Regulation of MEK/ERK pathway output by subcellular localization of B-Raf

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Abstract
The strength and duration of intracellular signalling pathway activation is a key determinant of the biological outcome of cells in response to extracellular cues. This has been particularly elucidated for the Ras/ Raf/MEK [mitogen-activated growth factor/ERK (extracellular-signal-regulated kinase)/ERK signalling pathway with a number of studies in fibroblasts showing that sustained ERK signalling is a requirement for S-phase entry, whereas transient ERK signalling does not have this capability. A major unanswered question, however, is how a cell can sustain ERK activation, particularly when ERK-specific phosphatases are transcriptionally up-regulated by the pathway itself. A major point of ERK regulation is at the level of Raf, and, to sustain ERK activation in the presence of ERK phosphatases, sustained Raf activation is a requirement. Three Raf proteins exist in mammals, and the activity of all three is induced following growth factor stimulation of cells, but only B-Raf activity is maintained at later time points. This observation points to B-Raf as a regulator of sustained ERK activation. In the present review, we consider evidence for a link between B-Raf and sustained ERK activation, focusing on a potential role for the subcellular localization of B-Raf in this key physiological event.

Introduction
During vertebrate development, morphogen gradients are important in specifying the formation of different cell types and imposing tissue ontogeny [1]. This is achieved through exquisite control of intracellular signal transduction pathway activity such that target cells can make appropriate alterations in gene expression in response to the extracellular stimulus. The strength and duration of intracellular signalling pathway activity has long been associated with the imposition of distinct biological outcomes in response to extracellular stimuli [2]. The best-characterized signalling pathway in this regard is the Ras/Raf/MEK [mitogen-activated growth factor/ERK (extracellular-signal-regulated kinase)/ERK pathway that is activated by nearly all growth factors and cytokines; mechanisms controlling the magnitude and duration of this pathway is the subject of the present review.

In a seminal review article published in 1995 [2], a model was proposed for the role of the duration of ERK activation in cell signalling decisions, based on observations by a number of research groups using the rat PC12 phaeochromocytoma cell line. The model proposed that PC12 cells can enact differentiation or proliferation in response to receptor tyrosine kinase activation, purely on the basis of the duration of ERK activation. In this particular system, stimulation of cells with EGF (epidermal growth factor) or insulin elicited proliferation and was associated with transient induction of ERK activation, whereas NGF (nerve growth factor) triggered differentiation of sympathetic neurons and was associated with sustained ERK activation. Sustained ERK activation was associated with the presence of ERK in the nucleus, whereas transient activation did not lead to nuclear ERK activation and it was suggested that this difference allowed for a qualitative difference in transcription factor activation that in turn determined biological output.

After the establishment of this paradigm in PC12 cells, the role of the duration of ERK activation in other cellular systems was investigated. In fibroblasts, a number of studies made a link between sustained ERK activation and cell proliferation [3–7]. In the hamster CCL39 fibroblast cell line, it was demonstrated that thrombin induces a proliferative response by eliciting sustained ERK activation and the expression of a subset of AP-1 (activator protein 1) proteins [3], whereas mimetic thrombin peptides failed to elicit S-phase progression and only transiently induced ERK activation. A similarly elegant study in Swiss 3T3 cells demonstrated that PDGF (platelet-derived growth factor) can induce sustained ERK activation, high levels of AP-1 proteins and proliferation, whereas the transient ERK activation elicited by EGF was not sufficient to drive proliferation in the same cells [7,8]. Similar correlations were made in other cell types, including macrophages [9].

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and T-lymphocytes [10], whereas studies in Xenopus have demonstrated the importance of ERK signal duration in mesoderm formation in vivo [11]. The overall view from these investigations is that normal cells use ERK signal duration to harness distinct biological outcomes in response to a specific extracellular stimulus.

In work led by John Blenis and colleagues, the mechanism by which a cell distinguishes between transient and sustained ERK activation was elucidated [7,8]. Their studies centred on the IEG (immediate-early gene) product c-Fos, a very unstable protein that accumulates at sustained time points. It was shown that transient ERK phosphorylates the extreme C-terminus of c-Fos at Ser234, leading to increased stability of the protein. This phosphorylation event also primed c-Fos for interacting with ERK through a DEF (docking site for ERK, F XF) domain at the F343TYP346 sequence within the C-terminal region of c-Fos and allowed for further phosphorylation of the more proximal Thr 325/Thr331 by ERK. By creating a phospho-specific antibody for Thr325, it was demonstrated that maximal phosphorylation of this residue in c-Fos occurred at sustained time points following PDGF treatment of Swiss 3T3 cells. It was thus suggested that the DEF domain and phosphorylation of Thr325/Thr331 are required to stabilize c-Fos, leading to its accumulation [12]. Since c-Fos is an important component of the AP-1 transcription factor, an increase in its expression levels results in greater expression of downstream target genes such as CCND1 (cyclin D1), promoting G1–S cell-cycle progression. In follow-up work, the same authors showed the presence of DEF domains in other IEG-encoded proteins including Fra-2 and c-Myc and similarly demonstrated their regulation by persistent ERK signalling [8].

Retention of ERK activity in the nucleus

The prevailing model for the spatiotemporal regulation of ERKs proposes that MEK acts to anchor ERK in the cytoplasm in resting cells but, on mitogen stimulation, this complex dissociates, and allows entry of active ERK into the nucleus, where it is able to activate nuclear substrates [13]. Mechanisms involved in terminating the activity of ERK in the nucleus have been the subject of investigation for a number of years. A key publication in 1998 showed that inhibitors of protein synthesis prevented the nuclear accumulation of ERK and it was also shown that proteasomal inhibitors prolonged the nuclear localization of ERKs after mitogenic stimulation [14]. Taking these data together, it was suggested that the nuclear-anchoring and retention of ERKs requires the de novo synthesis of short-lived proteins.

By monitoring phospho-specific staining of ERKs compared with total ERK staining, it was demonstrated subsequently that, during prolonged stimulation, ERKs become inactive within the nucleus and that inactivation is mediated by vanadate-sensitive protein phosphatases [15]. The dual-specificity nuclear-specific ERK phosphatase DUSP5 (dual-specificity phosphatase 5) has since been shown to be a good candidate for this [16]. Induction of DUSP5 mRNA and protein is dependent on ERK1/2 activity and the accumulation of the DUSP5 protein is regulated by proteasomal degradation [17]. Nuclear anchoring of ERK by DUSP5 requires interaction via a KIM (kinase-interacting motif) within its N-terminal catalytic domain. These data have allowed a model to be proposed whereby active ERK translocation to the nucleus leads to stimulation of DUSP5 expression that localizes to the nucleus and retains ERK2 in the nucleus and inactivates it here. Sustained ERK activation has been proposed to be a balance between binding of IEG proteins through their DEF domains and binding of ERK to DUSP5 through its KIM [12]. If the concentration of one of these binding partners increases, then this equilibrium shifts and ERK activity in the nucleus is affected as a consequence.

The above model presupposes that ERK activity in the nucleus is entirely dependent on a pool of active ERK that has translocated into the nucleus after being activated by the upstream Ras/Raf/MEK pathway in the cytoplasm. However, this may not be the complete story as there is considerable experimental evidence to suggest that MEK activity is also present in the nucleus. The fact that MEK contains a NES (nuclear export signal) suggests that it must reside in the nucleus at some point, and disruption of this NES shows that MEK translocates to the nucleus in response to growth factor stimulation [18,19]. In addition, on expression of inactive DUSP5 mutant in cells that retain ERK2 in the nucleus, ERK2 is readily phosphorylated and activated [16], suggesting that the cytoplasm may not be the exclusive site of ERK activation as discussed further below.

Role of Raf in ERK signal duration

A major point of regulation of ERK activity in the cell is at the level of Raf, of which there are three family members in mammals: A-Raf, B-Raf and C-Raf. All three are able to bind Ras and MEK, although they have differing affinities and activities towards MEK, with the consensus being B-Raf>C-Raf>A-Raf in this regard [20–22]. A wealth of biochemical data have indicated that the Raf proteins reside in the cytoplasm in an inactive conformation, bound to 14-3-3 protein dimers. Following growth factor stimulation, Raf binds Ras, leading to displacement of 14-3-3, and this event leads to translocation of Raf to the plasma membrane. Here, Raf is subjected to additional events including phosphorylation that lead to its activation, triggering sequential MEK/ERK activation [20]. Despite this well-accepted model, various studies suggest that Raf may also be activated by alternative mechanisms. Specifically, it has been shown that RAFs form hetero- and homo-dimers that have differing level of intrinsic kinase activity [23]. Under conditions where B-Raf activity is suppressed, this can lead to the formation of a C-Raf–B-Raf heterodimer that activates C-Raf through phosphorylation of its activation segment by the residual kinase activity of B-Raf [24]. This event occurs independently of Ras in the cytoplasm of the cell, but requires 14-3-3 binding to C-Raf.
**Figure 1 | B-Raf and ERK activation**

(A) Kinase assays of three Raf proteins immunoprecipitated from MEFs following a time course of growth factor (GF) stimulation. Shown are diagrammatic representations of data published by Huser et al. [26]. B-Raf has considerably higher activity than A-Raf or C-Raf, as demonstrated on the left. By increasing the sensitivity of the assay, C-Raf and A-Raf activity are detectable, as shown on the right. B-Raf has a high basal activity in unstimulated cells and its activity increases ∼2-fold immediately following GF stimulation. Although its activity decreases at 10 min post-stimulation, its activity is sustained for the remainder of the time course. C-Raf activity peaks at 2 min post-GF stimulation, but decreases rapidly after this point, returning to baseline levels within 20 min. A-Raf activity is weak, but follows a similar trend to C-Raf. (B) ERK phosphorylation analysis of Brat+/− and Brat−/− MEFs following a time course of GF stimulation. Soluble protein lysates were prepared from cells, and Western blots were performed and analysed with an antibody that detects phosphorylation of Thr202/Tyr204 of ERK1/2 or with an antibody against total ERK2. Brat−/− MEFs show substantially reduced levels of ERK phosphorylation, both in the transient and sustained phases of GF stimulation. (C) Detection of B-Raf in the nucleus. NIH 3T3 cells were transfected with a vector expressing a fusion cDNA between GFP and full-length human B-Raf. Cells were fixed and visualized using a fluorescence microscope. A typical example of data obtained is shown in the photomicrograph. GFP-B-Raf staining is observed to be exclusively cytoplasmic in most cells. However, a few cells within the culture express high levels of GFP-B-Raf in the nucleus. Scale bar, 50 μm.

In terms of the temporal control of ERK activation, Raf activity must be sustained in order for ERK activity to be sustained. The most effective way to measure Raf activity in cells is to perform a Raf immunoprecipitation after which the activity of the immunoprecipitated protein is measured using a MEK/ERK kinase cascade assay [25]. Following growth factor stimulation of MEFs (mouse embryonic fibroblasts) we found that B-Raf had considerably stronger activity than C-Raf, which had stronger activity than A-Raf [26] (Figure 1A). In addition, although the activities of A-Raf and C-Raf returned to baseline within a short time after growth factor stimulation, B-Raf activity was sustained for much longer (Figure 1A). These data suggest that sustained growth factor signals are transmitted through B-Raf, a result consistent with data in PC12 cells showing that B-Raf activity is sustained following NGF treatment [27,28].

We have also analysed ERK activation profiles of MEFs with knockout mutations of each of the three RAFs. Whereas Ara− and Craf− knockout MEFs do not demonstrate reduced levels of ERK phosphorylation [26,29], Brf− knockout...
generates a significant reduction in transient and sustained levels of ERK phosphorylation [30] (Figure 1B). Furthermore, combined knockout of Araf and Craf diminishes transient ERK phosphorylation in response to growth factor treatment, but sustained ERK phosphorylation is not affected [31]. Taken together, these data again support the view that B-Raf is the most important Raf isoform to sustain ERK activation, at least in MEFs.

### B-Raf subcellular localization

For B-Raf to contribute to the maintenance of ERK activity, it also needs to be in the appropriate subcellular compartment, specifically the nucleus. B-Raf is widely accepted as being a cytoplasmic protein that translocates to the plasma membrane upon growth factor stimulation and Ras activation [13]. This rubric has primarily been established from biochemical studies on C-Raf, but also from a comparative investigation of the regulation of the three Raf isoforms by oncogenic Ras [32]. This study showed that, upon expression of Myc-tagged human Raf expression vectors in MDCK (Madin–Darby canine kidney) cells, all three Rafs were located in the cytoplasm as assessed by immunofluorescence with the 9E10 anti-Myc antibody and, upon co-expression with a vector expressing G12VH-Ras, all three Rafs translocated to the plasma membrane. This analysis did not identify alternative locations for the Raf isoforms in the cell. However, a few other studies have since suggested that B-Raf may also be located in other compartments in the cell, including mitochondria, the nucleus, Golgi, centrioles, kinetochores and the proteasome. A summary of these various reports is provided in Table 1.

The detection of B-Raf in the nucleus has not so far been substantiated by additional studies. However, we have recently re-evaluated the subcellular localization of B-Raf in NIH 3T3 cells using a combination of subcellular fractionation and GFP (green fluorescent protein)-tagged fluorescence microscopy imaging and have detected clear evidence for the presence of this protein in the nuclear compartment in some cells. A typical fluorescence microscopy image of cells transfected with GFP–B-Raf is illustrated in Figure 1(C). These data show that, whereas B-Raf is exclusively cytoplasmic in the majority of cells, B-Raf is present in the nucleus in a few cells and, when present, it is expressed at extremely high levels, suggesting a role for stabilization of B-Raf in this compartment (Figure 1C). This observation is currently the subject of intensive investigation in our laboratories.

In addition, B-Raf in higher organisms contains a putative ERK-docking site KIM in its C-terminus, close to the 14-3-3-binding site and the ERK phosphorylation motif (SPKTP) (Figure 2A). It has been demonstrated that Ser\textsuperscript{750} and Thr\textsuperscript{753} within this latter motif in B-Raf are phosphorylated by ERK and impart negative-feedback regulation on B-Raf [33,34]. Thus, in a similar way to DUSP5, B-Raf may act as a nuclear anchor for ERK and, in so doing, may sequester ERK from DUSP5 and so propagate sustained ERK signalling. A model for this putative role of nuclear B-Raf in sustained ERK signalling is shown in Figure 2(B). This model proposes that ERK activity is sustained by a positive feedback of B-Raf by a possible mechanism involving phosphorylation of Ser\textsuperscript{750}/Thr\textsuperscript{753} of B-Raf by ERK and its consequent stabilization in the nucleus. This feedback would have to be positive rather than the negative feedback identified previously [33]. However, the existence of such positive and negative feedback is consistent with mathematical modelling of ERK activation in PC12 cells by different ligands showing that NGF treatment stimulates ERK activation to cause positive feedback at the level of Raf, whereas ERK activation by EGF inhibits Raf through a negative feedback [35,36]. The model also demands the presence of MEK in the nucleus to propagate the signalling link between B-Raf and ERK.

Further investigations will be needed to consolidate this model and address key issues raised by it such as how B-Raf enters the nucleus, whether it is active in this compartment and what the role of phosphorylation of the Ser\textsuperscript{750} and Thr\textsuperscript{753} is. The putative KIM in B-Raf is also identified as a canonical NLS (nuclear localization signal) when the pSORTII NLS

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**Table 1 | Summary of publications demonstrating alternative subcellular locations for B-Raf**

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>Details</th>
<th>Method of detection</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mitochondria</td>
<td>Exogenous and endogenous V\textsuperscript{600}E-B-Raf was detected in mitochondria in thyroid cancer cells.</td>
<td>Fractionation of endogenous and exogenous V\textsuperscript{600}E-B-Raf in thyroid cancer cells</td>
<td>[37]</td>
</tr>
<tr>
<td>Golgi</td>
<td>Exogenous wild-type B-Raf and V\textsuperscript{600}E-B-Raf were localized to Golgi where they were found to interact with RKTG (Raf kinase trapping to Golgi) protein</td>
<td>Immunofluorescence of Myc-tagged transfected proteins using an antibody against the Myc tag and co-localization with Golgin-97 and RKTG</td>
<td>[38]</td>
</tr>
<tr>
<td>Spindle structures</td>
<td>B-Raf was reported to localize to spindle poles and kinetochores at metaphase</td>
<td>Immunofluorescence of endogenous B-Raf in HFF cells using a rabbit polyclonal antibody against phosphorylated Thr\textsuperscript{599}/Ser\textsuperscript{602}</td>
<td>[39]</td>
</tr>
<tr>
<td>Proteasome</td>
<td>B-Raf was found to interact with PA28\textsubscript{α}, one of the subunits of the 11S regulator of proteasomes</td>
<td>Yeast two-hybrid analysis and co-immunoprecipitation of transfected tagged proteins in HEK (human embryonic kidney)-293 cells</td>
<td>[40]</td>
</tr>
<tr>
<td>Nucleus</td>
<td>B-Raf isoforms were detected in nuclear fractions of forebrain and cerebellum of the rat</td>
<td>Fractionation was used combined with Western blot analysis using the B-Raf IS-11 rabbit polyclonal antibody</td>
<td>[41]</td>
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Figure 2 | Model for possible role of nuclear B-Raf in sustained ERK signalling

(A) Amino acid sequence of C-terminus of B-Raf in several species. This region of the protein contains several important elements involved in the regulation of B-Raf, shown in the grey boxes. A putative ERK-docking site is identified at the KIM, this sequence is also predicted to be an NLS (http://www.psort.org). After a space of 19 amino acids, a 14-3-3-binding site motif is identified in B-Raf that includes Ser729 (of human B-Raf), a known regulatory phosphorylation site. After a further 14-17 amino acids, an ERK phosphorylation site that includes Ser750 and Thr753 (of human B-Raf) is identified, near the end of the protein. (B) Model for role of B-Raf in ERK signal duration. Growth factor (GF) stimulation leads to translocation of B-Raf to the plasma membrane where it binds Ras and is activated by events including phosphorylation. This leads to sequential activation of MEK and ERK within the cytoplasm. Activated ERK is able to translocate to the nucleus where it is able to induce IEG expression, including the ERK phosphatase DUSP5. DUSP5 is able to interact with ERK through its KIM, leading to ERK inactivation and termination of the signal. We propose a role for B-Raf in sustaining ERK activity in the nucleus. B-Raf may interact with ERK through its KIM, leading to ERK-mediated phosphorylation of B-Raf at Ser750 and Thr753, stabilization of B-Raf and propagation of sustained ERK signals through MEK. Whether ERK activity is transient or sustained is dependent on the relative equilibrium between binding of ERK to the KIM of B-Raf and DUSP5, as well as whether MEK is also located in the nucleus.

predictor algorithm is applied (http://www.psort.org) and it will be important to address whether this is a functional NLS in B-Raf and, if so, what the relationship is between B-Raf nuclear import and interaction with ERK.

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