Single-well simultaneous measurement of MAP2K MEK1 activity and interaction with the MAP kinase ERK2 using AlphaScreen and AlphaLISA platforms

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1 Abstract

Protein kinases play crucial regulatory roles in important biological processes and represent attractive pharmacological targets. Most of today's kinase inhibitors block target phosphorylation by competing with ATP for binding to the catalytic cleft of the kinase. There is increasing demand for less promiscuous compounds including allosteric and protein-protein interaction modulators representing technological challenges for several screening approaches. Here we describe how AlphaScreen® and AlphaLISA® platforms were used for the simultaneous detection of biomolecular interaction and phosphorylation. These technologies have proven to be versatile and powerful tools for monitoring protein-protein interactions as well as phosphorylation events. MEK1 and its substrate ERK2 were used as model. In their un-phosphorylated state, they form a complex that dissociates upon activation by phosphorylation. Data will be shown where MEK1 activity on ERK2 and binding are measured simultaneously. For a compound screen, such a twin detection set-up could provide immediate discrimination between ATP competitors, interaction inhibitors, and allosteric modulators.

2 Introduction

Activated by numerous stimuli, the ERK MAP kinase cascade is a key regulator of processes such as cell growth, survival and proliferation. At the bottleneck of this cascade are ERK1/2 and their upstream activators MEK1/2. We have used MEK1 and ERK2 as an experimental model to simultaneously monitor phosphorylation and interaction events.

Although, many reports on MEK1 and ERK2 regulation exist, phosphorylation and interaction events have always been observed separately. Here, we propose a method to monitor simultaneously both types of biomolecular events using Amplified Luminescence Proximity Homogenous Assay (Alpha) technology, with MEK1 and ERK2 as an experimental model.

MEK1 – ERK2 activation model. In the unactive state, unphosphorylated MEK1 and ERK2 are tightly bound. Activated by phosphorylation, MEK1 phosphorylates and activates ERK2, resulting in ERK2 activation and complex dissociation.

Alpha technology relies on the bridging of Donor and Acceptor beads. Excitation of Donor beads at 680 nm generates the release of singlet oxygen that can diffuse for 200 nm before losing its capacity to trigger light emission from Acceptor beads. This makes the technology suitable for observing many biochemical phenomena such as phosphorylation reactions and protein-protein interactions.

Both AlphaScreen and AlphaLISA Acceptor beads are activated by singlet oxygen to produce light. In contrast, the fluorophores used in each Acceptor bead differs, thus conferring different emission properties to AlphaScreen and AlphaLISA Acceptor beads. We took advantage these properties to simultaneously monitor phosphorylation and interaction events.

3 Materials

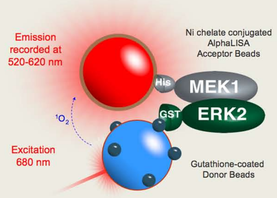
Recombinant protein kinases were purchased as listed (right). Mouse anti-dual phosphorylated ERK1/2 antibody was purchased from Cell Signaling Technology; ERK activation inhibitory peptide from Invitrogen; U0126 and SL-327 from EMD. AlphaScreen and AlphaLISA beads as well as the EnVision® 2101 and 2103 readers and filters were from PerkinElmer.

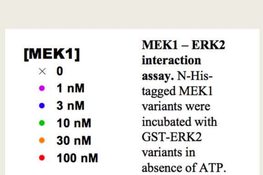
Kinase	Activity	Tag	Supplier
ERK2	Active	GST-N	Carna Bio
ERK2	Unactive	untagged	Signal Chem
ERK2	Unactive	GST-N	Carna Bio
MEK1	Active	His-N	Carna Bio
MEK1	Unactive	His-N	Signal Chem

4 Methods

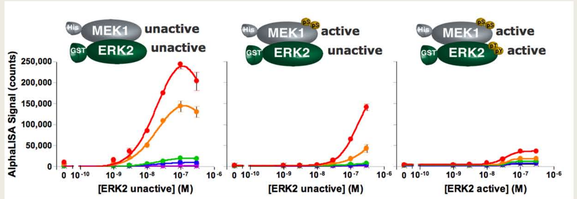
All assays were performed in 384 well plates (PerkinElmer) at 23°C in kinase buffer containing 20 nM Tris pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1mM DTT and 0.01% Tween20. Kinase reactions were started by adding ATP and incubated for 2h before addition of detection reagents. Donor and Acceptor beads were used at 20 µg/mL for each type and assays were read using the AlphaScreen protocol. For phosphorylation-interaction assays, 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 readers, first with a dysprosium 572 nm narrow bandwidth filter, then with a europium 615 nm narrow bandwith filter.

5 MEK1-ERK2 Interaction



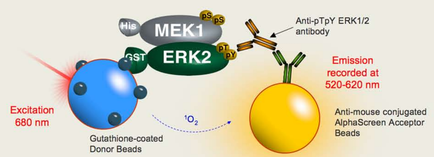


MEK1 – ERK2 interaction assay. N-His-tagged MEK1 variants were incubated with GST-ERK2 variants in absence of ATP.

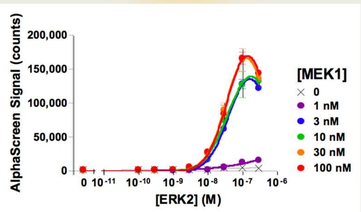


MEK1-ERK2 interactions can be detected with an antibody-free AlphaLISA set-up, and activation state of either protein greatly influences binding.

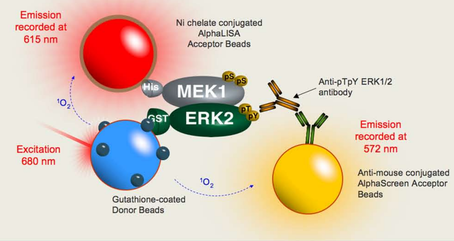
6 ERK2 Phosphorylation by MEK1

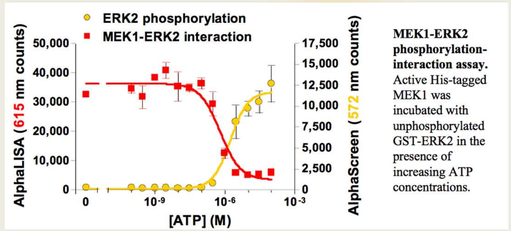


MEK1 kinase assay on ERK2. His-tagged active MEK1 was incubated with unphosphorylated GST-ERK2 in the presence of 10 µM ATP.



7 Phosphorylation-Interaction Assay

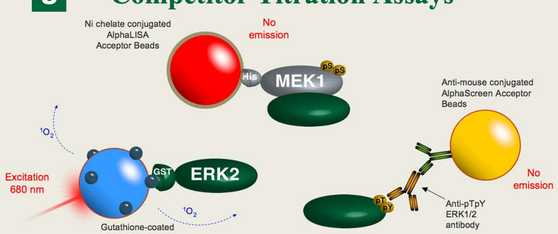


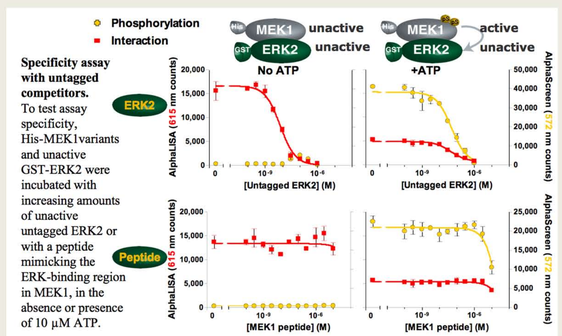


MEK1-ERK2 phosphorylation-interaction assay. Active His-tagged MEK1 was incubated with unphosphorylated GST-ERK2 in the presence of increasing ATP concentrations.

We here provide the first direct evidence that ERK2 phosphorylation triggers its dissociation from active MEK1. Both biomolecular events are intrinsically linked with interaction IC_{50} matching ERK2 phosphorylation EC_{50}.

8 Competitor Titration Assays

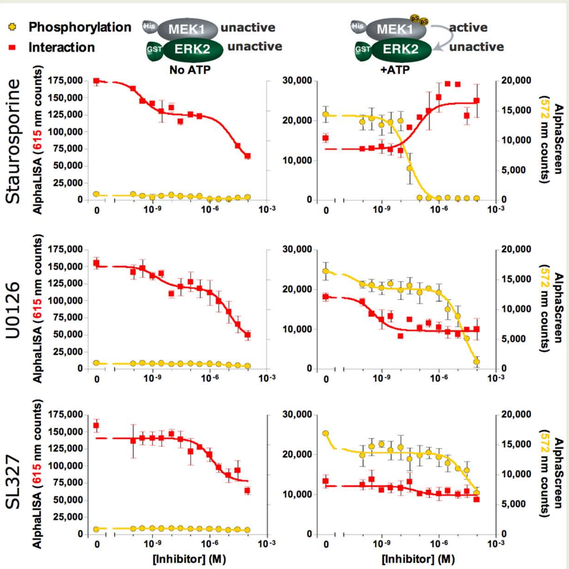




Specificity assay with untagged competitors. To test assay specificity, His-MEK1variants and unactive GST-ERK2 were incubated with increasing amounts of unactive untagged ERK2 or with a peptide mimicking the ERK-binding region in MEK1, in the absence or presence of 10 µM ATP.

ERK2 phosphorylation is abolished by disrupting its interaction with upstream activator MEK1. Full-length untagged ERK2 protein inhibits ERK2-MEK interaction with more potency than the MEK1 peptide.

9 Pharmacological MEK1 inhibition



His-MEK1 – GST-ERK2 phosphorylation-interaction assay on ERK2. His-MEK1 variants and unactive GST-ERK2 were incubated with increasing amounts of pharmacological kinase inhibitors, in the absence or presence of 10 µM ATP.

Although all three inhibitors affected unactive MEK1 and ERK2 binding, their active MEK1 inhibition mechanism greatly differs. The poorly selective ATP competitor staurosporine perturbs ERK2 phosphorylation while partially rescuing its interaction with MEK1. U0126, a reported Raf and MEK1/2 inhibitor displays allosteric effects on MEK1. In contrast, the MEK1/2 inhibitor SL327 affected MEK1 catalytic activity, but not its interaction with ERK2.

10 Summary

- MEK1-ERK2 binding was measured with AlphaLISA beads
- The phosphorylation/activation states of both proteins affects their physical interaction
- ERK2 phosphorylation was detected with AlphaScreen beads
- Phosphorylation and interaction events were simultaneously monitored by combining AlphaScreen and AlphaLISA beads
- These biomolecular events have also been monitored with the kinase pairs Raf1-MEK1 and Akt1-GSK3β (data not shown)
- Upon ERK2 phosphorylation, there is a marked decrease of its association with MEK1
- Phosphorylation and binding can be disrupted with a specific peptide or, more potently, by untagged ERK2
- Simultaneous monitoring of phosphorylation and interaction events allowed us to delineate distinct mechanisms of action for three MEK1 inhibitors: Staurosporine, U0126 and SL327

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