effects of the PP2Cα inhibitors remain to be tested, the Wip1 small molecule inhibitors slowed growth of breast cancer cells and reduced xenograph tumor development in mice (Belova et al., 2005). Therefore, these inhibitors can be used as lead compounds for the design of additional cell-permeable inhibitors that are specific for PPM family members, which may ultimately prove to exhibit therapeutic value.

Regulatory mechanisms for PP2C remain largely unknown; however, a recent report indicated that PP2Cα is subject to allosteric activation via binding of an insulin mediator mimetic, *d*-chiro-inositol-galactosamine (Brautigan et al., 2005). Treatment of severely diabetic rats with *d*-chiro-inositol-galactosamine enhanced insulin signaling and decreased blood sugar levels, presumably as a result of increased PP2Cα dephosphorylation and activation of glycogen synthase and/or pyruvate dehydrogenase. Because allosteric activation of PP2Cα correlates with increased sensitivity to insulin, PP2Cα may also prove to be a desirable target for the design of new diabetes drugs.

Summary of Future Prospects of Targeting Phosphatase Catalytic Subunits for Drug Development

Research efforts to design small-molecule PSTP inhibitors/activators that target the catalytic site will continue to use available structural information from phosphatase catalytic subunit-inhibitor crystals. Like the norcantharidin studies discussed earlier, compounds binding the catalytic site will probably be used to construct pharmacophores upon which SAR studies can be used to improve phosphatase selectivity/specificity. Selectivity may also be improved by targeting additional sites that are less conserved and adjacent to the active site, like the RVxF-binding site of PP1c (see *PP2A/PP1 Catalytic subunits as Drug Targets*). In this respect, phosphatase catalytic subunit drug design will probably follow in the footsteps of kinase inhibitors that have been designed to contact regions other than the well conserved ATP-binding pocket.

Targeting Serine/Threonine Phosphatase Complexes for Drug Development

Because serine/threonine phosphatases are involved in numerous cellular processes, inhibiting or activating all forms of a select phosphatase subtype using a catalytic subunit inhibitor may not only be unnecessary for achieving the desired therapeutic effect but also produce unwanted toxic side effects. Thus, the ability to directly target a specific phosphatase holoenzyme [i.e., catalytic subunit plus associated regulatory subunit(s) plus] to modulate the phosphostate and function of a specific substrate may be the Holy Grail in the field of phosphatase drug discovery. Two obvious approaches to accomplish this objective are 1) disruption of the interaction between the phosphatase (catalytic subunit or regulatory subunit) and its substrate and 2) disruption of the interaction between the catalytic subunit and regulatory subunit.

Disrupting Phosphatase Complexes: Is It Possible?

Before discussing whether or not it is plausible to disrupt specific macromolecular phosphatase complexes, one must first consider whether small molecules are capable of disrupting protein-protein interactions. Indeed, recent studies have revealed several examples of molecules that inhibit multiprotein complexes. The nutlin compounds, which mimic the chemical structure of p53, bind MDM2 at the same site as p53 and prevent the interaction of p53 with MDM2. Impaired formation of the p53-MDM2 complex promotes stability of the p53 tumor suppressor and, ultimately, cell cycle arrest and apoptosis (Vassilev et al., 2004). This antitumor response, prompted by nutlins, occurs in mouse models, indicating that the specific disruption of an enzyme-substrate interaction can be of therapeutic value (Vassilev et al., 2004).

The disruption of specific phosphatase complexes to achieve therapeutic benefits is an idea that is gaining ground in the biomedical community, and small-molecule or peptide antagonists have been identified for several phosphatase complexes (Tables 1 and 2 and Fig. 1, A–C). A recent study exploited cell-permeable peptides that mimic the structure of "phosphatase-interacting motifs" in specific subcellular targets of PP1 and PP2A; these peptides bind the phosphatase in the same manner as the substrate (Guergnon et al., 2006). The determination of phosphatase binding sites within a PP1 interacting protein (Bad) and a PP2A interacting protein (CD28) allowed for the construction of short corresponding peptides fused with a basic stretch of amino acids to confer cell permeability. The introduction of either one of these peptides into cells in culture induced cell death. Although these peptides interact with their targeted phosphatase subunit (PP1c and $PP2A_{B55}$, respectively), it remains to be determined whether an actual decrease or disruption of these

TABLE 1
Apparent therapeutic benefits of small molecule antagonists of phosphatase complexes

Protein Complex	Protein Complex Antagonists	Consequence of Disrupted Complex	Apparent Therapeutic Value
PP1-GADD34 (Boyce et al., 2005)	Salubrinal	↑ eIF2α phosphorylation	↓ HSV replication
PP1-HDAC 1/6 (Chen et al., 2005)	Trichostatin A	↓ Akt activity	↓ Cell growth and transformation
PP2B-NFAT (Clipstone and Crabtree, 1992)	FK506, Cyclosporin A	↓ PP2B activity	Immunosuppression
PP2A-Akt-βArr2 (Beaulieu et al., 2008)	Lithium	↑ Akt activity	Mood stabilizer

complexes occurred in the affected cells. Nevertheless, the manipulation of phosphatase-substrate interactions not only seems to be feasible but may also be of therapeutic value.

Impairing the formation of phosphatase-substrate complexes may be one way of achieving a therapeutic benefit; however, another viable strategy is the targeted disruption of individual phosphatase holoenzymes [i.e., catalytic subunit and associated regulatory subunit]. Various regulatory subunits serve to target both PP1 and PP2A holoenzymes to a specific substrate (Janssens and Goris, 2001). Separating the regulatory subunit from the holoenzyme can prevent the association of the catalytic subunit with its substrate and/or result in dissociation of the catalytic subunit from its substrate. For example, synthetic peptides containing the RVxF (PP1c-binding) motif have been used to disrupt PP1c complexes, and the introduction of these peptides into neuronal cells has been shown to influence synaptic transmission (Yan et al., 1999; Morishita et al., 2001).

Several viruses induce cell transformation through the disruption of phosphatase holoenzymes. Both SV40 and polyoma small tumor antigens (e.g., small t) interact with the PP2A AC dimer and displace the B/PR55 and B′/PR56 subunits from the PP2A heterotrimer (ABC), resulting in inhibition of PP2A activity (Pallas et al., 1990; Yang et al., 1991; Ruediger et al., 1999). The introduction of small t into cultured cells causes a decrease in $AB_{PR55}C$ and $AB_{PR56}C$ ho-

TABLE 2
Phosphatase complexes that could be targeted for therapeutic benefits

Protein Complex	Potential Consequence of Disrupted Complex	Potential Therapeutic Value
PP1-GL (Cohen, 2002)	↑ Glycogen synthase	↓ Blood glucose levels
PP5-DNA-PKc (Wechsler et al., 2004)	↑ DNA-PKc phosphorylation	↑ Radiation sensitivity
PP2A-SET (Neviani et al., 2005)	↓ BCR/Abl activity	↓ Leukemogenesis
PP2A-Raf (Adams et al., 2005)	↓ MAPK activity	↓ Cell proliferation
PP2A-IKK (Kray et al., 2005)	↓ IKK activity	↓ Inflammation
PP2A-SERT (Bauman et al., 2000)	↑ [5-HT] in synapses	Mood stabilizer

DNA-PKc, DNA-dependent protein kinase catalytic subunit; 5-HT, 5-hydroxytryptamine.

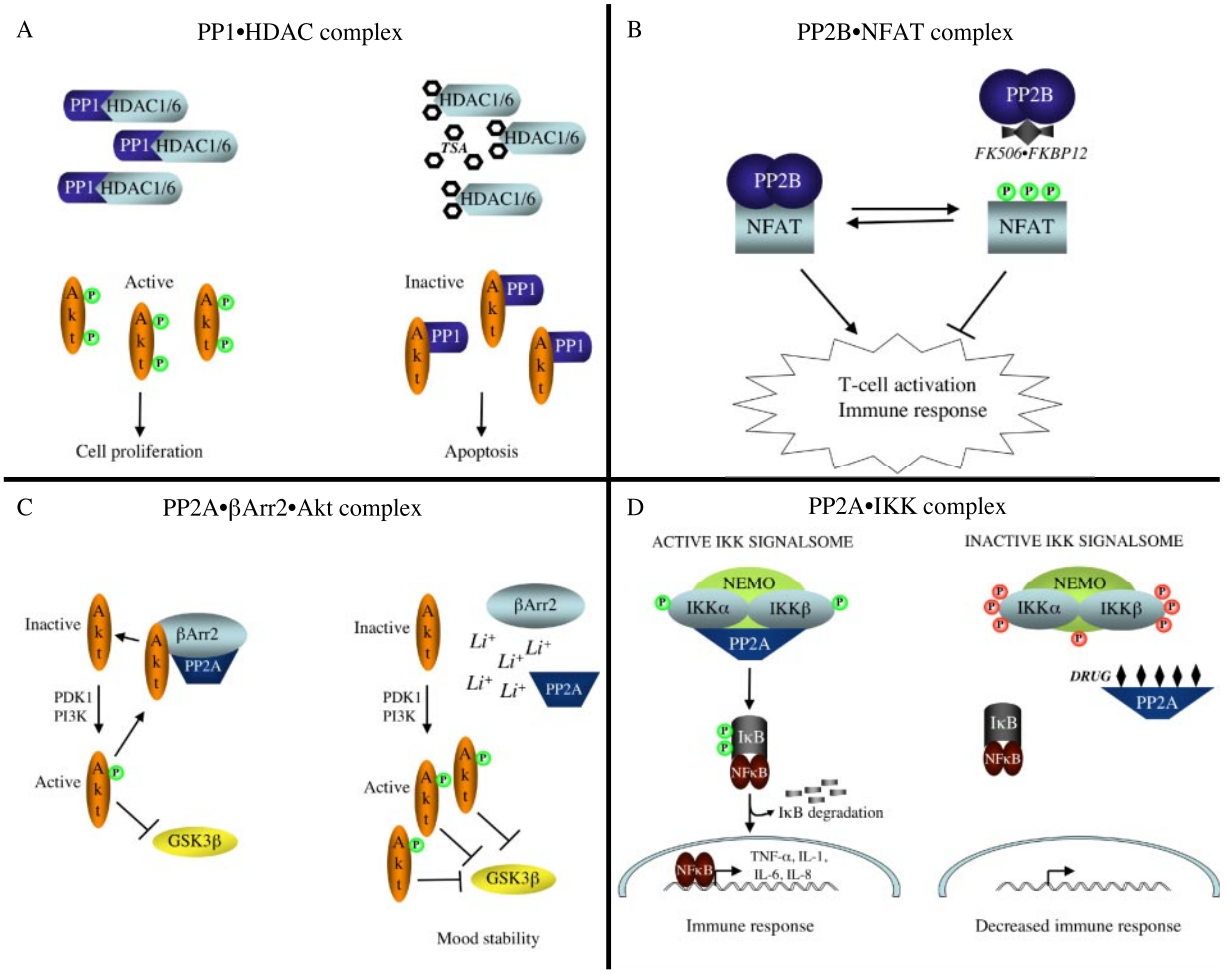

Fig. 1. Consequences of the targeted disruption of select phosphatase complexes. A to C, phosphatase complexes that can be disrupted by small molecules. The PP2A-βArr2-Akt complex (C), which is essential for dopaminergic signaling, can be disrupted by lithium salts. [Adapted from Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, Wetsel WC, Lefkowitz RJ, Gainetdinov RR, and Caron MG (2008) A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* **132:**125–136. Copyright © 2008 Elsevier. Used with permission.] D, a phosphatase complex that could potentially be targeted for therapeutic disruption. See text for further discussion of A, B, and D.

loenzymes and an increase in AC-small t complexes (Sontag et al., 1993; Chen et al., 2004). However, the disruption of $AB_{PR56}C$ holoenzymes, and not $AB_{PR55}C$ holoenzymes, most likely accounts for the small t-mediated induction of cell proliferation (Chen et al., 2004). Ultimately, fluctuations in the cellular levels of $AB_{PR56}C$ holoenzymes could affect PP2A regulation of the c-Myc transcription factor—a well known oncoprotein. Dephosphorylation of c-Myc by $AB_{PR56}C$ leads to its ubiquitination and subsequent degradation; a mutant form of c-Myc that is resistant to PP2A activity transforms cells in a similar manner to small t transformation (Arnold and Sears, 2006). Altogether, these studies illustrate how altering cellular levels of specific forms of phosphatase holoenzymes can profoundly affect major cellular processes.

Disrupting the PP2B-NFAT Complex

With the great success of the immunosuppressive drugs CsA and FK506 (Fig. 1B), one might question why the PP2B-NFAT complex should be considered for selective disruption by small molecule inhibitors. As discussed in an earlier section (*PP2B Catalytic Subunit as a Drug Target*), certain toxic side effects of CsA and FK506 have directed research toward the development of compounds that specifically inhibit PP2B activity without affecting the functions of the CsA and FK506-binding partners, cyclophilin and FKBP12, respectively. A possible way to achieve specific inhibition of PP2B activity toward NFAT is to block their association, thereby preventing NFAT activation of cytokine gene expression.

One such study uncovered several small molecule antagonists of the PP2B-NFAT interaction, termed INCAs (Inhibitors of NFAT-Calcineurin Association), from a library of more than 16,000 small organic compounds (Roehrl et al., 2004). The INCA compounds bind directly to the NFAT binding site of PP2B and inhibit NFAT activation and subsequent cytokine gene expression in T cells. Although no direct evidence was presented to show a decrease in PP2B-NFAT complexes within the INCA-treated T cells, the desired effect of blocking T-cell activation was accomplished. Thus, the selective targeting and interference of a defined protein-protein interaction, such as PP2B-NFAT, may represent a significant improvement in the design of novel immunosuppressive drugs.

Disrupting the PP1-GADD34 Complex

Apoptosis can be triggered by various signaling events but perhaps one of the least characterized apoptosis-activating pathways is the unfolded protein response, which is induced by extreme ER stress and is critical for the maintenance of cell homeostasis; aberrant regulation of this pathway is linked to several diseases, including Alzheimer's and diabetes (Marciniak and Ron, 2006). A recent screen for chemical compounds that protect the cell from ER stress-induced apoptosis yielded a novel small molecule termed salubrinal (sal) (Boyce et al., 2005). Because sal was shown to promote sustained eIF2α phosphorylation without activating any of four eIF2α kinases, its effect on eIF2α dephosphorylation was scrutinized. Further studies revealed that sal blocks eIF2α dephosphorylation by the PP1-GADD34 complex, which is activated during ER stress (Boyce et al., 2005). A decrease in PP1-GADD34 complexes was also observed in sal-treated cells compared with control cells. Current studies are focused on testing whether this change in the composition of PP1-GADD34 complexes is a result of the direct binding of sal to PP1 or GADD34. Regardless of the outcome, it is evident that small-molecule disruption of a specific phosphatase complex can override a programmed cell response.

The clinical implications from these findings also include a potential treatment for the herpes simplex virus (HSV). Boyce et al. (2005) reported that the sustained dephosphorylation of eIF2α caused by HSV infection can be blocked after sal treatment of HSV-infected cells; the sal-induced increase in eIF2α phosphorylation seems to be due to an inhibition of GADD34/PP1-mediated dephosphorylation of eIF2α. Because sal inhibits HSV replication in both cultured cells and a mouse cornea infection model (Bryant et al., 2008), interference of the PP1-GADD34 complex could become a goal in the design of future anti-HSV drugs.

Disrupting the PP1-HDAC Complex

Histone deacetylases (HDACs) have become a popular target for antitumor drugs, and several HDAC inhibitors are currently in clinical trials (Lin et al., 2006). Although the molecular mechanism(s) underlying the antitumor properties of HDAC inhibitors has not yet been fully elucidated, recent studies indicate that some HDAC inhibitors disrupt PP1-HDAC complexes, resulting in increased dephosphorylation or acetylation of specific substrates (e.g., Akt and tubulin) (Brush et al., 2004; Chen et al., 2005). Treatment of glioblastoma and prostate cancer cells with the HDAC inhibitor trichostatin A (TSA) disrupts the PP1-HDAC6 interaction and promotes increased PP1 association with Akt (Chen et al., 2005). Consistent with this result is the observation that TSA treatment decreased levels of phospho-Akt in target cells—an effect that can be reversed with PP1, but not PP2A, inhibitors (Chen et al., 2005). The dephosphorylation/inactivation of Akt leads to a decrease in cell proliferation, which may explain the therapeutic benefit of HDAC inhibitors (Fig. 1A). Disruption of PP1-HDAC complexes by TSA and other HDAC inhibitors has also been linked to the potentiation of CREB-mediated gene transcription (Michael et al., 2000; Canettieri et al., 2003); however, it remains to be determined whether these changes in gene transcription influence cell growth and proliferation. Furthermore, the tumor suppressor Rb protein has been shown to associate with PP1-HDAC complexes (Guo et al., 2007), but the sensitivity of this complex to TSA or other HDAC inhibitors has not yet been explored. Thus, it seems that the antitumor effects of HDAC inhibitors could, in part, be due to the inhibition of one protein-protein interaction (PP1-HDAC6), which facilitates another protein-protein interaction (PP1-Akt).

Disrupting the PP2A-Raf1 Complex

Raf1 (a.k.a. c-Raf) is an important MAP kinase kinase kinase that functions in the Ras-Raf1-MEK-ERK pathway to transmit mitogenic, differentiative, and oncogenic signals to the downstream kinases MEK (a MAP kinase kinase) and ERK (a MAP kinase). Raf1 is subject to complex regulatory steps that include inter- and intramolecular protein interactions as well as direct phosphorylations (Chong et al., 2003). A key regulator of Raf1 is the Ras, which in its active form recruits Raf1 to the plasma membrane. Ras binding promotes conformational changes in Raf1 that relieve autoinhibition and facilitate phosphorylation of activating sites within the kinase catalytic domain. Although the inactivation mechanism of Raf1 is not fully understood, it is thought

to involve phosphorylation of inhibitory residues and dephosphorylation of activating residues. Recent studies have revealed that two members of the PPP family of phosphatases, PP2A and PP5, associate with Raf1 and function as positive (PP2A) and negative (PP5) modulators of Raf1 activation by dephosphorylating the inhibitory and activating phosphosites, respectively (Abraham et al., 2000; Ory et al., 2003; Adams et al., 2005; von Kriegsheim et al., 2006).

Early investigations demonstrated that the PP2A catalytic subunit associates with several MAPK family members, including Raf1 (Abraham et al., 2000), MEK1 (Liu and Hofmann, 2004), ERK (Liu and Hofmann, 2004), and kinase suppressor of Ras-1 (Ory et al., 2003). Surprisingly, PP2A seems to function as both a positive and negative regulator of the mitogen-activated Ras-Raf1-MEK-ERK signal transduction pathway. Furthermore, recent studies have demonstrated that distinct PP2A holoenzymes differentially modulate multiple steps in this signal transduction pathway (Silverstein et al., 2002; Ugi et al., 2002; Adams et al., 2005; Letourneux et al., 2006). Bα- and Bδ-containing holoenzymes function as positive regulators of Raf1-MEK-ERK signaling by associating with Raf1 and catalyzing the dephosphorylation of an inhibitory site in this kinase (p-S259), resulting in Raf1 activation and consequential phosphorylation/activation of MEK and ERK (Adams et al., 2005). Another recent study has uncovered a negative role for B′-containing PP2A holoenzymes in the activation of ERK-PP2A holoenzymes containing a B′ (a.k.a. B56) family member associate with ERK and trigger the dephosphorylation/inactivation of this kinase (Letourneux et al., 2006).

Because Raf1 is centrally involved in Ras signaling, it can also contribute to oncogenic processes. In fact, deregulated or constitutively active Raf1 proteins can themselves cause cell transformation (Heidecker et al., 1990); Raf1 mutations have also been identified in breast, thyroid, and lung carcinomas, and bone sarcomas (Beeram et al., 2005). Because the Raf1-associated PP2A functions as a positive modulator of Raf1 activation, it is tempting to speculate that the pharmacological manipulation of the Raf1-PP2A interaction could have therapeutic value. For example, disruption of PP2A-Raf1 complexes could be therapeutic for pathological conditions characterized by abnormally high Raf1 activity. Specifically, targeting the Bα/Bδ-binding site on Raf1 or the Raf1-binding site on PP2A with a small molecule antagonist could serve to down-regulate levels of phospho-MEK and phospho-ERK, ultimately suppressing cell growth and proliferation.

Disrupting the PP2A-IKK Complex

The IκB kinase (IKK) complex plays a central role in the activation of NF-κB, a family of transcription factors that regulates the expression of numerous genes involved in the control of cell growth and survival (Hayden and Ghosh, 2008). A variety of stimuli can activate the IKK/NF-κB signal transduction cascade, including tumor necrosis factor-α (TNF), interleukin-1, T- and B-cell mitogens, bacterial products, viral proteins, as well as physical and chemical stress. Activation of the prototypic IKK complex, which is composed of two highly homologous kinases (IKKα and IKKβ) and a regulatory subunit (IKKγ), occurs after signal-induced phosphorylation of T-loop serines within IKKα and IKKβ (Delhase et al., 1999). The principal substrate of IKK is IκB, an inhibitory subunit of NF-κB. When phosphorylated by IKK, IκB is targeted for degradation via the 26S proteasome and NF-κB is free to translocate to the nuclear compartment where it activates transcription units containing the κB-binding site. The post-inductive repression of IKK activity is thought to involve inhibitory phosphorylations of IKK subunits and PP2C- or PP1-mediated dephosphorylation of the T loop serines (Delhase et al., 1999; Prajapati et al., 2004; Li et al., 2008).

Another important player in the IKK activation process is PP2A. Like the Ras-Raf1-MEK-ERK pathway, PP2A has been shown to play a crucial regulatory role at many steps in the IKK–NF-κB signal transduction pathway (Sun et al., 1995; DiDonato et al., 1997; Yang et al., 2001; Fu et al., 2003; Li et al., 2006). Although in vitro studies have revealed that PP2A dephosphorylates and inactivates IKK (DiDonato et al., 1997; Li et al., 2006), Kray and colleagues reported that PP2A functions as a positive modulator of cellular IKK activity via its association with the regulatory IKKγ subunit of the IKK complex (Kray et al., 2005). Removal of the PP2A-binding site in IKKγ impairs TNF-induced activation of IKK, indicating that proper induction of IκB kinase activity is contingent upon formation of IKK-PP2A complexes. Although the precise mechanism of PP2A action on IKK remains unclear, the IKK-associated PP2A may induce the activation of IKK by dephosphorylating inhibitory phosphosites within IKKβ and IKKγ subunits that include multiple serine residues positioned in the C-terminal region of IKKβ and Ser-68 in IKKγ (Delhase et al., 1999; Palkowitsch et al., 2008).

Because dysregulation of IKK activity has been implicated in several human pathologies, including immune and inflammatory diseases (Courtois and Gilmore, 2006; Karin, 2006), it is not surprising that the IKK complex has become an increasingly popular drug target. Moreover, recent studies linking chronic inflammation and persistent activation of the NF-κB pathway to tumorigenesis have underscored the importance of targeting molecular components of this pathway for the treatment of various cancers (Greten et al., 2004; Pikarsky et al., 2004). Given that the IKK-associated PP2A functions as positive modulator of IKK activity (Kray et al., 2005), one would predict that the specific disruption of this kinase-phosphatase interaction should attenuate IKK-dependent NF-κB activation (Fig. 1D). Identification of the precise protein-protein interaction domains within IKK and PP2A should facilitate further studies aimed at the development of small molecule antagonists of this interaction, which may prove to be of therapeutic value for pathological conditions characterized by persistent IKK/NF-κB activation.

Summary

The use of natural compounds indicates that the manipulation of ser/thr phosphatase activity has a broad range of potential therapeutic uses—from treating cancer to promoting a desired immunosuppressive effect. The emerging wealth of structural data for phosphatase holoenzymes will allow for an improved design on natural compounds to obtain more specific catalytic subunit inhibitors. Furthermore, discoveries of small molecule antagonists for macromolecular complexes open up new possibilities for the selective targeting and disruption of specific phosphatase complexes to achieve therapeutic benefits. The list of phosphatase com-

plexes that could potentially be targeted for drug development is continuously growing (Tables 1 and 2). Further studies of the regulation and function of phosphatase macromolecular complexes will lay the foundation for future studies aimed at developing specific antagonists for these complexes, which may ultimately prove to be valuable therapeutic agents for a number of human diseases.

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