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Insights into the molecular function of the inactivating mutations of B-Raf involving the DFG motif

Sonia Moretti [a], Valentina De Falco [d], Anna Tamburrino [d], Flavia Barbi [a], Maria Tavano [a], Nicola Avenia [b], Fausto Santeusanio [a], Massimo Santoro [d], Antonio Macchiarulo [c,*], Efisio Puxeddu [a,*]

[a] Dipartimento di Medicina Interna, Università di Perugia, Perugia, Italy
[b] Centro di Riferimento Regionale per l'Endocrinochirurgia, Università di Perugia, Perugia, Italy
[c] Dipartimento di Chimica e Tecnologia del Farmaco, Università di Perugia, Perugia, Italy
[d] Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano", Università "Federico II", Naples, Italy

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ABSTRACT

BRAF gene mutations have been associated with human cancers. Among the naturally occurring mutations, two that involve amino acids of the conserved DFG motif in the activation loop (D594V and G596R), appear to be inactivating. Aim of this study was to analyze the molecular mechanisms involved in the loss of function of B-Raf inactivating mutation G596R. Furthermore, the ability of the B-Raf DFG motif mutants to generate heterodimers with C-Raf and the possible functional consequences of the B-Raf/C-Raf heterodimer formation was examined. Wet molecular experiments in HEK293T cells demonstrate that B-Raf^{G596R} is a kinase-impaired mutant. Molecular dynamics simulations show that the loss of function of B-Raf^{G596R} depends on a restraining effect of Arg596 on the catalytic residue Asp594, which results in the loss of the appropriate spatial localization and/or conformation of the latter necessary for anchoring ATP to the enzyme. Exploration of B-Raf/C-Raf heterodimer formation indicates the occurrence of functioning heterodimers in the case of all the DFG B-Raf mutants, independently from the expected differences in spatial conformation of the activation loop, although the transforming activity of the mutants appear negligible. In conclusion, this study delivers novel information on the functional properties of the B-Raf DFG motif inactivating mutants and on the mechanisms driving B-Raf/C-Raf heterodimerization and consequent C-Raf transactivation.

1. Introduction

The activation of Ras–Raf–Mek–Erk pathway in response to extracellular signals, plays a pivotal role in the regulation of cell proliferation, survival, and senescence [1,2]. While Ras is a small G protein that is anchored to the inner part of the cell membrane, Raf, Mek, and Erk are cytosolic protein kinases whose sequential activation by each other, leads to a three-tiered signaling cascade.

In particular, three Raf isoforms are known in mammals: A-Raf, B-Raf, and C-Raf, with *BRAF* being frequently mutated in cancer (see below).

The kinase activity of B-Raf is regulated by an equilibrium between the inactive and active conformations of the enzyme that show different substrate accessibility to the catalytic site. In particular, the inactive conformation of B-Raf is endowed by a closed conformation of the activation loop (residues Asp593-Glu622 of the C-terminal lobe) which prevents the access of the substrate to the catalytic site. Conversely, the active conformation of B-Raf is characterized by an open conformation of the activation loop that makes the catalytic cleft accessible to the substrate. The crystal structures of B-Raf in the inactive [3] and active [4] conformations support the notion that the shift of the activation loop between a closed and open state is the only event needed to activate the enzyme. Under basal conditions, stabilizing interactions between the activation loop and the glycine-rich loop of the N-terminal lobe favor the inactive state of the enzyme. Upon the occurrence of post-translational modifications and/or point mutations, unfavorable interactions of these regions promote a conformational transition of B-Raf to the catalytically active form [3].

BRAF gene is mutated in approximately 7% of human cancers [5]. Among these, melanoma and papillary thyroid carcinoma (PTC) show the highest rate of *BRAF* mutations [6,7]. The majority of point mutations so far identified, is localized in the glycine-rich loop and the activation loop [3,8]. Although the most common mutations of B-Raf activate the enzyme, such as the frequent replacement of a glutamic acid for valine at position 600 (B-Raf^{V600E}), at least five other cancer-associated mutants (B-Raf^{G466E}, B-Raf^{G466V}, B-Raf^{G474R}, B-Raf^{D594V}, and B-Raf^{G596R}) show an impaired B-Raf kinase activity *in vitro* [3,9–12].

* Corresponding authors. E. Puxeddu is to be contacted at Dipartimento di Medicina Interna, Via E. dal Pozzo, 06126 Perugia, Italy. Tel.: +39 075 5783406; fax: +39 075 5730855. A. Macchiarulo, Dipartimento di Chimica e Tecnologia del Farmaco, Via del Liceo 1, 06123 Perugia, Italy. Tel.: +39 075 5855114; fax: +39 075 5855114.
E-mail addresses: antonio@chimfarm.unipg.it (A. Macchiarulo), efisio.puxeddu@unipg.it (E. Puxeddu).

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G466 and G474 are positioned in the glycine-rich loop or in its proximity, respectively, whereas D594 and G596 are located in the activation segment of B-Raf where they form the kinase conserved DFG motif (residues 594–596). While it is likely that all these mutations affect the conformational equilibrium of the enzyme between the inactive and catalytically active forms, a clear explanation of the inactivity was provided only for B-Raf^{D594V}, with the mechanism being ascribed to the key role that the kinase-conserved aspartate plays in chelating the magnesium atom that anchors the ATP to the enzyme [13,14].

Interestingly, although all these mutants appear kinase impaired for three of them (B-Raf^{G466E}, B-Raf^{G466V}, and B-Raf^{G596R}) evidence is provided for their ability to activate to some extent Mek and Erk, due to their capacity to heterodimerize with C-Raf and to induce its transactivation [3,15]. More recent data suggest that B-Raf/C-Raf heterodimerization, either involving B-Raf^{WT} or B-Raf inactivating mutants, occurs as part of the physiological activation process and that the heterodimer has distinct biochemical properties that may be important for the regulation of some biological processes [16]. Mutational experiments and peptide arrays provided evidence for the existence of molecular regions in the kinase domains of both C-Raf and B-Raf responsible for high- and medium-affinity heterodimerization [16].

In order to shed further light on the function and on the potential transforming properties of B-Raf inactivating mutants, in this work we investigate B-Raf^{G596R} and B-Raf^{D594V} using *in silico* molecular dynamics, and a number of *in vitro* and *in vivo* experimental studies.

2. Materials and methods

2.1. Plasmids

pEFP vector-expressing myc-tagged B-Raf^{WT} and B-Raf^{V600E} were kindly provided by Dr. Jeffrey A. Knauf (MSKCC, New York City, USA). pEFP vector-expressing myc-tagged B-Raf^{V599Ins}, B-Raf^{D594V}, B-Raf^{G596R}, B-Raf^{G596E}, B-Raf$^{D594V+V600E}$, B-Raf$^{G596R+V600E}$, B-Raf$^{G596E+V600E}$, and B-Raf^{K483M} were obtained from pEFP-BRAF^{WT} or pEFP-BRAF^{V600E}, as appropriate, by site-directed mutagenesis, using the QuickChange XL mutagenesis kit (Stratagene, La Jolla, CA, USA), according to manufacturer's instructions.

pEGFP-C1 vector-expressing GFP-tagged B-Raf^{V600E}, B-Raf^{G596R} and B-Raf$^{G596R+V600E}$ were obtained by insertional cloning of PCR-generated full length cDNA of the *BRAF* mutants.

pEFP vector-containing myc-tagged C-Raf was kindly provided by Dr. Christopher J. Marshall (Cancer Research UK Centre for Cell and Molecular Biology, London, UK).

2.2. Cell cultures

HEK293T and COS-7 cells were from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Paisley, PA, USA).

NIH-3T3 cells were from American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM supplemented with 10% calf serum (CS) (HyClone, Logan, UT, USA).

Transient transfections were carried out with the ExGene 500 reagent according to manufacturer's instructions (Fermentas, Burlington, ON, Canada). Cells were seeded at a density of 1.5×10^6/100 mm dish the day before transfection, transfected with 2 μg of DNA and harvested 48 h later.

Stable transfections of NIH-3T3 cells were carried out with the lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In detail, cells were seeded at a density of 1.5×10^6/100 mm dish the day before transfection, transfected with 5 μg of the GFP-tagged B-Raf expression constructs, divided 1:5 24 h later and put into selection medium (DMEM with 10% calf serum and 800 μg/ml of G418 (Gibco, Paisley, PA, USA)) 48 h after transfection. The selection was prolonged for 10 days and then the cells were assayed for transgene expression by Western blotting using an anti-GFP antibody (Sigma, St. Louis, MI, USA).

2.3. Kinase assays

For the B-Raf kinase assays, transiently transfected HEK293T cells were cultured for 30 h in complete medium, and then kept for 18 h in serum-deprived medium before harvesting. In detail, cells were lysed in a buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (vol/vol) Triton X-100, 50 mM NaCl, 5 mM ethylene glycol-bis(b -amino-ethyl ether) N, N, N', N' tetraacetic acid (EGTA), 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride, 0.2 mg each of aprotinin and leupeptin per ml. Lysates were clarified by centrifugation at 10,000×*g* for 15 min and the supernatant processed. B-Raf kinase was immunoprecipitated with the anti-myc tag (9E10) antibody (Santa Cruz, CA, USA) and resuspended in a kinase buffer containing 25 mM sodium pyrophosphate, 0.02–3 mM ATP and 1 μg of recombinant GST-Mek (Upstate Biotechnology Inc., Lake Placid, NY, USA). After 15 min incubation at 30 °C, reactions were stopped by adding 2× Laemmli buffer. Proteins were then subjected to 10% SDS gel electrophoresis. The degree of Mek phosphorylation was analyzed by Western Blotting using an anti-phospho Mek1/2 (MAP kinases 1 and 2) antibody (#9121) (Cell Signaling, Beverly, MA, USA).

In order to find the more efficient kinase assay conditions, *in vitro* pMEK assays of B-Raf^{WT} and B-Raf^{V600E} were repeated in the presence of three increasing concentrations of ATP (0.02, 0.8 and 3 mM), and the best condition was chosen for the further tests.

2.4. Immunoblotting experiments

Immunoblotting experiments were performed according to standard procedures. Anti-phospho p44/42 MAPK (#9102), specific for MAPK (Erk1/2) phosphorylated at Thr202/Tyr204, anti-phospho Mek1/2 (MAP kinases 1 and 2) (#9121), specific for Mek1 and Mek2 phosphorylated at Ser217/Ser221, and anti-Mek1/2 (#9122) were purchased from Cell Signaling (Beverly, MA, USA). Anti-myc tag (9E10) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Reporter assays

For reporter assays HEK293T cells were co-transfected with B-Raf expressing vectors and 0.1 μg of the pdAP1-luc reporter (containing the luciferase (*Firefly* luciferase) reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP1 binding elements [17]) and 0.01 μg of phRL, which expresses *Renilla* luciferase from *Renilla reniformis* for normalization. The total amount of plasmid DNA was adjusted with pcDNA3-β-Galactosidase. *Firefly* and *Renilla* luciferase activities were assayed using the Dual-Luciferase Reporter System (Promega Corporation, Madison, WI, USA). Light emission was quantified using a Berthold Technologies luminometer (Centro LB 960). Experiments were performed in triplicate and data were represented as average ± S.D.

2.6. Molecular dynamics simulations

The atomic coordinates of B-Raf^{WT} and B-Raf^{V600E} were retrieved from the protein data bank (pdb codes: 1uwh, 1uwj) and the inhibitor BAY43-9006 was removed while keeping the crystal water [18].