A Homogenous Multiplexing Assay Studying the Phosphorylation-Interaction Interplay Between MAP2Ks and MAP kinases

Mathieu Arcand, Roger Bossé, Philippe Roby & Sophie Dahan

1 Abstract

By multiplexing AlphaScreen® and AlphaLISA® beads, we have recently observed in a single well the direct dissociation of MAP kinase ERK2 from MAP2K MEK1 upon phosphorylation. Furthermore, we have used this experimental approach to discriminate an allosteric inhibitor from an ATP competitor. Here, we use these tools to further study the protein-protein interactions between MEK1 and ERK2 with respect to phosphorylation state-dependent and -independent effects.

We first determined that the dissociation between the two proteins is solely dependent on their activation state and does not involve a phosphorylation feedback loop. Second, by co-titration experiments on full-length recombinant proteins, we confirmed that phosphorylation of MEK1 and/or ERK2 reduces their binding. However, this interaction is more sensitive to the phosphorylation of MEK1 than that of ERK2. Interestingly, the binding between unphosphorylated MEK1 and ERK2 is impaired by increasing ATP concentrations. This interaction modulation requires the intact catalytic domain of MEK1 but not that of ERK2. Other phosphonucleotides such as ADP, ATP-γ-S, and UTP can also modulate MEK1-ERK2 binding, whereas AMP and GTP have no effect. Finally, ATP can modulate the interaction between ERK2 and its non-cognate MAP2K MKK6 also in a phosphorylation-independent manner.

The above results suggest that nucleotides can influence the conformation of MAP2Ks. These changes affect MAP kinase binding and our Alpha technologies platform provided a sensitive approach to detect this subtle modulation in protein-protein interactions. More so, multiplexing phosphorylation and interaction events allows screening for small molecules that modulate catalytic activity and enzyme-substrate interactions, either individually or together.

2 Introduction

MAP kinase pathways are essential to numerous biological processes that include growth and inflammation. These pathways are tightly regulated by interplay between phosphorylation events, protein-protein interactions and subcellular localization. For example, in the ERK MAP kinase pathway, the upstream MAP2K, MEK1, binds to MAP kinase ERK2. This interaction occurs in the cytoplasm with both proteins unphosphorylated. Upon sequential phosphorylation, the kinases become active; this in turn causes their dissociation and subsequent ERK2 nuclear localization.

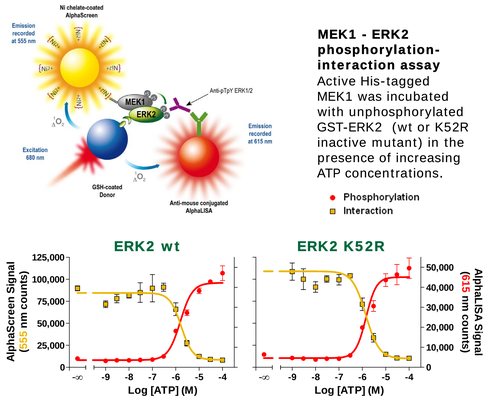
Model for ERK2 activation by MEK1
MEK1 is activated by phosphorylation and phosphorylates in turn ERK2, thereby causing their dissociation.

3 Materials & Methods

All recombinant protein kinases were purchased from Carna Bio except N-His-MEK1 from SignalChem and C-His-MEK1 (wt and K97R) from Millipore. Phosphorylation-specific antibodies were purchased from Cell Signaling Technology. AlphaScreen and AlphaLISA beads as well as the EnVision® Multilabel Plate Reader and filters were from PerkinElmer.

All assays were performed in 384 well OptiPlates™ (PerkinElmer) at 23°C in kinase buffer containing 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1mM DTT and 0.01% Tween20. All kinases and substrates were used at 100 nM. Kinase reactions were started by adding ATP and incubated for 2h before addition of detection reagents. Donor beads were used at 40 µg/mL and AlphaScreen and AlphaLISA Acceptor at 20 µg/mL each. Assays were read twice by unmodified EnVision reader, first with a Texas Red 555 nm filter, then with a Europium 615 nm narrow bandwidth filter.

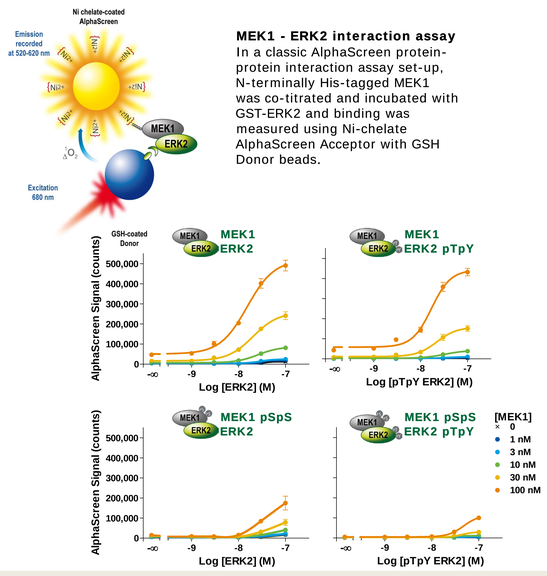
4 ERK2 Dissociates from MEK1 Upon Phosphorylation



MEK1 - ERK2 phosphorylation-interaction assay
Active His-tagged MEK1 was incubated with unphosphorylated GST-ERK2 (wt or K52R inactive mutant) in the presence of increasing ATP concentrations.

ERK2 dissociates upon phosphorylation by MEK1. Catalytically-inactive ERK2 mutant (K52R) behaves as wild type (wt) protein suggesting that dissociation of the proteins is not due to MEK1 feedback phosphorylation by ERK2.

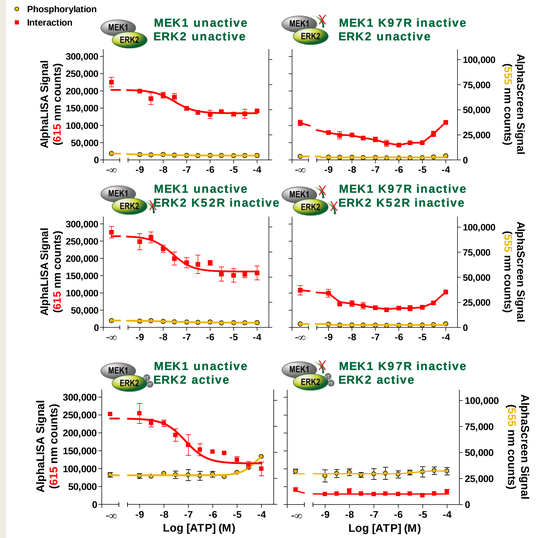
5 Phosphorylation States Alter MEK1-ERK2 Interaction



MEK1 - ERK2 interaction assay
In a classic AlphaScreen protein-protein interaction assay set-up, N-terminally His-tagged MEK1 was co-titrated and incubated with GST-ERK2 and binding was measured using Ni-chelate AlphaScreen Acceptor with GSH Donor beads.

Though not abolished, MEK1-ERK2 binding is impaired by phosphorylation of either counterpart, but more severely by that of MEK1 than of ERK2.

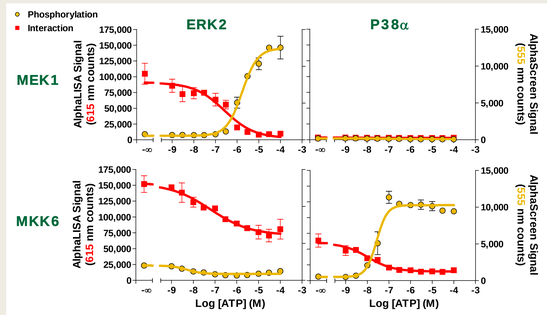
6 ATP Modulates MEK1-ERK2 Binding



MEK1 - ERK2 phosphorylation-interaction assay C-His-tagged MEK1 variant were incubated with unphosphorylated GST-ERK2 (wt or K52R inactive mutant) in the presence of increasing ATP concentrations. Detection occurred as in (4) except AlphaScreen and AlphaLISA beads were swapped.

Increasing ATP concentrations reduce MEK1-ERK2 interaction. This effect requires the intact catalytic domain of MEK1 but not of ERK2.

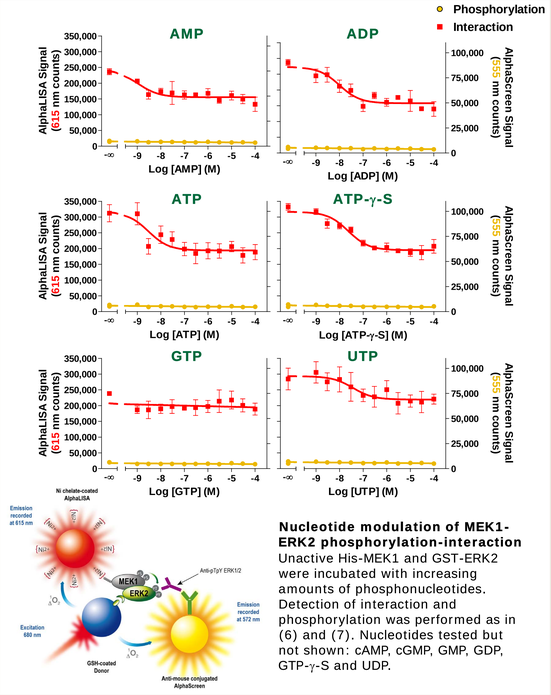
7 ATP Modulates MKK6– ERK2 Binding



MAP2K– MAP kinase phosphorylation-interaction assay Active N-His-MAP2K were incubated with cognate or non-cognate GST-MAP kinase in the presence of ATP. Detection of interaction was performed using Ni AlphaLISA beads and phosphorylation detection using an anti-pTpY ERK2 or anti-pTpYP38 antibody captured by Anti-mouse AlphaScreen Beads.

ATP decreases MKK6-ERK2 binding in a phosphorylation-independent manner.

8 Phosphonucleotide Effects on MEK1-ERK2 Binding



Nucleotide modulation of MEK1-ERK2 phosphorylation-interaction
Unactive His-MEK1 and GST-ERK2 were incubated with increasing amounts of phosphonucleotides. Detection of interaction and phosphorylation was performed as in (6) and (7). Nucleotides tested but not shown: cAMP, cGMP, GMP, GDP, GTP-γ-S and UDP.

Adenosine and uracil nucleotides affect the MEK1-ERK2 interaction whereas guanosine and cyclic nucleotides do not.

9 Summary

- Combined use of AlphaScreen and AlphaLISA beads allowed simultaneous monitoring of substrate phosphorylation and enzyme interaction
- AlphaScreen and AlphaLISA beads can be used interchangeably
- ERK2 dissociates from MEK1 solely upon phosphorylation
- MEK1-ERK2 interaction is perturbed by phosphorylation of ERK2 but more significantly by phosphorylation of MEK1
- ATP modulates MEK1-ERK2 binding
  - In a phosphorylation-independent manner
  - This requires the kinase integrity of MEK1 but not of ERK2
  - MKK6-ERK2 interaction is also ATP-sensitive
  - Other nucleotides exert similar effects on MEK1-ERK2
- Our assays provide a sensitive platform to detect protein-protein interactions that can be modulated by small molecules such as nucleotides in a phosphorylation-dependent or independent manner

PerkinElmer, Inc., 940 Winter Street, Waltham, MA USA (800) 762-4000 or (+1) 203 925-4602 www.perkinelmer.com