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The RAF family: an expanding network of post-translational controls and protein-protein interactions

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ABSTRACT

Protein kinase RAF is strategically located in the "Ras - MAP-kinase signal transduction pathway", a principle system which transmits signals from growth factor receptors to the nucleus, resulting in cell proliferation. Growth factor responses are mediated in part by activation of Ras, which in turn activates RAF to phosphorylate MEK, its downstream substrate. MEK activates MAP-kinase to influence nuclear events. It is clear, however, that a network of signals other than those carried by Ras plays a role in RAF regulation. These orthogonal influences are mediated by: serine/threonine kinases, tyrosine kinases, and protein - protein interactions. As a further complication to the RAF network, three isoforms of RAF have been established which have divergent N-terminal regulatory domains. Whereas these divergent regulatory domains implicate isoform-specific functions, no clear evidence or hypothesis for distinct functions for individual isoforms has been presented. Recently, "isoform-specific protein interactions" have been identified among numerous proteins interacting with RAF. These studies may serve to delineate independent functions for RAF isoforms.

INTRODUCTION

Since their discovery in the early 1980's, RAF proteins[1] have fascinated signal transduction experts and cell biologists. A number of reviews have dealt with var-

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ious aspects of RAF including structure, function, Ras binding and activation[1-5]. The field has grown rapidly in the past two years, and during this period over 250 publications have appeared with the word "RAF" in the title. This review will focus on controls over RAF activity mediated by protein - protein interactions and by post-translational phosphorylation. The theme we will attempt to develop is that RAF plays a central role uniquely situated at a crossroads of signal transduction. An attempt to portray such an intricate and complex system will be made with the use of various analogies intended to provoke clarity and perspective.

RAF is a key intermediate of the growth factor -Ras- MAP kinase pathway (Fig 1) essential for cell proliferative responses. RAF isoforms are cytosolic proteins which serve as serine/threonine kinases. A variety of growth hormones, upon binding to their respective receptors, stimulate receptor dimerization and autophosphorylation at key tyrosine residues. Phosphotyrosine groups on the cytoplasmic domain of the receptor trigger formation of multi-component signal-transduction complexes which include members of Grb and SOS protein families. The complexes subsequently activate the small molecular weight GTPase Ras, by promoting the exchange of GDP for GTP. GTP-bound Ras activates RAF and initiates the subsequent phosphorylation/activation cascade through MEK and MAP-kinase. MAP-kinase phosphorylates nuclear transcription factors and thereby activates the expression of genes necessary for cell division.

Historical perspective of the RAF family

The identification of a truncated RAF in murine sarcoma virus 3611 as an oncogene[6] provided a key insight into the importance of this protein. More recently, antisense oligonucleotides specific to C-RAF isoform inhibited the proliferation of transformed cells[7, 8] and of growth factor stimulated cells[9], re-enforcing the influence of this system on cell growth. Genetic[10] and biochemical[11] studies place RAF in the Ras-MAP-kinase pathway between Ras and its downstream substrates - the MEK family of protein kinases.

The RAF family consists of at least three isoforms: A-RAF, B-RAF and C-RAF. Three isoforms of RAF were identified by cross hybridization[12] and enzymes from numerous species have been cloned including C. elegans, chicken, mouse, rat, baboon and human[13-15]. RAF-related pseudogenes exist in both human and baboon[12, 15-17]. The genes for RAF proteins are located on chromosomes: X, 7, and 3 (for A-, B-, and C-RAF, respectively)[12, 16, 17]. Genomic sequences are available which include information on promoter structure, introns, and exons (GenBank accession numbers: L24038, X65187, SEG_ HUMRAF1).

[1] RAF isoforms will be referred to as A-, B, and C-RAF (also referred to in the literature as Raf-1 or Mif-1) and sequence numbers, unless stated otherwise, refer to the human C-RAF sequence (Genbank #XO3484), historically, the most intensively studied isoform.

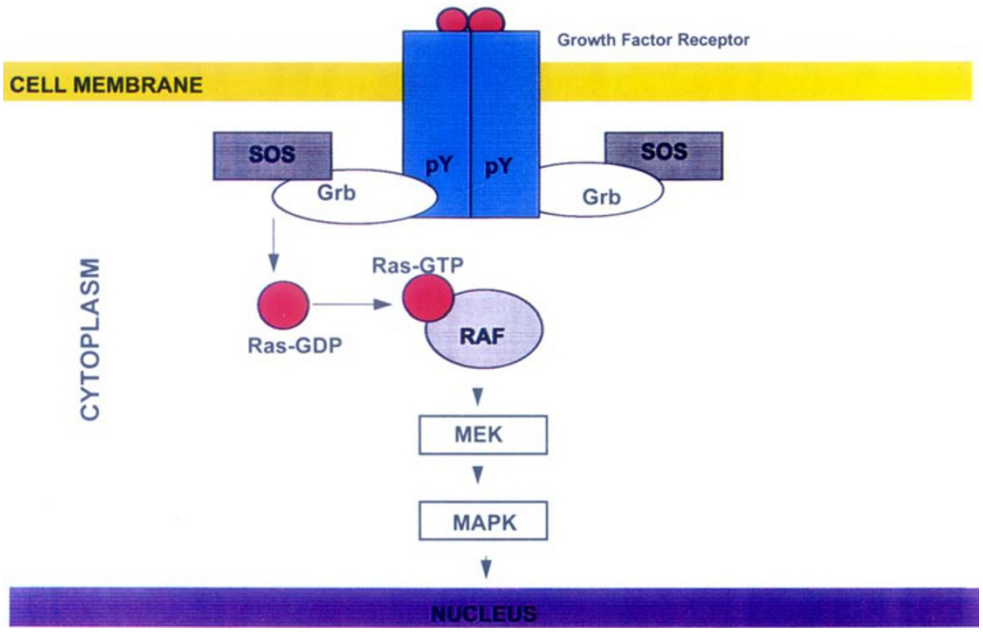
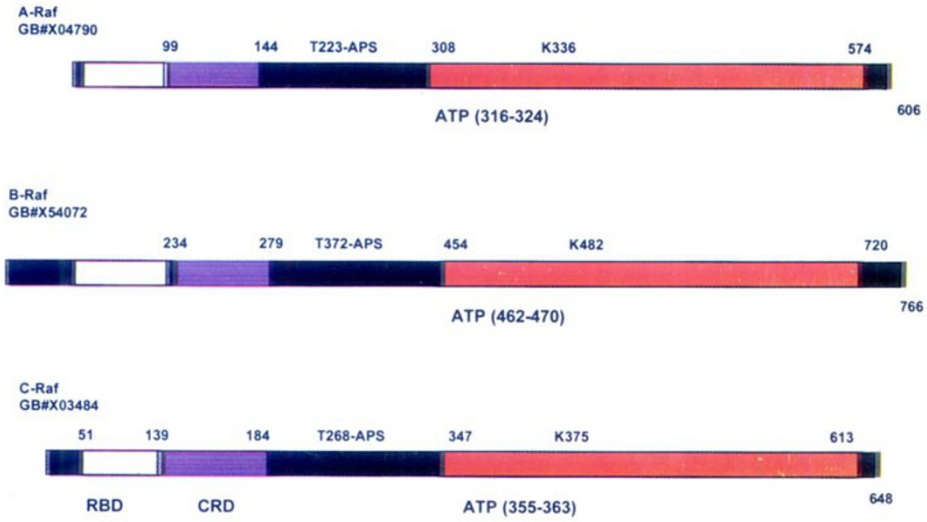


Fig 1. The schematic representation of Ras-RAF-MEK-MAPK signal transduction pathway. The signaling through the pathway is initiated by binding dimeric growth factor to corresponding growth factor receptor. Dimerization of the receptor leads to its autuphosphorylation on Tyr residues. Phosphotyrosine residues initiate the formation of the Grab-SOS signal transduction complex, which eventually lead to exchange of GDP to GTP in small molecular weight GTPase Ras. Ras-GTP molecule is capable of activating RAF, which triggers the cascade of consecutive phosphorylation/activation events and eventually lead to the phosphorylation and activation of the transcription factors. The proteins from each step of the pathway have multiple isoforms which precise functions are not understood.

An important issue is why has nature evolved three variations of this important kinase? The amino acid sequence of the three isoforms are aligned in Fig 2a. The C-terminal kinase domain is highly conserved among isoforms. The N-terminal regulatory domain is less conserved. Individual isoforms are highly conserved among different species from chicken to human. In fact, the conservation of a particular isoform between different species is higher than that between isoforms within a single species (Tab 1). This information clearly suggests that distinct roles for the individual RAF isoforms have evolved. Although information about RAF isoform cellular localization, which will be discussed below, is provocative, our understanding of the role of the individual isoforms remains unresolved.

2A. SEQUENCE ALIGNMENT OF RAF KINASE ISOFORMS



2B. Three RAF-Kinase lsoforms

Fig 2. A. The sequence alignment of three RAF isoforms. Identical amino acids are in red, conserved substitutions are in blue, non-conserved amino acids are in black. Note the that highest number of identical amino acids are in the C-terminal catalytic region of RAF isoforms.

B. The schematic representation of three RAF isoforms. The Cysteine rich domain (CRD) is in purple, Ras binding domain (RBD) is in white, and serine/threonine protein kinase catalytic domain is in red. The numbers mark the beginning and the end of each domain in the isoform, and the total number of amino acids in each RAF isoform. Also are shown: Thr residue of the autophosphorylation site, ATP binding regions and the key catalytic Lys in the kinase domains.

A-RAF and C-RAF mRNAs have been detected in most tissues[29]. B-RAF isoform is predominantly expressed in adult mouse brain and testes. In addition, B-RAF mRNA is expressed as four alternatively spliced messages in mouse brain[30]. Both A- and C-RAF can co-exist in a single cell. Rat smooth muscle A10 cells contain both A- and C-RAF as judged both by Western and Northern analysis[9]. Treatment of these cells with antisense to either isoform resulted in a non-additive decrease in cell proliferation[9].

All indications point to a dynamic regulation of RAF at the protein level as will be outlined below. There are no reports concerning the regulation of RAF activity at the level of protein expression or protein stability. C-RAF protein half-life in human coronary artery smooth muscle cells was reported to be 30 h[18]. C-RAF,

which is the most abundant RAF isoform, is a minor cellular component, estimated by labeling and immunoprecipitation experiments to represent 0.001% of the total cellular protein in CV1 kidney fibroblast cells[15].

Tab 1. Similarity of RAF Proteins

	Percent similarity (Identity)		
Three human isoforms	Full sequence	Regulatory domain	Kinase domain
A-RAF/C-RAF	75 (62)	64 (48)	88 (77)
A-RAF/B-RAF	74 (60)	62 (45)	87 (76)
B-RAF/C-RAF	75 (59)	62 (41)	91 (80)
Across Species	Full Sequence	Regulatory Domain	Kinase Domain
A-RAF Human / Rat	97 (95)	96 (92)	98 (99)
B-RAF Human / Chicken	97 (95)	95 (91)	100 (100)
C-RAF Human / Rat	99 (98)	98 (98)	100 (99)

Percent similarity accounts for conserved aminoacid substitutions within RAF sequence. Percent of identical aminoacids is given in brackets.

Structure of RAF

RAF can be pictured (Fig 2b) as a multi-domain protein containing a C-terminal kinase and a N-terminal regulatory domain containing several sub-domains. Analysis of the protein sequence[2] reveals a consensus serine/threonine kinase domain (aa 347-613 in the C-RAF sequence), an ATP-binding motif (aa 355-363) and a cysteine-rich domain (CRD, aa 139-184)[3]. A Ras-binding domain (RBD, aa 51-139) has been documented (Avruch and Morrison have reviewed the complex information on Ras-RAF interactions[3, 20]). High-resolution structure of the complex between RAF and Rap1a, a small molecular weight GTPase homologous to Ras, has been reported[21] (see also[22, 23]). Both the RBD and CRD participate in Ras binding.

[2] A useful Website for obtaining protein structural information of this type is the ExPASy site at the Geneva University Hospital and the University of Geneva h"ttp://expasy.hcuge.ch/www/expasy-top.html". ". Domain searches can be done using p"fscan" at h"ttp://expasy.hcuge.ch/sprot/prosite.html".

[3] This cysteine-rich domain is different from cysteine-rich domains present in extracellular domains of receptor proteins (such as Prosite T "NFR NGFR" or I"ntegrin Beta"), or to the cysteine-rich domains involved in binding DNA which are also referred to as zinc fingers (such as Prosite G"ATA ZN FINGER")[19]. The p"fscan" program run on C-RAF defines aa 138-184 as a P"KC-C1 domain', aa 139-184 as a D" AG_PE_"BIND" binding domain, aa 152-184 as a Z" F-Ring finger

Ras and Rap1a may compete for activation/inactivation of RAF by binding to the same binding site[24].

The cysteine-rich domain

The 45 amino acid (aa) region of C-RAF (aa 139-184) containing six cysteines and two histidines is referred to as a cysteine-rich domain (CRD) or a zinc finger[20] reviewed by Klug and Schwabe[19]. RAF CRD binds two zinc atoms[23]. This motif is similar to the diacylglycerol-binding N-terminal domain of protein kinase C (PKC). However, unlike PKC, RAF does not bind phorbol esters[25] (See Footnote 3).

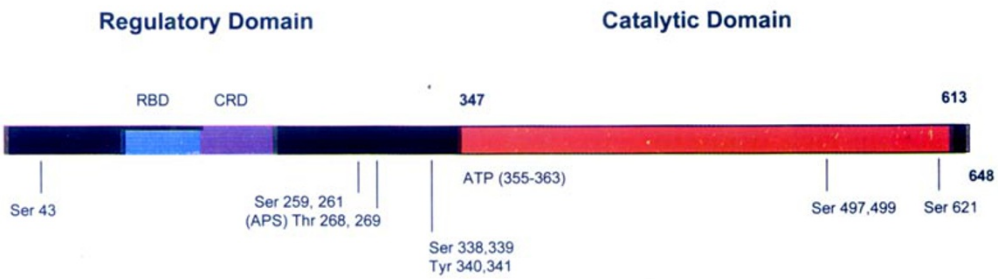
A growing body of evidence indicates that the RAF CRD is a major region for protein interaction and therefore regulation of RAF. Mutations of the CRD which affect RAF biological activities have been reported[20, 26]. The RAF CRD has been shown to bind phospholipids[27], a property that may be important during RAF translocation to the plasma membrane. The CRDs, analogous to the RAF CRD, are present in a variety of other kinases including KSR and 11 PKC isoforms[28], suggesting that the regulation of kinase activity through CRD is widespread. We will return to this aspect below.

Kinase substrates

The most established RAF substrates are the MEK family of protein kinases. MEK 1 kinase is phosphorylated *in vitro* by both C-RAF and B-RAF at Ser 218[31, 32]. MEK binds the catalytic domains of C-RAF and B-RAF, as demonstrated either with the yeast two-hybrid assay or with immunoprecipitation[33]. A-RAF was shown to bind and activate MEK1, but not MEK2[34]. IκB protein, a negative regulator of the NF-κB transcription factor, is phosphorylated by C-RAF in vitro leading to an increase in NF-κB transcriptional activity[35]. A systematic study of RAF-kinase substrate requirements or a detailed enzyme kinetic analysis of this system has not been performed. This lack of information is due to the difficulty in the purification of active RAF and in part due to the fact that signal transduction components exist naturally as multiprotein complexes.

Phosphorylation and post-translational control of RAF

Phosphorylation of RAF represents a primary mechanism by which various lateral systems influence RAF activity. This area has been one of intense study. Both serine/threonine and tyrosine phosphorylation have been reported (Fig 3, Tab 2). Phosphorylation can result both in negative or positive effects on RAF catalytic activity. As detailed in the Tab 2, different methods have contributed to this information, some of which distinguish modifications that effect enzyme activity and others that pinpoint changes correlated with mitogen stimulation.



CRD=Cysteine-Rich domain (139-184) RBD=Ras-Binding Domain(55-131) APS=Autophosphorylation site

C-Raf Domain Structure and Posphorylation Sites

Fig 3. The schematic representation of the most studied C-RAF isoform with different phosphorylation sites. Table 2 shows the kinases and conditions which cause C-RAF phosphorylation.

Tab 2. List of known phosphorylation sites of RAF isoforms

C-RAF site	-SEQUENCE- (Isoform)	Effect on Activity	*in vitro* (Involved Kinase)	Cell type detected	Reference
Ser 43	-QRRASDD- (B=C not A)	negative	PKA	quiescent 3T3, HFF	[36, 37, 39]
Ser 259, 261	-QRSTSTPN- (A=B=C)	positive	PKC α PKA	A431, PDGF-activated 3T3, HFF, (mitogen +)	[37, 39, 43, 67]
Thr 268, 269	-HMVSTTLPV- (A=C not B)	positive	C-RAF autophosphorylation, CAPK	Sf9*	[39, 68]
Ser 338, 339	-RDSSYYWE- (A=B=C)	positive		COS-7 with ras-V12	[42]
Tyr 340, 341	-RDSSYYWE- (A=C not B)	positive		Sf9 + v-src	[40]
Ser 497, 499	-RWSGSQQV- (B=C not A)	positive	PKC α	quiescent 3T3	[43]
Ser 621	-NRSASEP- (A=B=C)	negative	PKA	Sf9*	[39, 69]
{B-RAF} Thr 372	-VHINTIE-		B-RAF autophosphorylation		[70]

Sf9 is a baculovirus insect cell expression system. CAPK - ceramide-activated kinase

Ser 43 can be phosphorylated *in vitro* by protein kinase A (PKA) or under conditions of cAMP increase, both of which represent a negative regulatory influence[36-38]. Ser 43, Ser 259, and Ser 621 were found to be phosphorylated in insect Sf9 cells co-expressing activated PDGF receptors and C-RAF[39]. Tyr 340 and 341 were phosphorylated in insect cells when C-RAF was over-expressed together with the Src-family tyrosine kinases or with the JAK-2 kinase[40, 41]. Ser 338 and 339 were found to be phosphorylated in COS-7 cells when C-RAF was over-expressed with an oncogenic form of Ras[42]. The ability of PKC to phosphorylate RAF at Ser 259 and Ser 499 is well documented[43]. C-RAF autophosphorylates at Thr 268/269[39].

Many RAF isoforms have been cloned and expressed by the pioneering work from Ulf Rapp and colleagues. Importantly, expression of highly-active enzyme requires post-translational modification in the form of serine/threonine and tyrosine phosphorylations[39]. Co-expression of RAF with both a tyrosine kinase such as Src and with the Ras protein leads to maximal enzyme activity in a baculovirus expression system[44, 45]. Activation of RAF by phosphorylation allows for numerous and complex lateral influences on the RAF pathway. Like a river with numerous tributaries converging in a dynamic fashion, cell regulation through RAF is extensive and changeable.

RAF protein-protein interactions

RAF protein-protein interactions have been extensively studied by the yeast two-hybrid screening method[46], by immunoprecipitation[15, 47], and by binding techniques[48, 49]. Tab 3 lists sixteen proteins shown to interact with RAF.

RAF - Ras

GTPases of the Ras family are the most well-studied proteins which interact with RAF. RAF has higher affinity for GTP-Ras over GDP- Ras[27]. Over-expression of Ras activates RAF, and over-expression of the N-terminal domain of RAF blocks the Ras transforming ability[50]. Several Ras-related small molecular weight GTPases can interact with C-RAF including: Ha-Ras[46, 51], Rap-1A[52], R-Ras [53], Rheb[54].

Ras is normally farnesylated and palmitoylated in vivo at C-terminal cysteine residues[55, 56]. These modifications result in the trans-localization of Ras to the membrane. A proposed function for Ras is to target RAF to the membrane via this mechanism. Mutant RAF proteins, encoding C-terminal farnesylation signals to mimic the effect of Ras, were shown to be isoprenylated. These constructs were preferentially membrane localized and resulted in cell transformation.

RAF - 14-3-3 protein interaction

RAF has been shown to interact with 14-3-3 proteins[57]. These 14-3-3 proteins

Tab 3. List of Published Proteins Interacting with RAF

Protein name	Swiss Prot/ Gen Bank accession number	RAF isoform interaction domain	Method	Reference
Ha-Ras, R-ras, Rap1A, Rheb	P01112, P10301, P10113, S68419	C-RAF RBD domain, 55-131 C-RAF CRD, 139-185	*in vitro*, two-hybrid	[27, 54]
14-3-3 β, ζ, θ, h	P31946, P29312, P27348, Q04917	C-RAF CRD, 139-185 B-RAF N-termnus, 1-443	IP, two-hybrid, *in vitro*	[59, 71, 72]
β-subunit of trimeric G-protein		C-RAF	two-hybrid	[73]
BAG1	Q99933	C-RAF kinase domain, 303-648	two-hybrid, *in vitro*, IP	[74]
Bcl2	P10415	C-RAF kinase domain, 303-648	two-hybrid, in vitro	[75]
Fyn	P06241			[76]
β subunit of Casein kinase2	P13862	A-RAF kinase domain, 255-587	two-hybrid	[64, 65]
MEK1, MEK2	Q13233, P36507	C-RAF kinase domain, 303-648 B-RAF kinase domain, 333-767	two-hybrid, IP	[71, 77]
c-Myc	P01106	C-RAF	*in vitro*	[78]
IkB	P25963	C-RAF kinase domain, 303-648	*in vitro*, two-hybrid	[35]
Heat Shock Proteins hsp90 + p50	P08238	C-Raf	IP	[15, 79, 80]

IP = immunoprecipitation, Two-hybrid = the yeast Two- Hybrid analysis, "*in vitro*" refers to various other biochemical techniques such as "Far Western Analysis" or Affinity Chromotography methods.

are abundant cytosolic proteins which appear to act as co-factors to a variety of cytosolic proteins (reviewed in[58]). The interaction of RAF with 14-3-3 proteins is complex. The binding of a 14-3-3 protein to RAF is influenced by RAF phosphory-

lation on Ser259[3, 59, 60]. This serine is contained within a -RxSxSpxP- sequence which has been shown to be the 14-3-3 binding motif[60]. Conversely, phosphorylation of 14-3-3 protein by casein kinase I abolish 14-3-3 binding to RAF[61]. Over-expression of 14-3-3 protein activates signaling through RAF[62, 63]. However, recent publications argue that 14-3-3 proteins inhibit RAF activity in vivo, because a mutated RAF, that is unable to bind 14-3-3 protein, has enhanced transforming activity[26]. Reconciliation of these diverse observations and of the multiple contact regions is an ongoing effort.

Other RAF - protein interactions

Six proteins have been described to interact with the RAF catalytic domain (Tab 3), of the which only IκB are phosphorylated by RAF. In the two-hybrid assay, β subunit of casein kinase II binds to the C-terminal domain of A-RAF but not C-RAF[64, 65]. The functional significance of this interaction is not understood.

We have performed extensive yeast two-hybrid screens with both A-RAF and C-RAF in search of isoform-specific protein interactions. These studies identified 20 RAF-interacting proteins using the poorly conserved N-terminal regulatory domain of RAF as the bait (aa 1-314 of A-RAF and aa 1-353 of C-RAF)[28]. Several novel RAF-interacting proteins were identified and sequenced. Among these is a novel protein kinase referred to as hA38, a kinase which binds non-selectively to all three RAF isoforms. In addition, several isoform-specific RAF binding partners were identified. These include two mitochondrial membrane transport proteins which bind specifically to A-RAF. Subsequently, A-RAF was localized inside purified rat liver mitochondria by Western analysis[66]. This finding has led to the hypothesis that A-RAF isoform is selectively located in rat liver mitochondria where it may coordinate mitochondrial replication, energy production, or potentially apoptosis[66].

All 20 proteins we identified as binding to RAF N-terminal domain interacted with the RAF CRD. The fact that the CRD was sufficient for their interaction suggested that the CRD is a principle site for RAF protein-protein interactions. Closer inspection of the amino acid sequence of the CRD region from the three RAF isoforms (Fig 2a) reveals that this sequence is highly conserved. Of the 45 residues only 12 amino acids are different among the isoforms, however, seven of these changes result in changes of charge. Because we have determined that the CRD is sufficient to interact with all of the RAF binding partners[28], we infer that these specific residues play a key role in specifying isoform-specific interactions. The numerous proteins reported to interact with RAF suggest a busy and dynamic intersection with competition from multiple directions.

Cellular functions of the RAF network

An expanding list of receptor signals has been shown to activate RAF (Tab 4). Although a general list of this type probably contains unestablished "cause and ef-

fect" relationships, it attests to the broad influence of the RAF network. Included in this coverage are receptors for growth factors, for cytokines and for seven-transmembrane spanning receptors. The variety of signals which impinge upon RAF reiterates the fundamental importance of this protein to signal transduction. Because the decision for a cell to divide is a fundamentally profound one, a sophisticated integration of information and therefore control occurs in the cytosol at the level of RAF, prior to signal transmission into the nucleus.

Tab 4. Examples of cellular responses

Input or receptor	Cell type	Reference
Gamma irradiation	breast cancer cells	[81]
PACAP-like neuropeptide	Drosophila	[82]
12-O-tetradecanoylphorbol-13-acetate (TPA)	Madin-Darby canine kidney cells,3T3	[83, 84]
Interleukin-5	eosinophils	[85]
Insulin-like growth factor I	Balb/c, rat cardiac myocytes	[86, 87]
Ca^{2+}mobilizer thapsigargin	Balb/c	[88]
Trimeric G-Protein	HEK 293 cells	[89]
Bone morphogenetic protein 4	Xenopus oocytes	[90]
Bile acid secretion by tauroursodeoxycholaterat	liver	[91]
Angiotensin II and PDGF	smooth muscle	[92]
Fibroblast-derived growth factor	rat hippocampalneurons	[93]
Integrin mediated cell attachment		[94]
Prostaglandin F2-α	arat uterine	[95]
β-interferon,oncostatin M	HeLa cells	[96]
Lactosylceramide	smooth muscle cell proliferation	[97]
Prolactin	mammary cells	[98]
α l-adrenergic receptor agonist	rat ventricular myocytes	[99]
Bombesin and neuromedin B	Rat-1 cells	[100]
ICAM-1	B cell lymphoma line	[101]
Gonadotropin-releasing hormone	α T3-1 cell line	[102]
G-CSF	myeloid NFS-60 cells	[103]
TGF β1	rat hepatic stellate cells	[104]
Osmolarity	C6 glioma cells	[105]
Erythropoietin and inositolphosphate-glycan	erythroid progenitor cells	[106]

IP = immunoprecipitation, Two-hybrid = the yeast Two- Hybrid analysis, "*in vitro*" refers to various other biochemical techniques such as "Far Western Analysis" or Affinity Chromotography methods.

Summary and conclusions

This review has focused on the expanding number of complexities to the RAF network, particularly at the level of protein - protein interactions and at RAF phosphorylation by lateral systems. In view of these complexities, the importance of RAF in integrating information from numerous systems is obvious. More effort is needed to fully define the spectrum of proteins which interact directly with RAF and to delineate the unique functions of RAF isoforms.

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