# A-Raf

**Jens Rauch[1], Walter Kolch[2]**

[1]Systems Biology Ireland, University College Dublin, Dublin 4, IE, and The Beatson Institute for Cancer Research, Glasgow G61 1BD, UK. [2]Systems Biology Ireland, University College Dublin, 4, IE.

Correspondence should be addressed to Walter Kolch: walter.kolch@ucd.ie

**Abstract:**
A-Raf (v-raf murine sarcoma 3611 viral oncogene homolog) is a serine/threonine protein kinase of the Raf family that comprises A-Raf, B-Raf and C-Raf. Raf kinases are at the apex of the three-tiered Raf-MEK-ERK/MAPK pathway that features over 150 substrates and regulates many fundamental cellular functions, including proliferation, differentiation, transformation, apoptosis and metabolism. The only commonly accepted substrates for all three Raf kinases are MEK1/2, a pair of dual-specificity kinases that have ERK1/2 as substrates. A-Raf is the least studied member of the Raf family. A-Raf seems to be regulated similarly to C-Raf, with binding to activated Ras initiating the growth-factor-induced activation of A-Raf. In addition, A-Raf activity is regulated by phosphorylation, lipid interactions and protein-protein interactions. For instance, binding of the regulatory subunit of casein kinase II, CK2β, was shown to enhance A-Raf kinase activity. However, A-Raf is a poor MEK kinase with barely measurable catalytic activity, suggesting that A-Raf could have functions outside the MAPK cascade. A-Raf binding to mitochondrial membrane proteins suggests a potential role in mitochondrial transport and anti-apoptotic signaling pathways. Furthermore, the association of A-Raf with the pyruvate kinase M2, M2-PK, causing dimerization and inactivation of M2-PK, may link A-Raf signaling with energy metabolism and the Warburg effect in tumor cells. The generation of A-Raf knock-out mice revealed a role in neuronal migration and development. Recently, alternative A-Raf splice forms encoding truncated A-Raf proteins were identified. Owing to their ability to bind and block activated Ras, they function as physiological dominant-negative Ras inhibitors with roles in differentiation and transformation. A-Raf is expressed in most tissues, but expression levels differ dramatically. Elevated levels were reported in a number of malignancies, although no oncogenic mutations have been found.

**Alternative names for this molecule:** A-raf; A-Raf; A-RAF; Araf; ARAF; Araf1; ARAF1; PKS2; presumably for kinase sequence; RAFA1; v-raf murine sarcoma 3611 viral oncogene homolog

## Protein Function

A-Raf (v-raf murine sarcoma 3611 viral oncogene homolog; also known as presumably for kinase sequence 2 (PKS2), ARAF1, RAFA1 and ARAF) is a serine/threonine protein kinase. A-Raf is the least studied member of the Raf family that comprises A-Raf, B-Raf and C-Raf. The only commonly accepted substrates for all three Raf kinases are MEK1/2, a pair of dual-specificity kinases that have ERK1/2 as their substrates. Thus, Raf kinases are at the apex of the three-tiered ERK/MAPK pathway that features over 150 substrates and regulates many fundamental cellular functions, including proliferation, differentiation, transformation, apoptosis and metabolism (Yoon and Seger 2006). The main upstream input is provided by Ras GTPases and the Raf-MEK-ERK kinase cascade is considered a major effector of Ras.

The Raf family shares three conserved regions, named CR1, CR2 and CR3, which are separated by more variable sequences (Hagemann and Rapp 1999; Yuryev and Wennogle 1998). Whereas CR1 contains the Ras-binding domain and a cysteine-rich motif, CR2 is characterized by a short cluster of Ser and Thr residues. CR1 and CR2 have a regulatory function mediating binding to Ras and other regulators, and are thought to restrain the activity of the Ser/Thr kinase domain situated in the carboxy-terminal region CR3. Deletion of the amino-terminal regions CR1 and CR2 leads to a constitutively active A-Raf mutant.

From an evolutionary point of view, B-Raf seems to represent the oldest Raf gene. B-Raf retains regulatory features found in D-Raf and lin-45 (the single Raf ortholog in *Drosophila* and *C. elegans*, respectively), whereas C-Raf and A-Raf are more divergent (Marais and Marshall 1996; Wellbrock *et al*. 2004). A-Raf and C-Raf possess 85% homology in the central 100 amino acids (Wellbrock *et al*. 2004). Similar to C-Raf, there are two A-Raf genes in the human genome (Beck *et al*. 1987; Huebner *et al*. 1986), one of which is functional (*ARAF1*). The other one is a pseudogene

(*ARAF2*).The *ARAF1* gene was shown to be localized on the X chromosome (Grant *et al*. 1991).
A-Raf was initially identified by low-stringency screening of cDNA libraries as a paralog of C-Raf, which was isolated and characterized first (Beck *et al*. 1987; Huebner *et al*. 1986; Huleihel *et al*. 1986; Mark *et al*. 1986).
Like the other Raf family members, A-Raf was shown to bind lipids, which facilitate membrane association and regulate kinase activity (Johnson *et al*. 2005). Like C-Raf, A-Raf binds to monophosphorylated phosphoinositides (PI(3)P, PI(4)P and PI(5)P) and phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$). In addition, A-Raf also binds specifically to phosphatidylinositol bisphosphates ($PI(4,5)P_2$ and $PI(3,4)P_2$) and to phosphatidic acid. Lipid binding is thought to localize Rafs to specific microdomains within the plasma membrane, thus allowing it to carry out specific functions.

## Regulation of Activity

A-Raf is regulated by phosphorylation, lipid interactions and protein-protein interactions. The only known substrate is MEK. Given that A-Raf is only weakly activated by mitogenic signaling and has low kinase activity towards MEK, other kinase-independent functions are likely.
A-Raf is weakly activated by oncogenic H-Ras and Src (Marais *et al*. 1997) compared with the two other Raf isoforms (C-Raf and B-Raf), which are more strongly activated (with B-Raf being strongly activated by oncogenic Ras alone and C-Raf being activated by both Ras and Src). Ras-dependent activation of A-Raf is regulated by tyrosine phosphorylation of the amino acids Tyr 301 and Tyr 302 (Tyr 299 & Tyr 300 in mouse, respectively). Taken together, full A-Raf activation resembles C-Raf activation as it needs oncogenic Ras and other tyrosine kinases, such as Src (Kolch 2000; Marais *et al*. 1997).
Compared with equivalent protein amounts of the other Raf isoforms, A-Raf has the lowest kinase activity towards both MEK1 and MEK2 (approximately 20% of C-Raf and even less compared with B-Raf) (Han *et al*. 1993; Marais *et al*. 1997; McCubrey *et al*. 1998; Pritchard *et al*. 1995). According to Marais *et al*. (1997), no significant difference in activating either MEK1 or MEK2 could be observed. By contrast, Wu *et al*. (1996) reported that A-Raf activates MEK1 more robustly than MEK2. However, it is not entirely clear if this type of kinase activity, which was measured *in vitro*, reflects the *in vivo* activity due to interactions with other proteins and scaffolds.
A-Raf is considered a poor MEK kinase. Baljuls *et al*. (2007) reported that unique non-conserved amino-acid residues in the so-called amino-region (N-terminal of CR3) might be the reason for this low kinase activity. Substitution of the non-conserved amino acid Tyr 296 to glycine led to a constitutively active kinase, suggesting that the existence of a tyrosine residue at 296 is a major reason for the low kinase activity towards MEK (Baljuls *et al*. 2007).
Furthermore, in 2008, Baljuls *et al*. showed that A-Raf is regulated by several other phosphorylation events as well. Using mass spectrometry, novel phosphorylation sites of A-Raf were identified. Although Ser 432 (Ser 430 in mouse) is crucial for the binding of MEK and indispensable for A-Raf signaling, phosphorylation events in a novel regulatory domain (the IH-region) were shown to act in a stimulatory manner (Ser 257, Ser 262 and Ser 264 in human; Ser 255, Ser 260 and Ser 262 in mouse, respectively). It was suggested that the phosphorylation-induced negative surface charges of this region are responsible for the electrostatic destabilization of the interaction of A-Raf with the inner part of the plasma membrane. Phosphorylation of multiple amino acids in the IH-region would lead to the dissociation of A-Raf from the plasma membrane (Baljuls *et al*. 2008).
In addition, casein kinase II (CK2β) was shown to activate A-Raf (Hagemann *et al*. 1997). Co-expression experiments in Sf9 insect cells enhanced A-Raf activity towards MEK ten-fold. The physiological relevance of CK2β in A-Raf activation in mammalian cells remains to be proven. However, such a role is plausible, as CK2β binds to the KSR-1 scaffold protein, contributing to the activation of C-Raf and B-Raf (Ritt *et al*. 2007).
The cytokine IL-3 was shown to activate A-Raf activity, whereas inhibition of phosphoinositide 3 kinase blocked this activity. Cyclic AMP (cAMP) had no effect on A-Raf activity (Sutor *et al*. 1999).
Bogoyevitch *et al*. (1995) compared differentiated isoform-specific stimuli for Raf activity. Tissue plasminogen activator treatment in cardiac myocytes led to a sustained activation, but endothelin-1 only transiently activated A-Raf. Fetal calf serum, phenylephrine and carbachol are less potent activators of A-Raf.

## Interactions with Ligands and Other Proteins

**Ras proteins**

All three isoforms of the Raf family, including A-Raf, have been shown to interact with activated Ras family proteins using yeast two-hybrid or immunoprecipitation assays (Moodie *et al*. 1993; Vojtek *et al*. 1993). When A-Raf is co-expressed with activated H-Ras, it translocates from the cytoplasm to the plasma membrane (Marais *et al*. 1997). Furthermore, it was shown that activated Ras and activated Src synergize to activate A-Raf.

**MEK1 and MEK2**

A-Raf, like C-Raf and B-Raf, binds MEK1 and MEK2, but activation of catalytic activity to phosphorylate MEK occurs to a lesser degree (Bogoyevitch *et al*. 1995; Han *et al*. 1993; Marais *et al*. 1997; Wu *et al*. 1996; Yin *et al*. 2002b).

**Phosphatidylinositide 3-kinase**

A-Raf can associate with the p85 regulatory subunit of phosphatidylinositide (PI) 3-kinase (King *et al*. 2000). The formation of this complex does not require growth factor stimulation as the interaction was found in both quiescent and growth-factor-stimulated cells. Interaction was shown using phage display and co-immunoprecipitation. The interaction with A-Raf is mediated by the SH2 domain of the p85 PI 3-kinase subunit. It was shown subsequently that the interaction was phosphorylation-independent (Fang *et al*. 2002).

**Casein kinase II**

Boldyreff and Isinger (1997), and in parallel Hagemann *et al*. (1997), reported that the regulatory subunit of casein kinase II, CK2β, binds and activates A-Raf. Boldyreff and Issinger used yeast two-hybrid to identify a full-length A-Raf clone interacting with CK2β. Hagemann *et al*. used A-Raf as the bait in a yeast two-hybrid screen and identified CK2β as an interacting protein. Co-expression experiments with CK2β and A-Raf resulted in enhanced A-Raf activity. It was suggested that A-Raf might function as an alternative catalytic subunit of CK2β (Kolch 2000). However, whether this interaction is found in mammalian cells remains unclear (see above).

**Pyruvate kinase M2**

In another yeast two-hybrid screen, Le Mellay *et al*. (2002) used A-Raf as a bait and isolated pyruvate kinase M2 (M2-PK) as a directly interacting protein (see also Mazurek *et al*. 2007). A-Raf affects the activity of M2-PK by regulating the transition from the inactive dimeric form to the active tetrameric form of pyruvate kinase. This finding is important to start elucidating alternative A-Raf signaling pathways, although many of their mechanistic details are still unclear. M2-PK and A-Raf seem to cooperate to induce cell transformation as they promote loss of contact inhibition as shown by focus-formation assays. These findings suggest that A-Raf may be relevant to induce aerobic glycolysis and thus correlate A-Raf with energy metabolism and tumorigenesis.

**hTOM and hTIM**

Using yeast two-hybrid, Yuryev *et al*. (2000) showed an isoform-specific interaction of A-Raf with hTOM and hTIM, two proteins involved in the mitochondrial transport system. Using yeast two-hybrid, electron microscopy and fractionation of rat liver mitochondria, this lead to the discovery that A-Raf is located in mitochondria. In a second more exhaustive yeast two-hybrid screen, these findings were confirmed by the same authors (Yuryev and Wennogle 2003). The biological function of A-Raf in mitochondria is unknown. However, as A-Raf is a very poor MEK kinase, mitochondrial substrates of A-Raf seem plausible (O'Neill and Kolch 2004).

**Trihydrophobin 1**

A-Raf interacts *in vitro* and *in vivo* with trihydrophobin 1 (TH1) (Liu *et al*. 2004; Yin *et al*. 2002a; Yuryev and Wennogle 2003). TH1 is a widely expressed protein and is part of the negative elongation factor complex of proteins involved in repressing transcriptional elongation by RNA polymerase II. The interaction is independent of growth factor stimulation (detected in both quiescent and serum-stimulated cells), but enhanced after upstream activation. This suggests that the kinase activity of A-Raf might mediate the interaction. TH1 was also shown to inhibit A-Raf kinase activity. The physiological consequences still need to be determined.

**Kinase suppressor of ras 2**

Recently, A-Raf was identified as a dynamic interactor of the scaffold protein kinase suppressor of ras 2 (KSR-2) in HEK-293 cells treated with TNF-α using proteomics and mass spectrometry (Liu *et al*. 2009). The biological relevance of this interaction remains to be elucidated.

**Homodimers, heterodimers and trimers**

The ability of Raf proteins to form homodimers, heterodimers and trimers is well established in the literature. Rushworth *et al*. (2006) described such combinatorial interactions of A-Raf with C-Raf and B-Raf, which enhance the overall kinase activity of the complex. Importantly, Raf heteromeric complexes have distinct biochemical properties (i.e. elevated kinase activity towards MEK) compared with the monomeric or homodimeric Raf proteins and thus, may be important for

regulatory processes. Recently, a B-Raf/A-Raf complex was isolated using functional proteomics, indicating that several combinations of Raf complexes are found *in vivo* (Gloeckner *et al*. 2007). Although A-Raf interacts with oncogenic B-Raf (B-Raf V600E), kinase activity of B-Raf is unaffected (Karreth *et al*. 2009). A cooperative role for A-Raf and C-Raf was also reported for the transient activation of ERK leaving sustained activation unaffected (Mercer *et al*. 2005).

**Epidermal growth factor receptor and platelet-derived growth factor receptor**

Recently, A-Raf was shown to have distinct roles in signaling pathways mediated by epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Mahon *et al*. 2005). Although A-Raf is dynamically recruited to an EGFR-containing complex upon EGF stimulation, A-Raf is constitutively associated with PDGFR independently of PDGF stimulation. The results suggest that A-Raf undergoes specific regulation in response to EGF or PDGF stimulation (Mahon *et al*. 2005).

**14-3-3**

14-3-3 proteins participate in a plethora of cellular processes, including metabolism and apoptosis, by modulating enzyme activities, altering protein localizations and mediating protein-protein interactions. Recently, Fischer *et al*. (2009) compared the interaction of Raf family members with 14-3-3 isoforms. Whereas B-Raf and C-Raf bind to all seven 14-3-3 isoforms (β, γ, ε, σ, ζ, τ, η), A-Raf was shown to bind *in vitro* to a lesser degree to the ε, σ, and τ isoforms. A-Raf contains two putative 14-3-3 binding domains (around Ser 582 and Ser 214 in human, and Ser 580 and Ser 214 in mouse, respectively), but only the carboxy-terminal 14-3-3 domain was required for A-Raf activation.

**Mammalian sterile 20-like kinase**

We recently reported the interaction of another family member, C-Raf, with the pro-apoptotic kinase mammalian sterile 20-like kinase (MST2) (Matallanas *et al*. 2007; O'Neill *et al*. 2004). In quiescent cells, C-Raf counteracts MST-2-mediated apoptotic signaling by suppressing the activation of MST2. C-Raf inhibits the dimerization and phosphorylation of MST2 independently of C-Raf kinase activity. In comparison with C-Raf, A-Raf binds constitutively to MST2 (Rauch *et al*. 2010). In common with C-Raf, the interaction is independent of kinase activity. Interestingly, both proteins localize to the mitochondria in tumor cell lines as well as primary tumors (HNSCC). The significance of this colocalization at mitochondria is unclear so far, but in line with existing reports (Yuryev *et al*. 2000). It might explain why in human cancers A-Raf is more efficient in inhibiting MST2 pro-apoptotic activity than C-Raf (Rauch *et al*. 2010). The described anti-apoptotic, kinase-independent function of A-Raf would be consistent with the comparably low MEK kinase activity of A-Raf and suggests an inverse correlation between the kinase activity of Raf homologs and the capacity to interact with MST2.

**Other potential interacting proteins**

Yuryev and Wennogle (2003) also found other potential interaction partners using yeast two-hybrid, although these interactions were never confirmed by other methods. Amongst these interactors are geranylgeranyltransferase β (RABGGTβ), argininosuccinate synthetase (ASS), COP9 signalosome complex subunit 3 (COPS3), mitochondrial carbamoyl-phosphate synthetase (CPS1), uridine diphosphate glucose pyrophosphatase (NUDT14), lymphokine-activated killer T-cell-originated protein kinase (PBK), pre-mRNA-processing factor 6 (PRPF6) and negative elongation factor C/D (TH1L).

In another attempt to identify protein-protein interactions, Rual *et al*. (2005) used a stringent, high-throughput yeast two-hybrid system to test pairwise interactions on a proteome-wide scale. They found that A-Raf interacts with Kelch-like protein 12 (KLHL12).

The current literature reports A-Raf binding to a plethora of different proteins. It is unlikely that all these interactions take place at the same time in a given cell. It is more likely that A-Raf functions in different complexes at different localizations within a cell and that its action depends on the cellular system, on the state of the cell and tissue, as well as on the stimulus.

As a general source and overview for potential A-Raf-specific interactions apart from the current literature, information from the Protein Interaction Network Analysis Platform (PINA) and the STRING interaction network was used (Jensen *et al*. 2009; Wu *et al*. 2009).

## Regulation of Concentration

In general, A-Raf mRNA and protein levels seem to be elevated in a number of malignancies. Mark *et al*. (1986) reported that elevated levels of *A-Raf* mRNA in peripheral blood mononuclear cells isolated from two patients with angioimmunoblastic lymphadenopathy with dysproteinemia. A-Raf expression was found to be enhanced in a few tumor types, including astrocytic tumors (Hagemann *et al*. 2009), in which high expression of A-Raf also negatively correlated with patients' prognoses.

In addition, elevated levels of A-Raf mRNA were found in pancreatic ductal carcinoma (Kisanuki *et al*. 2005). Our own data show elevated A-Raf expression in head and neck squamous cell carcinomas and colon carcinomas (Rauch *et al*. 2010) .
Recent publications addressed the mutational status of A-Raf. Compared with B-Raf, which is a well-described target for mutations in human cancers (Wellbrock *et al*. 2004), mutations in A-Raf and c-Raf are rare to nonexistent (Fransen *et al*. 2004; Lee *et al*. 2005; Schreck and Rapp 2006).
Another potential regulatory mechanism for A-Raf was raised by Schreck and Rapp (2006), who mentioned a potential role of microRNAs in Raf regulation. According to the prediction of the microRNA target database (miRBase), A-Raf could be targeted by microRNAs that fine-tune its expression levels. This interesting hypothesis needs further clarification.
A recent report by Kawakami *et al*. (2003) showed an increased expression of A-Raf due to chromosomal aberration. As the human A-Raf gene is situated on the X chromosome, the duplication of X chromosomes, as occurs in testicular germ cell tumor-derived cell lines, leads to enhanced expression of A-Raf.
We recently showed that the splice factor heterogeneous nuclear ribonucleoprotein H (hnRNP H) is required for the correct transcription and expression of full-length A-Raf (Rauch *et al*. 2010). *In vivo* expression studies in colon specimens corroborated the over-expression of hnRNP H in malignant tissues and its correlation with A-Raf levels. This tight correlation of hnRNP H with A-Raf levels has been corroborated in other studies (Camats *et al*. 2008).

## Subcellular Localization

The data in the current literature support the localization of A-Raf in different subcellular compartments. Although initial reports showed an exclusive cytoplasmic localization, the recruitment of A-Raf to the inner part of the plasma membrane as a result of mitogenic stimuli conforms with knowledge about C-Raf and B-Raf.
Recent reports also showed a mitochondrial localization for A-Raf, which might have a role in anti-apoptotic signaling pathways (Yuryev *et al*. 2000; Yuryev and Wennogle 2003). Thus, the combination of subcellular localization and protein-protein interaction data supports the hypothesis that A-Raf is found in different complexes and has different roles in different cellular compartments (i.e. it undergoes spatial and temporal regulation).

## Major Sites of Expression

A-Raf cDNA was isolated from a murine spleen cDNA library. Using northern hybridization, a highly restricted tissue distribution was shown with highest expression levels observed in epididymis, ovary and intestine (Huleihel *et al*. 1986; Storm *et al*. 1990). A-Raf is expressed in most tissues, but the expression levels seem to be highly regulated and differ dramatically between tissues (Luckett *et al*. 2000). Whereas urogential tissues show a high expression, neuronal tissues express A-Raf only at low levels.

## Phenotypes

Pritchard *et al*. (1996) reported that the ablation of the *A-Raf* gene in mice causes neurological defects. A-Raf ablation in an inbred background resulted in intestinal and neurological abnormalities. A-Raf-deficient mice died 7-21 days post partum from megacolon, which is reminiscent of Hirschsprung's disease in humans, and was caused by a defect in the migration of visceral neurons controlling bowel contractions to their ultimate destinations. By contrast, in an outbred background, A-Raf$^{-/-}$ animals survived to adulthood. Although A-Raf ablation did not lead to intestinal abnormalities, the animals displayed a subset of neurological defects. In addition, Mercer *et al*. (2002) reported that the regulation of ERK and oncogene transformation are not impaired in A-Raf$^{-/-}$ mouse embryonic fibrobasts. These results, together with the low kinase activity towards MEK (Marais *et al*. 1997), suggest that A-Raf does not have a major role in MEK/ERK activation and that this function might be fully compensated by the other Raf family members. However, A-Raf seems to have a role in the development of the nervous system, possibly by regulating neuronal migration. Comparison of the knock-out phenotypes of all three Raf isoforms in mice indicates that C-Raf has a more general role in tissue formation, whereas A-Raf and B-Raf seem to have more specialized functions (Kolch 2000).

## Splice Variants

Two alternative splice forms of A-Raf, DA-Raf1 and DA-Raf2, were recently discovered (Nekhoroshkova *et al*. 2009; Yokoyama *et al*. 2007). They contain the amino-terminal Ras-binding domain, but lack the kinase domain owing to pre-terminal stop codons. These splice forms bind to activated Ras but, due to the lack of a kinase domain, act as dominant-negative antagonists of the Ras-ERK pathway. Consistent with this functional role, Yokoyama *et al*. (2007) reported that DA-Raf1 is a positive regulator of myogenic differentiation by inhibiting activation of the Raf-MEK-ERK pathway. Data from Nekhoroshkova *et al*. (2009) showed that DA-Raf2 binds and co-localizes with ARF6 on tubular endosomes and acts as a dominant effector of endocytic trafficking.

Antibodies

Reasonable quality A-Raf specific antibodies are available from a number of commercial sources, including BD Biosciences, Cell Signalling and Santa Cruz.

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