Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries

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AUTHOR SUMMARY

The response of living cells to their environment requires an array of sophisticated signal-processing circuits. Eukaryotic protein kinases are key signaling molecules that orchestrate these complex signal transduction pathways (1). To fully understand their functionality, they must be studied in their natural environment. This approach requires specific binding proteins that can fold inside the cell to create sensors for following signaling within the cell. Designed ankyrin repeat proteins (DARPins) (2) can constitute such molecules, because they correctly fold in the reducing environment of the cytoplasm.

Here, we demonstrate the selection of DARPins from a combinatorial library that specifically distinguish between the inactive (nonphosphorylated) and active (phosphorylated) forms of correctly folded mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 2 (ERK2). We rapidly obtained functional DARPins that can either inhibit or detect ERK2 activation in living cells, and we achieved this by focusing the selection on conformational epitopes that differ between both forms, i.e., are affected by the phosphorylation of ERK2 by upstream kinases.

We developed technology to select molecules from DARPin libraries that bind with high specificity to a variety of target molecules (2). Further, we demonstrated that these molecules can be selected for their ability to inhibit an enzyme within the cell (3). Here, we extended and refined our approach to ERK2, a member of the MAPK protein family, which comprises signal-transduction proteins of great biological and medical importance (4). Our goal was to address the question of whether DARPins are able to distinguish subtle structural differences between an inactive and an active MAPK. Such a discrimination would allow us to either inhibit or, alternatively, monitor ERK2 activation quantitatively in living cells and thus dissect kinase signaling pathways by complementing current gene knockout or antisense technologies.

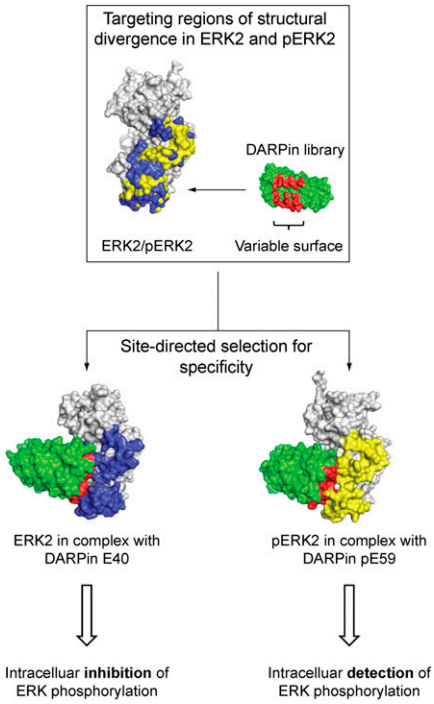

Fig. P1. Selection of binding proteins against structurally divergent regions in ERK2/pERK2 leads to ERK2-phosphorylation–specific probes for intracellular inhibition and detection of ERK activation. ERK2 is a member of the MAPK family. Upon phosphorylation, small conformational changes between the two ERK2 states (blue and yellow) served as epitopes for site-directed selection of ERK- and pERK2-specific binding proteins from DARPin libraries. Structural analysis of two selected DARPins in complex with their cognate ERK2 form (DARPin E40/ERK2 and DARPin pE59/pERK2) verified our strategy and gave insight into binding specificity. Both DARPins recognize conformational changes within the kinase activation loop and adjacent regions. Binding to such regulatory structural elements of PTMs resulted in intracellular inhibition or detection of ERK2 activation by phosphorylation-specific ERK kinase DARPins in a predictable manner.

Using the respective nondesired ERK2 form as a competitor, we used ribosome display to select DARPins that specifically recognize either the unphosphorylated (ERK2) or the phosphorylated form (pERK2) of the kinase. We determined that selected DARPins target a similar region in either ERK2 or pERK2. This finding is consistent with the results of a comparative structural analysis of inactive ERK2 and fully active pERK2 (5) revealing, upon phosphorylation of residues Thr-183 and Tyr-185, conformational rearrangements in a region that is limited to adjacent secondary structural elements. The primary sequences surrounding these residues are not conserved in other kinases, explaining kinase and conformation specificity.

We investigated the basis of this binding specificity at the atomic level by cocrystallization of ERK2 and pERK2 with two representative DARPins, E40 and pE59, which recognize ERK2 and pERK2 specifically. The structures of the complexes were solved by molecular replacement and refined to 1.9 Å for E40/ERK2 [Protein Data Bank (PDB) ID 3zu7] and 2.7 Å for pE59/pERK2 (PDB ID 3zuv) (Fig. P1). Analysis of the structures revealed that both DARPins target identical regions and residues of ERK2/pERK2, but specifically discriminate different spatial conformations in both kinase forms, which occur upon phosphorylation (5). The interaction surface in both complexes comprises three

Author contributions: L.K., P.P., A. Prinz, F.W.H., and A. Plückthun designed research; L.K., P.P., P.R., B.M., A. Prinz, P.R.E.M., M.K., and B.Z. performed research; L.K., P.P., P.R., B.M., A. Prinz, P.R.E.M., M.K., B.Z., F.W.H., and A. Plückthun analyzed data; and L.K., A. Prinz, P.R.E.M., F.W.H., and A. Plückthun wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3ZU7 and 3ZUV).

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See full research article on page E2248 of www.pnas.org.

Cite this Author Summary as: PNAS 10.1073/pnas.1205399109.

regions of ERK2/pERK2, the activation loop bearing residues Thr-183 and Tyr-185, the MAPK insertion, and α-helices G and 1L12. These structural elements retain the conformation of unbound pERK in the complex with DARPin pE59, emphasizing their rigidity in the activated state. In contrast, the activation loop of ERK2 adopts a conformation in the complex with DARPin E40 that is different from that found in free ERK2, consistent with the plasticity of the unphosphorylated loop. Consequently, the observed binding specificity of DARPins E40 and pE59 can be mainly attributed to their discrimination of the arrangement of the activation loop and its side chains as well as to the different chemical properties of residues Thr-183 and Tyr-185 upon phosphorylation. The high degree of selectivity of these two DARPins for their cognate ERK2 form was confirmed by surface plasmon resonance analysis of the binding affinities for ERK2 or pERK2. Further, the DARPins selectively precipitated the expected ERK2 forms from cell lysates. Thus, we conclude that our selection strategy successfully directed the DARPins to those regions for which conformational rearrangements were observed between free ERK2 and pERK2.

Finally, we demonstrated that DARPins E40 and pE59 retained their anticipated functionality in living cells, i.e., as an inhibitor (E40) or as a sensor (pE59) of ERK2 activation. By using bioluminescence resonance energy transfer (BRET) assays, we verified specific binding of DARPin E40 to ERK2 and DARPin pE59 to pERK2 within intact COS7 cells. Both DARPins did not interact with two other MAPK family members, JNK1 and JNK2. Western blot analysis showed that binding of the ERK-specific DARPin E40 efficiently blocks phosphorylation of residues Thr183 and Tyr185 in ERK2 after stimulation of ERK signaling. In contrast, the pERK2-specific DARPin reporter pE59 did not perturb ERK2 phosphorylation, but detected ERK2 activation after stimulation or inhibition of the ERK phosphorylation in COS7 cells.

The current study demonstrates that DARPins can be selected to reliably differentiate between the states of a posttranslationally modified protein and we provide an explanation of the basis of selectivity by determining the structures of both complexes. Our approach is generally applicable and could be easily extended to other proteins of interest with posttranslational modifications (PTMs) different from phosphorylation. Because PTMs are involved in many cell regulatory processes, binding proteins specific for PTMs themselves or the ensuing conformational changes would be of great value, both as detectors and as targeted inhibitors in living cells. In combination with the uniformity and stability of the DARPin scaffold, our findings highlight the potential of binding proteins for future use in functional assays and as affinity reagents in diagnostic microarrays to help understand the action of drugs.

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