A Cell-permeable, Activity-based Probe for Protein and Lipid Kinases*

Muh-ching Yee, Stefanie C. Fas, Michelle M. Stohlmyer, Thomas J. Wandless, and Karlene A. Cimprich‡

From the Department of Molecular Pharmacology, Stanford University, Stanford, California 94305-5441

Protein and lipid kinases are two important classes of biomedically relevant enzymes. The expression and activity of many kinases are known to be dysregulated in a variety of diseases, and proteomic tools that can assess the presence and activity of these enzymes are likely to be useful for their evaluation. Because many of the mechanisms by which protein kinases can become unregulated involve post-translational modifications or changes in protein localization, they can only be detected by examining protein activity, sometimes within the context of the living cell. Wortmannin is a steroid-derived fungal metabolite that covalently inhibits both protein and lipid kinases. Here we describe the synthesis of three wortmannin derivatives, biotin-wortmannin, BODIPY-wortmannin, and tetramethylrhodamine-wortmannin. We demonstrate that these reagents exhibit reactivity similarly as wortmannin and react with members of the phosphatidylinositol 3-kinase and PI3-kinase related kinase families in cellular lysates. Moreover, in some cases these reagents can differentiate between the active and inactive forms of the enzyme, indicating that they are activity-based probes. The reagents also exhibit complementary properties. The biotin-wortmannin reagent is effective in the isolation of labeled proteins; all three can be used for protein labeling, and BODIPY-wortmannin is cell-permeable and can be used to label proteins within cells.

In the post-genomic area, there has been increasing recognition of the need for reagents and tools that can analyze protein expression and activity in complex biological samples. Activity-based protein profiling (ABPP) has emerged as a promising new approach that allows the simultaneous analysis of many proteins, frequently from a common enzymatic family, within the whole proteome (1–4). This approach relies upon the development of bifunctional chemical probes that act in a covalent and site-directed manner with some subset of the proteome and contain a second functional group for either visualization and/or isolation of the probe-labeled proteins. Among the probes that have been developed for ABPP are molecules that target the serine hydrolyases (1, 3, 5, 6), cysteine proteases (7–9), oxidoreductases (10, 11), protein phosphatases (12), and metalloproteases (13). However, probes for many classes of enzymes remain to be identified.

Protein and lipid kinases are two important classes of biomedically relevant enzymes for which no cell-permeable ABPP probes exist. Because the expression and activity of many kinases are known to be dysregulated in a variety of diseases (14, 15), proteomic tools that can assess the presence and activity of these enzymes are likely to be useful for their evaluation. Moreover, many of the mechanisms by which protein kinases can become unregulated involve post-translational modifications or changes in protein localization. Thus, these mechanisms can only be detected by examining protein activity, sometimes within the context of a living cell.

One important class of kinases involved in a variety of physiological and pathological processes related to cancer, inflammation, immunology, and cardiovascular disease is the phosphoinositide 3-kinase (PI 3-kinase) family (14). Members of the PI 3-kinase family phosphorylate the 3′-hydroxyl position of the inositol head group of member phosphoinositides. Eight mammalian PI 3-kinases have been identified and categorized into three main classes (16). Another related family of kinases is the phosphoinositide 3-kinase-related kinase (PIKK) family. Though related in sequence to the lipid kinases, these enzymes act as protein kinases and have no apparent lipid kinase activity (reviewed in Refs. 17 and 18). In mammalian cells, members of this family include ATM, ATR, DNA-PKcs, mammalian target of rapamycin (mTOR), and SMG-1, and they function in many processes related to cellular stress responses including DNA damage checkpoints, double strand break repair, nutrient and growth factor sensing, and nonsense-mediated mRNA decay. Importantly, many of the PIKKs are also involved in physiological and pathological processes related to cancer, and the loss of at least one of these kinases, ATM, predisposes affected individuals to cancer (19, 20).

Wortmannin is a fungal metabolite (21) that was originally identified as a potent and selective inhibitor of PI 3-kinase family members (22, 23). A variety of recent studies have shown that wortmannin also inhibits members of the PIKK family, albeit with reduced potency (24). Wortmannin reacts in an irreversible manner with a critical lysine residue in the kinase domain of the PI 3-kinases, and this lysine is conserved in the different PI 3-kinase and PIKK family members (25).

* This work was supported by National Institutes of Health Grants GM062193 and GM068559 (the latter to T. J. W.), American Cancer Society Grant 99-241-01-CCG, and a Burroughs-Wellcome New Investigator Award in Toxicology (to K. A. C.). 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.

† A Leukemia and Lymphoma Society Scholar and to whom correspondence should be addressed: Dept. of Molecular Pharmacology, Clark Center, 318 Campus Dr., Stanford, CA 94305-5441. Tel.: 650-498-4720; Fax: 650-725-4665; E-mail: cimprich@stanford.edu.

‡ A Leukemia and Lymphoma Society Scholar and to whom correspondence should be addressed: Dept. of Molecular Pharmacology, Clark Center, 318 Campus Dr., Stanford, CA 94305-5441. Tel.: 650-498-4720; Fax: 650-725-4665; E-mail: cimprich@stanford.edu.

The abbreviations used are: ABPP, activity-based protein profiling; ATN, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; BODIPY, boron dipyrromethene difluoride; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; GST, glutathione S-transferase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PI, phosphoinositide; PIKK, PI 3-kinase-related kinase; PVD, polyvinylidene difluoride; TMR, tetramethylrhodamine.

Received for publication, April 29, 2005, and in revised form, June 21, 2005. Published, JBC Papers in Press, June 22, 2005, DOI 10.1074/jbc.M504730200
an irreversible, covalent inhibitor with a number of targets, wortmannin is an ideal starting point for development of a new ABPP probe. Here we describe the synthesis and characterization of three derivatives of wortmannin, namely biotin-wortmannin, BODIPY-wortmannin, and TMR-wortmannin. All three wortmannin derivatives react with members of the PI 3-kinase and PIKK families in cellular lysates, and one, BODIPY-wortmannin, is also cell-permeable.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Lines—**Wortmannin derivatives were stored at −20 °C in MeSO. Antibodies for ATM were a gift of Dr. Michael Kastan. Antibodies for DNA-PKcs (Serotec), FLAG (M5, Sigma), and N-Myc (Santa Cruz Biotechnology) are commercially available. ATR (26) and ATR-interacting protein (ATRIP) (27) antibodies have been provided by Dr. Jonathan Backer (Albert Einstein College of Medicine). N-Myc (Santa Cruz Biotechnology) are commercially available. ATR

**Preparation and Labeling of Cell Lysates—**Nuclear lysates were prepared as described using HeLa cell pellets from the National Cell Culture Center (Cat.). When whole cell lysates were prepared, cells were washed with PBS and then scraped into 1 ml of PBS with 0.5 mM EDTA and transferred to a microfuge tube. Cells were washed again with PBS before flash freezing or lysis. Cells were lysed in TGN (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween, and 0.2% Nonidet P-40) or MIP buffer (20 mM NaH₂PO₄, pH 7.2, 150 mM NaCl, 0.4% Nonidet P-40, and 5% glycerol) with phosphatase inhibitors (50 mM β-glycerophosphate, 1 mM NaF, and 1 mM Na₃VO₄) and protease inhibitors (10 µg/ml leupeptin and pepstatin and 0.1 mg/ml phenylmethylsulfonyl fluoride) unless otherwise indicated. Cell lysates were adjusted to 2 mg/ml before the addition of wortmannin derivatives. After labeling, lysates were quenched by the addition of one-sixth volume of 6× sample buffer (made without bromophen blue when used for fluorescent detection) and boiled.

**Kinase Assays—**Kinase reactions were performed on FLAG-ATR precipitates prepared as described (29). Kinase reactions contained GST-RAD17 (1.5 µg) in 27.5 µl of kinase buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, and 10 mM MnCl₂) with 1 µl (10 µCi) of [γ-³²P]ATP and 50 µM cold ATP. Reactions were incubated for 15 min at 30 °C and then stopped by the addition of 10 µl of 6× SDS sample buffer and heating for 5 min at 95 °C. GST-Rad17 SQ2 was prepared as described (30). Incubation with wortmannin/wortmannin derivatives was carried out in kinase buffer without ATP for 15 min at 30 °C.

**For DNA-PK kinase reactions, purified DNA-PK (100 units/ml, 0.5 µM) of Promega was incubated in a total volume of 30 µl containing 2 µl of E coli-R1 digested plasmid (100 ng/µl), 18.5 µl of buffer B (25 mM HEPES-KOH, pH 7.5, 12.5 mM MgCl₂, 20% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, and 50 mM NaCl), 7.5 µl of H₂O, and wortmannin/wortmannin derivative for 10 min at 22 °C. This mixture was then added 0.3 µl [γ-³²P]ATP, 0.8 µl of 10 mM ATP, 4 µl of buffer A (13 mM spermidine and 4 mM MgCl₂), 3 µl of buffer B, and 2 µg of GST-p53(1-101) (2 µg/µl) prepared as described (29). The mixture was incubated for 15 min at 30 °C and then stopped by the addition of 10 µl of 6× SDS sample buffer and heating for 8 min at 95 °C.

**In Vivo Labeling of Cells—**For competition experiments, cells were first incubated with wortmannin (30 µM) for 15 min. BODIPY-wortmannin was then added to the medium to 10 µM, and cells were incubated for another 30 min. After labeling, the medium was removed and cells were washed 2 times with PBS, scraped into 1 ml of PBS with 0.5 mM EDTA, transferred to a microfuge tube, and washed twice more with PBS before lysis.

**Visualization of Wortmannin Derivatives**

**Biotin-Wortmannin—**Before labeling with biotin-wortmannin, samples were precleared by incubating with streptavidin-Sepharose beads (60 µl of 2 mg/ml lysate to 20 µl of streptavidin-Sepharose; Amersham Biosciences) to reduce nonspecific binding to biotin. After biotin-wortmannin labeling, samples were run on 6% acrylamide gels and blotted to a PVDF membrane. The membrane was blocked by incubation in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat milk for 1 h at room temperature, and the membrane was then washed four times for 5 min each in TBST. Streptavidin-HRP (1 mg/ml stock in PBS; Molecular Probes) was added at 1:2000 in TBST, and the blot was incubated for 1 h at room temperature. The blot was washed 4 × 10 min in TBST and then developed with ECL Plus (Amersham Biosciences).

**TMR-Wortmannin—**After labeling and quenching, samples were run on an acrylamide gel. After electrophoresis, the gel, still sandwiched between glass plates, was scanned on an Amersham Biosciences Typhoon PhosphorImager at an excitation of 532 nm, and emission was detected using a 580-nm BP30 filter.

**BODIPY-Wortmannin—**Samples were run in the same manner as for TMR-wortmannin, except that the gel was scanned at an excitation of 488 nm and emission was detected using a 520-nm BP40 filter.

**RESULTS**

**Synthesis of Biotin-, BODIPY-, and TMR-Wortmannin Derivatives—**Structure-activity studies of wortmannin derivatives have revealed that, although the acylation of the C11 hydroxyl group appears to be important for activity, there is considerable flexibility in the size of the ester substituent that can be accommodated at this position (31). Based on these studies, we treated wortmannin with diethylamine, which resulted in rapid opening of the furan ring followed by decaylation of the C11 acetate functional group. Subsequent treatment with hydrochloric acid reformed the furan ring producing 11-O-deacetylwortmannin in high yield. This compound was acylated with a biotin derivative to produce biotin-wortmannin, in which the two compounds are linked by a short polyethylene glycol-derived linker (Fig. 1). As a control compound we prepared the methyl ester of the biotin derivative. Treatment of deacetylwortmannin with a BODIPY-derived carboxylic acid and a coupling reagent produced BODIPY-wortmannin with fluorescent properties similar to those of fluorescein. Finally, deacetylwortmannin was acylated with t-butoxycarbonyl-protected aminovaleric acid. Removal of the t-butoxycarbonyl group revealed a primary amine, which was directly acylated using an N-hydroxysuccinimide ester derivative of tetracyanmethyldihydramine to produce TMR-wortmannin.

**Biotin-Wortmannin Inhibits ATR and DNA-PK Kinase Activity—**Wortmannin is known to inhibit members of the PI 3-kinase and PIKK families. To determine whether our biotin-wortmannin reagent could inhibit these kinases, we performed in vitro kinase assays with two of these kinases. DNA-PK is a member of the PIKK family that is activated by double-stranded DNA ends and can phosphorylate a number of different proteins in vitro (17, 32). We tested the ability of wortmannin to inhibit purified DNA-PK in vitro using GST-p53 as a substrate (Fig. 2A). The phosphorylation of p53, which led to a decrease in the mobility of this protein by SDS-PAGE, was monitored by incorporation of [γ-³²P]ATP. Upon the addition of increasing concentrations of biotin-wortmannin, we observed decreased phosphorylation of p53 with significant inhibition at biotin-wortmannin concentrations of 1–2 µM. Detection of biotin-wortmannin-labeled DNA-PKcs using streptavidin-HRP was first observed at 1 µM and increased as the concentration of biotin-wortmannin increased. These results indicate that biotin-wortmannin inhibits DNA-PKcs kinase activity in vitro and further suggest that the interaction between biotin-wortmannin and DNA-PKcs is covalent. We also tested the ability of our biotin-wortmannin reagent to inhibit the kinase activity of ATR, another member of the PIKK family (Fig. 2B). In this case, HEK293T cells were transfected with FLAG-tagged ATR, and FLAG proteins were precipitated with an antibody to the FLAG epitope. GST-Rad17
was then added to each sample as the substrate, and the incorporation of \([\gamma-^{32}P]ATP\) was monitored by autoradiography. The GST-Rad17 phosphorylation signal decreased significantly starting at biotin-wortmannin concentrations of 20–35 \(\mu M\). Labeling of ATR was first observed at 10 \(\mu M\) and increased with increasing concentrations of biotin-wortmannin.

The reactivity of biotin-wortmannin was compared with the reactivity of wortmannin by determining the inhibitory concentration (50%) (IC50) of each compound for DNA-PKcs and ATR with these assays (data not shown). Three to four experiments were carried out for each condition in which the substrate phosphorylation was quantified. For the ATR kinase assay, these values were corrected for equal ATR expression by taking the anti-FLAG Western signal as a reference. Analysis of the resulting data indicates that biotin-wortmannin is less potent than wortmannin for both ATR and DNA-PKcs. Under the conditions of these experiments, the IC50 of wortmannin for ATR was 1.8 \(\mu M\), whereas the IC50 for biotin-wortmannin was 10.8 \(\mu M\). For DNA-PKcs, an IC50 of 0.14 \(\mu M\) was observed for wortmannin and an IC50 of 1.15 \(\mu M\) was observed for biotin-wortmannin. This observation indicates that the conjugation of wortmannin to biotin resulted in a 6- to 8-fold decrease in its potency.

**Identification and Isolation of Wortmannin-sensitive Kinases with Biotin-Wortmannin**—One application for use of the biotin-wortmannin reagent is in the isolation and identification of wortmannin-labeled proteins. To determine whether our biotin-wortmannin reagent could be used as an affinity reagent, we examined its ability to precipitate known wortmannin targets at concentrations ranging from 0.5 to 50 \(\mu M\) after incubation with streptavidin-Sepharose. Because of our interest in members of the PIKK family and their predominant localization to the nucleus, we used nuclear extracts for these experiments and tested for the presence of these family members in pull-down experiments. We found that ATM, ATR, and DNA-PKcs, three members of the PIKK family, were present in the biotin-wortmannin precipitates but not in precipitates pre-
pared using the biotin control compound (Fig. 3A). DNA-PKcs was observed in precipitates prepared from extracts treated with 0.5 μM biotin-wortmannin at 4 °C, whereas ATM was first observed in precipitates after treatment with 2 μM biotin-wortmannin reagent. ATR was only detected in precipitates obtained from extracts treated with 10 μM biotin-wortmannin, and, for efficient precipitation of ATR, incubation at 30 °C was necessary. This reactivity correlates with the known sensitivity of these kinases to wortmannin (24).

To determine whether this labeling occurred in a specific manner, we tested the ability of several agents to compete with biotin-wortmannin for the target proteins. To do so, a HeLa cell nuclear extract was incubated with either 150 μM biotin-wortmannin at 4 °C, whereas ATM was first observed in precipitates after treatment with 2 μM biotin-wortmannin reagent. ATR was only detected in precipitates obtained from extracts treated with 10 μM biotin-wortmannin, and, for efficient precipitation of ATR, incubation at 30 °C was necessary. This reactivity correlates with the known sensitivity of these kinases to wortmannin (24).

To determine whether this labeling occurred in a specific manner, we tested the ability of several agents to compete with biotin-wortmannin for the target proteins. To do so, a HeLa cell nuclear extract was incubated with either 150 μM biotin-wortmannin or 1 mM ATP prior to treatment with biotin-wortmannin (30 μM) and subsequent precipitation with streptavidin-Sepharose. The precipitated proteins were then analyzed by Western blot with antibodies to either DNA-PKcs or ATM. As shown in Fig. 3B, incubation of lysates with wortmannin before the addition of biotin-wortmannin significantly reduced the efficiency with which ATM and DNA-PKcs were precipitated. Prior denaturation by incubation at 95 °C also prevented the precipitation reaction, as no ATM or DNA-PKcs was detected in the precipitates. These results suggest that ATM and DNA-PKcs are specifically labeled by the biotin-wortmannin compound. As heat treatment also prevents wortmannin-mediated precipitation, we hypothesize that proper folding of the proteins is necessary for its specific reaction with the wortmannin compound. This hypothesis is consistent with data suggesting that wortmannin reacts with a specific lysine in the catalytic site of sensitive kinases (25).

**TMR-Wortmannin Targets DNA-PKcs and p110—**Another potential use for our labeled wortmannin derivatives is the labeling of complex mixtures of proteins in cellular extracts. To determine whether this was feasible with the TMR-wortmannin derivative, we first tested the ability of TMR-wortmannin to label protein in two cell lines derived from the same tumor, lacking DNA-PKcs (MO59J) and another containing this protein (MO59K) (33). As shown in Fig. 4A, incubation of TMR-wortmannin with whole cell extracts prepared from MO59K cells for 5 min at 25 nM led to labeling of a high molecular mass protein as well as several lower molecular mass proteins ranging between 95 and 110 kDa. However, in lysates prepared from MO59J cells incubated under the same conditions, no labeling of the high molecular mass band was observed. This observation strongly suggests that the high molecular mass protein is the 450-kDa catalytic subunit of DNA-PK.

The four lower molecular mass proteins observed upon the incubation of TMR-wortmannin with this whole cell lysate were of the size expected for members of the PI 3-kinase family of lipid kinases. Members of this family are potently inhibited by wortmannin, and labeling of these three/four bands could be blocked by prior incubation with low concentrations of wortmannin (data not shown; see also Fig. 5B), suggesting they might be members of this family. To directly determine whether the p110 protein could be labeled by wortmannin in cell lysates, we transfected cells with an expression vector for Myc-tagged p110. As shown in Fig. 4A, incubation of TMR-wortmannin with whole cell extracts prepared from MO59K cells for 5 min at 25 nM led to labeling of a high molecular mass protein as well as several lower molecular mass proteins ranging between 95 and 110 kDa. However, in lysates prepared from MO59J cells incubated under the same conditions, no labeling of the high molecular mass band was observed. This observation strongly suggests that the high molecular mass protein is the 450-kDa catalytic subunit of DNA-PK.

The four lower molecular mass proteins observed upon the incubation of TMR-wortmannin with this whole cell lysate were of the size expected for members of the PI 3-kinase family of lipid kinases. Members of this family are potently inhibited by wortmannin, and labeling of these three/four bands could be blocked by prior incubation with low concentrations of wortmannin (data not shown; see also Fig. 5B), suggesting they might be members of this family. To directly determine whether the p110 protein could be labeled by wortmannin in cell lysates, we transfected cells with an expression vector for Myc-tagged p110α either alone or together with a vector for hemagglutinin-tagged p85, the regulatory subunit of PI 3-kinase (Nmyc-p110) or with the catalytic and regulatory subunits of PI 3-kinase (Nmyc-p110 and HA-p85). Cell lysates were incubated with TMR-wortmannin as described for panel A, separated by SDS-PAGE (8%), and scanned on the PhosphoImager as described for panel A. The gel was also blotted to a PVDF membrane and probed with anti-Myc antibody to confirm the expression of N-Myc p110.

**Profiling Protein Kinases with Fluorescent Wortmannin Probes—**To test the ability of BODIPY-wortmannin and TMR-wortmannin to resolve activity and expression patterns between different cell types, we compared the profiles of wortmannin-labeled proteins from extracts of several different cell lines using a low concentration (100 nM) of each probe (Fig. 5A).
Although most of the proteins showed similar patterns of expression within this limited group of cell lines, some differences were noted. The 450-kDa protein identified as DNA-PKcs was expressed in all of the human cell lines studied with the exception of the MO59J cells, which are known to lack this protein. However, little labeling of DNA-PKcs was observed in the mouse 3T3 cell line, suggesting that mouse cells express low levels of this protein. Another protein of 60–65 kDa was found expressed in all of the human cell lines studied with the exception of the MO59J cells, which are known to lack this protein.

To examine the specificity of labeling with these reagents, we preincubated lysates with wortmannin prior to incubation with the labeled wortmannin derivative. As shown in Fig. 5A, a subset of the proteins labeled by BODIPY-wortmannin and TMR-wortmannin at 1 μM was not labeled when extracts were pretreated with an excess of wortmannin. Notably, most of the bands that disappeared when wortmannin was used as a competitor were also found to be labeled when a lower concentration (100 nM) of the fluorescent wortmannin derivatives was used as shown in Fig. 5A. As additional controls, we also determined the effect of staurosporine and LY294002 on the labeling of the PI 3-kinase family members (Fig. 5C). Staurosporine is a nonspecific kinase inhibitor, and LY294002 is a non-covalent inhibitor of lipid kinases; both have been shown to bind in the same site as wortmannin (35). Consistent with these structural studies, we found that LY294002 prevented labeling with TMR-wortmannin. Staurosporine also reduced labeling by TMR-wortmannin, albeit weakly, consistent with its lower affinity. These observations indicate that our wortmannin derivatives act in the active site of protein and lipid kinases. Taken together, they also indicate that the fluorescent probes will be useful for profiling the expression of a number of different lipid and protein kinases in cell extracts.

BODIPY-Wortmannin Is Cell Permeable—Optimal use of these wortmannin probes would allow for labeling of proteins within cells. To test the possibility that either TMR-wortmannin or BODIPY-wortmannin could be used for such experiments, we incubated cells with either 10 μM TMR-wortmannin or 10 μM BODIPY-wortmannin for 30 min and prepared cell lysates from the cells after washing. In both cases, a parallel set of samples was preincubated with wortmannin prior to the addition of the labeled probe to determine the specificity of labeling. We found that a number of proteins were labeled in cells treated with the BODIPY-wortmannin, though little labeling was observed in cells treated with TMR-wortmannin.
In this work, we report the synthesis and characterization of three new wortmannin derivatives that are linked via a water-soluble polyethylene glycol linker to biotin or to the fluorescent probes BODIPY or TMR. With these compounds, it is now possible to specifically profile members of the PI 3-kinase and PIKK families as a whole or in groups by varying parameters such as inhibitor concentration. Members of these protein and lipid kinase families play roles in important cellular processes such as signal transduction, cell cycle regulation, and DNA repair. These wortmannin derivatives allow isolation of the modified proteins, efficient detection, and labeling of wortmannin-sensitive proteins within the cell. Thus, these derivatives should be useful reagents for ABPP.

In vitro, the biotin-wortmannin probe appears to have a similar reactivity profile to wortmannin as determined using an anti-wortmannin antibody (data not shown), and the other two probes, BODIPY-wortmannin and TMR-wortmannin, exhibit similar reactivity. Furthermore, because the kinase activity of at least two members of the PIKK family can be inhibited with biotin-wortmannin and the labeling of several proteins can be blocked by prior incubation with wortmannin, it seems likely that these new probes react like wortmannin, with the lysine corresponding to Lys-802 (p110 numbering) in the active site of each kinase. These observations also suggest that attachment of the linker and different reporters (i.e., biotin, BODIPY and TMR) has minimal effect on probe specificity, although some differences were noted. However, the potency of the biotin-wortmannin compound is slightly reduced relative to that of wortmannin. A comparison of the IC50s for wortmannin and biotin-wortmannin for ATR and DNA-PKcs showed a 6- and 8-fold reduction in reactivity, respectively. This decrease is not surprising, as previous groups have shown a 4-fold decrease in activity for 11-O-desacetylwortmannin (31). In this context, it should be noted that some known wortmannin-sensitive kinases were not detectable under our conditions. For example, ATM and ATR were not readily detected under these conditions in whole cell lysates. This could be due to the low abundance of these proteins, their lower reactivity toward wortmannin, or both. In any case, it is likely that enrichment of certain fractions or use of a liquid chromatography-mass spectroscopy system for ABPP would address this limitation (36).

One important consideration involves the use of these compounds as profiling agents either in cells or in lysates. We have shown that BODIPY-wortmannin is cell-permeable, whereas TMR-wortmannin and biotin-wortmannin are only useful for lysates (Fig. 6 and data not shown). The cell-permeable nature of BODIPY-wortmannin makes analysis of wortmannin-sensitive proteins possible in cells. The activity of some proteins may vary under different conditions (e.g. stimulation, starvation and cell cycle stage) and in a manner that can only be detected in the context of a cell because of subcellular localization or

**DISCUSSION**

In vitro, the biotin-wortmannin probe appears to have a similar reactivity profile to wortmannin as determined using an anti-wortmannin antibody (data not shown), and the other two probes, BODIPY-wortmannin and TMR-wortmannin, exhibit similar reactivity. Furthermore, because the kinase activity of at least two members of the PIKK family can be inhibited with biotin-wortmannin and the labeling of several proteins can be blocked by prior incubation with wortmannin, it seems likely that these new probes react like wortmannin, with the lysine corresponding to Lys-802 (p110 numbering) in the active site of each kinase. These observations also suggest that attachment of the linker and different reporters (i.e., biotin, BODIPY and TMR) has minimal effect on probe specificity, although some differences were noted. However, the potency of the biotin-wortmannin compound is slightly reduced relative to that of wortmannin. A comparison of the IC50s for wortmannin and biotin-wortmannin for ATR and DNA-PKcs showed a 6- and 8-fold reduction in reactivity, respectively. This decrease is not surprising, as previous groups have shown a 4-fold decrease in activity for 11-O-desacetylwortmannin (31). In this context, it should be noted that some known wortmannin-sensitive kinases were not detectable under our conditions. For example, ATM and ATR were not readily detected under these conditions in whole cell lysates. This could be due to the low abundance of these proteins, their lower reactivity toward wortmannin, or both. In any case, it is likely that enrichment of certain fractions or use of a liquid chromatography-mass spectroscopy system for ABPP would address this limitation (36).

One important consideration involves the use of these compounds as profiling agents either in cells or in lysates. We have shown that BODIPY-wortmannin is cell-permeable, whereas TMR-wortmannin and biotin-wortmannin are only useful for lysates (Fig. 6 and data not shown). The cell-permeable nature of BODIPY-wortmannin makes analysis of wortmannin-sensitive proteins possible in cells. The activity of some proteins may vary under different conditions (e.g. stimulation, starvation and cell cycle stage) and in a manner that can only be detected in the context of a cell because of subcellular localization or
association with other proteins. This, in turn, may influence the reactivity of these proteins with the wortmannin probes. Thus, the ability to label proteins with BODIPY-wortmannin within a cell provides a potentially powerful tool for examining the biological activity of wortmannin-sensitive kinases within their native environment.

Our data suggest that these wortmannin probes can be used to monitor differences in the activation state of at least some of the proteins targeted. Although we saw no difference in the reactivity of P13-kinase family members with TMR-wortmannin in either starved or platelet-derived growth factor-stimulated cells (data not shown), we did see a modest but reproducible effect of phosphatase inhibitors on the labeling of DNA-PKcs with biotin-wortmannin. This suggests that the sensitivity of DNA-PKcs toward wortmannin is influenced by its phosphorylation state and is consistent with experiments that demonstrate the regulation of DNA-PKcs by phosphorylation (37, 38). Moreover, as our work was being written up, another group reported the synthesis of a related wortmannin-derived activity-based probe and showed that it inhibited Polo-like kinase (PLK) in an activity-dependent manner (39). It is likely that PLK is the 65-kDa protein observed in our samples that was labeled both in lysates and cells. Our probes are complementary to the TMR-derived probe described by Liu et al. (39), and we anticipate that the cell-permeable nature of our BODIPY probe will be particularly useful.

Although the full scope of these probes’ utility in profiling kinase activity is not yet clear, what is clear is that these probes will be useful for proteomic studies designed to profile protein levels and to identify new wortmannin-sensitive targets in mammalian cells and other species. The TMR-wortmannin probe was clearly superior to the biotinylated probe for the profiling studies. It was significantly more sensitive than both the BODIPY-wortmannin and biotin-wortmannin probes (data not shown) and, unlike the biotin-wortmannin probe, did not require pre-clearing of lysates to remove proteins that bind to streptavidin. However, the biotinylated derivative is useful for protein isolation. Although several of the proteins labeled in our lysates were readily identified, some were not, and the biotinylated derivative should be useful in isolation and characterization of these proteins.

In summary, we have shown that three wortmannin-derived, activity-based probes can be useful for profiling kinases both in lysates and living cells. These probes have complementary properties that should allow exploration and identification of wortmannin-sensitive kinases and their biological activity both in cell lysates and in their native environment of the cell.

REFERENCES

1. Liu, Y., Patricelli, M. P., and Cravatt, B. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14694–14699
2. Jessani, N., and Cravatt, B. F. (2004) Curr. Opin. Genet. Dev. 14, 54–59
3. Jessani, N., Humphrey, M., McDonald, W. H., Nissen, S., Masuda, K., Gan-gadharan, B., Yates, J. R., III, Mueller, B. M., and Cravatt, B. F. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13756–13761
4. Jeffery, D. A., and Bogoy, M. (2003) Curr. Opin. Biotechnol. 14, 87–95
5. Kidd, D., Liu, Y., and Cravatt, B. F. (2001) Biochemistry 40, 4005–4015
6. Patricelli, M. P., Giang, D. K., Stamp, L. M., and Burbaum, J. A. (2001) Proteomics 1, 1067–1071
7. Greenbaum, D., Medzhizhaysky, K. F., Burlingame, A., and Bogoy, M. (2000) Chem. Biol. 7, 569–581
8. Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997) EMBO J. 16, 2271–2281
9. Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Ressler, B. M. (2002) Chem. Biol. 9, 1149–1159
10. Adam, G. C., Cravatt, B. F., and Sorensen, E. J. (2001) Chem. Biol. 8, 81–95
11. Adam, G. C., Sorensen, E. J., and Cravatt, B. F. (2002) Nat. Biotechnol. 20, 895–899
12. Kumar, S., Zhou, B., Liang, F., Wang, W. Q., Huang, Z., and Zhang, Z. Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7943–7948
13. Saghatelian, A., Jessani, N., Joseph, A., Humphrey, M., and Cravatt, B. F. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10000–10005
14. Wymann, M. P., and Marone, R. (2005) Curr. Opin. Cell Biol. 17, 141–149
15. Noble, M. E., Endicott, J. A., and Johnson, L. N. (2004) Science 303, 1800–1805
16. Anderson, K. E., and Jackson, S. P. (2003) Int. J. Biochem. Cell Biol. 35, 1028–1033
17. Bakkenist, C. J., and Kastan, M. B. (2004) Cell 118, 9–17
18. Abraham, R. T. (2004) Science 303, 1379–1387
19. Kastan, M. B., and Bartek, J. (2004) Cell 127, 1363–1368
20. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999) Cancer Res. 59, 4375–4382
21. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirolo, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) Mol. Cell. Biol. 16, 1722–1733
22. Schmidt, D. R., and Schreiber, S. L. (1999) Biochemistry 38, 14711–14717
23. Bar-Shira, A., Pecker, I., Russell, P., Jorgensen, T. J., Tsarfati, I., and Shilo, Y. (1997) Oncogene 15, 159–167
24. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
25. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999) J. Biol. Chem. 274, 37535–37543
26. Creemer, L. C., Kirts, H. A., Vlahos, C. J., and Schultz, R. M. (1996) J. Med. Chem. 39, 5021–5024
27. Smith, G. C. M., and Jackson, S. P. (1999) Genes Dev. 13, 916–934
28. Lees-Miller, S. P., Godbout, R., Curtis, P., Hemming, H., and Norris, G. (1997) Trans. Brit. Mycol. Soc. 109, 365–368
29. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirolo, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) Mol. Cell. Biol. 16, 1722–1733
30. Schmidt, D. R., and Schreiber, S. L. (1999) Biochemistry 38, 14711–14717
31. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirolo, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996) Mol. Cell. Biol. 16, 1722–1733
32. Smith, G. C. M., and Jackson, S. P. (1999) Genes Dev. 13, 916–934
33. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, B. S., III, Barron, G. M., and Alahoumis-Turner, J. (1995) Science 267, 1183–1185
34. Yu, J., Zhang, Y., McIlroy, A., Rass, G. A., and Backer, J. M. (1999) Mol. Cell. Biol. 18, 1379–1387
35. Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., and Wymann, M. P., and Williams, R. L. (2000) Mol. Cell. Biol. 6, 909–919
36. Adam, G. C., Burbaum, J., Kozarich, J. W., Patricelli, M. P., and Cravatt, B. F. (2004) J. Am. Chem. Soc. 126, 1363–1368
37. Chen, D. W., Hestrin, S., Prithvirajan, S., Kuriyama, A., Story, M. D., Qin, J., and Chen, D. J. (2002) Genes Dev. 16, 2333–2338
38. Douglas, M., Sapkota, G. P., Horvitz, C. T., Goddard, A. A., Merkle, D., Meek, K., Alessi, D. R., and Wymann, M. P. (2002) Biochem. J. 368, 243–251
39. Liu, Y., Shreder, K. R., Gai, W., Cerrral, S., Ferris, D. K., and Rosenblum, J. S. (2005) Chem. Biol. 12, 99–107