Protein–Protein Interactions

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Phosphopeptide-Dependent Labeling of 14–3–3ζ Proteins by Fusicoccin-Based Fluorescent Probes**

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14–3–3 proteins play a critical role in serine/threonine kinase dependent signaling pathways through protein–protein interactions (PPIs) with multiple phosphorylated ligands.[1] A ligand-dependent 14–3–3 detection technique would facilitate elucidation of 14–3–3-related intracellular signaling networks. Herein, we describe phosphopeptide-dependent fluorescent labeling of 14–3–3ζ by using cell-penetrating probes derived from the diterpene natural product fusicoccin.

The 14–3–3 proteins are a family of dimeric conserved regulatory proteins that are expressed in all eukaryotic cells. Each of the highly helical monomers possesses a shallow groove (approximately 25 Å in length) that recognizes consensus phosphopeptide motifs containing either phosphoserine (pS) or phosphothreonine (pT) residues.[1b,c] Hundreds of intracellular ligand proteins that possess 14–3–3 consensus motifs have been identified, including proteins involved in signaling and cell-cycle control, such as the Raf family, p53, and Cdc25 phosphatases.[1b] A number of studies have revealed that the disruption of 14–3–3 PPIs results in suppression of tumor growth,[2] thus suggesting that 14–3–3 proteins could potentially serve as new therapeutic targets. However, many details regarding intracellular regulatory processes that involve 14–3–3 proteins remain unknown. Low-molecular-weight agents that detect 14–3–3 proteins are desirable for elucidating intracellular 14–3–3 functions, however, such chemical probes are not currently available. Recent studies have successfully identified small molecule stabilizers[3a] and inhibitors[3b–c] of 14–3–3 PPIs, thus providing useful reagents for cell-based analysis. We anticipated that development of a cell-penetrating chemical probe that is capable of detecting 14–3–3 proteins in response to their binding to phosphorylated ligands would also enhance our understanding of the role of 14–3–3 proteins in PPI networks.

The diterpene fusicoccin A[4] (FC; Figure 1 A) is a phytotoxin that is produced by *Phomopsis amygdali*. FC activates plant plasma membrane H^+-ATPase through the formation of a ternary complex with plant 14–3–3.[5] Recent collaborative studies led by Kato[6] to semisynthetically modify FC resulted in the identification of several potent FC analogues that are active against human cancer cells, however, the biology underlying this activity is not fully understood. Further development of FC-based antitumor agents for medicinal purposes will thus necessitate identification of their target(s) and elucidation of their mechanism of action.

Wittinghofer and co-workers[7] reported the crystal structure of plant 14–3–3 bound to FC and a consensus phosphopeptide (QSYpTV) derived from the C terminus of H^+-ATPase. Their study revealed that FC binds to a hydro-

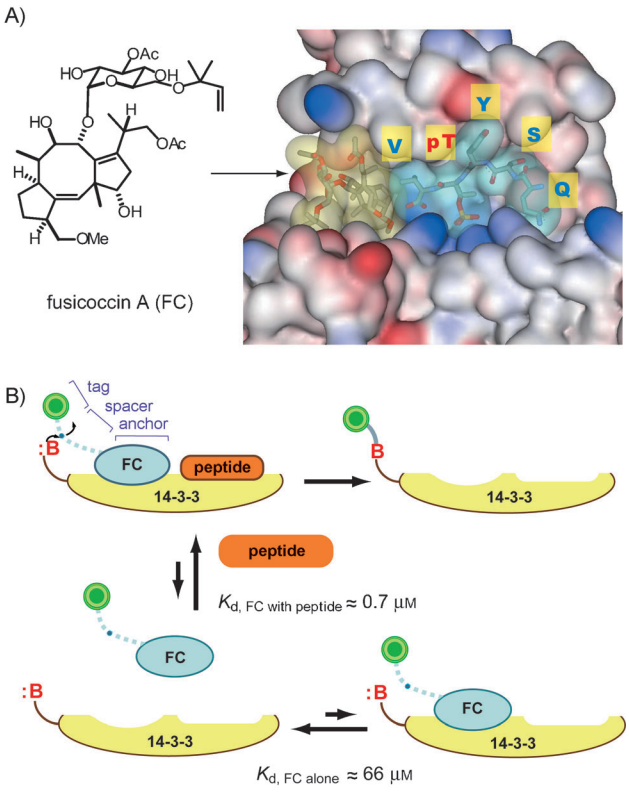

Figure 1. A) Chemical structure of fusicoccin A and crystal structure of the ternary complex of plant 14–3–3, FC, and the phosphopeptide QSYpTV (Ref. [7]). B) Schematic representation of the phosphopeptide-dependent 14–3–3 protein-labeling strategy. K_d values are cited from Ref. [7]. Q = glutamine, S = serine, V = valine, Y = tyrosine.

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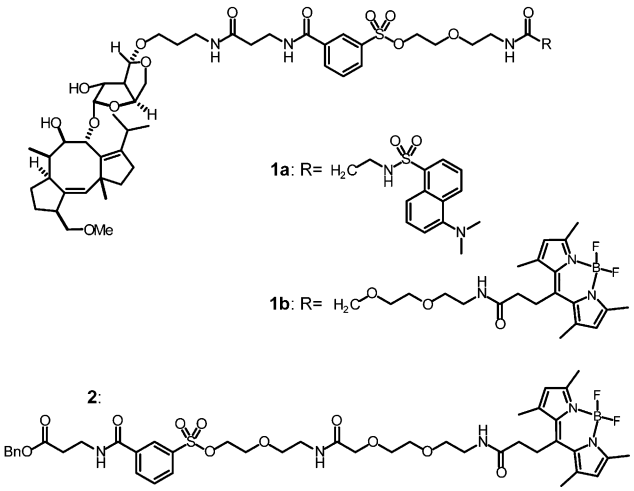
phobic cavity adjacent to the phosphopeptide binding pocket and forms the ternary complex (Figure 1A, right). Importantly, formation of the ternary complex significantly increases the affinity of both FC and QSYpTV to 14–3–3 by nearly two orders of magnitude. The key driving force behind the formation of the complex is the notable van der Waals interaction between the hydrophobic FC backbone and the isopropyl side chain of the V residue at position $i+1$, which is adjacent to the pT residue. These results suggest that FC stabilizes the PPI between 14–3–3 and any partner protein that bears a consensus motif with a V residue at position $i+1$, therefore, appropriately functionalized FC derivatives should be capable of detecting such interactions. As the first step toward the development of such a functionalized FC derivative, we rationally designed and evaluated an FC-based chemical probe that, after formation of a ternary complex with a 14–3–3 protein and a phosphopeptide, covalently attaches a fluorescent tag to the 14–3–3 surface. We then used the probe to detect endogenous 14–3–3 in cancer cells.

We hypothesized that a chemically modified FC bearing a fluorescent tag that is attached by a reactive spacer, and a phosphopeptide that contains a V residue at position $i+1$ would bind to 14–3–3 and form the ternary complex (Figure 1B). The formation of the complex would in turn trigger the spacer to react with nucleophilic residues on the protein surface to covalently attach the tag. Labeling through formation of a ternary complex with the consensus peptide should be kinetically more favorable than labeling with the probe alone, because of the stabilizing effect imparted by van der Waals interactions. Accordingly, selective labeling in the presence of the phosphopeptide ligand would be achieved.

Among the seven human isoforms of 14–3–3, the ζ isoform was of great interest to us because it has been implicated in lung[8a] and breast[8b] tumorigenesis. Histidine 164 (His164) of 14–3–3ζ is located in a flexible loop region, thus suggesting that it is vulnerable to labeling. We incorporated the recently developed bioorthogonal phenylsulfonylmethylene moiety (tosylate)[9] as the reactive spacer in order to make our probe react with His164. Thus, we synthesized FC-based fluorescent probes **1a** and **1b** by covalently linking the transformed glycosyl moiety of the FC analogue ISIR-042[6] to the reactive spacer, in which the terminus was attached to a dansyl or a BODIPY group, respectively (Scheme 1; see the Supporting Information for details). Based on a computer-generated docking model, a spacer with an appropriate length was chosen to bring the sulfonylmethylene moiety near His164 of 14–3–3ζ (Figure 2A).

Following the synthesis of the probes, labeling was evaluated by incubating compounds **1a**,**b** and **2** with recombinant 14–3–3ζ in the presence or absence of the PMA2 phosphopeptide[5b] ETIQQSYpTV, followed by SDS-PAGE (see Figure S1 in the Supporting Information for **1a**, and Figure 2B for **1b** and **2**). Clearly, **1a** and **1b** labeled 14–3–3ζ in the presence of the peptide (Figure 2B, lane 3 for **1b**), whereas no apparent fluorescent band was detected when the peptide was absent during labeling (Figure 2B, lane 2 for **1b**), thus demonstrating that the formation of the ternary complex is the determining factor for the labeling reaction. The yield of the labeled protein increased with the concentration of the



Scheme 1. Chemical structures of fluorescence-labeling reagents **1a** and **1b** attached to the fusicoccin derivative used in this study. **2** is a control compound.

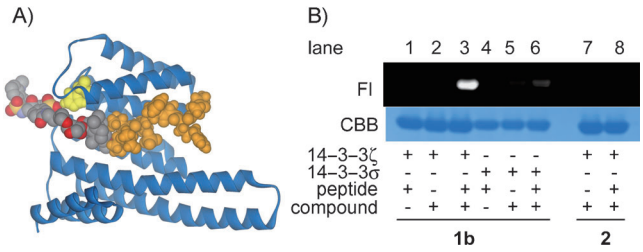


Figure 2. A) Superimposed model of **1a** (CPK), 14–3–3ζ protein (PDB 1YWT; blue ribbon, His164 highlighted in yellow CPK), and phosphopeptide MARSHpSVPA (orange CPK; Y in the original structure was modified to V). B) 14–3–3 proteins (40 μM) were incubated with compound (80 μM) in the presence or absence of PMA2 (80 μM) at 35 °C for 18 h. The SDS-PAGE gel image was visualized by fluorescence (FI) and staining with Coomassie brilliant blue (CBB).

phosphopeptide until equimolarity with 14–3–3ζ was reached (Figure S2), thus supporting a 1:1:1 model for the formation of the ternary complex. In the case of compound **1a**, the formation of a 1:1 adduct between the fluorescent tag and 14–3–3ζ was confirmed by using MALDI-TOF mass spectrometry (Figure S3).

The reaction with 14–3–3σ, which possesses an Asn instead of a His at position 164, resulted in a negligibly stained band (Figure 2B, lane 6), thus suggesting that labeling predominantly involves His164 of 14–3–3ζ. This site specificity was further confirmed by using site-directed mutagenesis of 14–3–3ζ. Substitution of Ala for His at position 164 decreased the degree of labeling (Figure S4). Furthermore, a control experiment involving compound **2** demonstrated that removal of the FC anchor completely precludes labeling (Figure 2B, lanes 7 and 8), thus further confirming that the FC anchor is essential for binding to 14–3–3.

We next evaluated whether compound **1b** recognizes the shape of the phosphopeptide ligand. We predicted that the residue at position $i+1$ of the phosphopeptide should have a major influence on the formation of the ternary complex

because this residue is responsible for the critical van der Waals interactions. To test this hypothesis, a series of consensus RSHpSXP hexapeptides,[10] each containing one of the 20 amino acids at position X ($X = i+1$) were evaluated for labeling (Figure 3). Use of peptides containing Val, Leu, or Ile at position $i+1$ resulted in high labeling efficiency,

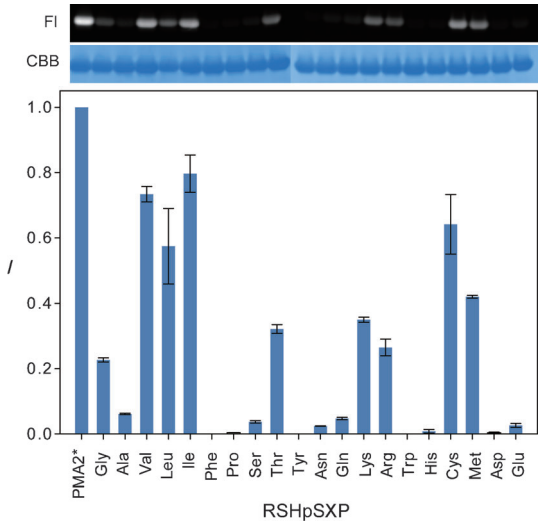


Figure 3. Various phosphopeptides (RSHpSXP, X = one of the 20 amino acids) were evaluated for use in the labeling reaction of 14–3–3ζ with **1b** under the same conditions as described in Figure 2. PMA2 was used as a positive-control standard. Top: fluorescent gel image and CBB-stained gel image. Bottom: Relative intensity of the fluorescent bands normalized against PMA2. Standard deviations are given for $n \geq 2$. H = histidine, P = proline, R = arginine.

presumably because the hydrophobic and branched side chains conform well to the FC backbone. The same trend was observed with the peptide containing Thr at position $i+1$, furthermore, it was approximately eight times more efficient at labeling than the peptide containing Ser, thus demonstrating that the methyl group of Thr contributes to the hydrophobic interaction with the FC moiety and compensates for unfavorable hydration caused by the hydroxy group, which is an effect that may be significant in the case of Ser. Surprisingly, labeling also proceeded efficiently with peptides containing Cys and Met at position $i+1$, thus suggesting that the sulfur atom does not interfere with the interaction. In contrast, peptides containing small or bulky side chains, such as Ala, Phe, Tyr, or Trp, were inefficient ligands, presumably because fewer van der Waals contacts and steric repulsion prevent binding of the FC anchor (Figure S5). Interestingly, while peptides containing the basic residues Lys and Arg were also effective ligands, peptides containing the acidic residues Glu and Asp were not effective ligands. These results suggest that electrostatic interaction with the acidic 14–3–3 protein might increase the affinity of the peptide ligand for the protein groove.

We then examined the labeling selectivity of 14–3–3ζ by using a cell lysate of *E. coli*, which expresses 14–3–3ζ with an N-terminal His_6 tag (His-14–3–3ζ; Figure 4). Although no reaction was detected when the lysate was treated with only

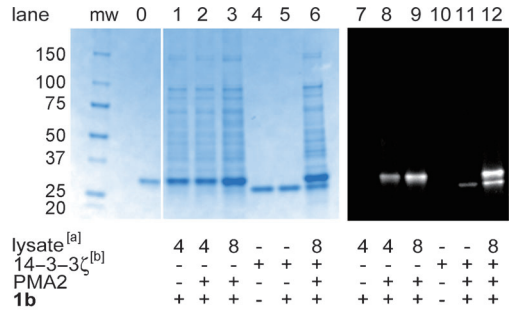


Figure 4. Labeling of the 14–3–3 protein was performed using *E. coli* cell lysate, **1b** (20 μM), and PMA2 (20 μM) in HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer (10 mM, pH 7.3) at 35 °C for 18 h. a) Total amount of added protein given in μg. b) 14–3–3ζ (10 μM) in which the His tag had been cleaved. mw: molecular-weight marker, lane 0: His-14–3–3ζ. mw and lanes 0–6: CBB-stained gel image; lanes 7–12: fluorescent gel image.

compound **1b** (Figure 4, lanes 1 and 7), an apparent fluorescent band corresponding to His-14–3–3ζ was observed when PMA2 peptide was added exogenously (Figure 4, lanes 8–9). In order to confirm that the His_6 tag in His-14–3–3ζ does not interfere with the labeling site, we tested lysate mixed with 14–3–3ζ in which the His tag had been cleaved. Both 14–3–3ζ and His-14–3–3ζ were almost equally labeled (Figure 4, lanes 6 and 12), thus confirming that selectivity based on His164 is not compromised by the His tag.

Finally, we sought to determine whether our FC-based probes could detect endogenous human 14–3–3 protein. We first evaluated the ability of compound **1b** to penetrate cells by using adhesive lung adenocarcinoma epithelial A549 cells, which are known to overexpress 14–3–3ζ.[8a] Compound **1b** was distributed throughout the cytosol, thus indicating that it readily enters cells (Figure 5A). For the in-cell detection experiment, we next chose a floating cell line, human leukemic monocyte lymphoma U937 cells, as it provides a sufficient amount of lysate for analysis. Cells were incubated with **1b** for 2 days in the presence of the protein kinase A activators 3-isobutyl-1-methylxanthine (IBMX) and forskolin.[11] The cell lysates were analyzed by fluorescence gel imaging and Western blotting with human 14–3–3ζ antibody (Figure 5B). No apparent fluorescent bands were detected in lysates of cells treated with **1b** in the absence of forskolin (Figure 5B, lane 7). In contrast, in cells incubated under hyperphosphorylation conditions, detection of endogenous 14–3–3 was dependent on the concentration of **1b** (i.e., detection at 8 and 16 μM, no detection at 4 μM; Figure 5B, lanes 8–10). Obviously, labeling of 14–3–3 with compound **1b** in U937 cells requires a high concentration of phosphorylated proteins, which most likely include the partner protein(s) that contribute to the formation of the ternary complex and consequently trigger intracellular 14–3–3 labeling. To the best of our knowledge, this is the first direct evidence indicating that 14–3–3 proteins are the primary target of FCs in human cells.

In conclusion, we developed natural-product-based fluorescent probes that are capable of labeling 14–3–3ζ in a site-specific, 14–3–3-selective, and, most importantly, highly ligand-dependent manner. The FC anchor precisely recog-

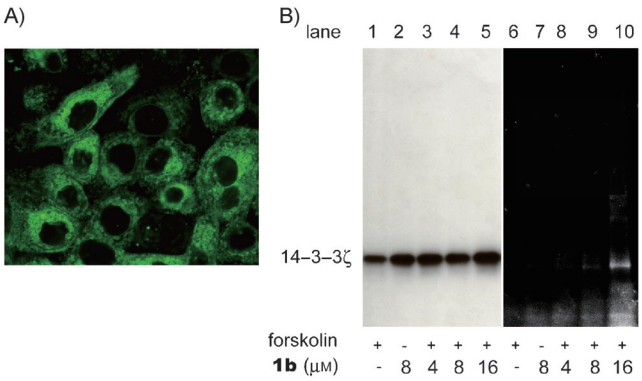


Figure 5. A) A549 cells were incubated with **1b** (50 μM) at 37 °C for 3 days. The sample was analyzed under a confocal microscope. B) U937 cells were treated with IBMX and then incubated with forskolin and **1b** (see the Supporting Information for details). The cell lysates were subjected to SDS-PAGE, and the gel was visualized by Western blotting with human 14–3–3ζ antibody (left) and by fluorescence (right).

nizes the structural difference of the residue at position $i+1$ in the phosphopeptide, thus enabling selective 14–3–3 labeling which depends on the shape of the ligand. This recognition-based selectivity of FCs will be an advantage further development of probes to detect 14–3–3 PPIs in a partner-protein-dependent manner. The FC derivative ISIR-042, which was used as the anchor here, has been developed for use as an antitumor agent.[6] The probes may also provide a robust tool for elucidating the mechanisms of FC antitumor activity, including identification of intracellular proteins involved in the formation of the ternary complex. Work toward these ends is currently in progress in our laboratory.

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