

B-RAF FORMS REGULATED HETERODIMERS WITH RAF-1

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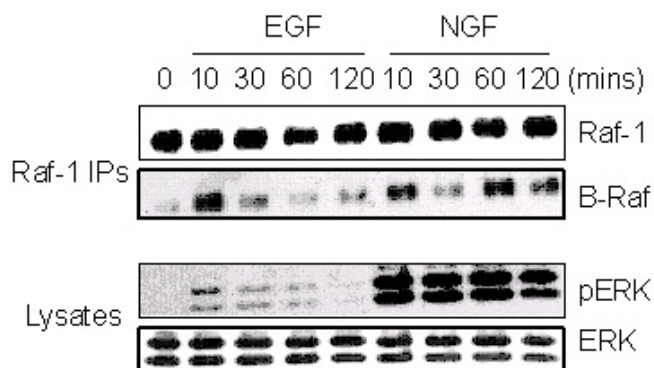
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INTRODUCTION. The MAPK pathway signalling cascade (RAS-RAF-MEK-ERK) plays a role in fundamental cellular processes such as proliferation, differentiation and apoptosis. Somatic mutations in the protein components of this pathway are linked with malignant transformation making it an important target for potential cancer therapy. For example, B-Raf has recently been found mutated in 66% of malignant melanomas (1). B-Raf has previously been hypothesised to be a member of a Raf-1 signalling complex (2), and here we show an endogenous interaction between Raf-1 and B-Raf that is mitogen-regulated and can be identified in all cell types analysed. Heterodimer formation is stabilised by pharmacological inhibitors of Raf or MEK suggesting that the disruption of the Raf-1/B-Raf complex is under negative feedback regulation. Further, we show that in addition to Raf-1, B-Raf binds to the Raf Kinase Inhibitor Protein (RKIP), and that RKIP can dissociate B-Raf/Raf-1 heterodimers.

METHODS. PC12 cells were starved and stimulated using EGF or NGF (Calbiochem) for the indicated times. Lysates were blotted for P-ERK (Sigma) production, then immunoprecipitated for Raf-1 (Santa Cruz) and probed for B-Raf (Santa Cruz); PC12 cells were starved and treated with the MEK inhibitor U0126 (Promega) for 30 minutes then stimulated with NGF. Lysates were immunoprecipitated for Raf-1 and probed for B-Raf; COS-1 cells were co-transfected with B-Raf, Raf-1 and either vector or RKIP. Cells were starved and stimulated with TPA for 10 minutes. Lysates were immunoprecipitated for B-Raf and probed for Raf-1.

RESULTS. Immunoprecipitation of Raf-1 shows B-Raf binding to Raf-1 in a regulated manner upon stimulation with either EGF or NGF (Fig 1). These two mitogens differ in the time they can sustain the interaction. EGF can stimulate efficient formation of the heterodimer for 30 minutes and NGF can sustain the interaction for up to 120 minutes. Pre-treatment of PC12 cells with a MEK inhibitor causes an increase in heterodimer production on NGF stimulation in comparison to



untreated cells (Fig 2). After 30 minutes a significant difference is seen. Total inhibition of P-ERK production in the lysate confirms U0126 is functionally inhibiting MEK activity. TPA treatment of COS-1 cells over-expressing B-Raf and Raf-1 stimulates heterodimer formation. Co-transfection of RKIP causes a decrease in heterodimers (Fig 3).

Fig 1: B-Raf forms heterodimers with Raf-1 in PC12 cells

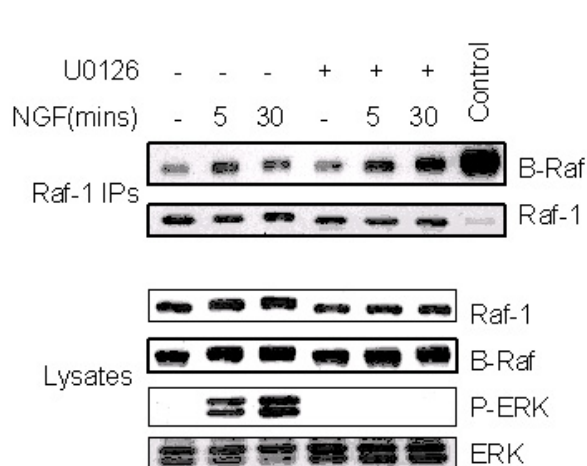


Fig 2: MEK inhibitor U0126 causes a increase in heterodimer formation

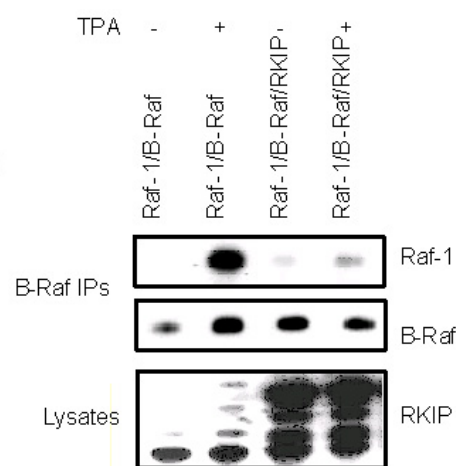


Fig 3: RKIP dissociates heterodimers

DISCUSSION. The kinetics of the formation of Raf-1/B-Raf heterodimers in PC12 cells in response to stimulation with EGF and NGF is consistent with the model that EGF causes a transient activation of the ERK pathway, while NGF provides a more sustained activation through B-Raf (3,4). This infers that there may be a direct correlation between heterodimer formation and ERK activity. Use of the MEK inhibitor suggested a mechanism for the control of the heterodimer through a negative feedback loop from ERK causing heterodimer dissociation. B-Raf has an ERK phosphorylation motif in its C-terminus containing a serine and threonine (5) and we are currently investigating the possibility that either or both of the sites when phosphorylated by ERK causes B-Raf to dissociate from Raf-1. We have however also found another more physiological regulator of the heterodimer in RKIP. RKIP binds to Raf-1 and MEK and selectively impairs the phosphorylation of MEK by Raf-1. We have now seen that RKIP also binds to B-Raf and has a similar effect on MEK phosphorylation (data not shown). In COS cells RKIP inhibits the formation of heterodimers, possibly by posing a steric obstacle to the association in the same way it does for Raf-1 and MEK. In summary, these results suggest two possible independent mechanisms for the regulation of the heterodimer – an ERK phosphorylation of B-Raf causing dissociation and an inhibitor of the association, RKIP. Clearly further work is required to elucidate the mechanisms at work here, and to find a functional role for the heterodimer.

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