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Raf-1 and B-Raf promote protein kinase C θ interaction with BAD

Alison Hindley [a], Walter Kolch [a,b,*]

[a] *Signalling and Proteomics Laboratory, The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK*
[b] *Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*

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Abstract

PKCθ regulates the proliferation, survival and differentiation of T-cells. Here we show that PKCθ interacts with Raf-1 and B-Raf kinases. Raf-1 enhanced the kinase activity of associated PKCθ, while PKCθ reduced the catalytic activity of associated Raf-1. In contrast, B-Raf binding did not affect PKCθ kinase activity, and PKCθ did not change B-Raf activity. Coexpression of mutationally activated Raf-1 in cells enhanced the phosphorylation of T538 in the PKCθ activation loop. PKCθ and Raf cooperated in terms of binding to BAD, a pro-apoptotic Bcl-2 family protein that is inactivated by phosphorylation. While neither Raf-1 nor B-Raf could phosphorylate BAD, they enhanced the ability of PKCθ to interact with BAD and to phosphorylate BAD *in vitro* and *in vivo*, suggesting a new role for Raf proteins in T-cells by targeting PKCθ to interact with and phosphorylate BAD.

Keywords: T-cells; Raf-1; B-Raf; PKCθ; BAD; Phosphorylation; Signal transduction

1. Introduction

Raf kinases are the entry point to the ERK/MAPK pathway, a three tiered kinase cascade where Raf phosphorylates and activates MEK which in turn phosphorylates and activates ERK. This pathway is involved in the regulation of many fundamental cellular processes including cell survival, proliferation, transformation and differentiation [1,2]. The Raf family has three members, A-Raf, Raf-1 and B-Raf. Raf-1 is ubiquitously expressed, while A-Raf and B-Raf expression appears more restricted [3]. A-Raf is mainly found in urogenital tissues, and B-Raf in haematopoietic and neuronal cells, although newer data suggest that they are more widely expressed [4]. Recently, B-Raf has received major attention as it is frequently mutated in melanoma and other cancers [5]. All three Raf kinases are activated by binding to Ras, but there are salient differences in the detailed mode of activation [6,7]. Raf-1 undergoes a multistep activation sequence that ensues with membrane translocation as a result of Ras binding. A-Raf activation probably is similar, whereas B-Raf activation is much simpler. Raf kinases share MEK as a common substrate, but differ in their specific activity with B-Raf being the most active and A-Raf the poorest MEK kinase.

Despite many attempts to identify other substrates, MEK is hitherto the only commonly accepted substrate for Raf kinases. However, accumulating evidence mainly from knock-out studies in mice has strongly suggested that Raf isozymes also serve isoform specific functions that are different from their well characterised role in the ERK pathway [8]. So far, Raf-1 has been convincingly demonstrated to function independently of the ERK pathway in apoptosis protection by inhibiting the pro-apoptotic kinases MST2 [9] and ASK1 [10], and in cell migration by regulating Rho kinase α (ROKα) [11]. Surprisingly, these roles are also independent of Raf-1 kinase activity and could be explained by Raf-1 acting as a scaffold or adaptor protein. Nevertheless, alternative Raf kinase substrates may exist. One of the proposed Raf-1 substrates includes BAD [12], a proapoptotic BH3 family member, which is inactivated by phosphorylation [13,14]. However, later reports could not confirm the evidence for a direct phosphorylation of BAD by Raf-1 [15] indicating that it may be a Raf-1 associated kinase that phosphorylates BAD. We have previously engineered Raf-1 to accept orthogonal ATP analogues and shown that novel

* Corresponding author. The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK. Tel.: +44 141 330 3983; fax: +44 141 942 6521.
E-mail address: wkolch@beatson.gla.ac.uk (W. Kolch).

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phosphorylations could be induced in cell lysates suggesting the existence of further Raf-1 substrates [16].

Here we have employed a bioinformatics approach to predict alternative Raf substrates. For this we used Scansite [17] to assemble a weighted matrix of a Raf consensus phosphorylation sites based on an alignment from MEK1 and MEK2 proteins from different species across all eukaryotic taxa. Besides MEK proteins the search revealed only two other main candidates, Adenylate Cyclase VI (ACVI) and PKCθ. ACVI has recently been described as a putative Raf-1 substrate [18,19], and here we characterise the relationship between PKCθ and Raf proteins in more detail. The phosphorylation site in the PKCθ activation loop was detected by a commonly used phospho-MEK antibody attesting the similarity of the phosphorylation motifs. However, while we could not obtain evidence that Raf kinases phosphorylate PKCθ, we found that Raf kinases enhance the ability of PKCθ to interact with BAD and phosphorylate BAD.

2. Materials and methods

2.1. Cell culture

COS-1 cells were cultured in Dulbecco's minimal essential medium (DMEM; Invitrogen) supplemented with 10% foetal calf serum (FCS) and 1% glutamine, and grown at 37 °C in 5% CO_2. For serum starvation cells were washed twice with PBS and incubated in DMEM supplemented with 0.2% FCS and 1% glutamine overnight. COS-1 cells were transfected with Effectene reagent (Qiagen) following the manufacturers instructions. T-cell lines Jurkat, CCRF-CEM and Molt-4 were maintained in RPMI (Invitrogen) supplemented with 10% FCS and 1% glutamine and grown at 37 °C in 5% CO_2. Sf-9 cells were maintained in TC100 medium (Invitrogen) supplemented with 10% FCS, 1% L-glutamine, 0.1% pluronic solution (Sigma) and 0.1% amphotericin B (Sigma) and grown at 27 °C.

2.2. Antibodies, reagents and plasmids

Raf-1 and PKCθ monoclonal antibodies (mAbs) were from BD Transduction Laboratories; B-Raf H145 polyclonal antibody was from Santa Cruz; MEK1/2 and phospho-MEK1/2 polyclonals, BAD polyclonal (detecting mouse BAD), phospho-BAD Ser136 polyclonal and phospho-BAD Ser112 mAb were from Cell Signalling Technology; FLAG M2 agarose was from Sigma; BAD antibody detecting human BAD was from AbCam. Rottlerin was from Calbiochem; TPA from Sigma. Raf-1, B-Raf and PKCθ mutants were created using the Quick Change kit from Stratagene. A mammalian PKCθ expression vector and the His-PKCθ virus were generously provided by Dr. A Altman. The mammalian expression vectors for B-Raf [20] and FLAG-tagged Raf-1 [21] were described previously. The GST-tagged expression vector for mouse BAD (pEBG-mBAD) was from Cell Signalling Technology. The GST-Raf-1 virus was described previously [22]. The GST-B-Raf virus was made as follows. Full length human B-Raf [20] was cloned as HindIII-NdeI into pSL1180 (Pharmacia). The GST-baculovirus vector was made by cutting out a 2.4 kb fragment of the Braf cDNA from pSL1180 with Bpu1102I (an internal B-raf site that cuts 3′ of the B-raf ATG)+XbaI (from polylinker). An NcoI-Bpu1102I oligoadapter (5′-CATGGCGGCG C-3′; 3′-CGCCGCGACTC-5′) was made. The pGST/AcC5 baculovirus vector was cut with NcoI+XbaI, and ligated to the oligoadapter and B-Raf cDNA fragment. Baculoviruses were generated as described previously [22].

2.3. Preparation of lysates, immunoprecipitations and pulldowns

Cells were washed with ice cold PBS and lysed in lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 0.5% NP40) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 1 mM Na_3V0_4, 10 mM β-glycerolphosphate, 2 mM sodium pyrophosphate, 5 μg/ml leupeptin and 2 μg/ml aprotinin), on ice for 20 min. Lysates were subsequently centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were added to proteinA or proteinG (for mAbs) agarose beads, and incubated with the appropriate antibody overnight at 4 °C. Immunoprecipitates were washed three times with ice cold lysis buffer. GST pulldowns were carried out by incubating cell lysates with glutathione sepharose beads (Sigma) and processing the beads as described above for immunoprecipitations.

2.4. Purification of Sf-9 expressed proteins

His-PKCθ, GST-B-Raf and GST-Raf-1 virus were used at a multiplicity-of-infection of 10 to infect Sf-9 cells plated on dishes at 0.5×10^6 cells/cm^2. 42–44 h post infection cells were harvested by centrifugation and lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% Triton-X100 supplemented with protease and phosphatase inhibitors (1 mM PMSF, 1 mM Na_3V0_4, 10 mM β-glycerolphosphate, 2 mM sodium pyrophosphate, 5 μg/ml leupeptin and 2μg/ml aprotinin). Lysates were incubated on ice for 20 min, then centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were added to glutathione sepharose or nickel sepharose as appropriate and incubated overnight at 4 °C. Then beads were washed three times in lysis buffer and two times with PBS containing 50% glycerol. Beads were stored in the 50% glycerol buffer at −20 °C.

2.5. Kinase assays

Raf kinase assays were using purified GST-MEK1 from Sf-9 cells as substrate. Briefly, Raf immunoprecipitates were washed three times in lysis buffer, then twice in kinase assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$) and adjusted to equal volumes. *In vitro* kinase assays were performed incubating the Raf immunoprecipitates, GST-MEK1, 100 μM ATP and 10 mM $MgCl_2$ in kinase assay buffer at 32 °C for 20 min. Reactions were resolved on 7.5% SDS-polyacrylamide gels and blotted. MEK1 phosphorylation was detected by Western blotting with a phospho-specific MEK antibody detecting the Raf specific phosphorylation sites S217/221. PKCè kinase assays were performed using myelin basic protein (MBP; Invitrogen) as substrate. Immunoprecipitates were prepared as described above, and *in vitro* kinase assays were performed by incubating the immunoprecipitates, 2 μg MBP, 100 μM ATP, 10 mM $MgCl_2$ and 0.08 MBq [^{32}P]-γ-ATP in kinase assay buffer at 30 °C for 10 min. Reactions were resolved on 10% SDS-polyacrylamide gels and blotted. MBP phosphorylation was detected by autoradiography and phosphoimager. BAD kinase assays were performed using purified mouse BAD (Upstate) as substrate. For some assays GST-tagged mouse BAD was used which was overexpressed in COS cells and purified by adsorption to glutathione sepharose beads as described above. PKCθ immunoprecipitates from mammalian cells, and His-PKCθ, GST-B-Raf or GST-Raf-1 proteins expressed in Sf-9 insect cells were prepared as described above. *In vitro* kinase assays were performed incubating kinase beads, 2 μg BAD, 100 μM ATP, 10 mM $MgCl_2$ in kinase assay buffer at 30 °C, for 30 min. Reactions were resolved on 10% SDS-polyacrylamide gels and blotted. BAD phosphorylation was detected by Western blotting with phospho-specific BAD antibodies to phospho-S112 and S136.

3. Results

3.1. PKCθ associates with Raf-1 and B-Raf

As the Raf substrates MEK1/2 associate with Raf proteins, we tested whether the Scansite predicted Raf substrate PKCθ can be co-immunoprecipitated with Raf proteins. For this purpose we co-expressed PKCθ and FLAG-tagged Raf-1 in COS cells and performed co-immunoprecipitation experiments (Fig. 1A). We also tested whether transfected PKCθ could co-immunoprecipitate with endogenous B-Raf (Fig. 1B). Both Raf-1 and B-Raf associated with PKCθ in this assay. We further examined the effect of mitogens on the interaction. In transient transfection experiments in COS cells neither serum, EGF, PDGF or TPA affected the association of Raf-1 with PKCθ (Fig. 1A and data