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

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ABSTRACT

In addition to act as an antiapoptotic protein, 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 abrogates 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 α 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 α protein half-life impairing HIF-1 α 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.

INTRODUCTION

Bcl-2 family proteins are defined by the sequence homology shared within four conserved bcl-2 homology (BH) domains, BH1, BH2, BH3 and BH4. Several antiapoptotic members, such as bcl-2 and bcl-xL, possess all four BH domains, whereas some proapoptotic members, such as bax and bak, conserve homology only in the BH1, BH2 and BH3 domains¹; however a structure-based alignment of globular bcl-2 family proteins, recently revealed that a conserved BH4 motif is present in some proapoptotic proteins, as well as antiapoptotic proteins². Whereas the BH1 and BH2 domains of bcl-2 are required for dimerization with proapoptotic members, the BH3 domain is crucial for homo-and hetero-dimerization between the bcl-2 family members. The amino(N)-terminal BH4 domain is critical for interaction of bcl-2 and bcl-xL with and/or regulation of several proteins involved in apoptosis³⁻⁶. All BH domains are necessary for the antiapoptotic function of bcl-2 and bcl-xL⁷. In addition to protecting from apoptosis and increasing survival, bcl-2 and bcl-xL are involved in several other important functions, including angiogenesis⁸⁻¹⁴. 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) gene through the hypoxia-inducible factor-1 (HIF-1)^{9;11}. Moreover, modulation of bcl-xL expression in glioblastoma cells regulates interleukin 8 protein expression through a nuclear factor-kappa B (NF-kB)-dependent mechanism^{10;15}. *In vitro* and *in vivo* inhibition of bcl-2 functions has a strong effect on HIF-1 α target genes that, in some cases, is functionally unrelated to the prosurvival effect of bcl-2¹⁶⁻¹⁸. These results, together with data demonstrating the ability of bcl-2 to orchestrate a contact-independent crosstalk between endothelial and tumor cells promoting tumor growth¹⁹, 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 more 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. Hypoxia-inducible

factor-1 (HIF-1) is a heterodimeric transcription factor, composed of α and β subunits, that plays a key role in cellular adaptations to hypoxia by controlling the expression of a series of genes involved in angiogenesis (such as VEGF, VEGF receptor-1, Chemokine receptor CXCR4), glucose metabolism (such as glucose transporter-1, hexokinase-2) and apoptosis (BNIP3, Wilms tumor suppressor). Whereas both α and β subunits are required for the formation of HIF-1 heterodimer, HIF-1 α is the key regulatory subunit responsible for HIF transcriptional function²⁰. Several major mechanisms underlying regulation of HIF-1 α protein have been delineated, involving multiple pathways and regulatory factors, some of them still to be fully characterized, such as PHD1-3/VHL, HSP90/Receptor of Activated protein Kinase C (RACK1), and HSP70/CHIP (Carboxyl terminus of Hsp70-Interacting Protein)^{21;22}. In addition to hypoxia, HIF-1 α expression can be regulated by certain oncogenes, including bcl-2^{9;11}. We recently demonstrated that bcl-2 induces HIF-1 α protein stabilization through the impairment of ubiquitin-dependent HIF-1 α degradation involving the molecular chaperone HSP90²³. 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. Our present study shows that bcl-2 is implicated in HIF-1 α 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

Previously, we showed that bcl-2 expression in tumor cells exposed to hypoxia increases the expression of VEGF gene through the activation of HIF-1^{9;11}. 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* bcl-2, or different point-mutated or deleted forms of this protein (Figure 1A,B). As shown by ELISA (Figure 1C), while under normoxia no differences were observed between the effect of *wild type* or mutated bcl-2 forced expression on VEGF protein level, under hypoxia the VEGF protein level increased in all cells compared to normoxia. In addition, under hypoxia higher levels of VEGF protein were observed in cells overexpressing bcl-2 *wild type* or deleted in the BH1 (BH1 del) or BH2 (BH2 del) domains when compared with control cells, besides higher level of bcl-2 protein was reached in cells overexpressing the *wild type* compared to 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 (BH4 del) relative to control cells. To better define the relevance of the BH domains in bcl-2-induced VEGF expression under hypoxia, we transiently transfected M14 cells with vectors encoding 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, G22-23, G6-7, G10-11, G16-17). As reported in Figure 1C all mutations in the residues of BH4 domain, abrogated the ability of the *wild type* 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.

Thus, demonstrating that VEGF activation by *bcl-2* under hypoxia involves its BH4 domain, but not BH1 or BH2. To confirm the results obtained from transient-transfection assay, we obtained stably transfected clones using expression vectors encoding *wild type* *bcl-2* (Bcl-2/clone5 and Bcl-2/clone 37), *bcl-2* deleted of BH4 (BH4 del/clone 8 and BH4 del/clone 42) or point-mutated within the BH1 [BH1(G145E)/clone 14 and BH1(G145E)/clone 25] or BH2 [BH2(W188A)/clone 6 and BH2 (W188A)/clone 16] domains (Figure 2A). The role of the different mutations on *bcl-2*-induced VEGF expression (Figure 2B) and VEGF transcriptional activity (Figure 2C) was evaluated under normoxia or hypoxia. Under normoxia, there were not differences in VEGF protein secretion in normoxia regardless *bcl-2* status. By contrast, under hypoxia *wild type* *bcl-2* overexpression significantly increased both the VEGF protein secretion (Figure 2B) and VEGF promoter activity (Figure 2C) when compared to 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 transfectants (Figure 2B,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. Altogether, these data demonstrate that the induction of VEGF expression by *bcl-2* under hypoxia requires the BH4 domain, but not the BH1 or BH2.

The BH4 domain is necessary for VEGF hypoxic induction by *bcl-2* in tumor cells of different origin

To extend the BH4-dependent role of *bcl-2* in the regulation of hypoxia-induced VEGF expression to other melanoma cells and other tumor histotypes, we transiently overexpressed *bcl-2* *wild type* or the same protein deleted of its BH4 domain, in different tumor cells (Figure 3). In normoxia, the levels of VEGF protein secreted were similar in all cells regardless of *bcl-2* status and expression. By contrast, the hypoxic induction of the VEGF protein was increased after forced expression of *wild type* *bcl-2* in PLF2 and JR1 melanoma cells when compared to control cells, while no

differences were observed after forced expression of the bcl-2 protein deleted of the BH4 domain (Figure 3A). Enhanced VEGF expression after overexpression of *wild type* bcl-2, but not of bcl-2 deleted of its BH4 domain, was also shown in colon (HT29), ovarian (CaOV3) and lung (H1299) carcinoma cell lines (Figure 3B), thus confirming the capability of bcl-2 to modulate, through its BH4 domain, VEGF expression under hypoxia in tumor cells of different origin.

The increase of HIF-1 α protein expression by bcl-2 is dependent on its BH4 domain

We have previously demonstrated that the modulation of bcl-2 expression in tumor cells regulates VEGF expression under hypoxia through a HIF-1-dependent pathway^{9;11;23;24}. Thus, 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 *wild type* or different mutants bcl-2. HIF-1 transcriptional activity and the hypoxic induction of HIF-1 α and HIF-1 β proteins control cells (puro), cells stably overexpressing bcl-2 *wild type* (Bcl-2/clone5 and Bcl-2/clone 37) or deleted of BH4 domain (BH4 del/clone 8 and BH4 del/clone 42) are reported in Figure 4A and Figure 4B, respectively. Under hypoxia *wild type* bcl-2 clones showed about 2-fold 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 *wild type* bcl-2 protein (data not shown). As shown in Figure 4B, after exposure to hypoxia, the HIF-1 α nuclear protein expression was detectable in control cells (puro), while it became more evident after forced expression of *wild type* bcl-2 (Bcl-2/clone5 and Bcl-2/clone 37). Similar to control cells, both clones carrying bcl-2 deleted of the BH4 domain (BH4 del/clone 8 and BH4 del/clone 42) showed a low HIF-1 α protein expression. Under hypoxia, high levels of HIF-1 α protein were also observed

in the cells transiently transfected with *wild type* 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 α protein were observed in cells transiently transfected with *wild type* 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 α 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 β expression was not modulated in any of the cell lysates regardless of the bcl-2 status (Figure 4B-D). In normoxia, levels of HIF-1 α protein were undetectable in all cells regardless of bcl-2 status and expression (data not shown).

The increase of HIF-1 α protein stability by bcl-2 is dependent on its BH4 domain

We previously reported that while bcl-2 overexpression under hypoxia did not alter HIF-1 α mRNA levels⁹, it affects HIF-1 α protein expression at the posttranscriptional level²³. RT-PCR analysis (**Figure 5A**) showed no difference in HIF-1 α mRNA levels between cells exposed to normoxia or hypoxia regardless of bcl-2 status, thus confirming that bcl-2 overexpression under hypoxia did not modulate HIF-1 α gene expression at transcriptional level and excluding the involvement of BH4 domain in the regulation of HIF-1 α mRNA levels. To elucidate the mechanism by which bcl-2 activates HIF-1, we examined the effects of ectopic expression of *wild type* bcl-2 or bcl-2 deleted in BH4 domain of bcl-2 on HIF-1 α protein at the posttranscriptional level. To this purpose, we firstly performed experiments using the proteasome inhibitor MG132 (**Figure 5B**). Since the blockade of proteasome function inhibits HIF-1 α proteasome-mediated degradation in normoxic conditions, the detected HIF-1 α protein levels will predominantly reflect the translation rate of the HIF-1 α protein. The accumulation of HIF-1 α protein after MG132 treatment was identical in all cells regardless bcl-2 status, indicating that neither *wild type* nor mutated form of bcl-2 does not affect HIF-1 α protein

synthesis (**Figure 5B**). To directly test whether bcl-2 status in hypoxic cells is associated with a modulation of HIF-1 α protein stability, we measured HIF-1 α protein half-life. As depicted in **Figure 5 C,D** a time-dependent decrease of HIF-1 α protein level was observed in the cells after treatment with the protein synthesis inhibitor Cyclohexamide (CHX) following hypoxia exposure. In particular, while HIF-1 α protein was still well detectable after 60 minutes treatment with the CHX in *wild type* bcl-2 transfectants (Bcl-2/37), HIF-1 α protein was strongly reduced already after 15 minutes from CHX adding and it was not detectable at 60 minutes of CHX treatment in both control cells (puro) and BH4 deleted bcl-2 overexpressing clone. Densitometric analysis revealed that, as previously reported²³, bcl-2 overexpression increased the HIF-1 α protein half-life from 15 \pm 5 min to 45 \pm 5 min under hypoxic conditions. By contrast, no significant difference in the HIF-1 α protein half-life was observed between control cells and stable clones expressing bcl-2 deleted of its BH4 domain (BH4 del/42, HIF-1 α protein half-life: 13 \pm 7 min). Similarly, while a reduced HIF-1 α ubiquitination is observed in Bcl-2/37, transfectant expressing *wild type* bcl-2, when compared to control cells (puro), similar modulation of HIF-1 α ubiquitination was observed in BH4 del/8, transfectant carrying the deletion of BH4 domain and control cells (puro) (**Figure 5E**). All together these results demonstrate that the degradation rate of HIF-1 α protein is faster in the control and bcl-2 deleted of BH4 domain overexpressing cells than in *wild type* bcl-2 transfectants, thus indicating an effect of bcl-2 on HIF-1 α protein accumulation through its BH4 domain.

The effect of bcl-2 mutations on HIF-1/VEGF signaling is independent of their relevance in apoptosis.

There are evidences that mutations or deletions of bcl-2 protein can affect bcl-2 ability to inhibit apoptosis^{7;25;26}, transient overexpression of *wild type* bcl-2 may induce rather than protect cells from apoptosis²⁷ and deletion of BH4 domain can convert bcl-2 into a proapoptotic protein⁶. 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 in terms of apoptotic cell death induced by camptothecin (CPT), a drug able to activate the apoptotic program in several experimental models²⁶. As reported in **Figure 6A-B**, Annexin V/Propidium Iodide staining analysis by flow cytometry shows that 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 *wild type* *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*, encoding *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, 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 *wild type* *bcl-2*, confirming that these residues are not required for *bcl-2* antiapoptotic activity^{6;7;28}. These results obtained in cells carrying *bcl-2* deleted of its BH4 domain and mutations into BH4 domain, were also confirmed by the activation of caspase-3(-like) proteases, as estimated by the cleavage of PARP after treatment with CPT (Figure 6C). Overexpression of *wild type* *bcl-2* but not of *bcl-2* deleted in BH4 domain prevented PARP cleavage induced by CPT treatment, indicating the BH4 domain was required to inhibit apoptosis. Further, PARP cleavage was markedly reduced only 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 ROS production under hypoxia. As depicted in Figure 6 A,B the exposure of cells to hypoxia for 24 h did not induce apoptosis either in control or in cells overexpressing *wild type* or mutated *bcl-2*. Less than 10% of apoptotic cells were observed regardless *bcl-2* expression. According with these results, hypoxia was not found to induce the generation of reactive oxygen species (ROS) (Supplementary Figure 1). Less than 5% of ROS was

observed after 24 h exposure to hypoxia, with no differences between control and cells overexpressing *wild type* or mutated bcl-2.

Since all mutations into BH4 domain of bcl-2 are relevant for HIF-1/VEGF induction by *wild type* form of bcl-2 under hypoxia, regardless their relevance in the ability of bcl-2 protein to block apoptosis, our data demonstrate that the proangiogenic function of bcl-2 is not related to its antiapoptotic role of bcl-2.

TAT-BH4 peptide alone is sufficient to enhance HIF-1/VEGF axis

The results described above indicate that the BH4 domain has a role in the hypoxic induction of HIF-1/VEGF by bcl-2. To confirm this hypothesis we determined whether the exogenous application of TAT-BH4 peptide, a cell-penetrating peptide of human immunodeficiency virus TAT fused to a synthetic peptide corresponding to the BH4 domain, is able to modulate VEGF protein expression and its promoter activity, HIF-1 α protein stabilization and transcriptional activity in M14 cells. Firstly, to confirm the anti-apoptotic activity of TAT-BH4 peptide^{29;30}, we evaluated by Annexin V/Propidium Iodide staining 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, where the TAT peptide is fused to a synthetic peptide corresponding to a mutated version of BH4 domain into amino acids 6-7, mutations that did not abolish TAT-BH4 anti-apoptotic effect (Figure 7A). 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. As shown by ELISA assay (Figure 7B), no modulation of VEGF protein secretion was

induced by TAT-BH4 peptide treatment under normoxia. On the contrary, under hypoxia the exogenous application of TAT-BH4 peptide at the doses of 2 μ M induced approximate 2-fold increase in the level of secreted VEGF protein when compared to M14 untreated or TAT-CTRL treated cells. Moreover, TAT-BH4 peptide treatment significantly enhanced VEGF promoter activity (Figure 7C) and HIF-1 α transcriptional activity (Figure 7D) under hypoxic conditions in a dose-dependent manner in the M14 line when compared to the untreated or TAT-CTRL treated cells. VEGF protein secretion, VEGF promoter and HIF-1 transcriptional activity were not increased after the treatment of M14 cells with mutant version of TAT-BH4 compared to control cells. As shown in Figure 7E, an increase in HIF-1 α protein levels was also observed after exposure to TAT-BH4 peptide under hypoxia when compared to TAT-CTRL treated cells, while the levels of HIF-1 α protein in cells after treatment with mutant version of TAT-BH4 was quite similar to TAT-CTRL treated cells. Similar results in terms of VEGF and HIF-1 α protein induction were also obtained using a BH4 antiapoptotic peptide³⁰ from bcl-xL (data not shown). Finally exogenous application of TAT-BH4 peptide increases HIF-1 α protein half-life (Figure 7F) and reduces HIF-1 α protein ubiquitination under hypoxic conditions (Figure 7G). Thus, demonstrating that the addition of TAT-BH4 alone managed the hypoxia-induced enhancement of VEGF protein secretion and promoter activity, HIF-1 α expression and HIF-1 transcriptional activity. Taken together, these findings indicate that the BH4 domain of bcl-2 is able to induce HIF-1/VEGF signaling, independently from its ability to prevent apoptotic cell death. Since we have obtained evidence that the localization of bcl-2 protein is important for its effect on HIF-1/VEGF signaling²³, we evaluated the presence of TAT-BH4 peptide in the cellular compartments where generally bcl-2 protein localizes³¹, such as the outer mitochondrial membrane, the endoplasmatic reticulum and the nuclear envelope, and the possible effect of hypoxia on TAT-BH4 localization. To this aim we performed laser scanning confocal microscopy experiments after treating cells with TAT-BH4 peptide labeled with 5(6)-carboxyfluorescein, in normoxic or hypoxic conditions for 24

h. Firstly, we confirmed by flow cytometric analysis the efficient incorporation of TAT-BH4 peptide into the cells: a dose-dependent increase of fluorescence intensity was observed after treatment with increasing doses of TAT-BH4 peptide (Figure 8A). As shown in Figure 8B, we confirmed by confocal microscopy that the peptides were internalized in cells, forming several aggregates. Using different organelle-specific labelings, 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

Some authors have reported that the deletion of BH4 domain converts bcl-2 into a dominant negative inhibitor of bcl-2 itself⁷, while other groups have suggested that bcl-2 deleted of its BH4 domain functions, like bax protein, to promote rather than inhibit cell death⁶. To test whether in our model the overexpression of bcl-2 deleted of BH4 domain protein could suppress the activity of endogenous protein, we assessed the BH4-dependent role of bcl-2 in the regulation of hypoxia-induced VEGF expression in the colon carcinoma cell line HT29, which is deficient for bcl-2 expression³². In particular, bcl-2 *wild type* or deleted of its BH4 domain was stably expressed in HT29 cells that do not express detectable levels of endogenous bcl-2, as confirmed by Western blot and RT-PCR analyses (Supplementary Figure 2). As shown in Figure 9 A,B *wild type* bcl-2-overexpressing HT29 cells (Bcl-2/1 and Bcl-2/2 clones) 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 (BH4 del/1 and BH4 del/2) expressed VEGF protein at a level that is comparable to that 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 (Figure 9 C,D) and HIF-1 α (Figure 9D) protein levels in HT29 cells was observed after hypoxic exposure to TAT-BH4 peptide when compared to 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). Thus, demonstrating that the effect of BH4 domain is sufficient to activate HIF-1/VEGF pathway even in absence of endogenous bcl-2 protein, and that bcl-2 deleted of its BH4 domain does not elicit a dominant-negative activity.

DISCUSSION

Recent studies, including ours, have demonstrated that bcl-2 not only functions as an antiapoptotic and a prosurvival molecule¹, but it also promotes tumor progression and angiogenesis in different tumor histotypes^{9;11;33}. By using antisense oligonucleotide or RNA interference directed to bcl-2 mRNA, or overexpressing bcl-2 protein, we have previously demonstrated the ability of bcl-2 protein to regulate the expression of VEGF gene through HIF-1 activation in melanoma⁹ and breast carcinoma¹¹ histotypes, independently of bcl-2 effect on cell survival¹⁶. More recently, we have also reported that bcl-2 induced HIF-1 α stabilization in response to hypoxia through the impairment of ubiquitin-dependent HIF-1 α degradation involving the molecular chaperone HSP90²³. In the present 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 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 *wild type* bcl-2 protein to induce VEGF promoter activity, HIF-1 α protein stabilization and ubiquitination under hypoxia.

Even though a recent prominent report suggested that there is a BH4 consensus sequence in some proapoptotic members, such as bax and bak², the BH4 domain is mainly found in the bcl-2 family members with death-repressing activity. BH4 is one of the principal domains for the full multifunctional 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, reduce cell proliferation, induce NF-kB activity and regulate DNA repair^{4;5;34}. In addition, some authors have suggested that bcl-2 deleted of its BH4 domain functions like bax to promote,

rather than inhibit, cell death⁶, while other groups have reported that BH4 deletion converts bcl-2 into a dominant negative inhibitor of bcl-2⁷. In our experimental model the bcl-2 protein deleted of BH4 domain did not function as a dominant negative inhibitor towards 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 *wild type* 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 α protein, or to modulate the activity of some proteins found to interact with the BH4 region. It has been documented that the BH4 domain of bcl-2 interacts with a number of different factors, such as Ras³⁵, Raf-1³⁶, c-Myc³⁷, NF-KB^{4,5}, FK-506 binding protein 38 (FKBP38)³⁸, and inositol 1,4,5-triphosphate receptor³⁹. Moreover, some bcl-2 protein interactors have been demonstrated to be involved in oxygen-dependent and independent regulation of the HIF-1 α protein. In particular, the bcl-2 mitochondrial chaperone, FKBP38, the orphan nuclear receptor, Nur77, and the molecular chaperone, HSP90, have been found to be implicated in HIF-1 α turnover^{22;39;40}. Very recently, we have demonstrated in a melanoma model that bcl-2 protein under hypoxic conditions interacts with both HIF-1 α and HSP90 proteins contributing to the enhancement of HIF-1 α protein stability²³. Besides, we cannot exclude that also other factors can be involved in bcl-2-induced HIF-1 α /VEGF regulation.

We have also evaluated the effect of exogenous application of the cell-permeable TAT-BH4 peptide in melanoma cells exposed to hypoxia. This peptide acts like full length *wild type* 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 α 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. We have also evidence that TAT-BH4 peptide localization was not responsible of the effects exerted by the TAT-BH4 peptide under hypoxic condition, in fact similar localization in the cytosol was found regardless oxygen tension. Our results also demonstrated that the overexpression of bcl-2 protein carrying point-mutations at or deletion of the BH1 or BH2 domains while required for the antiapoptotic activity of bcl-2, they are not indispensable for bcl-2-dependent VEGF/ HIF-1 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 *wild type* bcl-2, despite the lower deleted protein levels showed by the cells overexpressing BH1 and BH2 mutants in comparison to *wild type* bcl-2 overexpressing cells. So this finding indicates that those low levels of BH1/BH2 deleted proteins 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 to *wild type* bcl-2 protein expression. Besides, when we performed transient overexpression of bcl-2 constructs we generally reached higher level of proteins in cells overexpressing the *wild type* bcl-2 protein compared to deleted forms of protein. This observation, as also demonstrated by other authors⁶, may be due to an higher stability of full length *wild type* 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). In fact, 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 VEGF/HIF1 expression and activity 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, even though all these mutations damp bcl-2 antiapoptotic activity, only BH4 (G14-15) mutant affect 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 anti-apoptotic effect, it is not able to activate HIF-1/VEGF signaling. We further exclude 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, since transient transfection of *wild type* or mutated bcl-2 proteins did not induce “*per se*” cell death in our experimental model, and less than 10% of apoptotic cells were observed in all the cell lines tested at the end of hypoxia exposure, regardless of bcl-2 status expression.

By using the HT29 colon carcinoma cell line which is deficient for bcl-2 expression³², 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 fact, *wild type* bcl-2-overexpressing HT29 cells exposed to hypoxia secrete higher level of VEGF protein than the parental cells and, similarly to control cells, HT29 clones carrying bcl-2 deleted of the BH4 domain showed a low level of VEGF protein. 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 demonstrated that the ability of *wild type* bcl-2 to enhance angiogenesis through the BH4 domain is not related its ability to prevent 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 or HIF-1 α .

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). The cells were stably or transiently transfected with the expression vectors encoding the human *wild type* bcl-2 or different bcl-2 mutants (Figure 1A). Transfections of expression vectors and hypoxia treatment were performed as previously reported¹¹. Two *wild type* 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 (1µg/ml, Sigma-Aldrich, St. Louis, MI). TAT-BH4 Bcl-2 peptide (NH₂-GRKKRRQRRRGRTGYDNREIVMKYIHYKLSQRGYEW-COOH), TAT-BH4mut Bcl-2 peptide (NH₂-GRKKRRQRRRGGGGGYDNREIVMKYIHYKLSQRGYEW-COOH) corresponding to the mutated versions of BH4 (G6-7), and TAT-CTRL (NH₂-RKKRRQRRRGGLKNDDICLRVYTPVSILVNE-COOH) peptides in their cell-permeable form were obtained from PRIMM (Milan, Italy). For some experiments, TAT-BH4 peptide was labeled at amino-terminal with 5(6)-carboxyfluorescein. Camptothecin (CPT), Cyclohexamide (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^{9,24}. Nuclear and cytoplasmic fractions were prepared as previously reported²³. For immunoprecipitation assays and Western blot analysis, the cells were lysed in an 0.3% 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 Na₃VO₄, 0.3% CHAPS, and one tablet EDTA-free protease inhibitors [Roche] 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 µg of the HIF-1α antibody (clone H-206, Santa Cruz Biotechnology, Santa Cruz, CA) 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. Immunoprecipitation were also performed using multiple antibodies recognizing different epitopes on the bcl-2 (100, Santa Cruz Biotechnology) and HIF-1α (Santa Cruz Biotechnology; Novus Biologicals, Littleton, CO) protein. Antibodies against HIF-1α (clone 54, BD Pharmingen, San Jose, CA), HIF-1β (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), β-actin (clone AC-15, Sigma) and HSP72/73 (clone W27, Calbiochem, EMD Biosciences, La Jolla, CA) were used.

Reverse Transcriptase-Polymerase Chain reaction (RT-PCR). Total cellular RNA was isolated using Trizol reagent (Invitrogen). cDNA equivalent to 1µg of total RNA was generated by reverse transcription reaction using (oligo dT)₁₈ primer (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instruction and then 2µl of obtained cDNA were added to PCR reaction mix (Fermentas) containing exon-specific primers targeting HIF-1α (sense CCCAGATTCAGGATCAGACA, antisense CCATCATGTTCCATTTTTCGC) and β-actin (sense, AAGAGAGGCATCCTCACCT, antisense ATCTCTTGCTCGAAGTCCAG) 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 (Biorad). The intensity of the bands was quantified by densitometry using Molecular Analyst Software (Biorad) and the optical density of each HIF-1α amplified band was normalized to the intensity of each β-actin band.

Confocal analysis

Cells were treated with 0.5 μ M TAT-BH4 peptide modified at N-terminal with 5(6)-carboxyfluorescein and then exposed to normoxia or hypoxia. After 24h, 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) or anti-HSP60 (BD Transduction LaboratoriesTM, Franklin Lakes, NJ) 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). 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.

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

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TITLES AND LEGENDS TO FIGURES

Figure 1. The BH4 domain, but not BH1 or BH2, is necessary for VEGF induction by *wild type bcl-2* under hypoxia. (A) A schematic representation and names of structural constructs encoding the human *wild type* bcl-2 or different bcl-2 mutants that have been used throughout the manuscript: 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], both 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 analyses of bcl-2 protein expression on M14 cells untransfected (No DNA) or transiently transfected with empty vector (empty) or with vectors encoding *wild type* 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 *wild type* or point mutated bcl-2 protein. Western blots representative of three independent experiments with similar results are shown. β -actin is shown as loading and transferring control. (C) VEGF protein expression evaluated by ELISA on M14 cells transiently transfected with vectors encoding *wild type* or mutated bcl-2 exposed to normoxia or hypoxia. Results represent the average \pm SD of three independent

experiments. p values were calculated between control and *wild type* or mutated bcl-2 overexpressing cells (*p<0.01).

Figure 2. The BH4 domain, but not the BH1 or BH2, is necessary for VEGF induction by *wild type* 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 *wild type* 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 blot representative of three independent experiments with similar results are shown. β -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/ β galactosidase activities ratios). The results are reported as fold induction relative to normoxia. (B, C) Results represent the average \pm SD of three independent experiments. p values were calculated from control and bcl-2 *wild type* or mutated bcl-2 overexpressing cells (*p<0.01).

Figure 3. Forced expression of *wild type* 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,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 *wild type* (Bcl-2 wt) or bcl-2 deleted of the BH4 domain (BH4 del) and exposed to normoxia or hypoxia for 24 h. Results represent the average \pm SD of two independent experiments. p values were calculated from control and *wild type* or BH4-deleted bcl-2 overexpressing cells (*p<0.01).

Figure 4. The BH4 domain is necessary for HIF-1 protein expression and activity induced by *wild type* bcl-2 under hypoxia. (A) Hypoxic Responsive Element (HRE)-dependent transcriptional

activity in a control clone (puro), clones overexpressing *bcl-2 wild type* (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/ β galactosidase ratios). The results are reported as fold induction relative to normoxia and represent the average \pm SD of three independent experiments. p values were calculated between control and *wild type* or deleted *bcl-2* overexpressing cells (* $p < 0.01$). **(B-D)** Western blot analysis of HIF-1 α and HIF-1 β protein expression in the nuclear **(B)** or total **(C,D)** extracts of stably transfected M14-derived clones **(B)** or transiently transfected M14 cells **(C,D)** exposed to hypoxia for 24 h. **(C,D)** Expressing vector encoding *bcl-2 wild type* (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; 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. β -actin is shown as loading and transferring control.

Figure 5. The BH4 domain is necessary for HIF-1 α protein stabilization by *wild type* *bcl-2*. **(A)** RT-PCR evaluation of HIF-1 α mRNA level, **(B,C)** Western blot analysis of HIF-1 α protein expression after the exposure to MG132 proteosomal inhibitor (10 μ M) for 6 h **(B)** in normoxic conditions, or to hypoxia for 24 h **(C)** and treated for the indicated times with Cyclohexamide (CHX, 50mg/ml) of M14 melanoma control (puro), a clone overexpressing *wild type* *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 SD of fold induction versus control cells (puro) exposed to normoxia. Results represent the average \pm SD of three independent experiments. **(B,C)** Western blots representative of three independent experiments with similar results are shown. β -actin and HSP70 proteins are shown as loading and transfer control. **(D)** Densitometric analysis of the HIF-1 α protein level showed in

Western blot (C) was performed using Molecular Analyst Software and normalized to β -actin expression. Values are expressed as percentage of HIF-1 α protein after CHX treatment versus HIF-1 α protein expression in untreated cells (100%) (E) Western blot analysis of HIF-1 α ubiquitination on control, *bcl-2 wild type* and *bcl-2* deleted of the BH4 domain cells after exposure to hypoxia. Whole cell lysates were immunoprecipitated (IP) with anti-HIF-1 α 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 camptothecin (CPT, 0.5 μ M for 48 h). A representative experiment out of three performed with similar results is shown. Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ were considered early and late apoptotic cells, respectively. (B) Percentage of Annexin V⁺/PI⁻ (black bars) and Annexin V⁺/PI⁺ (white bars) cells was calculated by Cytofluorimetric analysis showed in Figure 6A using CellQuest Software. (C) Western blot analysis of PARP cleavage on the untransfected or transiently transfected cells treated with CPT (0.5 μ M for 48 h). Western blots representative of three independent experiments with similar results are shown. β -actin is shown as loading and transferring control. (A-C) Expressing 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; 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.

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 camptothecin (CPT, 0.5 μ M for 48 h) in presence of the different peptides (2 μ M). The percentage of the Annexin V⁺/PI⁻ (early apoptotic cells), and Annexin V⁺/PI⁺ (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-D)** The luciferase constructs containing the VEGF 1511-bp proximal promoter **(C)** or HIF-1-like (808-bp) **(D)** 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). The results are reported as the average \pm SD 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-1 α protein in total cell extracts of M14 cells after exposure to hypoxia in presence of different peptides (2 μ M). **(F)** Western blot analysis of HIF-1 α proteins on M14 cells after exposure to hypoxia in presence of peptide and then followed by Cyclohexamide (CHX, 50 μ g/ml) treatment for the indicated times. **(G)** Western blot analysis of HIF-1 α 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-1 α antibody and then Western blot analysis was performed using anti-Ubiquitin antibody. **(E,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.

Figure 8. Cellular localization of TAT-BH4 peptide in M14 cells exposed to normoxia and hypoxia. **(A)** Cytofluorimetric evaluation of TAT-BH4 peptide incorporation in M14 cells treated with 0,5 and 2 μ M of peptide. **(B)** Confocal laser scanning microscopy of immunofluorescence staining performed on M14 cells exposed to hypoxia or to normoxia for 24 h, in the presence of

TAT-BH4 peptide (green). Fixed cells were labelled with nuclear staining TO-PRO3® (blue), mitochondrial-specific anti-HSP60 antibody (red) or endoplasmic reticulum-specific anti-calreticulin antibody (red).

Figure 9. bcl-2 deleted of its BH4 domain is not a dominant-negative inhibitor of the endogenous *wild type* bcl-2 protein. (A) Western blot analysis of bcl-2 protein expression, and (B) VEGF protein expression evaluated by ELISA in HT-29 colon carcinoma cell line and its derivative clones overexpressing *wild type* bcl-2 (Bcl-2/1, Bcl2/2), 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 α and VEGF proteins in HT-29 cells after exposure to hypoxia in presence of 2 μ M TAT-BH4 or TAT-CTRL as control. (A,D) Western blot representative of three independent experiments with similar results are shown. β -actin is shown as loading and transferring control. (B,C) Results represent the average \pm SD of three independent experiments. (B) p values were calculated from control and *wild type* or mutated bcl-2 overexpressing cells expsed 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).

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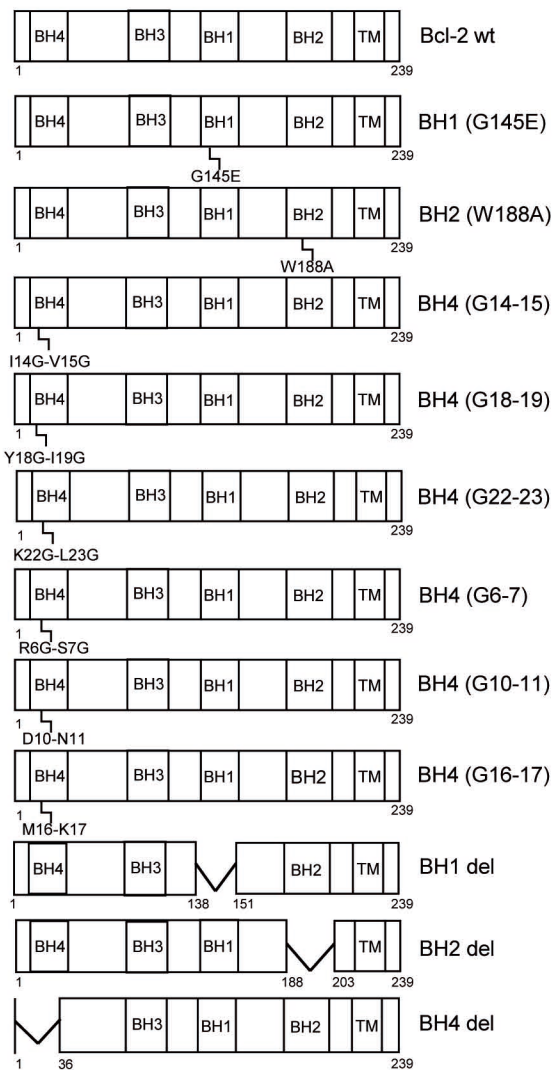
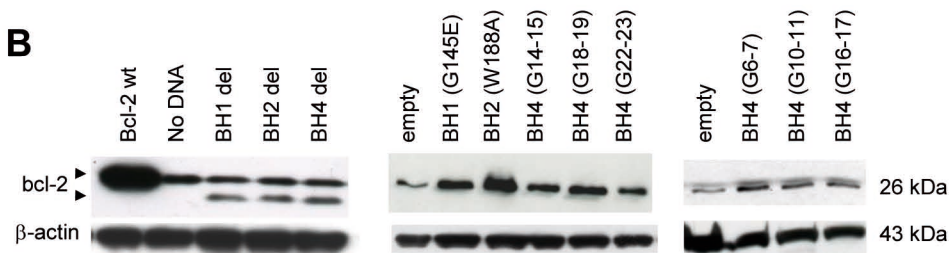
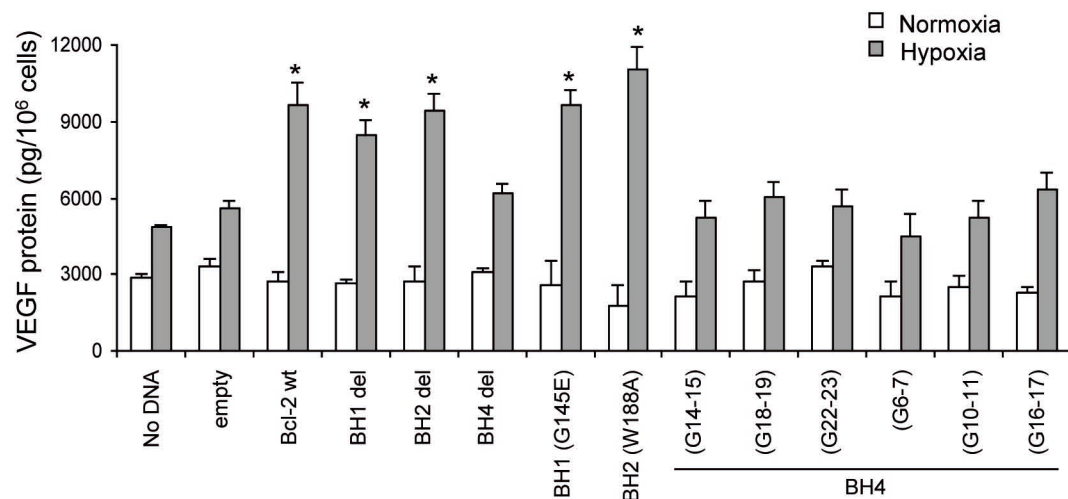
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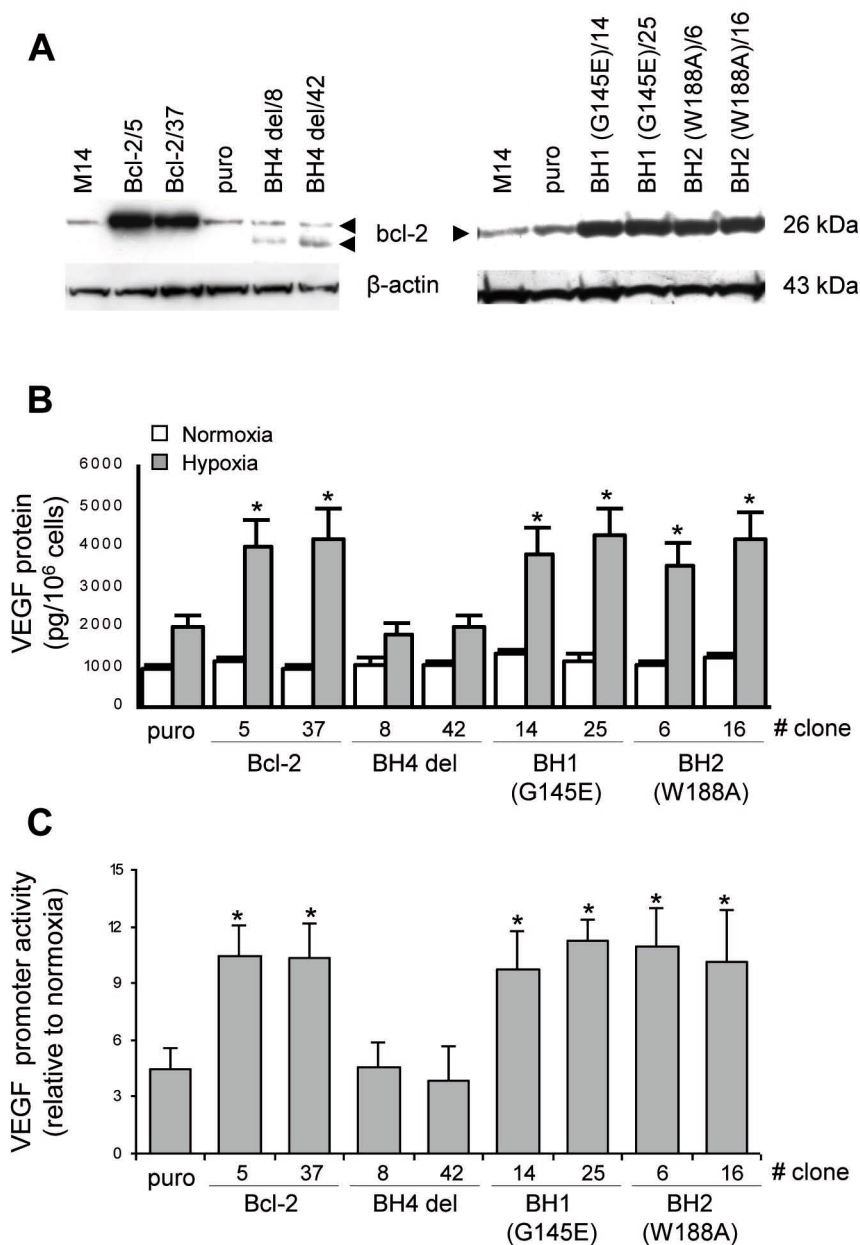
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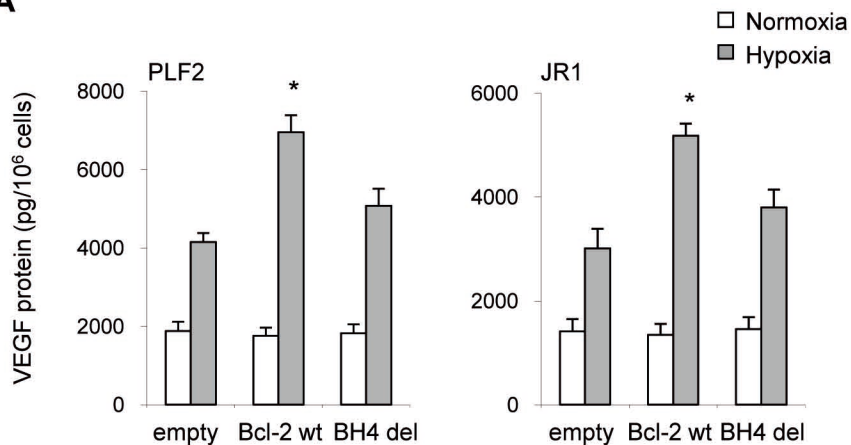
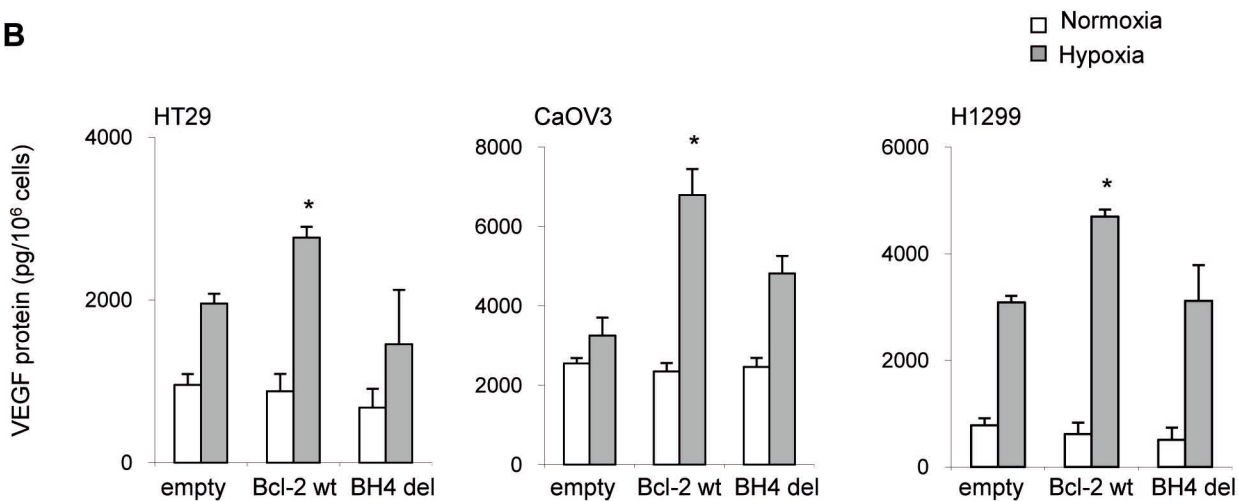
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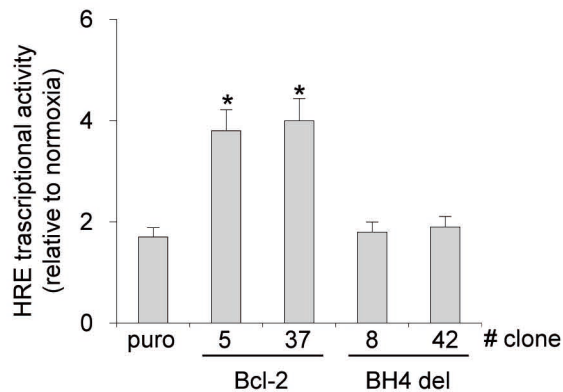
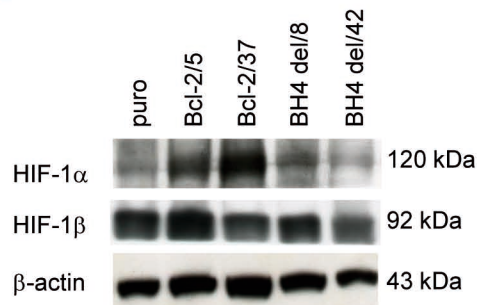
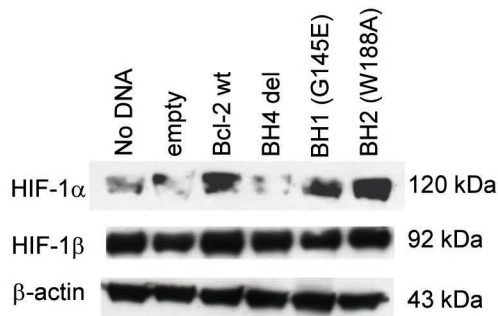
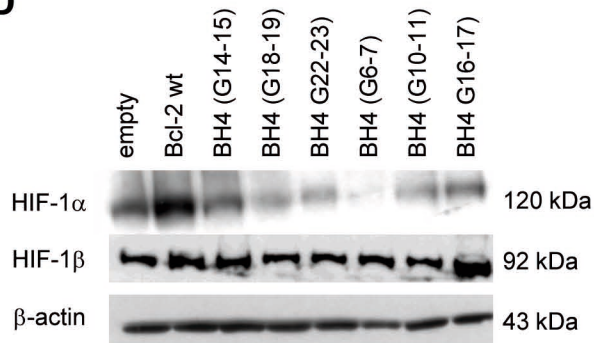
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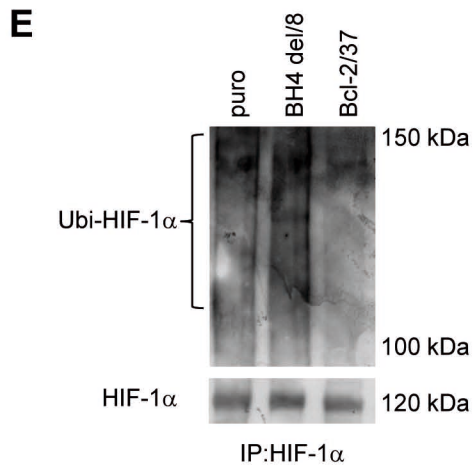
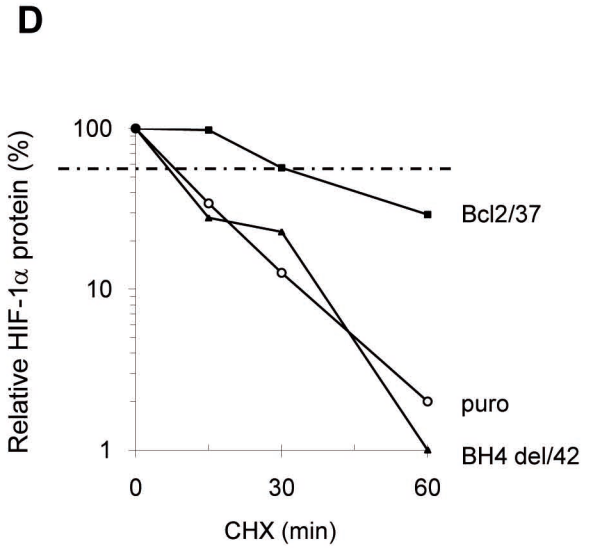
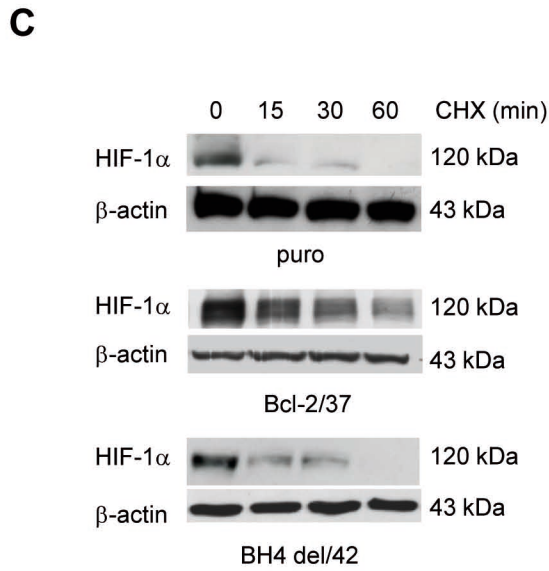
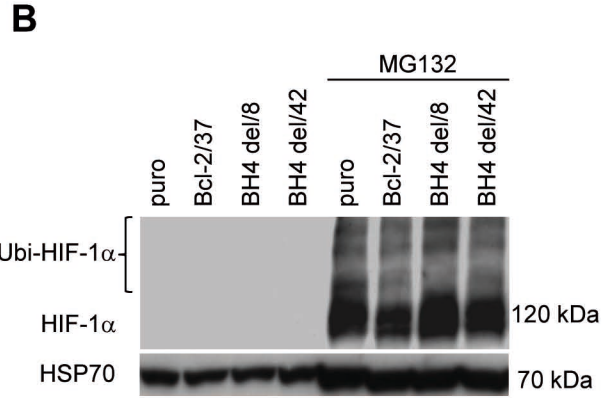
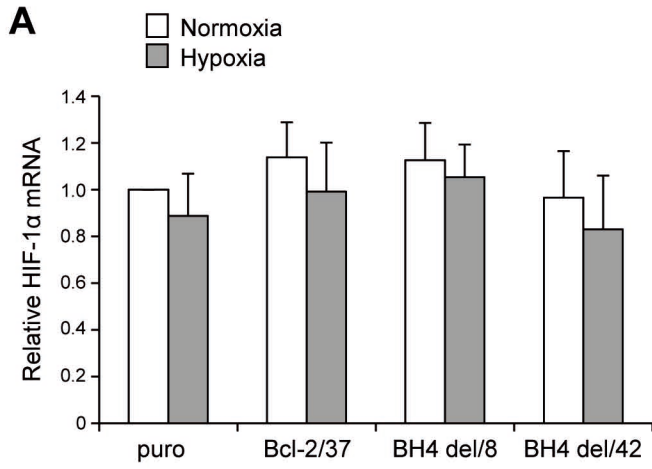
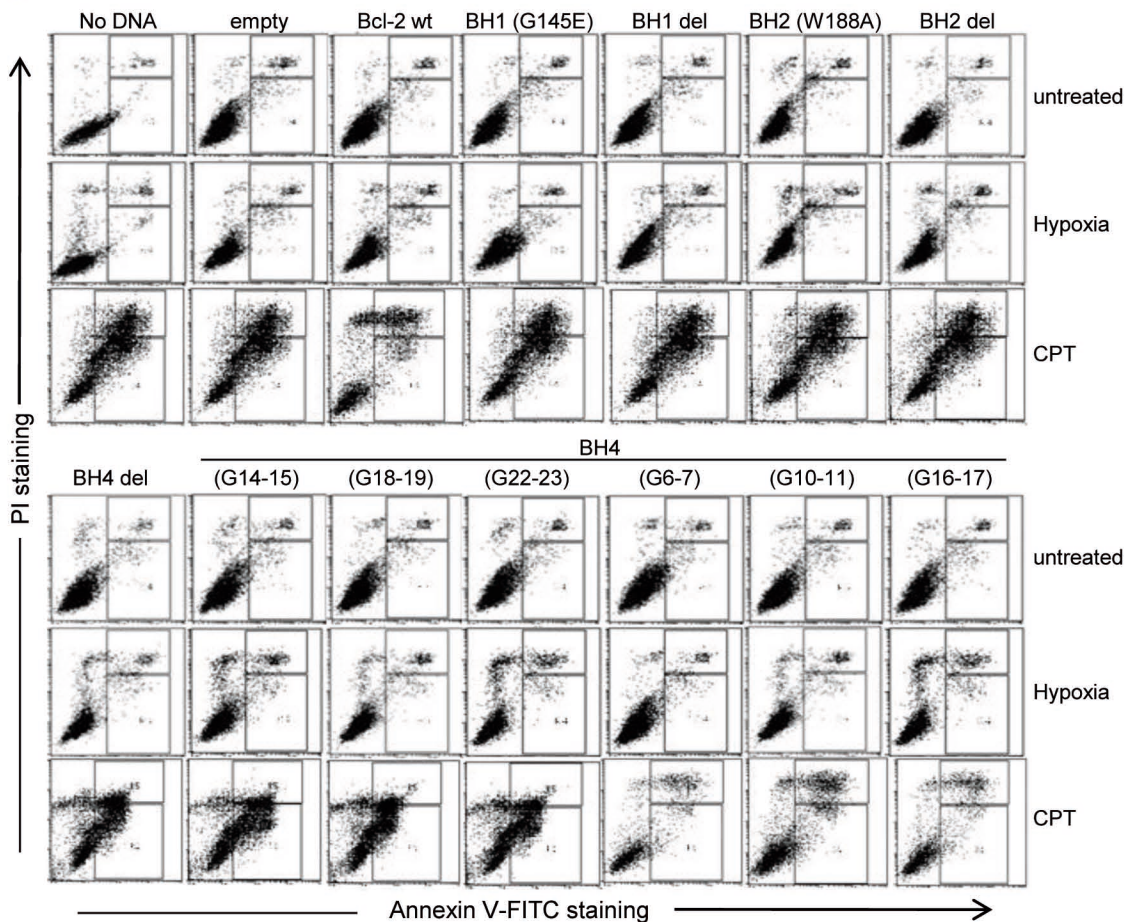
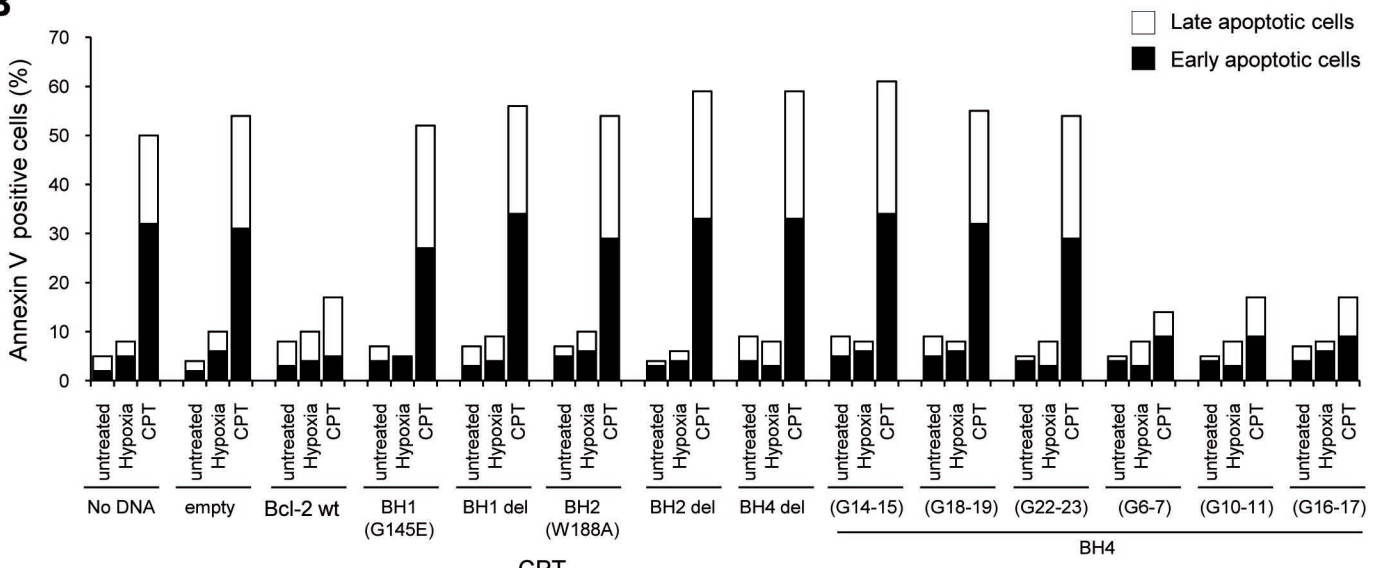
Figure 5

Figure 6

A



B



C

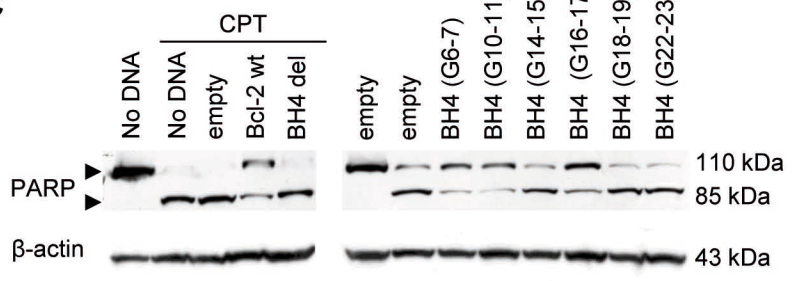


Figure 7

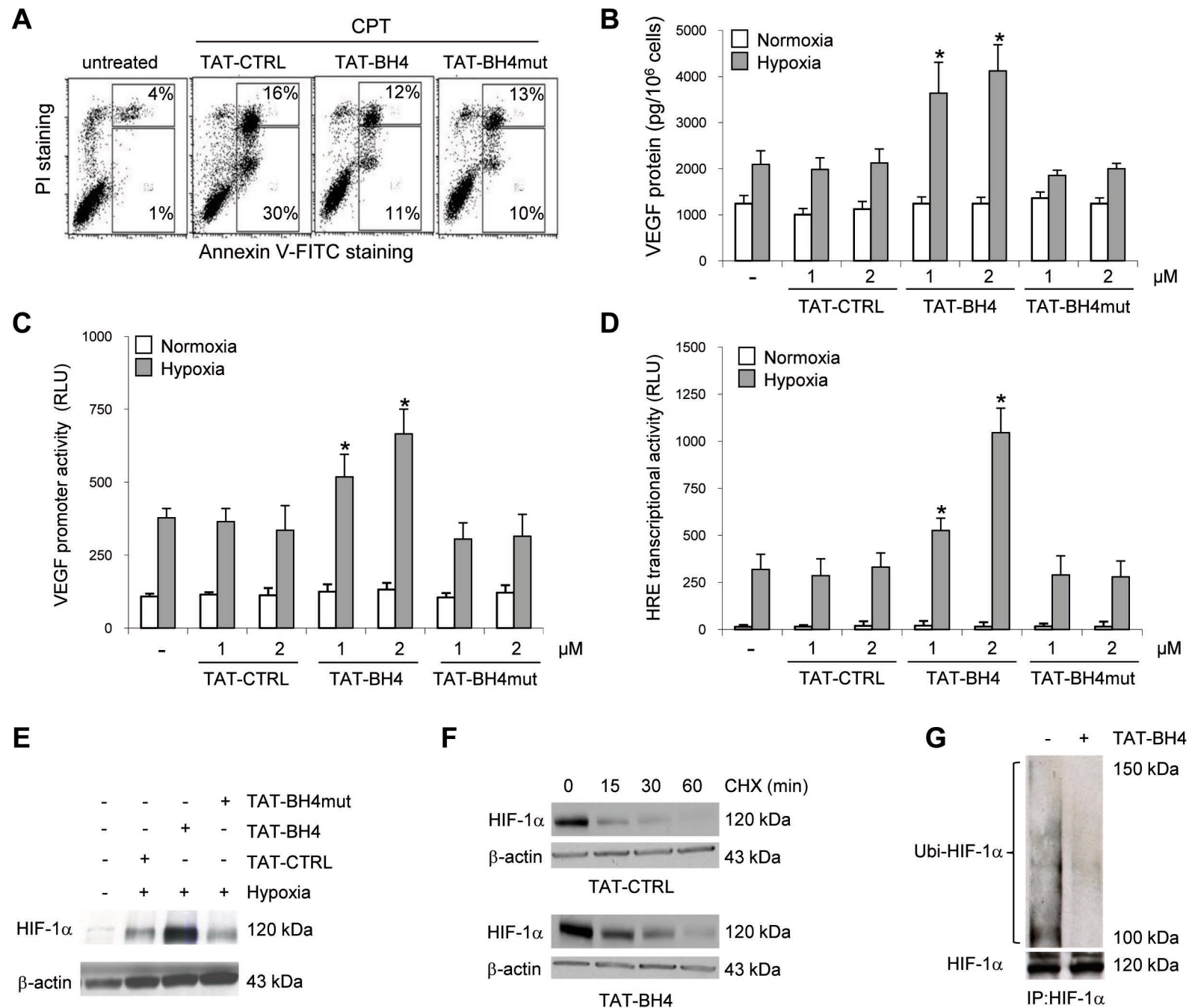


Figure 8

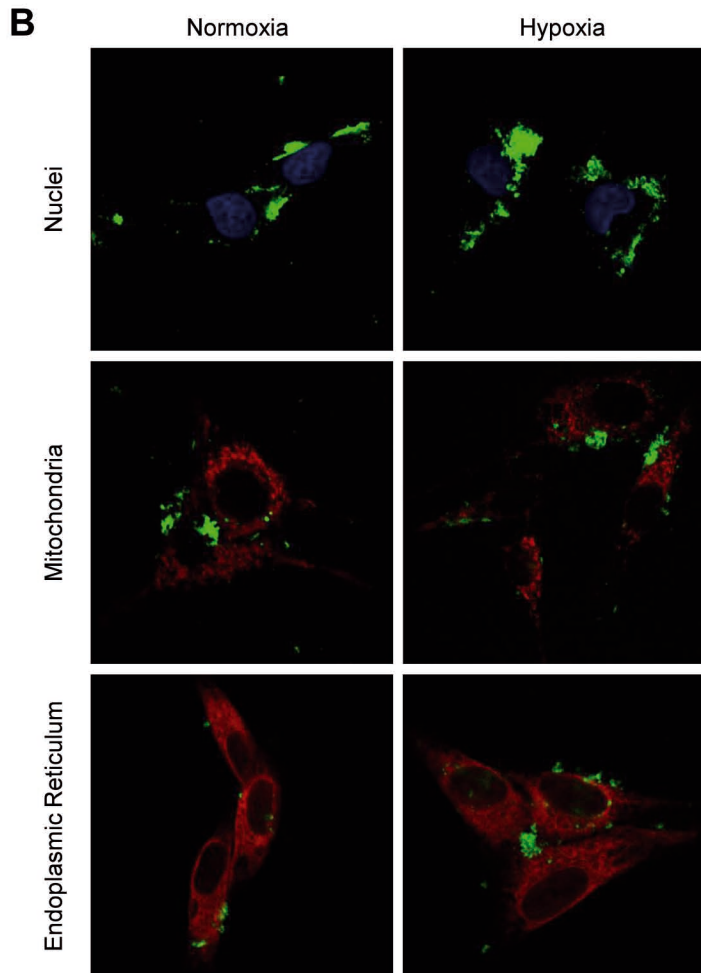
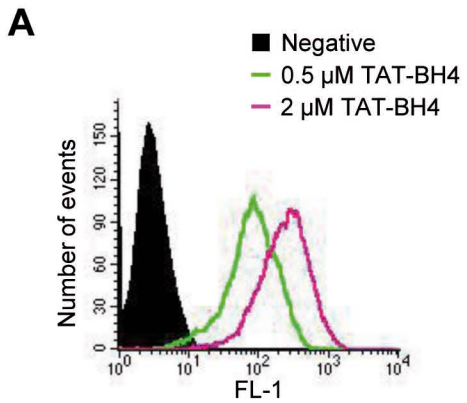


Figure 9