Enzyme superfamilies are composed of members that share a common catalytic mechanism, but not necessarily sequence or structural homology.\[1\] The characterization of sequence-unrelated members of enzyme classes is an experimentally challenging endeavor which often requires extensive biochemical studies on purified preparations of enzymes.\[2,3\] Given these issues, in conjunction with the abundance of unannotated proteins provided by recent genome-sequencing projects, it is likely that cryptic members of many enzyme classes still exist in eukaryotic and prokaryotic proteomes. Herein, we demonstrate that a chemical proteomics method referred to as activity-based protein profiling (ABPP)\[4,5\] can be used to identify sequence-unrelated members of enzyme superfamilies on the basis of their reactivity with “mechanism-based” probes.

In previous studies, we analyzed a panel of human cancer cell lines with a set of fluorophosphonate (FP) based ABPP probes, \[6\] which target the serine hydrolase (SH) superfamily of enzymes.\[7\] FPs are well-characterized affinity labels for SHs; they phosphonylate irreversibly the active-site serine nucleophile in these enzymes.\[8\] Numerous FP-labeled enzymes were identified in these experiments, including proteases, lipases, and esterases. Most of these enzymes were readily assigned to the SH superfamily on the basis of database (BLAST) searches, which identified conserved sequence elements shared by members of this enzyme class. Notably, however, one FP target, sialic acid 9-O-acetylesterase (SAE), which was selectively expressed in melanoma cell lines (Figure 1a), eluded such classification, as this enzyme shared no sequence homology with SHs or, for that matter, any other enzyme class. SAE was originally characterized by
Varki and colleagues as an enzyme that removes O-acetyl esters from the 9-position of naturally occurring sialic acids[9,10] and has been shown to be inhibited by diisopropyl fluorophosphate and the arginine-modifying reagents 2,3-butanedione and phenylglyoxal.[11] Nonetheless, to date the specific residues involved in SAE catalysis have remained obscure, and, as a consequence, so too has the mechanistic classification of this enzyme. To determine whether SAE might represent a novel member of the SH superfamily, we set out to identify and characterize the site of FP labeling in this enzyme.

To further characterize the SAE–FP interaction, we employed a gel-free version of ABPP that enables sites of probe labeling to be determined directly for enzymes in complex proteomes (Figure 1a).[12,13] In this method, probe-treated proteomes are digested with trypsin, and the probe-labeled peptides are captured by affinity chromatography and identified by liquid chromatography–tandem mass spectrometry.
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Comparative characterization of WT SAE and the S124A and S127A SAE mutants. SAE proteins were recombiantly expressed in COS-7 cells by transient transfection as myc-epitope-tagged fusion proteins (myc epitope: EKLLISEEDL) and affinity purified by using anti-myc agarose beads (Sigma; 50-μl beads with 0.75 mL of proteome in 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5 (1 mg protein mL⁻¹)). Following thorough washing of the beads with Tris (50 mM, pH 7.5, 2×0.5 mL), an aliquot of each SAE variant was treated with FP–Rh (4 μM) for 1 h. The reactions were then quenched with standard SDS-PAGE (sodium dodecylsulfate–polyacrylamide gel electrophoresis) loading buffer and analyzed in gel fluorescence scanning (a, upper panel) and western blotting with anti-myc antibodies (a, lower panel). The FP–Rh probe labeled both WT and S124A SAE, but not S127A SAE. Anti-myc blotting confirmed that each SAE protein was expressed at high levels in COS-7 cells. b) Comparison of the catalytic activity of WT SAE and the S124A and S127A SAE mutants. SAE variants were assayed on beads with a radiolabeled-substrate assay that follows the cleavage of [3H]acetate from [3H]Neu5,9Ac2 (10000 cpm reaction; cpm = counts per minute). This assay [11] and the biosynthetic preparation of [3H]Neu5,9Ac2 [9] have been described previously. Results represent the averages of two independent trials and have been normalized to account for the relative quantity of enzyme present in each reaction (as estimated by western blotting). Mock cells were transfected with empty vector (pcDNA3mycHis) and served as a background control for all experiments.
studies should clarify the roles that these arginine residues, as well as H377 and K381, play in the SAE catalytic mechanism.

In summary, the results reported herein indicate that SAE and its sequence homologues constitute a novel branch of the SH superfamily. This discovery may enable the design of specific inhibitors of SAE enzymes that incorporate functional groups, such as α-keto heterocycles and trifluoromethyl ketones, that show strong affinity for SH active sites. Such inhibitors would be of value for testing the functional significance of the 9-O acetylation of sialic acids, a modification that is expressed selectively on gangliosides in human melanoma cells. More generally, it is interesting to consider whether the assignment of unannotated enzymes to other mechanistic classes may also be facilitated by ABPP probes. On the one hand, the extrapolation of mechanistic information with active-site-directed probes that target multiple enzyme superfamilies that have resisted classification based on sequence comparisons.

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