

# Involvement of BH4 domain of bcl-2 in the regulation of HIF-1-mediated VEGF expression in hypoxic tumor cells

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In addition to act as an antiapoptotic protein, B-cell lymphoma (bcl)-2 can also promote tumor angiogenesis. In this context, we have previously demonstrated that under hypoxia bcl-2 promotes hypoxia-inducible factor-1 (HIF-1)-mediated vascular endothelial growth factor (VEGF) expression in melanoma and breast carcinoma. Here, we report on the role of the BH4 domain in bcl-2 functions, by showing that removal of or mutations at the BH4 domain abrogate the ability of bcl-2 to induce VEGF protein expression and transcriptional activity under hypoxia in human melanoma cells. We have also extended this observation to other human tumor histotypes, such as colon, ovarian and lung carcinomas. The involvement of BH4 on HIF-1 $\alpha$  protein expression, stability, ubiquitination and HIF-1 transcriptional activity was also demonstrated in melanoma experimental model. Moreover, we validated the role of the BH4 domain of bcl-2 in the regulation of HIF-1/VEGF axis, demonstrating that BH4 peptide is sufficient to increase HIF-1 $\alpha$  protein half-life impairing HIF-1 $\alpha$  protein ubiquitination, and to enhance VEGF secretion in melanoma cells exposed to hypoxia. Finally, we found that the mechanism by which bcl-2 regulates HIF-1-mediated VEGF expression does not require BH1 and BH2 domains, and it is independent of antiapoptotic and prosurvival function of bcl-2.

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B-cell lymphoma (bcl)-2 family proteins are defined by the sequence homology shared within four conserved bcl-2 homology (BH) domains, BH1, BH2, BH3 and BH4.<sup>1</sup> Whereas the BH1 and BH2 domains of bcl-2 are required for dimerization with proapoptotic proteins, the BH3 domain is crucial for homo- and hetero-dimerization between the bcl-2 family members. The amino-terminal BH4 domain is mainly found in the bcl-2 family members with death-repressing activity,<sup>1</sup> but it is also present in some proapoptotic molecules.<sup>2</sup> BH4 domain of bcl-2 and bcl-xL is critical for interaction with and/or regulation of several proteins involved in apoptosis<sup>3–6</sup> and all BH domains are necessary for the antiapoptotic function of bcl-2 and bcl-xL.<sup>7</sup> In addition to protecting from apoptosis and increasing survival, bcl-2 and bcl-xL are involved in several other important functions, including angiogenesis.<sup>8–16</sup> In this context, we have previously reported that bcl-2 expression in tumor cells exposed to hypoxia increases the expression of vascular endothelial growth factor (VEGF) through the hypoxia-inducible factor-1 (HIF-1).<sup>9,11</sup> *In vitro* and *in vivo* inhibition of bcl-2 functions has a strong effect on HIF-1 target genes that, in some cases, is functionally unrelated to the prosurvival effect of bcl-2.<sup>17–19</sup> These results, together with data demonstrating the ability of bcl-2 to orchestrate a contact-independent crosstalk between endothelial and tumor cells promoting tumor growth,<sup>20</sup> identify

a new function for bcl-2 and bcl-xL that goes beyond their role in cell survival. An important point, yet to be studied in depth, is which domain(s) of bcl-2 account for the regulation of angiogenesis by bcl-2 through a VEGF-mediated HIF-1-dependent pathway. We recently demonstrated that bcl-2 induces HIF-1 $\alpha$  protein stabilization through the impairment of ubiquitin-dependent HIF-1 $\alpha$  degradation involving the molecular chaperone heat shock protein 90 (HSP90).<sup>21</sup> In this work, we investigated the possible role of the BH domains of bcl-2 in the ability of this protein to regulate HIF-1-mediated VEGF expression in cooperation with hypoxia. This study shows that bcl-2 is implicated in HIF-1 $\alpha$  stabilization in hypoxic conditions, through a mechanism that involves the BH4 domain, but not the BH1 or BH2.

## Results

**The BH4 domain, but not the BH1 or BH2, is necessary for VEGF induction by *wild-type* bcl-2 under hypoxia.** To address the relevance of the different domains of bcl-2 in its ability to increase VEGF expression under hypoxia, M14 human melanoma cells were transiently transfected with expression vectors encoding human *wild-type* (*wt*) bcl-2, or different point-mutated or deleted forms of this protein (Figures 1a and b). As shown by enzyme-linked

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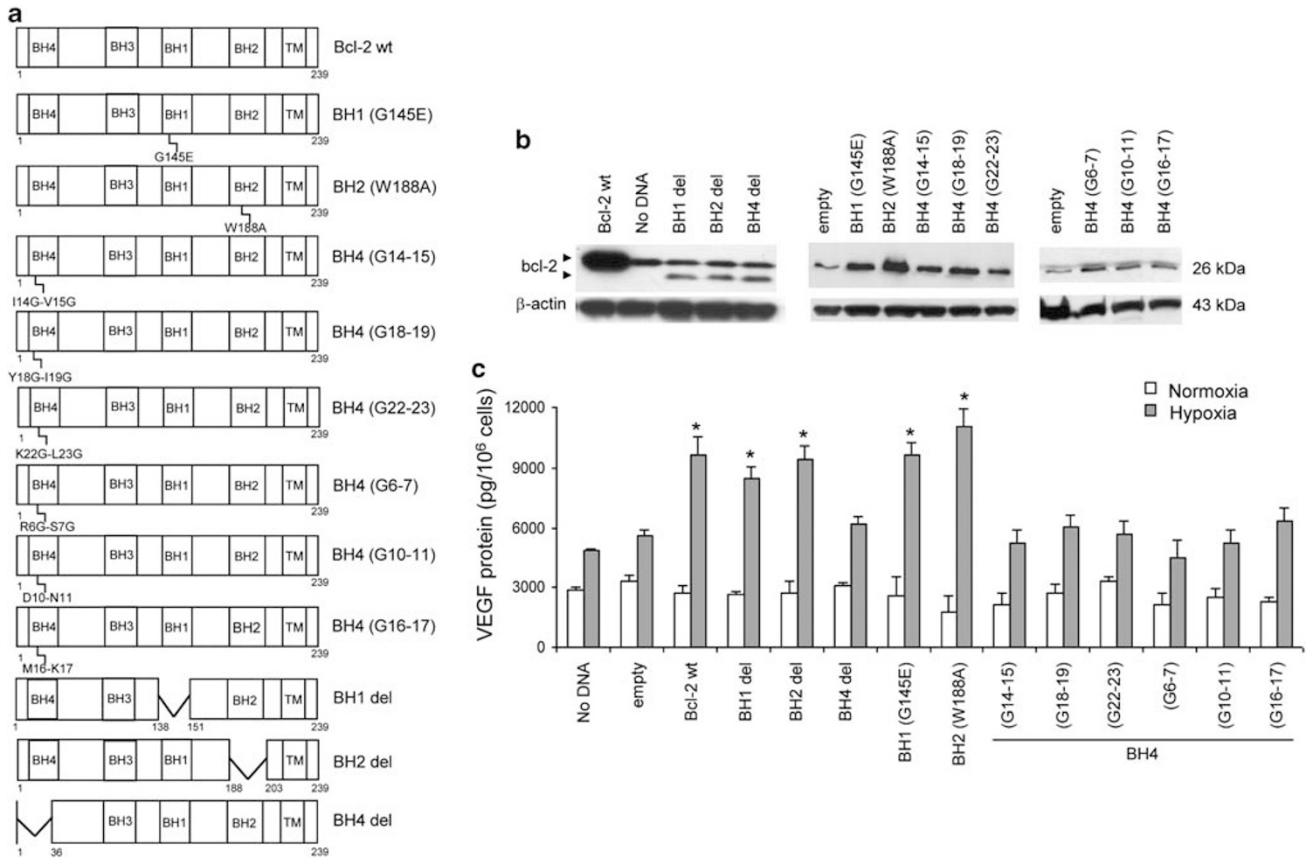
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**Abbreviations:** Bcl-2, B-cell lymphoma 2; BH, Bcl-2 homology; PARP, poly(ADP-ribose) polymerase; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelial growth factor; CPT, camptothecin; CHX, cyclohexamide; ROS, reactive oxygen species; HSP90, heat shock protein 90; ELISA, enzyme-linked immunosorbent assay; HRE, hypoxic responsive element

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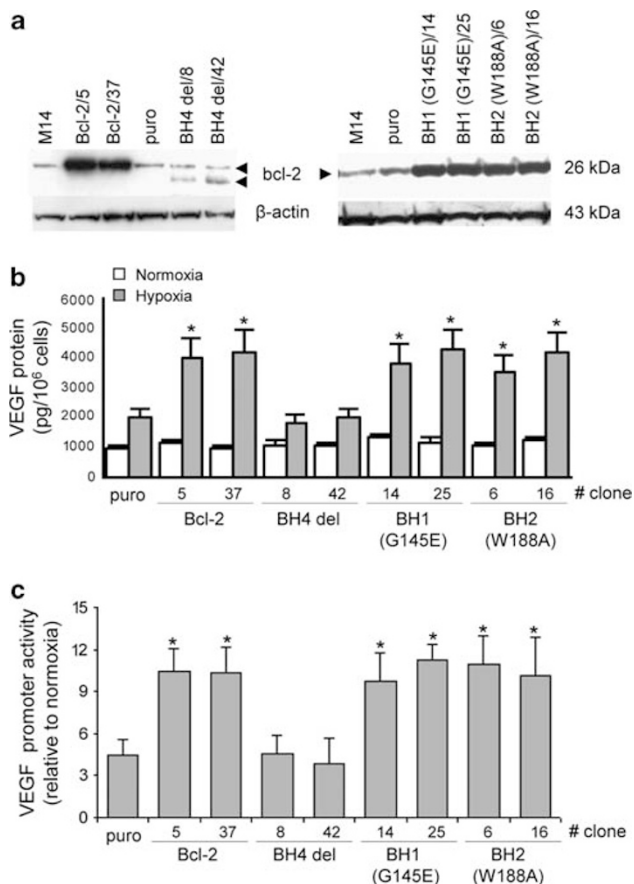


**Figure 1** The BH4 domain, but not BH1 or BH2, is necessary for VEGF induction by *wild-type* (*wt*) bcl-2 under hypoxia. **(a)** A schematic representation and names of structural constructs encoding the human *wt* bcl-2 or different bcl-2 mutants that have been used throughout the paper: the bcl-2 point mutants at the BH1 domain (G145E, amino acid residue 145 from glycine to glutamic acid), at the BH2 domain (W188A, amino acid residue 188 from tryptophan to alanine), and six different dicodon bcl-2 mutants at the BH4 domain (G14–15, amino acid residues 14 (isoleucine) and 15 (valine) replaced by glycines; G18–19, amino acid residues 18 (tyrosine) and 19 (isoleucine) replaced by glycines; G22–23, amino acid residues 22 (lysine) and 23 (leucine) replaced by glycines; G6–7, amino acid residues 6 (arginine) and 7 (serine) replaced by glycines; G10–11, amino acid residues 10 (aspartic acid) and 11 (asparagine) replaced by glycines; G16–17, amino acid residues 16 (methionine) and 17 (lysine) replaced by glycines); the bcl-2 deleted mutants of BH4 (residues from 1 to 36), BH1 (residues from 138 to 151) or BH2 (residues from 188 to 203) regions. All the mutations of bcl-2 protein, excluding G6–7, G10–11 and G16–17, impair its antiapoptotic activity. **(b)** Western blot analysis of bcl-2 protein expression on M14 cells untransfected (no DNA) or transiently transfected with empty vector (empty) or with vectors encoding *wt* or mutated bcl-2 protein. Two bands, indicative of the higher endogenous full-length and shorter exogenous bcl-2 protein, were observed in cells overexpressing bcl-2 deleted of BH1, BH2 or BH4 regions, while a single high band was observed in control cells (untransfected or transfected with empty vector) and in cells overexpressing *wt* or point-mutated bcl-2 protein. Western blots representative of three independent experiments with similar results are shown.  $\beta$ -actin is shown as loading and transferring control. **(c)** VEGF protein expression evaluated by ELISA on M14 cells transiently transfected with vectors encoding *wt* or mutated bcl-2 exposed to normoxia or hypoxia. Results represent the average  $\pm$  S.D. of three independent experiments. *P*-values were calculated between control and *wt* or mutated bcl-2-overexpressing cells (\**P* < 0.01)

immunosorbent assay (ELISA; Figure 1c), while under normoxia no differences were observed regardless bcl-2 status, under hypoxia the VEGF protein level increased in all cells compared with normoxia. In addition, under hypoxia higher levels of VEGF protein were observed in cells overexpressing bcl-2 *wt* or deleted in the BH1 or BH2 domains when compared with control cells, besides higher level of bcl-2 protein was reached in cells overexpressing the *wt* compared with mutated bcl-2 protein (Figure 1b), thus indicating that those low levels of BH1/BH2 deleted proteins are sufficient to sustain HIF-1/VEGF pathway activation in hypoxia. On the contrary, the level of the VEGF protein was not increased in cells expressing bcl-2 deleted in the BH4 domain relative to control cells. As reported in Figure 1c, all dicodon mutations in the residues of BH4 domain abrogated the ability of the *wt* form of bcl-2 to cooperate with hypoxia to

induce VEGF expression. On the contrary, point mutations within the BH1 or BH2 domains did not affect bcl-2-induced VEGF protein secretion.

The role of the different mutations on bcl-2-induced VEGF expression (Figure 2b) and VEGF transcriptional activity (Figure 2c) was also evaluated, under normoxia or hypoxia, using stably transfected clones expressing *wt* or mutated bcl-2 (Figure 2a). Under normoxia, there were not differences in VEGF protein secretion regardless bcl-2 status. By contrast, under hypoxia *wt* bcl-2 overexpression significantly increased both the VEGF protein secretion (Figure 2b) and VEGF promoter activity (Figure 2c) when compared with control cells. However, the deletion of the BH4 domain abrogates the ability of bcl-2 to cooperate with hypoxia to induce VEGF expression and promoter activity: both parameters were similar in the control clone and in bcl-2 deleted of BH4



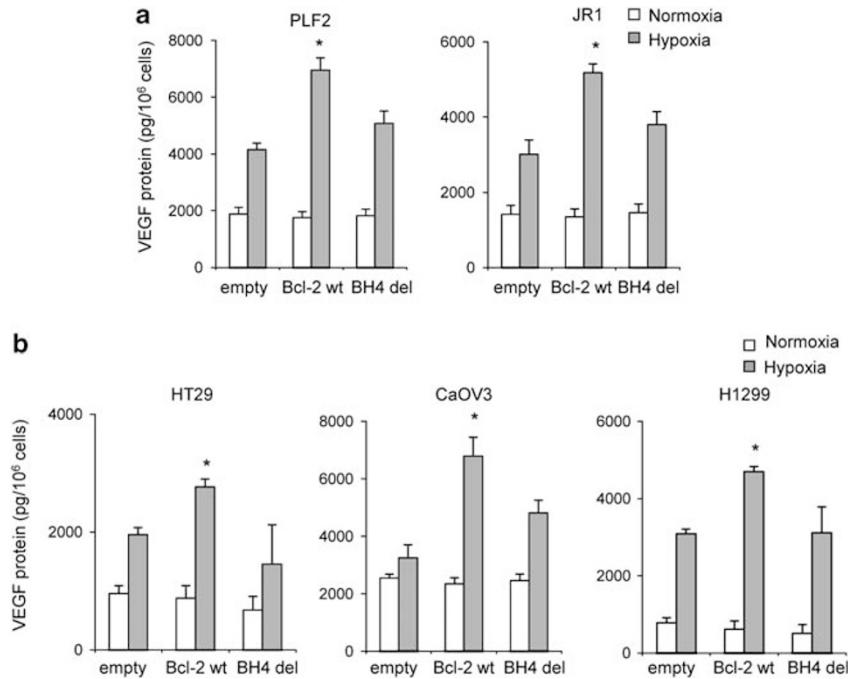
**Figure 2** The BH4 domain, but not the BH1 or BH2, is necessary for VEGF induction by *wild-type* (*wt*) bcl-2 under hypoxia. Western blot analysis of bcl-2 protein expression (a), VEGF protein expression evaluated by ELISA (b) and VEGF promoter activity (c) in M14 cells, a control clone (puro), clones overexpressing *wt* bcl-2 (Bcl-2/5, Bcl-2/37), bcl-2 deleted of the BH4 domain (BH4 del/8, BH4 del/42), bcl-2 with point mutations at the BH1 (BH1 (G145E)/14, BH1 (G145E)/25) or at the BH2 (BH2 (W188A)/6, BH2 (W188A)/16) domains. (a) Western blots representative of three independent experiments with similar results are shown.  $\beta$ -actin is shown as loading and transferring control. (c) The luciferase construct containing the VEGF 1511-bp proximal promoter sequence was transiently transfected into cells and then exposed to normoxia or hypoxia for 24 h. Luciferase values were normalized for transfection efficiency (luciferase/ $\beta$  galactosidase activities ratios). The results are reported as fold induction relative to normoxia. (b and c) Results represent the average  $\pm$  S.D. of three independent experiments. *P*-values were calculated from control and bcl-2 *wt* or mutated bcl-2-overexpressing cells (\**P*<0.01)

transfectants (Figures 2b and c). On the contrary, point mutations at the BH1 or BH2 domains did not affect bcl-2-induced VEGF protein secretion and promoter activity under hypoxia.

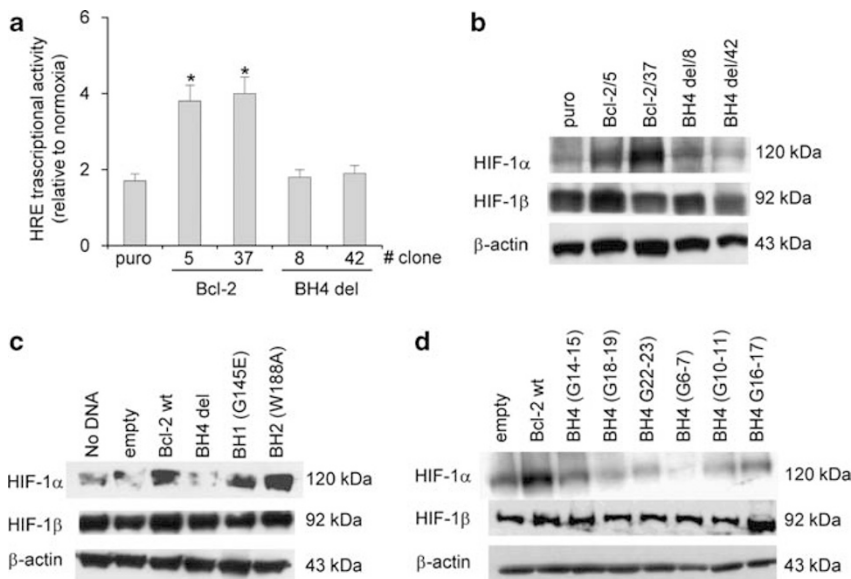
We also extended the BH4-dependent role of bcl-2 in the regulation of hypoxia-induced VEGF expression to other melanoma cells and other tumor histotypes (Figure 3). In normoxia, the levels of secreted VEGF protein were similar in all cells regardless bcl-2 status and expression. By contrast, the hypoxic induction of the VEGF protein was increased after forced expression of *wt* bcl-2 in melanoma (PLF2, JR1) (Figure 3a) and colon (HT29), ovarian (CaOV3) and lung (H1299) carcinoma (Figure 3b) cells when compared with control cells, while no differences were observed after forced expression of the bcl-2 protein deleted of the BH4 domain.

**The increase in HIF-1 $\alpha$  protein expression by bcl-2 is dependent on its BH4 domain.** We investigated on the role of different bcl-2 mutations on the bcl-2-induced HIF-1 transcriptional activity and HIF-1 proteins expression in melanoma cells, stably or transiently transfected with *wt* or different mutated bcl-2. Under hypoxia *wt* bcl-2 clones showed about twofold increase in the activity of the HIF-1-dependent gene transcription when compared with the transfected control cells, whereas the clones overexpressing bcl-2 deleted of its BH4 domain showed HIF-1 transcriptional activity similar to that observed in the transfected control cells (Figure 4a). Transfectants expressing point mutants within the BH1 or BH2 domains of bcl-2 showed HIF-1 transcriptional activity similar to that of the clones overexpressing the *wt* bcl-2 protein (data not shown). As shown in Figure 4b, after exposure to hypoxia, the HIF-1 $\alpha$  protein expression was detectable in control cells, while it became more evident after forced expression of *wt* bcl-2. Similar to control cells, both clones carrying bcl-2 deleted of the BH4 domain showed a low HIF-1 $\alpha$  protein expression. Under hypoxia, high levels of HIF-1 $\alpha$  protein were also observed in the cells transiently transfected with *wt* bcl-2 when compared with the cells transiently transfected with both empty vector and vector encoding bcl-2 deleted of its BH4 domain. On the other hand, similar levels of the HIF-1 $\alpha$  protein were observed in cells transiently transfected with *wt* bcl-2 and in those expressing bcl-2 point mutants within the BH1 or BH2 domains (Figure 4c). Besides, cells expressing bcl-2 dicodon mutants in the BH4 domain showed levels of the HIF-1 $\alpha$  protein comparable to those showed by cells transiently transfected with the empty vector (Figure 4d), independently from their response to apoptosis. Moreover, under hypoxia HIF-1 $\beta$  expression was not modulated in any of the cell lysates regardless the bcl-2 status (Figures 4b–d). In normoxia, levels of HIF-1 $\alpha$  protein were undetectable in all cells regardless bcl-2 status and expression (data not shown).

**The increase in HIF-1 $\alpha$  protein stability by bcl-2 is dependent on its BH4 domain.** To gain further insight into the mechanisms of bcl-2-induced HIF-1 $\alpha$  protein expression, we examined the HIF-1 $\alpha$  mRNA levels in melanoma cells stably transfected with *wt* or mutated bcl-2. No difference in HIF-1 $\alpha$  mRNA levels were observed between cells exposed to normoxia or hypoxia regardless bcl-2 status, thus confirming that bcl-2 overexpression under hypoxia did not modulate HIF-1 $\alpha$  gene expression at transcriptional level<sup>9</sup> and excluding the involvement of BH4 domain in the regulation of HIF-1 $\alpha$  mRNA levels (Figure 5a). Next, we examined the effects of ectopic expression of *wt* bcl-2 or bcl-2 deleted in BH4 domain on HIF-1 $\alpha$  protein at the posttranscriptional level using the proteasome inhibitor MG132 (Figure 5b). The accumulation of HIF-1 $\alpha$  protein after MG132 treatment was identical in all cells regardless bcl-2 status, indicating that neither *wt* nor mutated form of bcl-2 affect HIF-1 $\alpha$  protein synthesis (Figure 5b). To directly test whether bcl-2 status in hypoxic cells is associated with a modulation of HIF-1 $\alpha$  protein stability, we measured HIF-1 $\alpha$  protein half-life. As depicted in Figures 5c and d, a time-



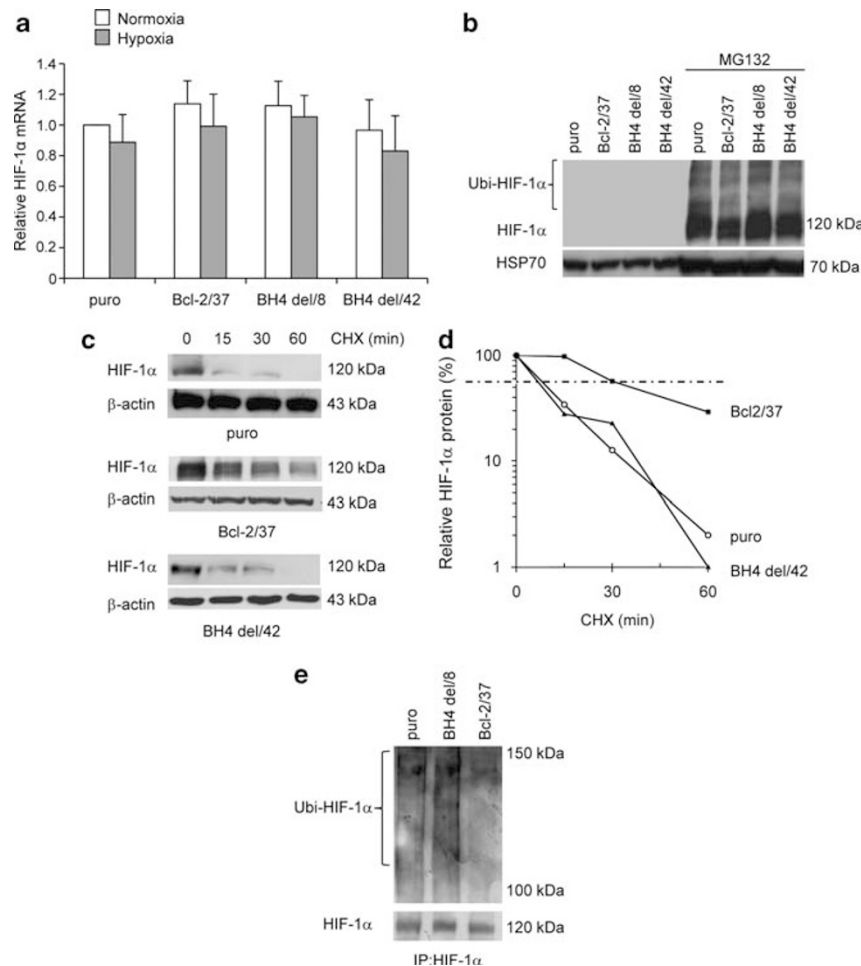
**Figure 3** Forced expression of *wild-type* (*wt*) bcl-2, but not bcl-2 deleted of BH4 domain, increases hypoxia-induced VEGF protein expression in several tumor cell lines of different origin. **(a and b)** VEGF protein expression evaluated by ELISA in human PLF2 and JR1 melanomas **(a)**, HT29 colon, CaOV3 ovarian and H1299 lung carcinoma cells **(b)** transiently transfected with control vector (empty) or constructs encoding bcl-2 *wt* (Bcl-2 *wt*) or deleted of the BH4 domain (BH4 del) and exposed to normoxia or hypoxia for 24 h. Results represent the average  $\pm$  S.D. of two independent experiments. *P*-values were calculated from control and *wt* or BH4-deleted bcl-2-overexpressing cells exposed at the same experimental conditions (\**P* < 0.01)



**Figure 4** The BH4 domain is necessary for HIF-1 protein expression and activity induced by *wild-type* (*wt*) bcl-2 under hypoxia. **(a)** Hypoxic responsive element (HRE)-dependent transcriptional activity in a control clone (puro), clones overexpressing bcl-2 *wt* (Bcl-2/5, Bcl-2/37) or deleted of the BH4 domain (BH4 del/8, BH4 del/42). The luciferase construct containing the HIF-1-like (808-bp) *consensus* sequence was transiently transfected into cells and then exposed to normoxia or hypoxia for 24 h. Luciferase values were normalized for transfection efficiency (luciferase/ $\beta$  galactosidase ratios). The results are reported as fold induction relative to normoxia and represent the average  $\pm$  S.D. of three independent experiments. *P*-values were calculated between control and *wt* or deleted bcl-2-overexpressing cells (\**P* < 0.01). **(b-d)** Western blot analyses of HIF-1 $\alpha$  and HIF-1 $\beta$  protein expression in the nuclear **(b)** or total **(c and d)** extracts of stably transfected M14-derived clones **(b)** or transiently transfected M14 cells **(c and d)** exposed to hypoxia for 24 h. **(c and d)** Expression vectors encoding bcl-2 *wt* (Bcl-2 *wt*) or mutants at residues into the BH1 (G145E), the BH2 (W188A), the BH4 domain (G14-15; G18-19; G22-23; G6-7; G10-11; and G16-17), and bcl-2 deleted of the BH4 domain (BH4 del) were used. The untransfected (no DNA) and the control vector transfected (empty) M14 cells were used as control. Western blots representative of three independent experiments with similar results are shown.  $\beta$ -actin is shown as loading and transferring control

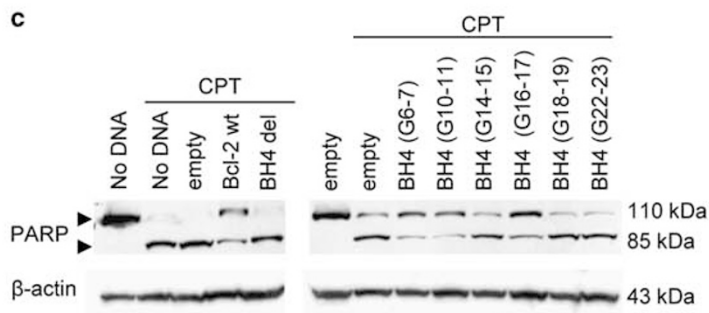
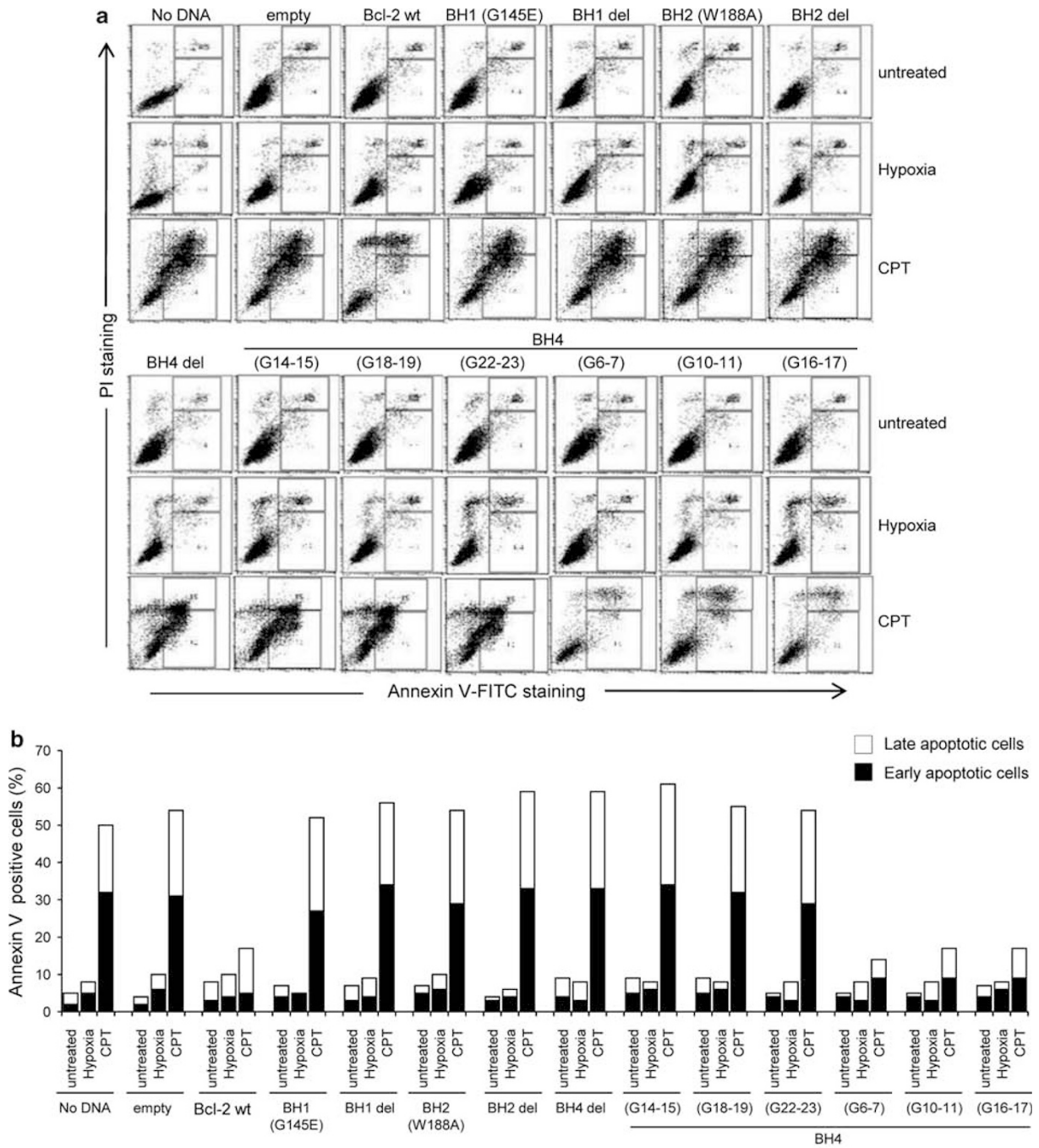
dependent decrease in HIF-1 $\alpha$  protein level was observed in the cells after treatment with the protein synthesis inhibitor cyclohexamide (CHX) following hypoxia. Densitometric analysis revealed that, as previously reported,<sup>21</sup> bcl-2 overexpression increased the HIF-1 $\alpha$  protein half-life from  $15 \pm 5$  to  $45 \pm 5$  min under hypoxic conditions. By contrast, no significant difference in the HIF-1 $\alpha$  protein half-life was

observed between control and stable clones expressing bcl-2 deleted of its BH4 domain (HIF-1 $\alpha$  protein half-life:  $13 \pm 7$  min). Similarly, while a reduced HIF-1 $\alpha$  ubiquitination was observed in transfectants expressing *wt* bcl-2, when compared with control cells, similar modulation of HIF-1 $\alpha$  ubiquitination was observed in transfectants carrying the deletion of BH4 domain and control cells (Figure 5e).



**Figure 5** The BH4 domain is necessary for HIF-1 $\alpha$  protein stabilization by *wild-type* (*wt*) bcl-2. (a) RT-PCR evaluation of HIF-1 $\alpha$  mRNA level, (b and c) western blot analyses of HIF-1 $\alpha$  protein expression after the exposure to MG132 proteasomal inhibitor ( $10 \mu\text{M}$ ) for 6 h in normoxic conditions (b), or to hypoxia for 24 h followed by CHX treatment ( $50 \mu\text{g/ml}$ ) for the indicated times (c) of M14 melanoma control (puro), a clone-overexpressing *wt* bcl-2 (Bcl-2/37), or bcl-2 deleted of the BH4 domain (BH4 del/8, BH4 del/42). (a) Values are expressed as means  $\pm$  S.D. of fold induction *versus* control cells (puro) exposed to normoxia. Results represent the average  $\pm$  S.D. of three independent experiments. (b and c) Western blots representative of three independent experiments with similar results are shown.  $\beta$ -actin and HSP70 proteins are shown as loading and transferring control. (d) Densitometric analysis of the HIF-1 $\alpha$  protein level showed in western blot (c) was performed using Molecular Analyst Software and normalized to  $\beta$ -actin expression. Values are expressed as percentage of HIF-1 $\alpha$  protein after CHX treatment *versus* HIF-1 $\alpha$  protein expression in untreated cells (100%). (e) Western blot analysis of HIF-1 $\alpha$  ubiquitination on control, bcl-2 *wt* and bcl-2 deleted of the BH4 domain cells after exposure to hypoxia. Whole cell lysates were immunoprecipitated (IP) with anti-HIF-1 $\alpha$  antibody and then western blot analysis was performed using anti-ubiquitin antibody

**Figure 6** The effect of bcl-2 mutations on HIF-1/VEGF signaling is independent of their impact in bcl-2-mediated antiapoptotic function. (a) Cytofluorimetric dot plot analysis of the annexin V-FITC *versus* propidium iodide (PI) staining performed on M14 cells transiently transfected with several bcl-2 expression vectors. After transfection, cells were exposed to normoxia (untreated), hypoxia for 24 h or treated with CPT ( $0.5 \mu\text{M}$  for 48 h). A representative experiment out of three performed with similar results is shown. Annexin V<sup>+</sup>/PI<sup>-</sup> and annexin V<sup>+</sup>/PI<sup>+</sup> were considered early and late apoptotic cells, respectively. (b) Percentage of annexin V<sup>+</sup>/PI<sup>-</sup> (black bars) and annexin V<sup>+</sup>/PI<sup>+</sup> (white bars) cells was calculated by cytofluorimetric analysis showed in Figure 6a using CellQuest Software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). (c) Western blot analysis of PARP cleavage on the untransfected or transiently transfected cells treated with CPT ( $0.5 \mu\text{M}$  for 48 h). Western blots representative of three independent experiments with similar results are shown.  $\beta$ -actin is shown as loading and transferring control. (a-c) Expression vectors encoding bcl-2 wild-type (Bcl-2 *wt*) or mutants at the residues into BH1 (G145E), the BH2 (W188A), at the BH4 domain (G14-15; G18-19; G22-23; G6-7; G10-11; and G16-17), or bcl-2 deleted of the BH4, BH1, BH2 domain (BH4 del, BH1 del, BH2 del) were used. The untransfected (no DNA) and the control vector transfected (empty) M14 cells were used as control



**The effect of bcl-2 mutations on HIF-1/VEGF signaling is independent of their relevance in apoptosis.** To exclude the possibility that the effect of bcl-2 on HIF-1/VEGF expression and activation could be a consequence of a different modulation of the apoptotic pathway by ectopic expression of different forms of bcl-2, we evaluated the response of cells to camptothecin (CPT), a drug able to activate the apoptotic program in several experimental models,<sup>22</sup> (Figures 6a and b). CPT treatment induced about 50% of apoptosis in untransfected cells or cells transfected with empty vector, while it induced a very low percentage of apoptosis in cells overexpressing *wt* bcl-2. CPT-induced apoptosis was also observed in cells transfected with expression vectors encoding BH1, BH2 and BH4 domain deletion mutants of bcl-2, bcl-2 single-point mutants of highly conserved residues within the BH1 (G145E) or BH2 (W188A) domains, or dicodon mutants into the BH4 domain (G14–15, G18–19 and G22–23). On the other hand, the rate of apoptotic events in the cells transfected with bcl-2 mutations into the residues 6–7, 10–11 and 16–17 of BH4 domain was quite similar to that observed following CPT treatment in cells overexpressing *wt* bcl-2, confirming that these residues are not required for bcl-2 antiapoptotic activity.<sup>6,7,23</sup> These results were also confirmed by the cleavage of poly(ADP-ribose) polymerase (PARP) after treatment with CPT (Figure 6c) in cells expressing bcl-2 deleted in BH4 domain but not in cells overexpressing *wt* bcl-2. Further, PARP cleavage was markedly reduced in the cells overexpressing bcl-2 protein mutated into amino acids 6–7, 10–11 and 16–17 within BH4 domain. Next, we evaluated whether mutated bcl-2 protein induced cell death and reactive oxygen species (ROS) production under hypoxia. The exposure of cells to hypoxia for 24 h did not induce either apoptosis (Figures 6a and b) or the generation of ROS (Supplementary Figure 1) either in control or in cells overexpressing *wt* or mutated bcl-2. In all cells, <10% of apoptosis and <5% of ROS were observed after 24 h exposure to hypoxia regardless bcl-2 status.

**TAT-BH4 peptide alone is sufficient to enhance HIF-1/VEGF axis.** To confirm the hypothesis that the BH4 domain has a role in the hypoxic induction of HIF-1/VEGF by bcl-2, we determined whether the exogenous application of TAT-BH4 peptide is able to modulate HIF-1/VEGF axis in M14 cells. First, to confirm the antiapoptotic activity of TAT-BH4 peptide,<sup>24,25</sup> we evaluated the response of M14 cells to CPT. Figure 7a showed that the addition of TAT-BH4 significantly decreased the percentage of apoptosis induced by CPT: approximately 30 and 11% early apoptotic cells were observed after treatment with CPT in presence of control (TAT-CTRL) or TAT-BH4 peptides, respectively. Further, we also tested the effect on VEGF/HIF-1 signaling of a mutant version of TAT-BH4. The percentage of early apoptotic events in the cells treated with CPT and exposed to the mutant version of TAT-BH4 was quite similar to that observed after treatment with CPT and TAT-BH4 peptide, confirming that mutant version of TAT-BH4 still retains the antiapoptotic activity showed by TAT-BH4 (Figure 7a). The exogenous application of TAT-BH4 peptide increased

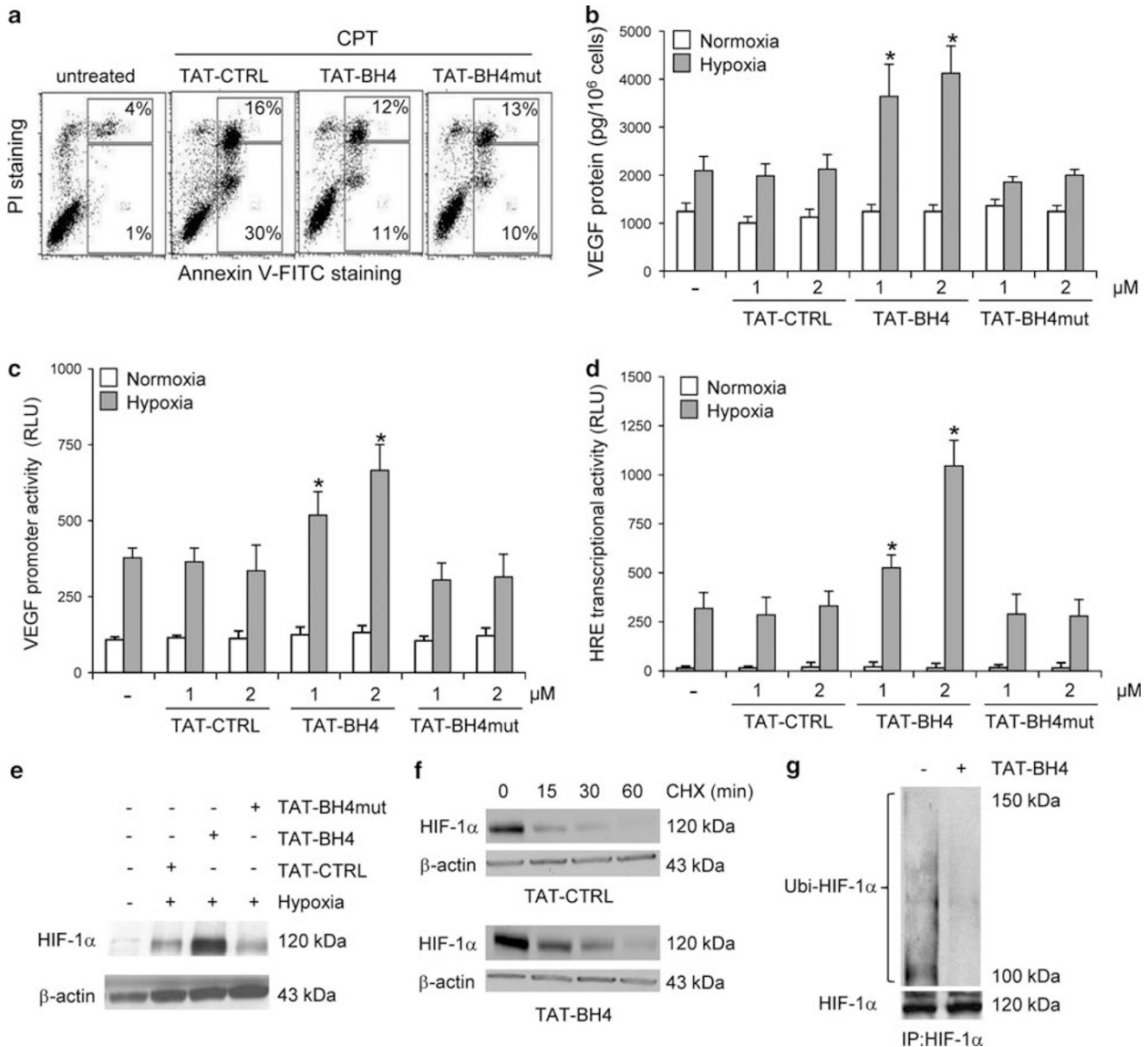
the level of secreted VEGF protein (Figure 7b), VEGF promoter activity (Figure 7c), HIF-1 transcriptional activity (Figure 7d) and HIF-1 $\alpha$  protein levels (Figure 7e) in the M14 cells when compared with the untreated or TAT-CTRL-treated cells only after exposure to hypoxia. On the contrary, these parameters were not affected by the treatment with the mutant version of TAT-BH4. Similar results in terms of VEGF and HIF-1 $\alpha$  protein induction were also obtained using a BH4 antiapoptotic peptide<sup>25</sup> from bcl-xL (data not shown). Finally, exogenous application of TAT-BH4 peptide increases HIF-1 $\alpha$  protein half-life (Figure 7f) and reduces HIF-1 $\alpha$  protein ubiquitination under hypoxic conditions (Figure 7g).

As we have obtained evidence that the localization of bcl-2 protein is important for its effect on HIF-1/VEGF signaling,<sup>21</sup> we evaluated the presence of TAT-BH4 peptide in the cellular compartments where generally bcl-2 protein localizes,<sup>26</sup> and the possible effect of hypoxia on TAT-BH4 localization by using a labeled version of peptide. First, we confirmed by flow cytometric (Figure 8a) and confocal microscopy (Figure 8b) analyses the efficient incorporation of TAT-BH4 peptide into the cells. Using different organelle-specific labeling, we found that TAT-BH4 peptide did not reside in the classical compartments in which full-length bcl-2 is generally found, such as nucleus, mitochondria or endoplasmic reticulum in normoxic conditions, and hypoxia did not alter this localization of TAT-BH4.

**The effect of BH4 domain on HIF-1/VEGF pathway activation is independent from the presence of endogenous bcl-2 protein.** To test whether in our model the overexpression of bcl-2 deleted of BH4 domain protein could suppress the activity of endogenous protein, bcl-2 *wt* or deleted of its BH4 domain was stably expressed in HT29 cells that do not express detectable levels of endogenous bcl-2,<sup>27</sup> as confirmed by western blot and RT-PCR analyses (Supplementary Figure 2). As shown in Figures 9a and b, *wt* bcl-2-overexpressing HT29 cells exposed to hypoxia for 24 h secrete higher level of VEGF protein than the parental cells. Both HT29 clones carrying bcl-2 deleted of the BH4 domain expressed VEGF protein at a level that is comparable to the one of control cells. Next, we evaluated whether exogenous application of TAT-BH4 peptide is sufficient to enhance HIF-1/VEGF axis also in a context of bcl-2-deficient background. An increase in both VEGF (Figures 9c and d) and HIF-1 $\alpha$  (Figure 9d) protein levels in HT29 cells was observed after hypoxic exposure to TAT-BH4 peptide when compared with untreated cells. Both VEGF promoter and HIF-1-dependent transcriptional activity were also increased after the treatment of HT29 cells with TAT-BH4 peptide, under hypoxic conditions (data not shown).

## Discussion

In this study, we investigated the possible role of the BH1, BH2 and BH4 domains of bcl-2 protein in the regulation of HIF-1-mediated VEGF expression under hypoxia. We demonstrated that the deletion of the BH4 domain abrogated the ability of bcl-2 to cooperate with hypoxia to induce VEGF protein expression in tumor cells with different origin. The

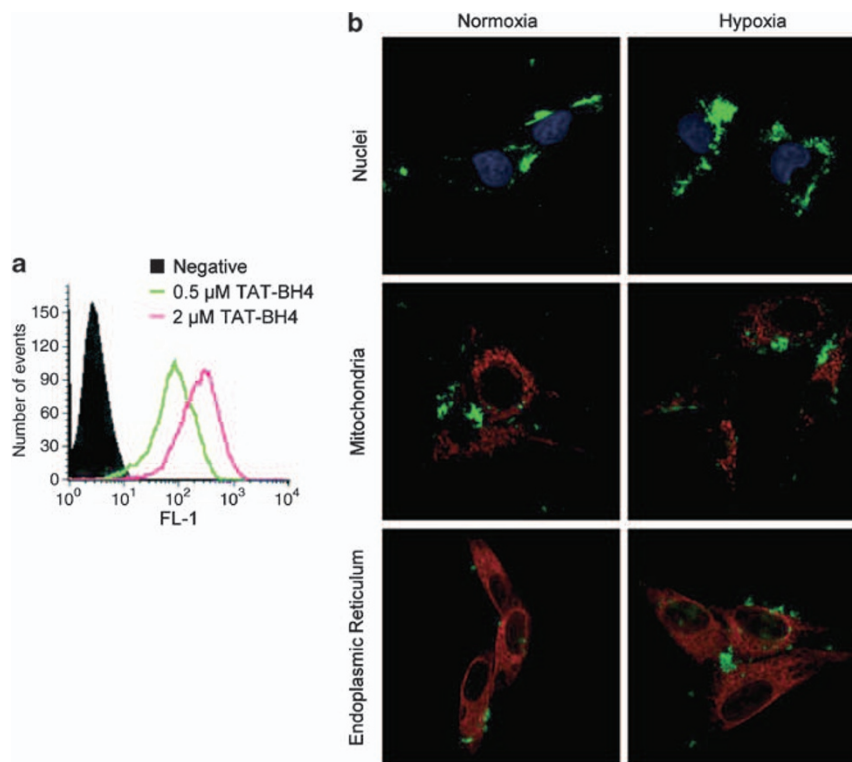


**Figure 7** VEGF protein expression is induced under hypoxia by exogenous application of the TAT-BH4 cell permeable peptide in M14 cells. (a) Cytofluorimetric dot plot analysis of the annexin V-FITC versus propidium iodide (PI) staining performed on M14 control cells (untreated), or treated with CPT (0.5 μM for 48 h) in presence of the different peptides (2 μM). The percentage of the annexin V<sup>+</sup>/PI<sup>-</sup> (early apoptotic cells) and annexin V<sup>+</sup>/PI<sup>+</sup> (late apoptotic cells) are shown. Each panel is representative of three separate experiments with comparable results. (b) VEGF protein expression evaluated by ELISA, (c) VEGF promoter activity, (d) hypoxic responsive element (HRE) transcriptional activity in M14 cells after exposure to hypoxia in presence of different peptides at the indicated concentrations. (c and d) The luciferase constructs containing the VEGF 1511-bp proximal promoter (c) or HIF-1-like (808-bp) (d) consensus sequences were transiently transfected and then cells were treated with peptides under hypoxia for 24 h. Luciferase values were normalized for transfection efficiency (luciferase/β galactosidase ratios). RLU, relative luciferase unit. The results are reported as the average ± S.D. of three independent experiments. *P*-values were calculated between control and treated cells exposed to hypoxia (\**P* < 0.01). (e) Western blot analysis of HIF-1α protein in total cell extracts of M14 cells after exposure to hypoxia in presence of different peptides (2 μM). (f) Western blot analysis of HIF-1α protein on M14 cells after exposure to hypoxia in presence of peptides and then followed by CHX (50 μg/ml) treatment for the indicated times. (g) Western blot analysis of HIF-1α ubiquitination after exposure of M14 cells to hypoxia in the presence or absence of TAT-BH4 peptide. Whole cell lysates were immunoprecipitated (IP) with anti-HIF-1α antibody and then western blot analysis was performed using anti-Ubiquitin antibody. (e and f) Western blots representative of two independent experiments with similar results are shown. β-actin is shown as loading and transferring control. (a-g) TAT-CTRL (control peptide), TAT-BH4 (peptide corresponding to wild-type BH4 domain of bcl-2) and TAT-BH4mut (peptide corresponding to a mutated versions of BH4 domain into amino acids 6-7) were used

relevance of BH4 domain on the regulation of HIF-1/VEGF axis under hypoxia was showed in a human melanoma model. In particular, deletion of or point mutations into the BH4 region resulted in impaired ability of *wt* bcl-2 protein to induce VEGF promoter activity, HIF-1α protein stabilization and ubiquitination under hypoxia.

BH4 is one of the principal domains for the full multi-functional ability of bcl-2, and it appears to mediate the antiapoptotic activity of bcl-2 independently from the interaction of this protein with other bcl-2 family members. The BH4 domain is necessary and sufficient for bcl-2 to prevent apoptosis, bind to bax, translocate into the nucleus,





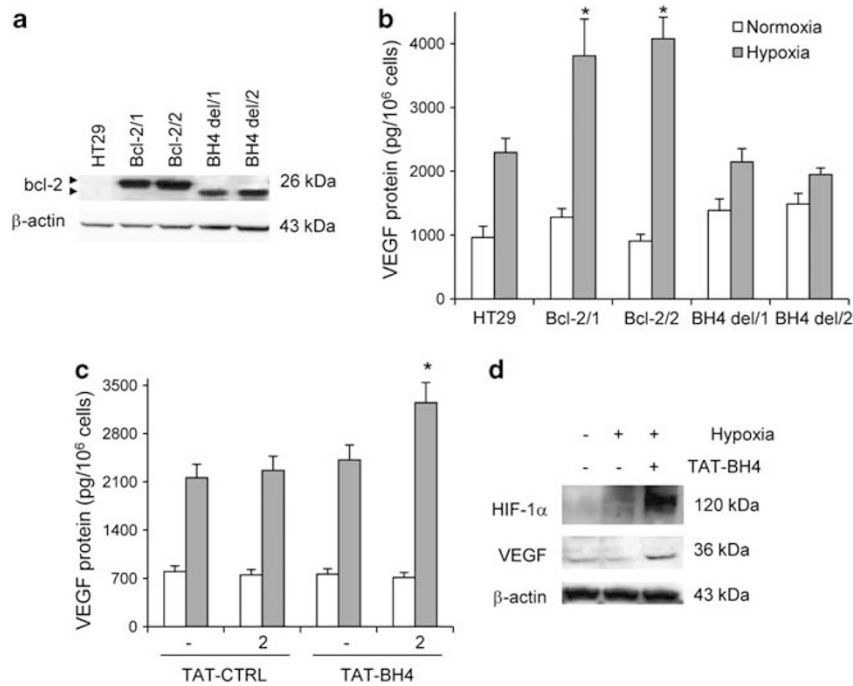
**Figure 8** Cellular localization of TAT-BH4 peptide in M14 cells exposed to normoxia or hypoxia. (a) Cytofluorimetric evaluation of TAT-BH4 peptide incorporation in M14 cells treated with 0,5 and 2  $\mu\text{M}$  of peptide. (b) Confocal laser scanning microscopy of immunofluorescence staining performed on M14 cells exposed to hypoxia or normoxia for 24 h, in the presence of TAT-BH4 peptide (green). Fixed cells were labeled with nuclear staining TO-PRO3 (blue), mitochondrial-specific anti-HSP60 antibody (red) or endoplasmic reticulum-specific anti-calreticulin antibody (red)

reduce cell proliferation, induce nuclear factor-kappa B activity and regulate DNA repair.<sup>4,5,28</sup> In addition, some authors have suggested that bcl-2 deleted of its BH4 domain functions like bax to promote, rather than inhibit, cell death,<sup>6</sup> while other groups have reported that BH4 deletion converts bcl-2 into a dominant-negative inhibitor of bcl-2.<sup>7</sup> In our experimental model, the bcl-2 protein deleted of BH4 domain did not function as a dominant-negative inhibitor toward the bcl-2 ability to protect from apoptosis. In fact, cells overexpressing bcl-2 protein, whose BH4 domain was deleted or presented point mutations, undergo to apoptosis after treatment with CPT, a drug able to induce apoptotic cell death in parental but not in bcl-2 *wt* overexpressing cells.

The molecular mechanism by which bcl-2 increases HIF-1-mediated VEGF expression in melanoma cells under hypoxia might be found in the ability of BH4 domain to directly or indirectly interact with HIF-1 $\alpha$  protein, or to modulate the activity of some proteins found to interact with the BH4 region.<sup>4,5,29–33</sup> Moreover, some bcl-2 protein interactors, such as the bcl-2 mitochondrial chaperone FKBP38, the orphan nuclear receptor Nur77 and the molecular chaperone HSP90, have been demonstrated to be involved in oxygen-dependent and -independent regulation of the HIF-1 $\alpha$  protein.<sup>33–35</sup> Even if we recently demonstrated that bcl-2 protein under hypoxic conditions interacts with both HIF-1 $\alpha$  and HSP90 proteins contributing to the enhancement of HIF-1 $\alpha$  protein stability,<sup>21</sup>

we cannot exclude that also other bcl-2 interactors can be involved in bcl-2-induced HIF-1 $\alpha$ /VEGF regulation.

We have also observed that TAT-BH4 peptide in hypoxic melanoma cells acts like full-length *wt* bcl-2 mimicking bcl-2 functions in terms of HIF-1/VEGF regulation. In fact, we showed that the TAT-BH4 peptide had a significant effect on VEGF protein induction and promoter activity, and regulated HIF-1 $\alpha$  protein expression increasing the half-life and decreasing the ubiquitination of the protein. Our results clearly indicate that the BH4 domain is sufficient to induce HIF-1-mediated VEGF protein expression under hypoxia. TAT-control as well as TAT-BH4 mutant peptides showed no such effects, confirming the specific effect of TAT-BH4 peptide. Further, TAT-BH4 peptide localization was not responsible of the effects exerted by the peptide under hypoxic condition, in fact similar localization in the cytosol was found regardless oxygen tension. Our results also demonstrated that amino acidic residues within BH1 or BH2 domains required for the antiapoptotic activity of bcl-2 are not indispensable for bcl-2-dependent HIF-1/VEGF induction under hypoxia. In fact, bcl-2 protein deleted in BH1 or BH2 domains is still able to cooperate with hypoxia to activate HIF-1/VEGF signaling, as observed for *wt* bcl-2, despite the lower deleted protein levels showed by the cells overexpressing BH1 and BH2 mutants in comparison with *wt* bcl-2-overexpressing cells. So this finding indicates that those low levels of BH1/BH2 deleted proteins



**Figure 9** Bcl-2 deleted of its BH4 domain is not a dominant-negative inhibitor of the endogenous *wild-type* (*wt*) bcl-2 protein. (a) Western blot analysis of bcl-2 protein expression, and (b) VEGF protein expression evaluated by ELISA in HT29 colon carcinoma cell line and its derivative clones overexpressing *wt* bcl-2 (Bcl-2/1, Bcl-2/2), or bcl-2 deleted of the BH4 domain (BH4 del/1, BH4 del/2). (c) VEGF protein expression evaluated by ELISA, and (d) western blot analysis of HIF-1 $\alpha$  and VEGF proteins in HT29 cells after exposure to hypoxia in presence of 2  $\mu$ M TAT-BH4 or TAT-CTRL as control. (a and d) Western blot representative of three independent experiments with similar results are shown.  $\beta$ -actin is shown as loading and transferring control. (b and c) Results represent the average  $\pm$  S.D. of three independent experiments. (b) *P*-values were calculated from control and *wt* or mutated bcl-2-overexpressing cells exposed at the same experimental conditions ( $*P < 0.01$ ). (c) *P*-values were calculated between control and TAT-BH4-treated cells exposed to hypoxia ( $*P < 0.01$ )

are sufficient to sustain HIF-1/VEGF pathway activation in hypoxia. The fact that the level of BH4 deleted protein obtained after overexpression is comparable with the ones of cells expressing BH1/BH2 deleted bcl-2 protein, is an indirect demonstration that the loss of bcl-2 activity showed after BH4 domain deletion is not due to the lower level of expression of bcl-2 mutated in comparison with *wt* bcl-2 protein expression. This observation, as also demonstrated by other investigators,<sup>6</sup> may be due to an higher stability of full-length *wt* bcl-2 protein or to a different percentage of transfected cells.

The role of the BH3-driven protein–protein interaction on bcl-2-dependent regulation of HIF-1/VEGF was excluded by using HA14-1, a BH3 mimetic antagonist of bcl-2 (Supplementary Figure 3). Exposure of bcl-2-overexpressing cells to HA14-1 did not affect the increased expression of VEGF observed after hypoxic exposure of bcl-2 transfectants, thus indicating that bcl-2-induced HIF1/VEGF pathway is not related to the ability of bcl-2 to interact with the BH3 domain of proapoptotic proteins. By other hand, mutations of bcl-2 protein into BH1 (G145E), BH2 (W188A) and BH4 (G14-15) domains, disrupting the heterodimerization of bcl-2 with BH3-containing pro-apoptotic proteins, confirm these results. In fact, although all these mutations damp bcl-2 antiapoptotic activity, only BH4 (G14-15) mutant affects bcl-2-induced VEGF/HIF1 expression and activity. Also the evidence that mutations on all the analyzed residues into BH4 abrogated bcl-2 ability to induce HIF-1/VEGF signaling under hypoxia,

whereas they differently affect bcl-2 ability to prevent apoptosis, sustained our hypothesis that the antiapoptotic function of bcl-2 is not required for its effect on angiogenesis. These results were also corroborated by the evidence that TAT-BH4 mutated peptide that still retains antiapoptotic effect, is not able to activate HIF-1/VEGF signaling. We further excluded the possibility that the effect of bcl-2 on the VEGF/HIF-1 pathway was attributed to the activity of bcl-2 as a prosurvival and antiapoptotic protein, because transient transfection of *wt* or mutated bcl-2 proteins did not induce ‘*per se*’ cell death in our experimental model, and  $< 10\%$  of apoptotic cells were observed in all the cell lines tested at the end of hypoxia exposure, regardless bcl-2 status expression.

By using the HT29 colon carcinoma cell line, which is deficient for bcl-2 expression,<sup>27</sup> we have also showed that bcl-2 deleted of its BH4 domain is not a dominant-negative inhibitor of endogenous bcl-2 protein in terms of VEGF expression. In the same context of bcl-2-deficient background, the application of TAT-BH4 peptide is sufficient to enhance HIF-1/VEGF axis. Thus, demonstrating that the effect of BH4 domain is not mediated by the presence of endogenous bcl-2 protein.

In summary, our results demonstrate that the ability of *wt* bcl-2 to enhance angiogenesis through the BH4 domain is not related to its role in preventing apoptosis, and indicate that the development of inhibitors based on the BH4 domain of bcl-2 can be useful to better define the functions of BH4 domain

and for the treatment of tumors associated with increased expression of VEGF and/or HIF-1 $\alpha$  proteins.

## Materials and Methods

**Cell cultures, reagents and transfections.** Human M14, PLF2 and JR1 (melanoma), HT29 (colon carcinoma), CaOV3 (ovarian carcinoma) and H1299 (lung carcinoma) were cultured in 10% FBS RPMI medium (Invitrogen, Carlsbad, CA, USA). The cells were stably or transiently transfected with the expression vectors encoding the human *wt* bcl-2 or different bcl-2 mutants (Figure 1a). Transfections of expression vectors and hypoxia treatment were performed as previously reported.<sup>11</sup> Two *wt* bcl-2-overexpressing (Bcl-2/clone 5 and Bcl-2/clone 37), two bcl-2 mutants at the BH1 domain (BH1(G145E)/clone 14 and BH1(G145E)/clone 25), at the BH2 domain (BH2(W188A)/clone 6 and BH2(W188A)/clone 16), two bcl-2 deleted of the BH4 domain (BH4 del/clone 8 and BH4 del/clone 42) clones, a control clone (puro) derived from the M14 line after stable transfection were used. Stable clones were cultured in the presence of puromycin (1  $\mu$ g/ml, Sigma-Aldrich, St. Louis, MI, USA). TAT-BH4 Bcl-2, a cell-penetrating peptide of human immunodeficiency virus TAT fused to a synthetic peptide corresponding to the BH4 domain (NH<sub>2</sub>-GRKKRRRQRGGRTGYDNRREIVMKYIHYKLSQRGYEW-COOH), TAT-BH4mut Bcl-2 peptide (NH<sub>2</sub>-GRKKRRRQRGGGGYDNRREIVMKYIHYKLSQRGYEW-COOH) corresponding to the mutated versions of BH4 (G6-7), and TAT-CTRL (NH<sub>2</sub>-RKKRRRQRGGGLKNDICLRVYTPVSVLVNE-COOH) peptide were obtained from PRIMM (Milan, Italy). For some experiments, TAT-BH4 peptide was labeled at amino-terminal with 5(6)-carboxyfluorescein. CPT, CHX, Z-leu-leu-leu-CHO (MG132) were purchased from Sigma-Aldrich.

**ELISA, western blot and flow cytometric analyses and promoter activity.** ELISA, flow cytometric analysis and evaluation of promoter activity were done as previously reported.<sup>9,36</sup> Nuclear and cytoplasmic fractions were prepared as previously reported.<sup>21</sup> For immunoprecipitation assays and western blot analysis, the cells were lysed in CHAPS buffer (40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na<sub>3</sub>VO<sub>4</sub>, 0.3% CHAPS, and one tablet EDTA-free protease inhibitors (Roche, Welwyn Garden City, UK) per 50 ml). Following centrifugation, the supernatant was precleared with protein A/G agarose beads coupled with rabbit IgG for > 2 h and then 1  $\mu$ g of the HIF-1 $\alpha$  antibody (clone H-206, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added, and incubated overnight at 4 °C with protein A/G-agarose beads (Amersham Biosciences Europe, Milan, Italy). Immunoprecipitates were washed four times in the lysis buffer before western blot analysis. For some immunoprecipitation experiments we used ExactaCruz reagents (Santa Cruz Biotechnology) to detect the bcl-2 protein without detection of the light chain of the immunoprecipitation antibody. Immunoprecipitations were also performed using multiple antibodies recognizing different epitopes on the bcl-2 (100, Santa Cruz Biotechnology) and HIF-1 $\alpha$  (Santa Cruz Biotechnology; Novus Biologicals, Littleton, CO, USA) proteins. Antibodies against HIF-1 $\alpha$  (clone 54, BD Pharmingen, San Jose, CA, USA), HIF-1 $\beta$  (clone 29, BD Pharmingen), PARP (clone 4C10-5, BD Pharmingen), bcl-2 (clones N-19 and C-2, Santa Cruz Biotechnology; clone 124, Dako, Milan, Italia), ubiquitin (clone P4G7, Santa Cruz Biotechnology),  $\beta$ -actin (clone AC-15, Sigma-Aldrich) and HSP72/73 (clone W27, Calbiochem, EMD Biosciences, La Jolla, CA, USA) were used.

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** Total cellular RNA was isolated using Trizol reagent (Invitrogen). cDNA equivalent to 1  $\mu$ g of total RNA was generated by reverse transcription reaction using (oligo dT)<sub>18</sub> primer (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instructions and then 2  $\mu$ l of obtained cDNA were added to PCR reaction mix (Fermentas) containing exon-specific primers targeting HIF-1 $\alpha$  (sense 5'-CCCAGATTCAGGATCAGACA-3', antisense 5'-CCATCATGTCCATTTTCGC-3') and  $\beta$ -actin (sense, 5'-AAGAGAGGCATCCTCACCT-3', antisense 5'-ATCTCTTGCTCGAAGTCCAG-3') for 25 cycles with an annealing temperature of 56 °C. PCR products were separated by 1.5% agarose gel electrophoresis and gel was scanned with Gel Doc XR (Bio-Rad, Hercules, CA, USA). The intensity of the bands was quantified by densitometry using Molecular Analyst Software (Bio-Rad) and the optical density of each HIF-1 $\alpha$  amplified band was normalized to the intensity of each  $\beta$ -actin band.

**Confocal analysis.** Cells were treated with 0.5  $\mu$ M TAT-BH4 peptide modified at amino-terminal with 5(6)-carboxyfluorescein and then exposed to normoxia or hypoxia. After 24 h, cells were fixed in 3.7% formaldehyde for 15 min at room

temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and then incubated with anti-calreticulin (Affinity Bioreagents, Golden, CO, USA) or anti-HSP60 (BD Transduction Laboratories, Franklin Lakes, NJ, USA) to localize endoplasmic reticulum and mitochondria, respectively. The cells were incubated with TRITC-conjugated goat anti-rabbit or anti-mouse (Jackson Lab, West Grove, PA, USA) antibodies. Nuclei were visualized using TO-PRO3 (Invitrogen). The images were scanned under a  $\times$  40 oil immersion objective and to avoid bleed-through effects, each dye was scanned independently by a Leica confocal microscope (laser-scanning TCS SP2) equipped with Ar/ArKr and HeNe lasers. The images were acquired and electronically merged utilizing the Leica confocal software (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Figures were processed using Adobe PhotoShop software (Adobe Systems Inc., San Jose, CA, USA).

**Statistical analysis.** Differences between groups were analyzed with a two-sided paired or unpaired *t*-test by use of GraphPad Prism 3.00 (GraphPad Software, San Diego, CA, USA). Results were considered to be statistically significant if *P* < 0.05. Experiments were replicated three times unless otherwise indicated.

## Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)