Probing the 14-3-3 Isoform-Specificity Profile of Protein–Protein Interactions Stabilized by Fusicoccin A

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ABSTRACT: Fusicoccin A (FC) is a fungal phytotoxin that stabilizes protein–protein interactions (PPIs) between 14-3-3 adapter proteins and their phosphoprotein interaction partners. Recently, FC has emerged as an important chemical probe of human 14-3-3 PPIs involved in cancer and neurobiology. These previous studies have established the structural requirements for FC-induced stabilization of 14-3-3-client phosphoprotein complexes; however, the effect of 14-3-3 isoforms on FC activity remains underexplored. This is a relevant question for the continued development of FC variants because there are seven isoforms of 14-3-3 in humans. Despite their sequence and structural similarities, a growing body of experimental evidence supports both tissue-specific expression of 14-3-3 isoforms and isoform-specific functions in vivo. Herein, we interrogate the isoform-specificity profile of FC in vitro using recombinant 14-3-3 isoforms and a library of fluorescein-labeled hexaphosphopeptides mimicking the C-terminal recognition domains of client proteins that are characterized targets of FC in vivo. Our results reveal modest isoform preferences for individual client phospholigands and demonstrate that FC differentially stabilizes PPIs involving 14-3-3σ. Together, these data support the feasibility of developing FC variants with enhanced isoform selectivity.

INTRODUCTION

14-3-3 proteins are a family of phosho-binding adapter molecules that form protein–protein interactions (PPIs) with hundreds of different client proteins.1,2 This large interactome is integrated with post-translational modifications to generate a dynamic signaling hub.3,4 At the molecular level, 14-3-3 PPIs are directed by phosphorylation of client proteins at serine or threonine residues within the consensus sequence RXXpZXP (X = any residue, pZ = phosphorylated S or T), although other recognition sequences exist.5 Once formed, this motif locates to an amphipathic groove on the surface of 14-3-3. The effect of 14-3-3 binding is dependent on the nature of the client and serves to finalize signal-induced events. Interactions with 14-3-3 proteins are known to modify protein trafficking or block interaction sites for effector proteins.6 In other settings, 14-3-3 proteins constrain binding partners into atypical conformations or serve as scaffolding to bring two clients proteins together.7,8 Despite this functional diversity, 14-3-3 PPIs have emerged as potential drug targets for cancer and neurological diseases.9,10 As such, the need for small-molecules to dissect the roles of individual 14-3-3 PPIs has become increasingly apparent.11 Fusicoccin A (FC) provides the best existing entry point to selective 14-3-3 PPI modulators (Figure 1).12 This phytotoxin is a well-characterized stabilizer of 14-3-3 PPIs in plants.13,14 More recently, FC has gained attention for its pro-apoptotic and neuroprotective properties in mammalian cell culture.15,16 Human 14-3-3 proteins are central in this pharmacology, as evidenced by studies connecting FC-induced stabilization of 14-3-3 PPIs in vitro to altered client function in vivo.17–23 These investigations have established that FC is incompatible
Table 1. Summary of Tissue Distribution and Isoform-Specific Functions of Human 14-3-3 Isoforms

| isoform (gene) | tissue localization | isoform-specific roles in disease | references |
|---------------|---------------------|----------------------------------|------------|
| σ (SFN)       | lung, breast, uterus, ovary, blood, skin, liver, pancreas, cornea | *epigenetically suppressed in epithelial carcinomas | 9, 28, 31 |
| β (YWHAB)     | brain, lung, colon, gastric lining, liver, bladder, kidney | *overexpressed in squamous cell carcinoma | 28, 35 |
| ε (YWAHE)     | brain (hippocampus), renal, liver, breast, gastric lining | *YWHAE deleted in Miller–Dieker syndrome | 10, 36 |
| ζ (YWHAZ)     | brain, lung, colon, head and neck, oral, ovary, esophagus | *overexpressed in cancer and correlates with poor prognosis | 28, 32, 33 |
| τ (YWAHQ)     | brain (frontal cortex), breast, lung, prostate | *overexpression protects from dopaminergic cell loss | 10, 34 |
| η (YWHAH)     | brain (frontal cortex), liver, prostate | *diminished expression in Alzheimer’s patients | 10, 34 |
| γ (YWHAH)     | brain, breast, liver, lung | *binding to α-synuclein disrupted in Parkinson’s disease | 10, 28, 37 |

The interaction profile of FC is broad, and therefore, the choice of client protein targets was carefully considered. There are at least 119 proteins in humans with a potential C-terminal recognition motif. In addition, certain noncanonical 14-3-3 PPIs are compatible with FC. Thus, to address the question of 14-3-3 isoform specificity, we focused on clients that are well-characterized targets of FC in vivo. These include the protein factor ERα, the potassium ion channel TASK3, and the platelet adhesion receptor GpIIb. As shown in Figure 2, we used synthetic N-fluorescein-tagged, C-terminal hexaphosphopeptides (ctp) that recapitulate the functional 14-3-3 recognition domains of these clients. Importantly, this phospholigand library was also variable with regard to relative hydrophobicity, number of charged residues, and identity of the phosphorylated side chain (pZ = S or T), and identity of the C-terminal residue (B = V or L).

**Figure 2.** N-Fluorescein-labeled hexaphosphopeptides used as 14-3-3 client phospholigands in this study.
and FC locate to the amphiphilic 14-3-3 phospho-binding groove (green), which comprised α-helices αC, αE, αG and αI.

The phosphopeptide partially fills this channel and is held in place via contacts between the phosphothreonine and a triad of polar residues (i.e., R56, R129, and Y130) of 14-3-3σ that form a conserved phospho-binding pocket. Moving up the binding groove, hydrogen bonds (2.5 − 2.9 Å) formed between the phospholigand and K49 and N175 of 14-3-3σ are apparent. These 14-3-3σ/ERα contacts are expected to be conserved for all C-terminal phospholigand binding to 14-3-3σ. Importantly, FC locates to a hydrophobic surface created by the union of 14-3-3σ and the phospholigand. Binding of the natural product is supported by simultaneous hydrophobic contacts with 14-3-3σ and ERα.26 In contrast, interactions between FC and 14-3-3σ are limited to the convex periphery of the natural product. These include three hydrogen bonds: (1) K122 of 14-3-3σ and the C3 alkyl group on FC; (2) D215 of 14-3-3σ and the C8 alcohol of FC; and (3) a water-mediated hydrogen bond formed between N42 of 14-3-3σ and the C9 glycoside of FC.

Having identified the key contacts in the 14-3-3σ-ERα-FC ternary complex, we carried out a multiple sequence alignment (Figure S1) to assay sequence homology across human 14-3-3 isoforms. As shown in Figure 3B, we found that residues within the phospholigand-binding groove are almost identical between isoforms. This similarity in 14-3-3σ contrasts with the diversity of C-terminal client protein sequences found to be compatible with FC.26 Nevertheless, the residues of 14-3-3σ contacting FC (including D122, D215, and N42) and the client phosphopeptide (including R56, R129, Y130, K49, and N175) are strictly conserved across all of the human isoforms.

Analyzing Dose–Response (EC50) Across 14-3-3 Isoforms. To evaluate the extent to which sequence homology...
within the 14-3-3 phospho-binding groove correlates with similarities in FC activity, we determined EC_{50} values for FC across the primary cytosolic 14-3-3 isoforms (i.e., β, ε, ζ, σ, and τ) using the suite of client phospholigands shown in Figure 2. As expected, each PPI in this series afforded a dose–response curve that allowed us to extract relative EC_{50} values for FC (Table 2). With both ERα-ctp (1) and Task3-ctp (2) as phospholigand 2, 14-3-3ζ gave the highest affinity (apparent K_d = 1.3 ± 0.1 μM), whereas 14-3-3ε showed the lowest affinity (apparent K_d = 6.0 ± 0.4 μM). Although this 4.6-fold difference in isoform preference is smaller than observed with phospholigand 1, it lies outside of the standard deviation of our measurements. Finally, for phospholigand 3, we observed that 14-3-3β exhibited the highest affinity (apparent K_d = 18 ± 3 μM), whereas 14-3-3σ showed the lowest affinity (apparent K_d = 47 ± 6 μM). It should be noted, however, that the standard deviation of data collected using 3 was larger than with other phospholigands, presumably because this peptide binds to 14-3-3 weakly compared to 1 and 2. Consistent with the titrations shown in Table 1, these data support isoform-dependent interactions for certain clients.

Table 2. Compiled EC_{50} Values (μM) for FC Across the Dominant Cytosolic Human 14-3-3 Isoforms^a,b

| 14-3-3 isoform | ERα-ctp (1) | Task3-ctp (2) | GplBz-ctp (3) |
|----------------|-------------|---------------|---------------|
| β              | 1.8 ± 1.1   | 3.0 ± 1.1     | 20 ± 1.2γ   |
| ε              | 2.8 ± 1.1   | 1.3 ± 1.1     | 78 ± 1.3γ   |
| ζ              | 3.5 ± 1.2   | 2.1 ± 1.1     | 7.6 ± 1.1   |
| σ              | 3.6 ± 1.1   | 3.2 ± 1.1     | 4.2 ± 1.1   |
| τ              | 2.8 ± 1.1   | 2.2 ± 1.1     | 38 ± 1.2γ   |

aReported EC_{50} values represent the average of two independent experiments. bThe [FC] was varied from 48 nM to 100 μM; [phospholigand] = 100 nM; [14-3-3] = 600 nM. Values represent the upper limit of the EC_{50}.

clients, the EC_{50} values for FC were similar across all isoforms, demonstrating a lack of isoform-specific association of FC with these binary protein complexes. In contrast, using the GplBz phospholigand (3), we observed more significant isoform-specific differences in EC_{50} values for FC. The lowest EC_{50} value, 4.2 ± 1.1 μM, was obtained with 14-3-3ε. This measurement was consistent with EC_{50} values determined using 14-3-3ε and clients 1 (3.6 ± 1.1 μM) and 2 (3.2 ± 1.1 μM). Conversely, the highest EC_{50} value, 78 ± 1.1 μM, was obtained with 14-3-3τ. This approximately 18-fold difference lies well outside the standard deviation of our measurements and demonstrates that, for some clients, FC exhibits clear isoform-specific activity.

Trends in Affinities of 14-3-3-Phospholigand Complexes. Having established that FC exhibits isoform-specific activity, we investigated the extent to which the affinity of individual phospholigands varies across 14-3-3 isoforms in the presence of FC in this series. A similar trend was observed for phospholigand 3; 14-3-3β showed the highest affinity (apparent K_d = 0.87 ± 0.2 μM) and 14-3-3ε had the lowest affinity (apparent K_d = 1.7 ± 0.2 μM), resulting in a 2.0-fold isoform preference.

Table 3. Intrinsic Affinity (Apparent K_d μM) of 14-3-3-Phospholigand Complexes^a,b

| 14-3-3 isoform | ERα-ctp (1) | Task3-ctp (2) | GplBz-ctp (3) |
|----------------|-------------|---------------|---------------|
| β              | 0.72 ± 0.1  | 2.4 ± 0.2     | 18 ± 3        |
| ε              | 3.4 ± 0.2   | 6.0 ± 0.4     | 28 ± 5        |
| ζ              | 1.5 ± 0.2   | 1.3 ± 0.1     | 20 ± 2        |
| σ              | 6.6 ± 0.6   | 2.1 ± 0.1     | 47 ± 6        |
| τ              | 1.1 ± 0.1   | 2.2 ± 0.2     | 20 ± 3        |

aReported K_d (μM) values represent the average of two independent experiments. bThe [14-3-3] was varied from 40 nM to 160 μM; [phospholigand] = 100 nM.

results of these experiments revealed clear differences in 14-3-3 isoform specificity for individual clients. Titrations with phospholigand 1 showed that 14-3-3β had the strongest affinity (apparent K_d = 0.72 ± 0.1 μM), whereas the same interaction using 14-3-3ε was comparatively weaker (apparent K_d = 6.6 ± 0.6 μM). This represents an approximately 9-fold preference between the two isoforms. On the other hand, with...
isofrom-dependent effects on FC activity, and they are clearly more substantial for 14-3-3σ than other isoforms evaluated in this study. Notably, the hydrophobic ERα sequence (1) was the most effectively stabilized by FC in complex with 14-3-3σ. Consequently, these trends should be considered in the future for design and biological evaluation of 14-3-3 PPI stabilizers based on the FC scaffold.

**CONCLUSIONS**

In summary, we examined the 14-3-3 isofrom-specificity profile of fusicochin A (FC), a natural product stabilizer of 14-3-3 functions in vivo. A sequence analysis of human 14-3-3 isoforms, along with inspection of available crystallographic data, demonstrated that the residues contacting both FC and client phospholigands bound in the 14-3-3 binding groove are strictly conserved across the different isoforms. Based on this observation, it was anticipated that FC-induced stabilization of 14-3-3-client phospholigand complexes would be very similar across the 14-3-3 isoforms. Nevertheless, isofrom preferences were observed using a series of phospholigands mimicking the 14-3-3 interaction motifs of characterized FC targets in vivo. These isofrom-dependent interactions were most notable in the absence of FC; however, our data suggest that FC-induced stabilization of PPIs involving 14-3-3σ depend on the nature of the client. This outcome is intriguing given that 14-3-3σ mediates cell death pathways and is suppressed in several cancers. It is also notable that the biological role(s) of 14-3-3σ are distinct relative to the other 14-3-3 family members (Table 1). Although the observed isofrom specificity of FC was modest, this selectivity might be more significant in vivo, where full-length client proteins can make additional contacts with 14-3-3 and FC. Consequently, isofrom specificity might be exploited via the rational design of non-natural FC variants provided the salient structure–activity relationships responsible for isofrom selectivity can be identified.

**EXPERIMENTAL SECTION**

**Materials.** FC was purchased from Enzo Life Sciences and used directly. N-Fluorescein-labeled hexaphosphopeptides that mimic the reported C-terminal recognition motifs of ERα (1), Task3 (2) and GpIBα (3) were obtained from Peptide 2.0 in >99% purity and used as received. pET-22b(+) expression vectors used for recombinant protein expression were obtained from Genscript. All other chemicals and consumables were purchased and used as received.

**Proteins.** Cloning, expression, and purification of human 14-3-3β (UniProt ID P31946), 14-3-3ε (UniProt ID Q04917), 14-3-3ζ (UniProt ID P63104), and 14-3-3τ (UniProt ID P27348) were performed as previously described for 14-3-3σ (UniProt ID P31947). Protein concentrations were determined spectrophotometrically. The purity of recombinant protein was analyzed by SDS-PAGE (Figure S2).

**Fluorescence Polarization (FP) Assays.** FP measurements were performed using a filter-based microplate reader (BioTek Synergy H1MF) with a fluorescein filter set (λex = 485/20 nm, λem = 535/25 nm) and an integration time of 50

![Figure 4](https://dx.doi.org/10.1021/acsomega.0c01454)
ms in black, flat-bottom 96 well plates (FLUOTRAC, medium binding). The measured polarization values were converted to anisotropy (A = 2P/3-P) and processed using GraphPad Prism.

For the determination of EC₅₀ values, a solution of 100 nM fluorescein-labeled phosphogalactin and 600 nM 14-3-3 was titrated with FC in buffer A containing 10 mM HEPES (pH 6.5), 150 mM NaCl, 0.1% (v/v) Tween 20, and 0.1% BSA. To obtain relative EC₅₀ values, the anisotropy signal (mA) was corrected for FC contribution in the absence of 14-3-3 (control) and plotted against logarithmic FC concentration. The reported EC₅₀ values were obtained by fitting the data into a four-parameter logistic curve, abbreviated 4PL, using the equation Y = bottom + (top − bottom)/(1 + 10^((log EC₅₀ − X)*HS)). In this model, the EC₅₀ value is the concentration that provokes a response half-way between the basal (bottom) response and the maximal (top) response. Y is the anisotropy signal, X is the concentration of FC on log scale, and HS is the Hill slope, which was constrained to a constant value of 1.

For the determination of apparent Kₚ values, a solution of 100 nM fluorescein-labeled phosphogalactin was titrated with 14-3-3 in buffer A in the presence and absence of 80 μM FC. The anisotropy signal was corrected for background signal at zero 14-3-3 concentration prior to fitting the data. The reported Kₚ values were determined, as previously described, by plotting fraction bound (q) as a function of 14-3-3 protein concentration. The value q was calculated from the equation q = (Y − Y₀)/(ΔY), where Y is the fluorescence anisotropy signal at each 14-3-3 concentration, Y₀ is the initial fluorescence anisotropy signal in the absence of 14-3-3, and ΔY is the change in fluorescence anisotropy signal. For titrations in the absence of FC and titrations performed with low-affinity ligand 3 in the presence of FC, data were fitted to the hyperbolic equation q = P₀/(Kₚ + P₀), where P₀ is the total 14-3-3 concentration, using the assumption that [P₀] ≈ [P]free which is applicable for weak binding. For titrations performed with ligands 1 and 2 in the presence of FC, the hyperbolic assumption is invalid because the concentration of the constant species is comparable to the Kₚ value. Therefore, these data were fit to the quadratic equation q = [(P₀ + L₀ + Kₚ) − (P₀ + L₀ + Kₚ)² − 4P₀P₀(L₀)]/(2L₀), where P₀ is the total 14-3-3 concentration and L₀ is the total ligand concentration. We developed a python script to solve this equation, which is provided in the SI.

Data Analysis. Reported values for EC₅₀ and Kₚ represent the average of two independent experiments, where individual measurements were collected in triplicate and averaged. The reported error represents standard deviation. Fold-stabilization (S⁾ was calculated by dividing the apparent Kₚ in the absence of FC by the Kₚ of a replicate experiment in the presence of 80 μM FC. The error was propagated using the standard equation for the division of measured quantities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01454.

SDS gel image of recombinant human 14-3-3 isoforms, processed FP data, and sequence alignment for 14-3-3 isoforms (PDF)

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Notes

The authors declare no competing financial interest.

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