Trifunctional Chemical Probes for the Consolidated Detection and Identification of Enzyme Activities from Complex Proteomes*§

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Chemical probes that covalently modify the active sites of enzymes in complex proteomes are useful tools for identifying enzyme activities associated with discrete (patho) physiological states. Researchers in proteomics typically use two types of activity-based probes to fulfill complementary objectives: fluorescent probes for rapid and sensitive target detection and biotinylated probes for target purification and identification. Accordingly we hypothesized that a strategy in which the target detection and target isolation steps of activity-based proteomic experiments were merged might accelerate the characterization of differentially expressed protein activities. Here we report the synthesis and application of trifunctional chemical proteomic probes in which elements for both target detection (e.g. rhodamine) and isolation (e.g. biotin) are appended to a sulfonate ester reactive group, permitting the consolidated visualization and affinity purification of labeled proteins by a combination of in-gel fluorescence and avidin chromatography procedures. A trifunctional phenyl sulfonate probe was used to identify several technically challenging protein targets, including the integral membrane enzyme 3β-hydroxysteroid dehydrogenase/Δ5-isomerase and the cofactor-dependent enzymes platelet-type phosphofructokinase and type II tissue transglutaminase. The latter two enzyme activities were significantly up-regulated in the invasive estrogen receptor-negative (ER(−)) human breast cancer cell line MDA-MB-231 relative to the non-invasive ER(+) breast cancer lines MCF7 and T-47D. Collectively these studies demonstrate that chemical proteomic probes incorporating elements for both target detection and target isolation fortify the important link between the visualization of differentially expressed enzyme activities and their subsequent molecular identification, thereby augmenting the information content achieved in activity-based profiling experiments. Molecular & Cellular Proteomics 1:828–835, 2002.

Proteomic research aims to develop and apply methods to characterize the molecular and cellular function of the greater than 30,000 protein products encoded by the human genome (1, 2). These efforts typically strive to provide a global analysis of either protein expression or protein function. Conventional approaches for the characterization of protein expression rely on two-dimensional gel electrophoresis, protein staining, and mass spectrometry (MS)¹ methods for the separation, detection, and identification of proteins, respectively (3). Although two-dimensional gel electrophoresis-MS methods are capable of determining the relative abundance and modification states of numerous proteins from endogenous sources (4), these approaches offer only an indirect estimate of protein function and may fail to detect critical posttranslational forms of regulation such as those mediated by protein-protein and/or protein-small molecule interactions (5).

Recently strategies have emerged to profile the activity of enzyme superfamilies in complex proteomes using affinity-tagged chemical probes (6). These active site-directed probes profile proteins on the basis of function rather than abundance and are therefore capable of distinguishing, for example, active proteases from their inactive zymogens and/or inhibitor-bound forms (7, 8). To date, most efforts to create activity-based proteomic probes have exploited well known affinity labels as reactive groups, resulting in the generation of distinct sets of reagents that profile serine hydrolases (7, 9) and subclasses of cysteine proteases (8, 10). Recently serine hydrolase-directed probes were used to generate enzyme activity profiles that classified human breast and melanoma cancer cell lines into subtypes based on tissue of origin and state of invasiveness (11), indicating that the information content achievable in activity-based proteomic experiments is of sufficient quantity and quality to depict higher order cellular properties.

To accelerate the discovery of activity-based proteomic probes for enzyme classes lacking cognate affinity labeling reagents, we have introduced a non-directed or combinatorial strategy in which libraries of candidate probes are screened

¹ The abbreviations used are: MS, mass spectrometry; 3HSD1, 3β-hydroxysteroid dehydrogenase/Δ5-isomerase-1; TriPS, trifunctional phenyl sulfonate probe; ER, estrogen receptor; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; NHS, N-hydroxysuccinimide; pPFK, platelet-type phosphofructokinase; PS-rhodamine, rhodamine-conjugated phenyl sulfonate probe; tTG, type II tissue transglutaminase; FTMS, Fourier transform MS; DHB, 2,5-dihydroxybenzoic acid.
against complex proteomes for activity-dependent protein reactivity (12, 13). Through a two-tiered strategy utilizing rhodamine-conjugated probes for rapid and sensitive target detection and biotin-conjugated probes for target isolation and molecular identification, members of a probe library bearing a sulfonate ester reactive group were found to label in an activity-based manner enzymes from at least six mechanistically distinct classes (13). During these studies, however, we noted that certain sulfonate targets evaded molecular characterization. These proteins tended to exhibit "difficult" properties, such as membrane association, context-dependent labeling, and/or co-migration with endogenous biotinylated proteins, that frustrated efforts to proceed from the stage of target detection to target identification. Accordingly we hypothesized that a method in which the target detection and target purification steps of activity-based proteomic experiments were consolidated might facilitate the characterization of such recalcitrant protein targets. Here we report the synthesis of a class of trifunctional chemical proteomic probes in which both rhodamine and biotin tags are coupled to a sulfonate ester reactive group, thereby permitting the simultaneous visualization and affinity isolation of activity-based protein targets by in-gel fluorescence scanning and avidin chromatography, respectively. Using these trifunctional probes, we report the molecular characterization of several protein targets previously resistant to characterization by the two-tiered strategy described above. These targets include the integral membrane enzyme 3β-hydroxysteroid dehydrogenase/Δ5-isomerase and two cofactor-dependent enzymes, platelet phosphofructokinase and type II tissue transglutaminase. Notably, the latter two enzymes were significantly up-regulated in the invasive estrogen receptor-negative (ER(−)) human breast cancer cell line MDA-MB-231 relative to non-invasive ER(+) cell lines MCF7 and T-47D.

**EXPERIMENTAL PROCEDURES**

**Chemical Synthesis of Trifunctional Sulfonate Ester Probes**—To permit the generation of libraries of trifunctional probes, a synthetic strategy was elaborated for the late stage incorporation of the reactive group. All reactions were carried out under an atmosphere of argon unless specified. Commercial reagents of high purity were purchased and used without further purification unless otherwise noted.

To a solution of carboxylic acid (2) (Bachem, Torrance, CA; 0.06 g, 0.120 mmol, 1.0 equivalents (eq)) in N,N-dimethylformamide (3 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.032 g, 0.170 mmol, 1.4 eq) and N-hydroxsuccinimidine (NHS) (0.032 g, 0.280 mmol, 2.3 eq). After stirring for 12 h at 25 °C, the reaction mixture was poured into saturated aqueous NaHCO3 solution (5 ml), and the product was extracted with ethyl acetate (3 × 5 ml). The organic layer was washed with water (15 ml) and saturated aqueous NaCl (15 ml), dried (MgSO4), and concentrated under reduced pressure. The crude NHS ester (0.070 g, 0.120 mmol, 3.5 eq) was dissolved in methanol (2 ml) followed by the addition of 5-(biotinamido)-pentylyamine (Pierce; 0.015 g, 0.034 mmol, 1.0 eq). After stirring for 2 h at 25 °C, the solvent was evaporated under reduced pressure, and the remaining residue was washed with ethyl acetate (2 × 4 ml), solubilized in a minimal volume of chloroform, and transferred to a clean glass vial, and the solvent was evaporated. The process was repeated to rid the desired biotinylated intermediate of excess reagents and byproducts, affording the desired chemical probe (250 μg stock in dimethyl sulfoxide), and the reactions were incubated for 1 h at 25 °C before quenching with 1 mL of SDS-PAGE loading buffer (reducing). Quenched reactions were separated by SDS-PAGE (30 μg of protein/gel lane) and visualized in-gel using a Hitachi FMBio IIe flatbed laser-induced fluorescence scanner (MiraiBio, Alameda, CA). Labeled proteins were quantified by measuring integrated band intensities (normalized for volume).

**Cancer Cell Line Preparation**—Breast cancer cell lines were grown to 80% confluency in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum and harvested, sonicated, and Dounce homogenized in 50 mM Tris-HCl, pH 8.0 (Tris buffer). After centrifugation at 100,000 × g (40 min), the supernatant was collected as the soluble fraction, adjusted to 2 mg of protein/ml with Tris buffer, and labeled as described above.

**Enrichment and Molecular Characterization of Sulfonate-reactive Proteins**—For affinity isolation of protein targets directly from tissue or cell lines fractions, −8 mg of total protein was used as starting material (equivalent to ~2 × 107 cells). Samples diluted to 2.5 ml with Tris buffer were labeled with the TriPS probe (5 μM) for 2.5 h at 25 °C and then applied to a PD-10 size exclusion column and eluted with 3.5 ml of Tris buffer. For unsolubilized membrane samples, Triton X-100 was added to a final concentration of 1.0%, and the samples were rotated for 1 h prior to passage over a PD-10 column and elution with Tris buffer with 0.1% Triton X-100. Desalted samples were fractionated by
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Q-Sepharose chromatography, and fractions containing the desired targets were affinity-isolated using avidin-agarose beads (Sigma) as described previously (7, 12). Affinity-isolated proteins were separated by SDS-PAGE, excised from the gel, and digested with trypsin. The resulting peptides were analyzed by matrix-assisted laser desorption mass spectrometry (Kratos Axima CFRIA MALDI-TOF instrument, Kratos Analytical, Chestnut Ridge, NY). The MS data were used to search public data bases to identify the sulfonate-labeled proteins.

Recombinant Expression of Enzymes in Eukaryotic Cells—cDNAs corresponding to each sulfonate target were purchased as expressed sequence tags (Invitrogen), sequenced, and transiently transfected into COS-7 cells following methods described previously (9). Transfected cells were harvested by trypsinization, resuspended in Tris buffer, sonicated, and Dounce-homogenized. The soluble fraction was separated by centrifugation at 100,000 × g (45 min), adjusted to 1 mg of protein/ml with Tris buffer, and labeled as described above.

Type II Tissue Transglutaminase (tTG) Activity Assay—Soluble fractions of breast cancer cell lines (1 mg of protein/ml) were pretreated with 4 mM CaCl₂ and/or GTP as indicated for 20 min at 25 °C followed by treatment with 250 μM 5-(and-6)-carboxytetramethylrhodamine-cadaverine (Molecular Probes). After incubation for 1 h at 25 °C, the reactions were quenched with 1 volume of standard 2 × SDS-PAGE loading buffer (reducing) and separated by SDS-PAGE (15 μg of protein/gel lane). Samples were then visualized by in-gel fluorescence. For determination of the IC₅₀ value for GTP inhibition of tTG activity, inhibition curves were generated for three distinct protein bands cross-linked to the rhodamine reporter group, and the estimated IC₅₀ values from these curves were averaged to provide the reported value.

RESULTS
Labeling and Affinity Enrichment of Protein Targets with Trifunctional Chemical Proteomic Probes

To date, strategies for activity-based proteomics have typically utilized a two-tiered platform in which, first, proteomic samples are treated with fluorescently tagged chemical probes and separated by one-dimensional or two-dimensional gel electrophoresis, providing a rapid and sensitive method to detect labeled enzyme activities by in-gel fluorescence scanning (11, 13–15). A second series of proteome labeling experiments using biotinylated probes is then required to permit the affinity isolation of protein activities by avidin chromatography procedures. The molecular identity of the purified protein activities can then be determined by standard tryptic digestion-mass spectrometry techniques. For low abundance targets of chemical proteomic probes, an anion exchange chromatography step may be required to enrich these proteins prior to avidin-based affinity purification.

In the course of screening cell and tissue proteomes with rhodamine-tagged sulfonate probes, we detected several labeled protein activities for which molecular identities were sought. Although many of these sulfonate-reactive proteins could be affinity purified with biotinylated probes, permitting their identification by mass spectrometry methods (13), some sulfonate targets proved resistant to molecular characterization by these methods. These sulfonate-reactive proteins generally represented lower abundance targets that displayed one or more of the additional challenging properties: 1) membrane association, 2) context-dependent labeling, and/or 3) migration on SDS-PAGE in the vicinity of endogenous biotinylated proteins. For example, an analysis of a panel of human breast carcinoma cell lines uncovered two 75–80-kDa phenyl sulfonate-reactive proteins enriched in the ER(−) invasive line MDA-MB-231: an 80-kDa protein that exhibited ATP-sensitive labeling (Fig. 1, single arrowhead) and a 75-kDa protein that displayed calcium-dependent labeling (Fig. 1, double arrowhead). Initial attempts to label and affinity-isolate these proteins with biotinylated probes, either directly from the crude cytosolic preparation or following prefractionation by Q anion exchange chromatography, were unsuccessful. One challenge facing these analyses was that the 75–80-kDa sulfonate targets migrated in the vicinity of endogenous biotinylated proteins (7, 14), complicating target detection by avidin blotting methods. Additionally we noted that both the 75- and 80-kDa proteins were unreactive with the phenyl sulfonate probe following desalting of the crude proteomic preparation (Fig. 2A), indicating that these proteins required additional cytosolic factors to maintain activity. This context-dependent reactivity displayed by the 75–80-kDa proteins precluded their enrichment by chromatography methods prior to probe
FIG. 2. Labeling and affinity enrichment of protein targets with trifunctional chemical proteomic probes. A, heat-sensitive sulfonate targets in the MDA-MB-231 soluble proteome are labeled by both the rhodamine-tagged (PS-Rhodamine) and trifunctional (TriPS) phenyl sulfonate probes. Desalting the proteome prior to treatment with probe blocks the labeling of both the calcium-dependent 75-kDa and ATP-sensitive 80-kDa sulfonate targets. Δ, heat-denatured proteome. B, pretreatment of the MDA-MB-231 soluble proteomic fraction with TriPS followed by Q-Sepharose anion exchange chromatography and SDS-PAGE analysis identifies fractions enriched for the 75- and 80-kDa sulfonate targets (F 9–15). Avidin-based affinity purification greatly enriched these TriPS-labeled targets from the pooled Q fractions (right panel). Fluorescent gel images are shown in grayscale.

labeling. Attempts to label cytosolic preparations with biotinylated sulfonate probes prior to Q chromatography and then analyze the resulting column fractions by avidin blotting were hindered by the limited sensitivity, dynamic range, and throughput of this screening method (data not shown). To circumvent these shortcomings, a trifunctional probe was synthesized in which both rhodamine and biotin substituents were coupled to the protected bifunctional linker (i.e., the protected bifunctional linker 3). The resulting column fractions were treated with the TriPS probe, followed by Q chromatography and SDS-PAGE analysis of the resulting fractions, providing a straightforward method by which to visualize fractions that were enriched for the labeled 75- and 80-kDa targets (Fig. 2B). These fractions were combined and treated with avidin-agarose beads as described previously (7, 12). Elution of bound proteins by heating in 1 volume of standard SDS-PAGE loading buffer provided a greatly enriched sample of TriPS-labeled targets (Fig. 2B). Protein bands corresponding to the 75- and 80-kDa targets were excised from the gel, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry, resulting in their identification as tTG and platelet-type phosphofructokinase (pPFK), respectively. A more detailed characterization of these sulfonate targets is described below. Collectively these results highlight the value of trifunctional chemical probes as tools that simplify the transition from target detection to target identification in activity-based proteomic experiments.

Purification and Characterization of Membrane Proteins with Activity-based Chemical Proteomic Probes

To test whether the devised methods would apply to the isolation and characterization of membrane-associated as well as soluble proteins, we pursued the identification of a 40-kDa phenyl sulfonate target selectively expressed in mouse testis membranes (13). Following treatment with the TriPS probe, testis membrane proteins were solubilized with Triton X-100 and separated by Q chromatography (Fig. 3A). Fractions enriched in the 40-kDa sulfonate target were sub-
Avidin-based affinity purification greatly enriched this TriPS-labeled protein from the pooled Q fractions (right panel). B, 3HSD1-transfected (3HSD1) but not mock-transfected (Mock) COS-7 cells possess a 40-kDa sulfonate-reactive membrane protein. The sulfonate labeling of both endogenous 3HSD1 from mouse testis membranes and recombinant 3HSD1 from transfected COS-7 cells was reduced in the presence of the 3HSD1 cofactor NAD⁺ (1 mM). C, quantitation of the inhibition of 3HSD1 labeling in mouse testis membranes by 1 mM NAD⁺ (arbitrary units, n = 3). Δ, heat denaturation. D, relative reactivity of members of the sulfonate ester probe library with 3HSD1 in mouse testis membrane (arbitrary units, n = 3). Phenyl probe responds to PS-rhodamine. Fluorescent gel images are shown in grayscale.

Sulfonate Targets Up-regulated in the Invasive Human Breast Cancer Line MDA-MB-231

pPFK—Comparative profiling of ER(+) and ER(−) human breast carcinoma cell lines revealed that the 80-kDa sulfonate target identified as pPFK was significantly up-regulated in the invasive ER(−) line MDA-MB-231 relative to the ER(+) lines MCF7 and T-47D (Fig. 4A). Recombinant expression of pPFK in COS-7 cells confirmed the sensitivity of this enzyme to sulfonate labeling (Fig. 4B). Additionally, the sulfonate reactivity of both native and recombinantly expressed pPFK was strongly inhibited by the addition of 1 mM ATP (Figs. 1 and 4B), a known allosteric inactivator of the enzyme (17). These data indicate that sulfonate labeling of pPFK can detect changes in the activity of the enzyme resulting from posttranslational regulatory events like the presence of a natural allosteric inhibitor.

tTG—Comparative profiling of human breast cancer lines also identified a calcium-activated sulfonate target that was dramatically up-regulated in MDA-MB-231 cells (Fig. 5A). This protein was identified as tTG, a cytosolic enzyme with dual functionalities as a transamidating enzyme and as a signal-transducing GTP-binding protein (18, 19). The transamidating
activity of tTG is calcium-dependent and results in acyl transfer between the γ-carboxamide of glutamine residues and the ε-amino group of lysine residues to form new intra- or inter-protein amide bonds (20). Thus, considering that both the sulfonate reactivity and transamidating activity of tTG were dependent on calcium, probe labeling of this enzyme appeared to serve as an effective measure of its state of activity. To explore this notion further, we compared the effects of GTP, a known allosteric inactivator of tTG (21, 22), on the transamidating activity and sulfonate labeling of the enzyme in human breast cancer cell proteomes.

The transamidating activity of tTG can be visualized in complex proteomes using small molecule amine substrates conjugated to reporter groups like biotin or fluorescein (23, 24). Here we applied rhodamine-cadaverine to lysates of breast cancer cell lines in the presence or absence of calcium chloride and detected tTG-catalyzed transamidation events between this small molecule amine and cellular proteins by SDS-PAGE and fluorescence scanning (Fig. 5B). In the presence of calcium, numerous rhodamine-cross-linked proteins were observed in MDA-MB-231 cytosolic extracts, but not in MCF7 or T-47D extracts (Fig. 5B), confirming the selective expression of tTG activity in the MDA-MB-231 line as was predicted from the sulfonate labeling profiles of these cell lines (Fig. 5A). The addition of GTP strongly inhibited tTG-catalyzed protein transamidation in the MDA-MB-231 cytotoxic extract (Fig. 5B), exhibiting an estimated IC₅₀ value of 109 ± 16 µM. Notably, GTP also blocked the sulfonate labeling of tTG (Fig. 5C) with a similar IC₅₀ value of 46 ± 5 µM. Collectively these findings indicate that sulfonate esters act as effective activity-based probes for tTG, capable of distinguishing active from inactive or allosterically inhibited forms of the enzyme.

DISCUSSION

Here we have described the synthesis and application of a class of trifunctional activity-based proteomic probes that contain discrete chemical groups for: 1) active site-labeling (sulfonate ester), 2) target detection (rhodamine), and 3) target purification (biotin). Trifunctional probes were used to identify several protein targets that had resisted molecular characterization by a more conventional two-tiered strategy in which separate classes of probes were used for target detection (rhodamine-conjugated probes) and target isolation (biotin-conjugated probes) (13). Notably, each of the sulfonate-reactive proteins identified with trifunctional probes displayed one or more challenging biochemical properties that typically confounded molecular analysis, including membrane association (3HSD1), co-migration with endogenous biotinylated proteins (pPFK and tTG), and/or context-dependent labeling (pPFK and tTG). Trifunctional probes are of particular value for characterizing such difficult protein targets because these reagents permit the simultaneous detection and affinity isolation of labeled proteins by in-gel fluorescence scanning and avidin chromatography, respectively. Thus, enzymes that are of low abundance, associated with membranes, and/or require an ill-defined mixture of cofactors for activity can be labeled with trifunctional probes in crude cellular extracts and purified by avidin or sequential Q-avidin chromatography, and their enrichment can be monitored by fluorescence scanning. The superior sensitivity, speed, and dynamic range of fluorescence detection relative to avidin blotting methods makes such postlabeling, multistep purification procedures straightforward to conduct. Additionally, using in-gel fluorescence detection methods, trifunctional probe-reactive proteins can be distinguished from closely migrating endogenous biotinylated proteins that often complicate avidin enrichments from whole proteomes (7). Nonetheless, compared with fluorophore-tagged probes, trifunctional probes are more expensive to synthesize and more sterically encumbered and therefore may not be as suitable as fluorophore-conjugated probes for initial comparative profiling experiments involving a large number of proteomic samples.

Two of the sulfonate targets identified in this study, pPFK and tTG, were significantly up-regulated in the invasive ER(−) human breast cancer cell line MDA-MB-231 relative to the noninvasive ER(+) lines MCF7 and T-47D. To our knowledge, neither of these proteins has been previously identified as a constituent of the MDA-MB-231 proteome. pPFK, also referred to as PFK type C, catalyzes the first committed step in aerobic glycolysis (25). Many tumor types demonstrate high levels of PFK activity, which appears to support their characteristically enhanced levels of aerobic glycolysis (26).
Notably, pPFK has been shown to correlate with malignancy in ascitic tumor cells in mice (27), suggesting that this enzyme may represent a general marker of advanced stages of cancers. pPFK activity is regulated in a posttranslational manner by several cytosolic factors, including ATP, which acts as an allosteric inhibitor (17). Importantly, we observed that the sulfonate labeling of pPFK was also inhibited by ATP, indicating that this probe-enzyme reaction occurs in an activity-dependent manner.

Like pPFK, tTG is up-regulated in certain types of cancers, including drug-resistant cell lines like NCI/ADR (28) and PC-14 (29). Curiously, however, tTG is also associated with apoptosis where increased transglutaminase activity has been proposed to contribute to cell death (30, 31). Thus, it remains unclear why certain cancer cells would overexpress an enzyme that promotes apoptosis. One possible explanation, as has been suggested for the NCI/ADR line, is that these cells possess reduced intracellular calcium stores and therefore fail to activate ITG (29). Alternatively, however, recent evidence suggests that the activation of tTG in response to apoptotic stimuli may protect cells from death through the transamidation of the retinoblastoma gene product, which inhibits its degradation by caspases (32). Thus, the up-regulation of ITG in invasive cancer cells, like MDA-MB-231 cells, may contribute to an apoptosis-resistant phenotype. Regardless, we anticipate that the sulfonate probes described herein should serve as important new tools for profiling tTG activity in complex proteomes. In support of this notion, the sulfonate reactivity of ITG was purely activity-based with labeling being dependent on the presence of the physiological activator calcium and blocked by the natural allosteric inhibitor GTP.

In summary, trifunctional chemical probes offer significant advantages for the emerging field of activity-based proteomics as these reagents greatly facilitate the molecular characterization of probe-labeled targets. Previous methods have benefited from the sensitivity of fluorescent probes for target detection and the specificity of biotin-avidin interactions for target isolation, but the uncoupled nature of the visualization and identification steps has frustrated efforts to identify certain difficult protein targets, including low abundance, membrane-associated, and/or cofactor-dependent enzymes. Through linking both rhodamine and biotin tags to an active enzymes as these reagents greatly facilitate the molecular characterization of probe-labeled targets. Previous methods have benefited from the sensitivity of fluorescent probes for target detection and the specificity of biotin-avidin interactions for target isolation, but the uncoupled nature of the visualization and identification steps has frustrated efforts to identify certain difficult protein targets, including low abundance, membrane-associated, and/or cofactor-dependent enzymes. Through linking both rhodamine and biotin tags to an active site-directed reactive group, trifunctional probes overcome this shortcoming by consolidating the fluorescence detection of labeled proteins with avidin-based affinity chromatography procedures. Using trifunctional probes, we identified several challenging protein targets, including the integral membrane enzyme 3HSD1 and two cofactor-dependent enzymes, pPFK and tTG, that were up-regulated in invasive breast cancer cells. Notably, in the case of both pPFK and tTG, natural allosteric regulators of enzyme activity were found to exhibit commensurate effects on probe labeling, highlighting the ability of chemical probes to provide an accurate readout of the functional state of enzymes in complex proteomes. As more activity-based chemical probes are developed through both directed (7–10, 14, 15) and combinatorial methods (12, 13), their trifunctional variants will likely play important roles in accelerating the discovery of new protein targets associated with discrete physiological and/or pathological states.

Acknowledgments—We thank Greg Hawkins and Mark Humphrey for technical assistance and members of the Cravatt and Sorensen laboratories for helpful discussions.

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