An engineered survival-selection strategy for synthetic binding scaffolds specifically targeting post-translationally phosphorylated proteins

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Abstract

Protein phosphorylation plays an important role in the regulation of protein function and many cellular processes. Aberrant phosphorylation has been shown to be a cause of cell death as well as maligination. As such, there is an urgent need for affinity reagents that target phospho-modified sites on individual proteins. Currently, generation of phospho-specific antibodies relies primarily on hybridoma technology which requires phospho-epitope mapping through mass spectrometry, selection of the phospho-epitope to be targeted, and synthesis of a short phosphopeptide to be injected. As an alternative to immunization, protein display technologies (*e.g.*, phage, yeast, and ribosome display) have been employed as a viable approach to specifically select for binders against phospho-modified sites on individual targets. Though up-front phospho-amino acid identification is eliminated in protein display technologies, purification of kinases is still required. Another challenge is that the resulting antibody fragments require intradomain disulfide bonds for conformational stability, thus precluding their use as "intrabodies" in the reducing intracellular environment where most phosphoproteins of interest naturally reside. This bottleneck can be overcome by using alternative non-antibody binding scaffolds for molecular recognition such as designed ankyrin repeat proteins (DARPins), which do not contain disulfide bonds and can be expressed in soluble form with high yields in the cytoplasm of living cells thus allowing for intracellular applications. However, a drawback to the synthetic library approaches reported to date is that they rely on in vitro selection methods, such as phage display or ribosome display, which are technically demanding and labor intensive, and are implemented in cell-free environments that may not accurately reflect the complex conditions inside of a cell. To address the shortcomings of existing technologies, we recently adapted a previous genetic assay termed FLI-TRAP (*f*unctional *l*igand-binding *i*dentification by *T*at-based *r*ecognition of *a*ssociating *p*roteins) for selection of phospho-specific binders directly in living cells in a manner that greatly simplifies the process by which synthetic libraries are interrogated. Here, we extended this FLI-TRAP to affinity-mature a binding scaffolds called DARPins targeting phosphorylated human ERK2. Following just a single round of survival-based enrichment using FLI-TRAP, we selected phospho-specific DARPin mutants with greater binding affinity against pERK2, but not their non-phosphorylated counterparts. In addition, we reprogram the substrate specificity of the same DARPin towards non-cognate ERK2. Collectively, these results establish our genetic selection as a useful and potentially generalizable protein engineering tool for studying phospho-specific binding proteins and customizing their affinity and selectivity.