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Site-Selective Protein Immobilization by Covalent Modification of GST Fusion Proteins

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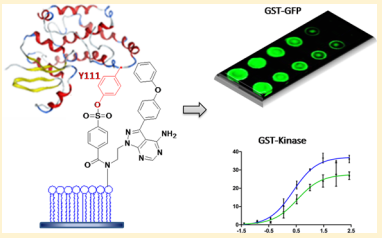
Supporting Information

ABSTRACT: The immobilization of functional proteins onto solid supports using affinity tags is an attractive approach in recent development of protein microarray technologies. Among the commonly used fusion protein tags, glutathione *S*-transferase (GST) proteins have been indispensable tools for protein−protein interaction studies and have extensive applications in recombinant protein purification and reversible protein immobilization. Here, by utilizing pyrimidine-based small-molecule probes with a sulfonyl fluoride reactive group, we report a novel and general approach for site-selective immobilization of *Schistosoma japonicum* GST (*sj*GST) fusion proteins through irreversible and specific covalent modification of the tyrosine-111 residue of the *sj*GST tag. As demonstrated by *sj*GST-tagged eGFP and *sj*GST-tagged kinase activity assays, this immobilization approach offers the advantages of high immobilization efficiency and excellent retention of protein structure and activity.



INTRODUCTION

The immobilization of proteins onto solid supports, including microarrays, chips, and beads, is a powerful approach for illuminating protein−protein or protein−ligand interactions and for probing novel protein functions in a high-throughput manner.[1] Unlike DNA microarrays, it has been challenging to implement a universal method for protein immobilization because of intrinsic difficulties in achieving selective and uniform orientation of proteins onto solid surfaces.[2] Conventional immobilization methods mainly rely on three mechanisms: physical attachment, covalent linking, and bioaffinity immobilization.[3] However, nonselective physical adsorption or covalent binding may occlude active sites or even denature proteins. Protein immobilization by the expression and coupling of recombinant fusion tags has therefore emerged as a more attractive approach, as it allows definite protein orientation and offers flexibility in fine-tuning the bioconjugate topology. For example, immobilization of the *Schistosoma japonicum* glutathione *S*-transferase (*sj*GST) tag onto glutathione (GSH)-conjugated beads, the His tag onto Ni-NTA chips, or the biotin tag onto streptavidin-coated solid supports have been widely adopted as noncovalent protein immobilization methods that maintain the activity of the tagged proteins.[4−7] However, such noncovalent interactions may remain sensitive to pH or to other undermining factors in experimental systems.[8] It is therefore highly desirable to develop effective strategies for stable protein immobilization with maximal activity retention.

Site-selective covalent immobilization of fusion tags has recently attracted much attention because it enables stable, functional, homogeneous, and high-density immobilization of various proteins,[8−11] where a protein of interest is fused to a tag protein that covalently and selectively reacts with an immobilized substrate molecule. For example, Kindermann et al. reported a covalent approach for the immobilization of the hAGT tag by irreversibly linking the alkyl group of O^6-benzylguanine derivatives onto one of the hAGT cysteine residues.[10] Recently, various studies have focused on the regioselective capture of GST-tagged proteins because this fusion tag has been recognized for its high substrate affinity and practical ease for affinity purification of native recombinant proteins.[11−14] Most GST tag immobilization methods are based on reversible GSH−GST binding. However, in 2013, Viswanathan et al. reported an immobilization strategy that covalently appended GST onto glass surfaces at an alkyne-modified C-terminal cysteine residue by a Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition ("click") reaction.[11] Here, we present a facile approach that uses pyrimidine-based sulfonyl fluoride compounds for the specific and irreversible immobilization of *sj*GST-tagged proteins by one step, site-selective covalent binding (Figure 1A).

RESULTS AND DISCUSSION

Specific Labeling of *sj*GST by the G1 Series of Molecules. Compound **G1-H** and its corresponding probe **G1-alkyne** are pyrimidine-based small molecules with a sulfonyl fluoride group. These compounds could be generated via relatively straightforward synthetic procedures with good yields, and detailed procedures are provided in the Supporting Information. Although originally intended to selectively label

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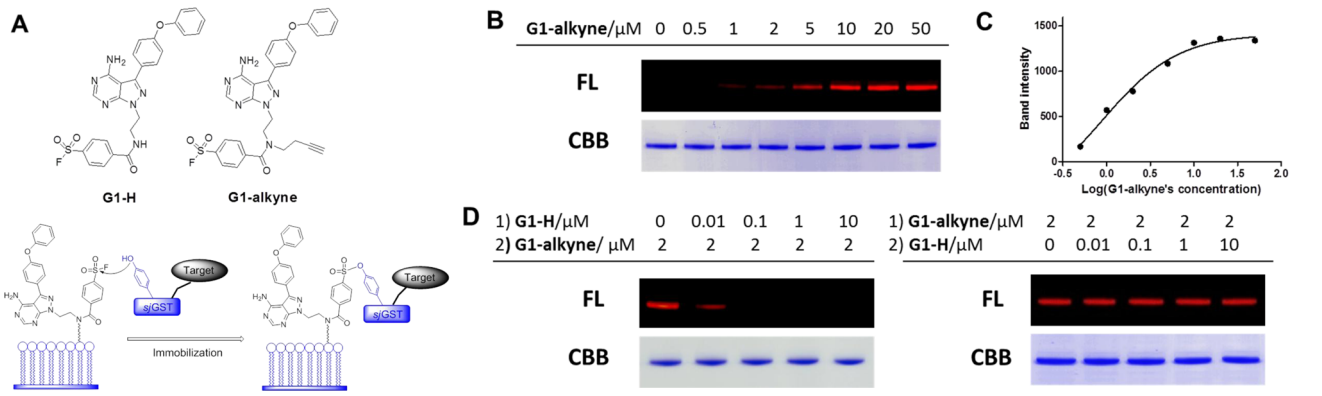

Figure 1. (A) Chemical structures of **G1-H** and **G1-alkyne** and site-selective covalent immobilization of proteins with fused *sj*GST tags. (B) **G1-alkyne** labeling of recombinant *sj*GST protein (0.5 μg each lane): fluorescence scanning (FL, upper) and Coomassie blue staining (CBB, lower). (C) Densitometry of fluorescent bands. (D) Competition experiments with addition of different concentrations of **G1-H** before (left) and after (right) **G1-alkyne** treatment (0.5 μg *sj*GST each lane).

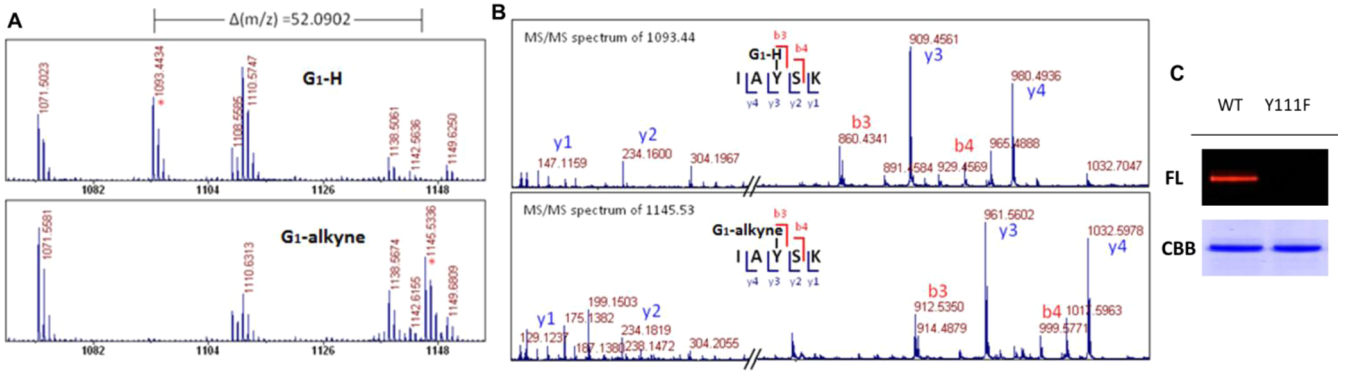

Figure 2. MALDI-TOF-MS reveals tyrosine-111 as the binding site of **G1** compounds. (A) A mass shift of 52 Da was detected between **G1-H**- and **G1-alkyne**-treated *sj*GST peptides after trypsin digestion. (B) MS/MS analysis of the peaks at *m*/*z* values of 1093 and 1145. (C) An *sj*GST Y111F mutant abolished labeling by **G1-alkyne**.

serine residues on proteins, they were serendipitously discovered to label the *sj*GST tags of recombinant proteins, thus prompting us to examine in detail their binding to GST proteins. During the course of our study, Gu et al. reported that, using simple sulfonyl fluoride-based probes derived from 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), tyrosine residues in the substrate binding pocket (H-site) of multiple classes of GST proteins can be labeled along with other proteins in cell lysates.[15]

Recombinant *sj*GST protein was incubated with increasing concentrations of **G1-alkyne** at room temperature for 1 h, followed by "click" chemistry with TAMRA-N_3. The mixtures were then subjected to SDS-PAGE in reducing conditions for in-gel fluorescence scanning. The fluorescence of the protein bands increased as more **G1-alkyne** probe was used, whereas Coomassie blue staining indicated that an equal amount of recombinant protein was loaded in each lane (Figure 1B). Maximum labeling of *sj*GST was achieved at approximately 10 μM **G1-alkyne** as assayed by gel densitometry (Figure 1C).

Next, competition experiments were performed to further examine the binding of the **G1** series of compounds with *sj*GST. Recombinant *sj*GST protein was incubated with different concentrations of **G1-H** before or after treatment with 2 μM of **G1-alkyne**. The subsequent click reaction, SDS-PAGE, and fluorescence scanning steps were performed in the same manner as above. This experiment showed that **G1-alkyne** labeling could be completely blocked by 0.1 μM of **G1-H** (Figure 1D, left). However, once labeled by the **G1-alkyne** probe, high concentrations of **G1-H** could no longer disrupt the linkage (Figure 1D, right). These results suggested that *sj*GST is specifically and irreversibly bound to **G1** molecules.

Next, we attempted to identify the exact binding site of both **G1** compounds on *sj*GST by MALDI-TOF-MS/MS. Recombinant *sj*GST protein was separately treated with **G1-H** and **G1-alkyne** and digested with trypsin. After ZipTip desalting, the samples were subjected to mass spectrometry analysis. Comparison of the mass spectra of samples treated with **G1-H** and **G1-alkyne** revealed a peak shift from 1093.47 to 1145.53, which was consistent with the molecular weight difference between **G1-H** and **G1-alkyne** (52 Da) (Figure 2A). Thus, these two peaks were chosen for further MS/MS analysis, which revealed that both compounds were bound to the tyrosine-111 residue in the IAYSK peptide fragment (Figure 2B). We generated an *sj*GST mutant (Y111F) by substituting this tyrosine residue with phenylalanine. This substitution completely abolished labeling by **G1-alkyne**, further establishing tyrosine-111 as the specific **G1** binding site (Figure 2C).

In comparison with the AESBF-derived probes that labeled various classes of GST proteins,[15] we found that our probe labels only the Mu and Alpha classes of human GST in addition to *sj*GST (Figure 3A). Furthermore, we tested how **G1-alkyne** labeled a *sj*GST-spiked mammalian proteome. **G1-alkyne** was incubated at 5 μM with human embryo kidney-293T (HEK-293T) cell lysate spiked with up to 5% of recombinant *sj*GST

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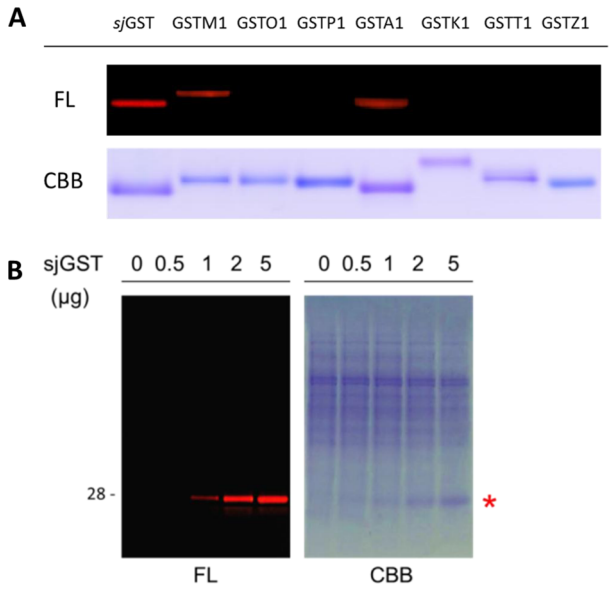

Figure 3. (A) **G1-alkyne** labeling of *sj*GST and seven classes of human GSTs. (B) **G1-alkyne** labeling of *sj*GST-spiked HEK293T cell lysate. The total amount of lysate in each sample was 100 μg. The gel was visualized by fluorescence scanning (left panel) and Coomassie blue staining (right panel).

protein before being subjected to the click reaction, SDS-PAGE and fluorescence scanning. In sharp contrast to previously reported probes that labeled many proteins but especially serine proteases,[16−18] we only detected labeling of the spiked *sj*GST protein. Approximately 2% of *sj*GST in HEK-293T cell lysates was sufficient for selective labeling (Figure 3B), indicating that the pyrimidine group improved the selectivity of **G1** series compounds. Such enhanced labeling selectivity very likely arises from the added structural complexity of **G1** probes, which would mitigate the chances of unintended labeling at sites other than Y111 in the GST tag.

Protein Immobilization Studies. After confirming the covalent binding between **G1** compounds and *sj*GST protein, we next assessed whether our compounds can be reliably applied for site-selective protein immobilization. First, **G1-biotin**, a biotinylated probe of **G1**, was synthesized and captured on streptavidin-sepharose beads (Figure 4A). These beads were used to mimic covalently immobilized **G1** probes based on the strong binding affinity between streptavidin and biotin. Recombinant *sj*GST protein was captured and eluted to evaluate the binding ability of the beads. GST protein was captured by **G1-biotin**-bound beads and was eluted by boiling (Figure 4B, lane 2). Pretreatment of *sj*GST with a high concentration of free GSH (20 mM) blocked the binding of *sj*GST to **G1-biotin**-bound beads (Figure 4B, lane 3). However, once covalent immobilization occurred, it was stable against high-concentration GSH treatment (Figure 4B, lane 4). In comparison, *sj*GST protein reversibly bound to GSH-sepharose beads was eluted by 20 mM of free GSH (Figure 4B, lane 5).

To mimic semi-high-throughput screening conditions, recombinant *sj*GST-tagged enhanced green fluorescent protein (*sj*GST-eGFP) was incubated with **G1-biotin** and captured on a streptavidin-coated 96-well microplate. After careful washing with PBS buffer, the immobilized GFP protein was detected with a fluorescence scanner. By either incubating increasing amounts of *sj*GST-eGFP with 0.5 μM of **G1-biotin** (Figure 4C, upper panel) or by incubating *sj*GST-eGFP (1.5 μg) with increasing amounts of **G1-biotin** (Figure 4C, middle panel), the fluorescence intensities increased accordingly, indicating that the fluorescence properties of eGFP protein were well-maintained after immobilization. As expected, immobilization could be blocked by preincubating *sj*GST-eGFP with free **G1-H** (Figure 4C, lower panel). Collectively, these experimental results indicated that *sj*GST-eGFP could be dose-dependently

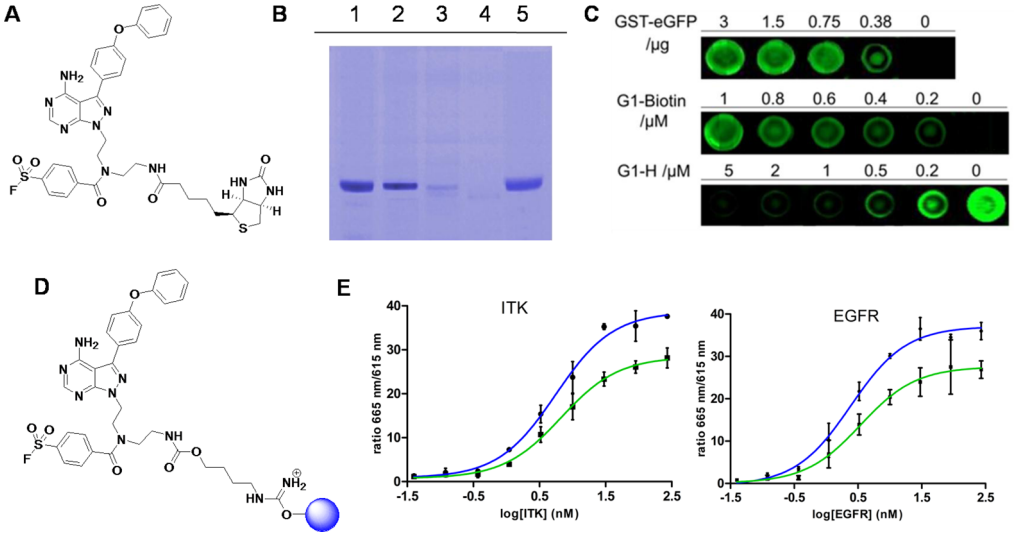

Figure 4. (A) Chemical structure of **G1-biotin**. (B) Pull-down experiments: (1) recombinant *sj*GST without capture-and-elute; (2) *sj*GST captured by **G1-biotin**-bound beads and eluted by boiling; (3) *sj*GST preincubated with 20 mM GSH then treated with **G1-biotin**-bound beads and eluted by boiling; (4) *sj*GST treated with **G1-biotin**-bound beads as in (2) but eluted with 20 mM GSH; (5) *sj*GST bound to GSH-sepharose and eluted with 20 mM GSH. (C) GST-eGFP immobilization on a **G1-biotin** pretreated, streptavidin-coated 96-well microplate (upper panel). Greater amounts of GST-eGFP were captured with increasing amounts of **G1-biotin** (middle panel). Preincubation of GST-eGFP with increasing amounts of **G1-H** gradually diminished immobilization (lower panel). (D) Structure of **G1-H**-immobilized sepharose beads. (E) HTRF-based enzyme activity assays for soluble (cyan) and immobilized (green) ITK (left) and EGFR (right) kinases.

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immobilized by **G1** compounds onto solid supports and that it retained its native fluorescence after immobilization.

Finally, as a simple demonstration that the biological activity of proteins can be maintained after immobilization, we tested the enzymatic activities of *sj*GST-tagged kinases before and after immobilization. A derivative of **G1-H** was coupled onto activated CH-sepharose 4B (Sigma) beads (Figure 4D). Various amounts of recombinant *sj*GST-tagged interleukin-2-inducible T cell kinase (ITK) and *sj*GST-tagged epidermal growth factor receptor (EGFR) were immobilized onto **G1**-bound beads, and their kinase activities were measured by a homogeneous time-resolved fluorescence (HTRF)-based enzyme assay (Supporting Information). Compared with soluble *sj*GST-tagged kinases subjected to the same assay conditions, the activities of the immobilized enzymes were largely retained, with approximately 70% of kinase activity in solution maintained after immobilization (Figure 4E). Therefore, our immobilization approach may provide high yields of active proteins.

CONCLUSIONS

We have discovered a novel series of pyrimidine-based small molecule compounds that specifically and covalently label the *sj*GST protein at its tyrosine-111 residue. The binding reaction was rapid, mild, and efficient. By linking this type of molecule to solid supports, we have demonstrated a novel approach for site-specific protein immobilization through direct, irreversible and site-selective conjugation using the well-established fusion tag *sj*GST. Importantly, native protein structure and activity were robustly preserved by this labeling strategy. This one-step method may be readily exploited for protein chips and high-throughput screening platforms that require the efficient immobilization of bioactive proteins.

ASSOCIATED CONTENT

Supporting Information

Synthesis of compounds and detailed experimental procedures of biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Yiqing Zhou and Tianlin Guo contributed equally to this work. Y.Z., T.G., and Z.P. designed the study. Y.Z., T.G., G.T., and H.W. performed experiments. Y.Z., T.G., N.W., and Z.P. prepared the manuscript.

Notes

The authors declare no competing financial interests.

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