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Vaccinia-Related Kinase 2 Modulates the Stress Response to Hypoxia Mediated by TAK1[▽]

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Hypoxia represents a major stress that requires an immediate cellular response in which different signaling pathways participate. Hypoxia induces an increase in the activity of TAK1, an atypical mitogen-activated protein kinase kinase kinase (MAPKKK), which responds to oxidative stress by triggering cascades leading to the activation of c-Jun N-terminal kinase (JNK). JNK activation by hypoxia requires assembly with the JIP1 scaffold protein, which might also interact with other intracellular proteins that are less well known but that might modulate MAPK signaling. We report that TAK1 is able to form a stable complex with JIP1 and thus regulate the activation of JNK, which in turn determines the cellular stress response to hypoxia. This activation of TAK1-JIP1-JNK is suppressed by vaccinia-related kinase 2 (VRK2). VRK2A is able to interact with TAK1 by its C-terminal region, forming stable complexes. The kinase activity of VRK2 is not necessary for this interaction or the downregulation of AP1-dependent transcription. Furthermore, reduction of the endogenous VRK2 level with short hairpin RNA can increase the response induced by hypoxia, suggesting that the intracellular levels of VRK2 can determine the magnitude of this stress response.

Hypoxia represents one of the most severe stresses to which cells must adjust, triggering a response whose magnitude is likely to be modulated depending on the protein interacting with the main response pathway. The cellular response to hypoxia is mediated by at least two routes: the first route involves the Hif pathway, which activates the transcription of genes coding for proteins that try to improve oxygenation of the tissue (38, 41). Alternatively, there is also a stress response that might contribute to either survival or apoptosis of the hypoxic cell, depending on the magnitude and duration of oxygen deprivation. This latter response is mediated by the mitogen-associated protein kinases (MAPKs), an effect that might be inhibited in order to promote cell survival (24). Very little is known regarding the implication of MAPK routes in response to hypoxia and how this response might be regulated.

c-Jun N-terminal kinase (JNK) is primarily a stress response kinase and can be activated by proinflammatory cytokines and growth factors coupled to membrane receptors or through nonreceptor pathways by stimuli such as heat shock, UV irradiation, protein synthesis inhibitors, and conditions that elevate the levels of reactive oxygen intermediates (18). The molecular initiators and modulators of MAPK activation from endogenous signals that do not start at membrane receptors are less well known. Several studies have reported that hypoxia induces *JUN* and *FOS* gene expression in selective brain areas (7), in PC12 cells (39), and in HeLa cells (33). Hypoxia also stimulates JNK and activates the transcription of AP1-inducible genes in endothelial cells (2), cardiac myocytes (52), PC12 cells (37), and HeLa cells (33). Nevertheless, the kind of response varies depending on the magnitude of the stimulus, the type of tissue, and the cellular context. Thus, in neurons cultured from the developing rat forebrain, sequential activation of JNK and AP1 was found to be involved in the apoptosis induced by reoxygenation after hypoxia (7); but in cardiac myocytes, the JNK response improves cell survival under hypoxic conditions (12, 13). Therefore, the modularity of signaling pathways permits great flexibility and thus, depending on the molecular context, the particular biological response will be specific in each cell (23).

There are multiple MAPKs (9, 49) that can form complexes with different scaffold proteins (23), one of which is represented by the JIP family (58). JIP forms a complex with these kinases that is phosphorylated sequentially, and the third one phosphorylates the target transcription factor. Among the kinases that are representative of the three steps are MLK, which is an initial kinase; MKK7 (31, 49), which is a middle kinase; and JNK (9, 21, 53), which is the last kinase in the complex and phosphorylates the transcription factor c-Jun. JIP1 can also target MAPK to dephosphorylation, instead of activation, and thus downregulates the signaling pathway (56); but how this negative regulation is performed is not known.

A new family of kinases, known as the vaccinia-related kinase (VRK) family, has been identified in the human kinome that is composed of three members (29). Two of them, VRK1 and VRK2, are catalytically active serine-threonine kinases but diverge in their C-terminal regions (34). Very little is known regarding their functions, despite their expression in most cell types, of which VRK1 is the most characterized. VRK1 has been shown to phosphorylate p53 (3, 28, 51), forming an autoregulatory loop (50), ATF-2 (44), and c-Jun (43). All these targets are transcription factors implicated in different stress responses. Also, VRK1 phosphorylates Baf, which is implicated in the assembly of the nuclear envelope (14, 36). The

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structurally more distant VRK3 modulates MAPK signaling by interacting with and activating the VHR dual-phosphatase activity (20).

The human VRK2 gene generates by alternative splicing two isoforms, A and B, consisting of 508 and 397 amino acids, respectively, which differ in their C-terminal regions. The VRK2A isoform is expressed in all cell types, it is anchored to cellular membranes by a hydrophobic tail in its C terminus, and it is located mostly in the endoplasmic reticulum (6, 35) and mitochondria, although the relative distribution varies depending on cell type (6). The shorter VRK2B isoform lacks the membrane anchor region, is more restricted in its expression, and is located in both the cytosol and the nuclei, functionally replacing VRK1 in those cells where this latter protein is cytosolic (6). VRK2 interacts with JIP1 and prevents the incorporation of JNK into the JIP1 signalosome, resulting in reduced c-Jun-dependent transcription (S. Blanco, M. Sanz-Garcia, C. R. Santos, and P. A. Lazo, submitted for publication). If the cellular response to hypoxia is partly channeled via complexes of MAPKs with scaffold proteins, it is possible that the level of VRK2 protein might affect this response.

In this work, we have analyzed the role that the interaction between the atypical TAK1 and the JIP1 scaffold proteins plays in the cell response to hypoxia and the effect that VRK2 proteins play in the modulation of this response by interacting with elements of this signaling pathway.

MATERIALS AND METHODS

Plasmids. The mammalian expression plasmid GST-JIP1 and all its deletion variants were from Roger Davis (University of Massachusetts) (54). Plasmids expressing murine hemagglutinin (HA)-TAK1 (variant a) and Flag-TAB1 were from K. Matsumoto (22). The plasmid encoding murine HA-TAK1 (residues 1 to 480) was made by digesting plasmid HA-TAK1 with PstI. Plasmids expressing murine Flag-TAK1 (variant b) and Flag-TAK1ΔC (residues 1 to 300) were from G. Gross (16). Plasmids expressing HA-TAK1^{K63W} (kinase-negative mutant) were from Shunsuke Ishii (RIKEN, Tsukuba, Japan). The plasmid pFlag-JNK was made by PCR from plasmid pHA-JNK from S. Gutkind. The mammalian expression constructs pCEFL-HA-VRK2A and -VRK2B and pCEFL-GST-VRK2A and -VRK2B and inactive kinase VRK2(K169E) have been described previously (6). Glutathione *S*-transferase (GST) fusion proteins spanning VRK2 regions 1 to 320, 256 to 508, and 364 to 508 were made by PCR from full-length VRK2A. The plasmid p-sh-RNA-VRK1 carrying specific short hairpin RNA (shRNA) for VRK1 was reported previously (51), and plasmids p-sh-RNA-VRK2-230, p-sh-RNA-VRK2-438, and p-sh-RNA-VRK2-1335, which carry specific VRK2 shRNA with the sequences 5′-GAAGATTGGCTCTGGAGGA-3′, 5′-GGTATCCGAATGTTGGATG-3′, and 5′-ATACACTTCCACAGTCAGC-3′, respectively, were cloned into plasmid pSUPERIOR-Retro-puro following the manufacturer's instructions (Oligoengine, Seattle, WA). The pAP1-Luc reporter was from Stratagene (San Diego, CA). The plasmid pRL-tk, from Promega Biotech (Madison, WI), was used for internal control in luciferase assays.

Cell culture, transfection, and transcriptional assays. Cos1 and HeLa cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics in 5% CO_2 humidified atmosphere. For assays of transcriptional activity using a reporter plasmid, Cos1 cells were transfected with 0.8 μg of the synthetic reporter plasmid pAP1-Luc, 10 ng of pRL-tk (Promega), 50 ng of pHA-TAK1a, 50 ng of pFlag-TAB1, and the indicated amounts of the specific kinase constructs or the shRNA-expressing plasmid indicated for each experiment. The plasmid DNA was mixed with 6 μl of JetPEI transfection reagent (Polytransfection, Ilkirch, France). Twenty-four hours after transfection, the medium was changed to serum-free DMEM, cells were lysed 48 h after transfection, and luciferase activity was determined with a dual-luciferase reporter reagent from Promega. Seven hours before lysis, cells were treated with 300 μM of deferoxamine mesylate (DFO) (Sigma) or were incubated under hypoxic conditions ($pO_2 \approx 0.5\%$) in DMEM without serum at pH 7.5, which had been previously gassed with a mixture of 95% N_2 and 5% CO_2.

For assays of transcriptional activity using shRNA, HeLa cells were plated in 35-mm-diameter dishes and transfected with 0.2 μg of pAP1-Luc, 10 ng of pRL-tk, and 6 μg of the specific shRNA-expressing plasmids. Cells were treated as indicated above, and luciferase activity was determined 48 h posttransfection with a dual-luciferase reporter reagent from Promega.

Antibodies and reagents. The HA epitope was detected with a monoclonal clone, HA.11, from Covance (Berkeley, CA). The Flag epitope was detected with a rabbit polyclonal antibody from Sigma. GST fusion proteins were detected with a polyclonal antibody (sc-138) from Santa Cruz Biotechnology. Actin was determined with a monoclonal antibody (clone AC-15) from Sigma. Human JIP1 protein was detected with rabbit polyclonal (M-300) antibody from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against human VRK2 have been described previously (6). Human JNK1 was detected with a monoclonal antibody from BD Pharmingen. JNK phosphorylated in Thr183 and Tyr185 was detected with a monoclonal antibody (G7) from Santa Cruz Biotechnology. Human TAK1 was detected with a monoclonal antibody (C-9) from Santa Cruz Biotechnology. Phospho-TAK1 antibody, from Cell Signaling, detects TAK1 phosphorylated in Thr184 and Thr187. Phospho-MKK7 antibody, from Cell Signaling, detects MKK7 phosphorylated in Ser271 and Thr275. A goat horseradish peroxidase–anti-mouse antibody was from GE Healthcare. A sheep horseradish peroxidase–anti-rabbit antibody was from Sigma. DFO was from Sigma.

Immunoprecipitation, pull-down experiments, and immunoblot analysis. For pull-down experiments, Cos1 cells were grown in 100-mm-diameter dishes and transfected with different fragments of fusion proteins in vectors for mammalian expression. The amounts and types of the specific plasmids are indicated for each individual experiment. All TAK1 expression plasmids, deletion mutants, and the coactivator pFlag-TAB1 were used at 50 ng, 3 μg of plasmids expressing JIP1, 4 μg of pFlag-JNK, and 4 μg of plasmids expressing full-length or deletion mutants of VRK2. Cells were harvested 48 h posttransfection and lysed with buffer containing 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 10% (vol/vol) glycerol, and 1% Triton X-100, with inhibitors of proteases and phosphatases (1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM Na orthovanadate). The GST fusion proteins were brought down with 20 μl of glutathione-Sepharose beads (GE Healthcare) for 12 h at 4°C with gentle shaking. The washed beads were loaded in a sodium dodecyl sulfate-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore), and the Western blot was analyzed for the indicated proteins with the corresponding antibody, in individual experiments.

For the pull-down or immunoprecipitation assay of endogenous proteins, HeLa cells were grown in 100-mm-diameter dishes and transfected with the plasmids indicated for each experiment. Twenty-four hours after transfection, cells were grown in serum-free DMEM and then treated as indicated for each experiment. Whole-cell extracts were prepared 48 h after transfection, as described before. Immunoprecipitation and pull-downs were performed using 1 mg of the whole-cell protein extract. GST-VRK2A proteins were brought down with 20 μl of glutathione-Sepharose beads (GE Healthcare). HA-TAK1 was immunoprecipitated with the monoclonal anti-HA antibody from Covance, endogenous TAK1 was immunoprecipitated with a monoclonal anti-TAK1 antibody from Santa Cruz Biotechnology (clone C-9), and endogenous JIP1 was brought down with the polyclonal anti-JIP1 antibody from Santa Cruz Biotechnology (clone M-300). Antibodies were then brought down with GammaBind G Sepharose (GE Healthcare). The washed beads were analyzed as described before.

Detection of protein complexes by gel filtration chromatography. For isolation of endogenous protein complexes, HeLa cells were grown for 24 h in serum-free medium and then treated with 300 μM DFO at different times. Protein extracts were prepared as described before. Insoluble material was removed by centrifugation at 16,000 × *g* for 20 min. The supernatant, containing 1.5 mg of dissolved protein, was fractionated by high-performance liquid chromatography gel filtration through a Superose 12 10/300 GL column (GE Healthcare). High-performance liquid chromatography was performed with an HP model 1100 machine from Agilent Technologies (Germany) equipped with ChemStation software and developed with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM KCl at a flow rate of 0.1 ml/min. Fractions (0.2 ml) were collected, precipitated, resolved on a 10% polyacrylamide gel, and immunoblotted. Molecular weight markers used to calibrate the column were bovine thyroglobulin (670,000), apoferritin from horse spleen (440,000), alcohol dehydrogenase from yeast (150,000), bovine serum albumin (66,000) and bovine carbonic anhydrase (29,000), all from Sigma. The effluent was monitored by its absorbance at 280 nm.

RESULTS

The response to hypoxia is enhanced by TAK1. It has been previously described that the transcription factor AP1 is activated by hypoxia, via the JNK pathway, although the signaling connection between hypoxia and the stress response is still not well known (15, 42, 57). To characterize how hypoxia activates the stress response pathway that leads to AP1-dependent transcription, Cos1 cells were transfected with a reporter plasmid, pAP1-Luc, and a MAPK kinase kinase (MAPKKK) expression plasmid, HA-TAK1, and its coactivator Flag-TAB1, since TAK1 might need the presence of the coactivator TAB1 to be activated (17). Hypoxic conditions were induced in two ways, by the addition of deferoxamine (57), an iron-chelating agent, or by growing cells under hypoxic conditions (0.5% oxygen atmosphere). Both of the low-oxygen environments, with the presence of TAK1/TAB1, resulted in a strong increase in the activation of AP1-dependent transcription; this response was blocked when the $TAK1^{K63W}$ kinase-dead protein was used (Fig. 1A). In this assay, activation of the AP1 reporter by UV light was used as a negative control of TAK1 requirement. In this latter case, activation of the AP1-dependent transcription did not require TAK1 since it was also detected in its absence or in the presence of the TAK1 kinase-dead mutant (Fig. 1A), indicating that UV-induced activation is independent of TAK1. The Cos1 cell line has no detectable endogenous TAK1 (Fig. 1B), and in this situation, it responds to UV but not to DFO or hypoxia; therefore, the latter need the transfection of TAK1. These results indicated that TAK1 was mediating this hypoxic response and that AP1-dependent transcription was not activated by alternative routes in this system.

The stress response to hypoxia requires the scaffold JIP1 protein. The transmission of stress signals involves the assembly of complexes with scaffold proteins of the JIP family (32). However, TAK1 is not a typical MAPKK (22). Therefore, it needs to be ascertained that assembly of the signaling complex is a required component. For this aim, Cos1 cells were transfected with TAK1/TAB1 plasmids, with and without a form of JIP1 scaffold protein (JIP1ΔJBD) that lacks the JNK-binding domain and that functions as a dominant negative of the MAPK signaling complex (55). If the effect of DFO requires the assembly of a JNK-JIP1 complex, the cell response should be blocked by this JIP1 deletion mutant. Indeed, the cell response to DFO (and low-oxygen conditions, data not shown) in the presence of TAK1/TAB1 was blocked by the dominant negative JIP1ΔJBD protein (Fig. 1C, top), confirming that these are steps implicated in the response to hypoxia. The correct expression of the proteins was determined by immunoblot analysis (Fig. 1C, bottom panel). The JIP1 requirement indicates that assembly of the MAPK cascade is necessary for this response to hypoxia.

TAK1 and JIP1 interact to form a stable complex. The previous data suggested that the TAK1 kinase mediates a hypoxia response signal that probably implicates the assembly of JIP1 complexes. However, although TAK1 is able to activate downstream signaling by complexes assembled on the JIP1 scaffold protein, so far it is not known if TAK1 can indeed form a stable complex with JIP1 (19).

To address this issue, Cos1 cells were transfected with mammalian expression pGST-JIP1 constructs that cover different

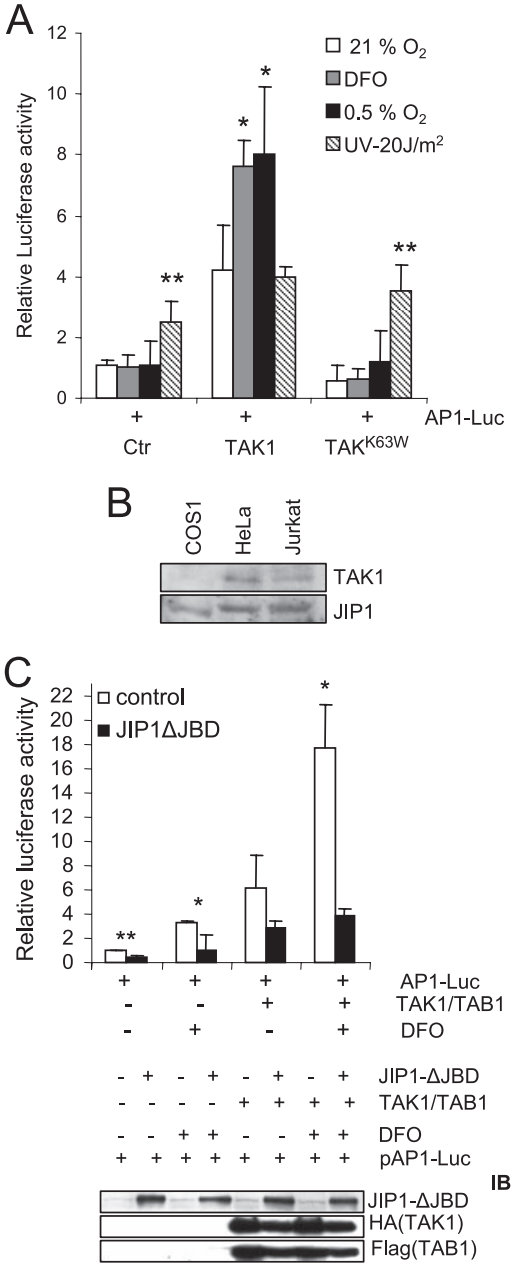

FIG. 1. Implication of TAK1 in the response to low oxygen levels. (A) Cos1 cells were transfected with 50 ng of pCMV-HA-TAK1 or 100 ng of the kinase-dead pCMV-HA-$TAK1^{K63W}$ mutant, 50 ng of the coactivator pCMV-Flag-TAB1, 0.8 μg of pAP1-Luc, and 10 ng of pRL-tk. After 24 h, the culture medium was changed to DMEM serum-free medium, and 16 h later, cells were incubated in serum-free medium at pH 7.5 in normoxia (at 21% oxygen), with 300 μM DFO, or in hypoxia (0.5% oxygen). As a control, cells were exposed to UV light (80 J/m2) in normoxia and 10% fetal bovine serum DMEM (striped bars). Cells were maintained under these conditions for 7 hours before lysis. Ctr, control with empty vector. (B) Immunoblot to show the level of endogenous TAK1 and JIP1 protein in different cell lines. (C) Implication of JIP1 in the transcriptional response to hypoxia. Cos1 cells were transfected with the plasmids indicated above and the dominant negative pGST-JIP1-ΔJBD (1 μg). Twenty-four hours after transfection, serum was removed, and 16 h later, 300 μM deferoxamine was added to the medium, and cells were lysed at 48 h. Luciferase activity was normalized against that of *Renilla* luciferase activity. The results are the means of three experiments with their standard deviations analyzed by Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. Western blotting analysis is also shown (control).

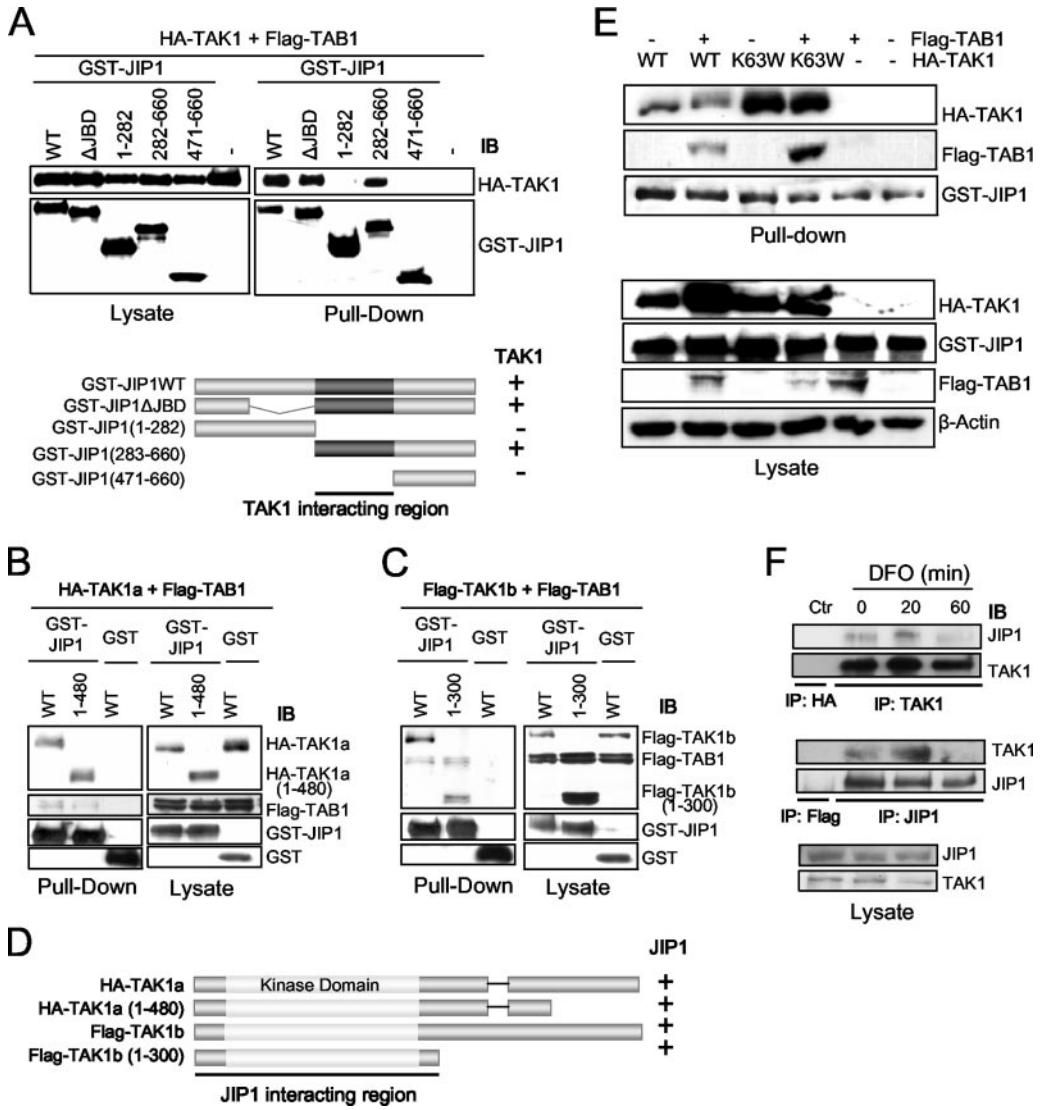

FIG. 2. Interaction between TAK1 and JIP1. (A) Mapping the region of JIP1 that interacts with TAK1. Cos1 cells (10^6) were cotransfected with constructs of JIP1 fused to GST expressing different regions of the JIP1 protein and with plasmids pHA-TAK1 (50 ng) and pFlag-TAB1 (50 ng). The correct expression levels of the proteins were checked in the lysates (left blot). The lysates were used for a pull-down assay with glutathione-Sepharose beads, and the associated proteins were detected in Western blots (right blot). The diagram shows the region of JIP1 needed for interaction with TAK1. (B and C) Mapping the region of TAK1 that interacts with JIP1. Cos1 cells were cotransfected with constructs of GST-JIP1 or GST as control and with 50 ng of plasmids expressing different regions of HA-TAK1 (variant a) and Flag-TAK1 (variant b) and pFlag-TAB1 (50 ng). The expression of each protein was checked in the lysates, which were used for a pull-down assay as described before. The diagram shows the region of TAK1 needed for the interaction with JIP1. (D) Diagram illustrating the region of TAK1 implicated in its interaction with JIP1. (E) Requirements of TAK1 needed for the TAK1-JIP1 interaction. Cos1 cells were cotransfected with wild-type (WT) or the kinase-dead mutant $TAK1^{K63W}$ (K63W) forms of HA-TAK1 with and without the coactivator Flag-TAB1 and the full-length pGST-JIP1 constructs. The correct expression of the proteins was determined in the lysates (bottom blot) that were used for the pull-down assay of associated proteins (top blot). (F) Reciprocal immunoprecipitation to show TAK1-JIP1 interaction. The upper panel shows the immunoprecipitation with anti-TAK1. The middle panel shows the immunoprecipitation with anti-JIP1. The level of endogenous proteins is shown in the lower panel. HeLa cells were grown in serum-free medium for 24 h before lysis, and some plates were treated with 300 μM of DFO for 20 or 60 min. TAK1 was immunoprecipitated with a monoclonal antibody, and as a control, the monoclonal antibody anti-HA was used (top panel). JIP1 was immunoprecipitated with the polyclonal antibody anti-JIP1, and the anti-Flag polyclonal antibody was used as a control (middle panel). The coimmunoprecipitated proteins were identified with a specific polyclonal or monoclonal antibody for TAK1 and JIP1, respectively.

regions of the JIP1 molecule, residues 1 to 282, 282 to 660, and 471 to 660, a deletion of the JNK binding domain (ΔJBD), and the full-length protein (55). These constructs were cotransfected with HA-TAK1 plus Flag-TAB1. Cell lysates were used for a pull-down, and the associated proteins were identified by immunoblotting (Fig. 2A), although additional cellular proteins may also be present in these complexes. JIP1 stably interacts with TAK1 by a region located between residues 283 and 471. This region of JIP1 is different from the one that interacts with other MAPKs such as MLK and JNK (54). Then, a similar assay was performed in order to determine the TAK1 region required for this interaction with JIP1. Cos1 cells were

transfected with the plasmid expressing GST-JIP1 and constructs that carried the full length and the N-terminal region (residues 1 to 480) of murine HA-TAK1 (variant a) (Fig. 2B) or the full length and the N-terminal region (residues 1 to 300) of murine Flag-TAK1 (variant b) (16). These constructs were cotransfected with Flag-TAB1 (Fig. 2C). Both TAK1 variants interact by their N-terminal domains, the first 300 amino acids, with JIP1 (Fig. 2D).

Next, it was determined if this JIP1-TAK1 interaction could be affected by the presence of the TAB1 coactivator or the kinase activity of TAK1, for which a kinase-inactive ($TAK1^{K63W}$) form was used. The cells were cotransfected with different combinations of plasmids, and their correct levels of expression were confirmed in cell lysates (Fig. 2E, bottom panel) before they were used for a pull-down assay. The proteins associated with GST-JIP1 in the pull-down assay were identified in an immunoblot (Fig. 2E, top panel). TAK1 was able to bind JIP1 independently of the presence of TAB1 (Fig. 2E, lanes 1 and 2) and did not require its kinase activity (Fig. 2E, lanes 3 and 4), and TAB1 did not directly interact with JIP1, since its interaction appears to depend on the presence of TAK1 (Fig. 2E, lanes 2, 4, and 5). The complex with the inactive TAK1 appeared to form a more stable complex, probably because it is locked in a conformation in which the signal cannot be transmitted.

Finally, we determined whether endogenous JIP1 associates with endogenous TAK1 (levels shown in Fig. 2F, bottom panel) and whether hypoxic conditions can affect this association. For this aim, HeLa cells were exposed to DFO for different times (20 to 60 min), lysed, and immunoprecipitated with either anti-JIP1 or anti-TAK1 antibody, followed by immunoblotting with the antibody for the corresponding partner. The endogenous TAK1 interacted with endogenous JIP1 even in the absence of any stimuli (Fig. 2F, second lane). The TAK1-JIP1 interaction increases within 20 min following DFO exposure (Fig. 2F, third lane). Nevertheless, this association was transient, and TAK1 disassociated from JIP1 after 1 h of DFO treatment (Fig. 2F, fourth lane). These results confirmed that endogenous TAK1 interacts with endogenous JIP1 and that this interaction is transiently enhanced during hypoxia.

TAK1 effect on JNK activation and binding to JIP1. The following step was performed to determine how the TAK1-JIP1 interaction might affect the binding of JNK to JIP1, the last step in the cascade. To address this issue, we performed a dose-response assay of JNK binding to JIP1 in the presence of increasing amounts of TAK1. The amount of phosphorylated JNK increased in the presence of both TAK1 and TAB1 in a dose-dependent manner in the whole lysate, suggesting that TAK1 contributes to JNK activation (Fig. 3, lysate). Regarding the pulled down proteins, as the amount of TAK1/TAB1 increased, so did the JIP1-bound JNK and its phosphorylated form (Fig. 3, left box). If TAB1 was not included in the assay, the binding of JNK to JIP1 was not affected (Fig. 3, central box) and there was a reduced level of phosphorylated JNK. Thus, the presence of TAB1 is essential for the JNK-JIP1 interaction induced by TAK1. When the kinase-dead protein $TAK1^{K63W}$ was used, the binding of JNK to JIP1 and the phosphorylation of JNK were lower than that with wild-type TAK1 (Fig. 3, right box). These data suggest that an active TAK1 and the presence of its coactivator TAB1 are required

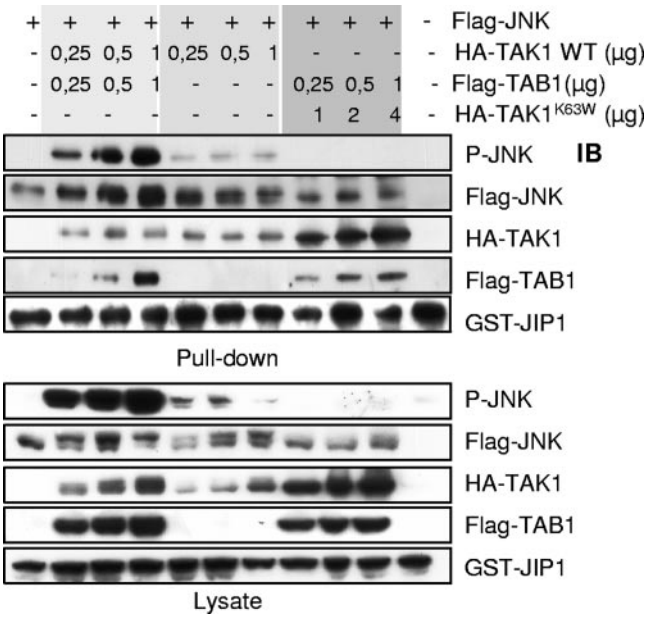

FIG. 3. Effect of TAK1 and TAB1 on JNK binding to JIP1. Cos1 cells were transfected with the indicated amount of plasmids (HA-TAK1, Flag-TAB1), p-Flag-JNK (4 μg), and pGST-JIP1 (2 μg). The correct expression levels of the proteins were determined in the lysates (bottom blot) that were used for a pull-down assay of associated proteins (top blot) and analyzed by immunoblot with specific antibodies.

for maximal activation, detected as a phosphorylated JNK bound to JIP1, and thus contribute to the activation of the TAK1-JIP1-JNK signaling module.

Hypoxia activates TAK1 and promotes the assembly of an oligomeric complex. We postulate that TAK1 can form a stable complex with JIP1 and thus contribute to the activation of this signaling module (54). This signalosome is formed by TAK1 as the upstream MAPKKK, MKK7 as the MAPKK, and JNK as the last kinase in the cascade (54). To establish that hypoxia was able to induce the activation of the MAPK pathway initiated in TAK1, a time course assay of the activation was performed. For this, HeLa cells were exposed to DFO for different times, and then whole-cell extracts were analyzed for the phosphorylation level of each endogenous kinase (Fig. 4A). We detected the autophosphorylation of TAK1 in Thr184 and Thr187 within its activation loop (46); next, we also detected the phosphorylation of MKK7 in Ser271 and Thr275 as the middle kinase, and finally, we detected the phosphorylation of JNK in residues Thr183 and Tyr185 (Fig. 4A). The three kinases were clearly phosphorylated within the first 30 min of exposure to DFO, and this effect was transient and lasted for 1 h, decreasing afterward to basal levels (Fig. 4A). This rapid and transient phosphorylation was closely correlated with the inducible interaction between TAK1 and JIP1 (Fig. 2F), suggesting that hypoxia also promoted the assembly of large complexes formed by TAK1, MKK7, JNK, and JIP1.

Based on these data, we postulated that hypoxia might promote the interaction of the three kinases with JIP1, forming a signaling complex, leading to the phosphorylation of each consecutive kinase, and thereby causing the activation of the signalosome. Consequently, we tested whether hypoxia promoted

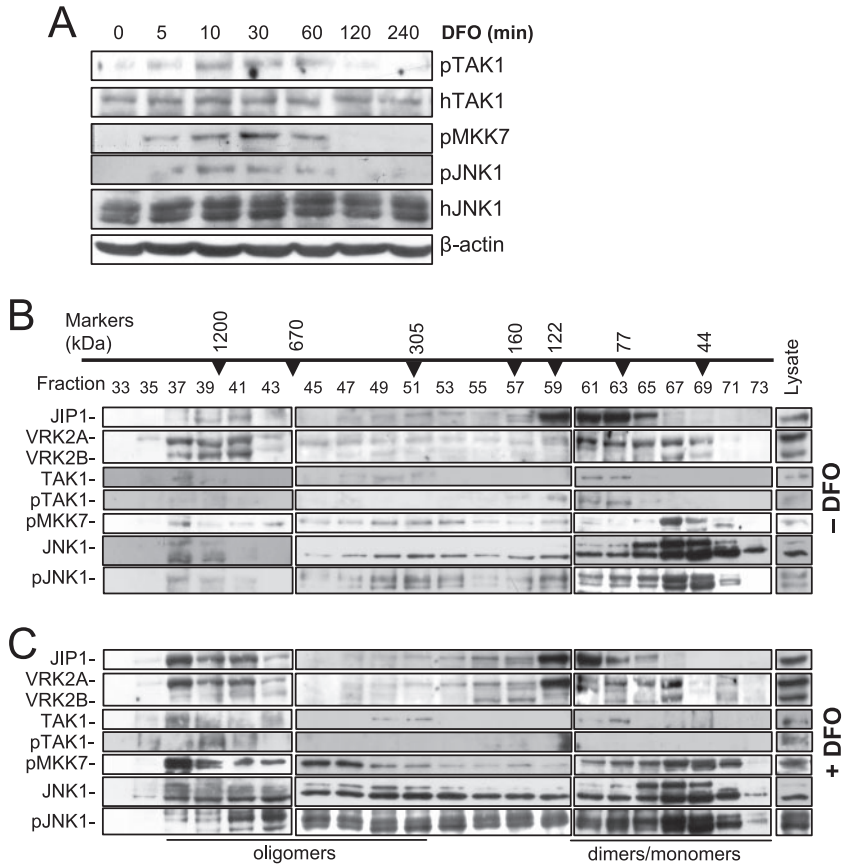

FIG. 4. Oligomerization of the signalosome in the presence of hypoxia. (A) Kinetics of TAK1, MKK7, and JNK activation by DFO treatment. HeLa cells were grown in serum-free DMEM and treated with 300 μM of DFO, and then protein extracts were collected at different time points. The activation levels of each endogenous kinase were detected with the specific antibodies described in Materials and Methods. (B and C) Composition of endogenous signalosome formed by JIP1, TAK1, MKK7, and JNK without (B) or with (C) DFO stimulation. HeLa cells were treated as described before, and the whole-cell extracts were prepared 30 min after DFO stimulation. Then, cell extracts were fractionated in a Superose 12 10/300 GL column that fractionates proteins ranging from 50 to 1,500 kDa. The fractions were analyzed by Western blotting with antibodies for the specific endogenous proteins.

an increase in the association of these kinases to the signalosome. For this aim, HeLa cells were treated for 30 min with DFO, and the extracts of these cells and of control cells without DFO treatment were used for fractionation by gel filtration in a Superose 12 10/300 GL column. In the control cells without any stimulus, most of the JNK protein remained in the small molecular size fractions (Fig. 4B). However in cells treated with DFO, there was a displacement toward the high molecular size fractions of JNK (including pJNK) and other proteins of the signalosome, including JIP1, TAK1, and MKK7 (Fig. 4C) and their phosphorylated forms. These results are consistent with the interpretation that hypoxia can induce the assembly of the signalosome and therefore the activation of the signaling route.

VRK2 interferes with TAK1 signaling and with the response to hypoxia. The regulation of these signaling complexes depends on interactions of the module with other proteins (23). In order to ascertain if VRK2 could also modulate the stress signal induced by hypoxia, the AP1-dependent activation of transcription was analyzed. For this aim, Cos1 cells were transfected with a reporter, pAP1-Luc, and plasmids expressing TAK1 and TAB1 and were treated with DFO to induce hypoxia (Fig. 5A). This stimulatory effect was almost completely blocked by overexpression of either the common VRK2A or the rare VRK2B (6), as well as their kinase-dead mutants (K169E substitution). These results indicated that the effect of both VRK2 isoforms is dependent on their interaction with the signaling system and does not require the kinase activity, thus ruling out additional effects by phosphorylation of either JIP1 or other cellular proteins mediated by VRK2 proteins. The downregulatory effect on transcription induced by the maximum amount of VRK2A or VRK2B was lost if the cells were cotransfected with shRNA specific for both isoforms of VRK2 (Fig. 5A).

Next it was determined if VRK2 proteins could also modulate the hypoxic response induced by an atmosphere of 0.5% O_2. To characterize this potential effect, Cos1 cells were transfected with TAK1/TAB1 expression plasmids in the presence of active or kinase-dead (K169E substitution) VRK2 isoforms (6), and cells were incubated under hypoxic conditions. The expected activation of transcription induced by hypoxia was blocked by both isoforms of VRK2, and this effect was also independent of their kinase activity (Fig. 5B). This response to the low oxygen level was also blocked by the use of specific

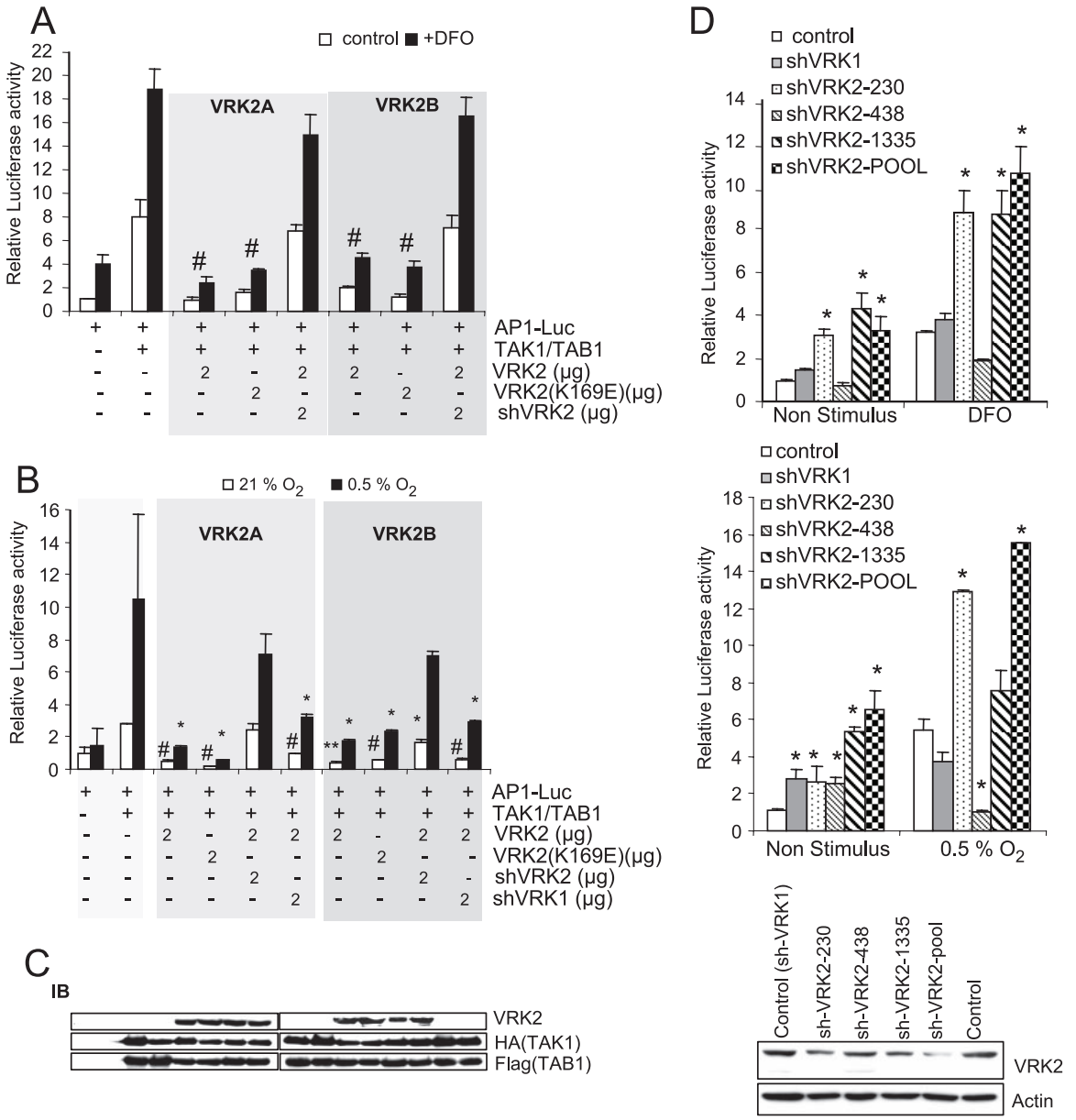

FIG. 5. Effect of VRK2 on the hypoxia response. (A) Activation of AP1-dependent transcription by hypoxia mediated by TAK1. Cos1 cells were transfected with 0.8 μg of the reporter AP1-Luc plasmid, 10 ng of pRL-tk, 2 μg of plasmid expressing the VRK2 isoforms, the active (pCEFL-VRK2A or pCEFL-VRK2B) or kinase-dead variants (K169E substitution), and pHA-TAK1 (50 ng) and pFlag-TAB1 (50 ng) where indicated. RNA silencing of VRK2 was induced with the plasmid pSUPERIOR-shVRK2-230. DFO was added to the culture at 300 μM. The results are the means of six experiments. #, $P < 0.0005$. The correct expression of the proteins was also determined, by immunoblotting. (B) Effect of the lack of oxygen. Cos1 cells were transfected as described before and 7 h before lysis were incubated in normoxia (21% oxygen) or hypoxia (0.5% oxygen) in serum-free DMEM. Luciferase activity was determined as described before. The results are the means of three experiments. *, $P < 0.05$; **, $P < 0.005$; #, $P < 0.0005$. (C) Immunoblot illustrating the protein levels in transfection experiments of Cos1 cells used in panels A and B. (D) Reduction of endogenous VRK2 increases the DFO (top) or hypoxia (middle) response. Human HeLa cells were transfected with 0.2 μg of reporter pAP1-luc, 10 ng of pRL-tk, and 6 μg of the plasmids expressing shRNA constructs specific for VRK1 (control) or VRK2. Forty hours after transfection, the cells were maintained in DMEM with 0% fetal bovine serum to reduce background activity and were stimulated with 300 μM DFO (right side) or without DFO (left side). Extracts were collected 6 h after stimulation and processed for dual luciferase determination. The results from six experiments are shown. **, $P < 0.05$. At the bottom is shown an immunoblot used to determine the level of VRK2 detected with a specific polyclonal antibody against human VRK2. The initial level of VRK2 protein in nontransfected cells is also shown (control).

shRNA for human VRK2 (Fig. 5B), and the shRNA specific for the closely related VRK1 (plasmid p-shRNA-VRK1), used as a negative control, had no effect, although it has been shown to be highly effective in downregulating the level of endogenous VRK1 protein (51). The expression of the plasmids used in the transfections of cells used for exposure to DFO or hypoxia is shown in a Western blot of one of the transfections (Fig. 5C).

The dependence of this hypoxic response on endogenous VRK2 protein levels was also studied using the same type of assay but with the human HeLa cell line. Plasmids expressing shRNA for human VRK1 or VRK2 were transfected, and their effects on the AP1-dependent transcriptional response to DFO (Fig. 5D, top) or hypoxia (Fig. 5D, middle) were analyzed. Two of the shRNA for VRK2 (sh-VRK2-230 and sh-VRK2-1335) resulted in an increase in the response to hypoxia, while the shVRK1 and one of the shVRK2 had no effect, which is consistent with their effects on protein levels as determined in an immunoblot, in which the two shRNA, one for VRK1 and the other for VRK2, that were ineffective did not alter the VRK2 protein level (Fig. 5D, bottom panel). These results indicate that the effect of VRK2 was more general, since it could also be detected in a different cell line by modulating its endogenous levels, and it depends on the protein levels instead of the kinase activity.

VRK2A and TAK1 interact to form a stable complex. It is possible that VRK2A or VRK2B, in addition to its interaction with JIP1 (Blanco et al., submitted), might also directly interact with TAK1 as part of the multiprotein complex, and that would reveal a novel interaction that can contribute to determining how the signalosome is assembled or modulated. The assembly of the signaling complex suggests that interactions might occur among more than two proteins in the complex. To further characterize the assembly of the complex formed by VRK2A or VRK2B, JIP1, and TAK1, we determined the potential interaction between VRK2A and TAK1. For this aim, different constructs of VRK2 fused to GST were used to perform a pull-down assay of cells transfected with HA-TAK1a (Fig. 6A, top panel). We clearly detected the interaction of the full-length VRK2A (508 amino acids), as well as of a carboxy-terminal construction of VRK2A (residues 364 to 508). Nonetheless, the N-terminal region (residues 1 to 320) or isoform VRKB (amino acids 1 to 396) did not interact with TAK1, suggesting that the minimum region necessary for this interaction is located between residues 397 and 508 of VRK2A, probably proximal to residue 396, since GST fusion to residue 364 weakens the interaction, probably by distorting this region (Fig. 6A, bottom panel). It is known that the VRK2 C-terminal region has a low complexity and might have alternative folding conformations, of which only some might be able to interact with TAK1. This would explain the weaker interaction observed for this construct.

Next, it was determined which region within TAK1 was necessary for interaction with VRK2A by using a similar approach. Cos1 cells were transfected with GST-VRK2A and TAK1 constructs carrying either the wild-type or the N-terminal domain (residues 1 to 480) and the full-length murine HA-TAK1 (variant a) (Fig. 6B, left) and the N-terminal region (residues 1 to 300) and the full-length murine Flag-TAK1 (variant b) (Fig. 6B, right), all of them cotransfected with Flag-TAB1. VRK2A interacted with the full-length and the N-terminal domain of TAK1 variant a (Fig. 6B, left) but did not interact with variant b (Fig. 6B, right) of TAK1, which contains an insertion of 27 residues in its C-terminal domain (Fig. 6B, bottom), probably because the interacting peptide is disrupted by this insertion. These results suggest that the VRK2A-TAK1 interaction is very specific, since it only occurs between the VRK2A isoform and TAK1; however, both the VRK2A and the TAK1a isoforms are ubiquitously expressed (6, 16).

Finally, it was determined if the interaction occurs with endogenous proteins and whether DFO stimulation also affects the TAK1-VRK2 interaction, as is the case with the JIP1-TAK1 association. An immunoprecipitation of endogenous proteins could not be performed because antibodies compete for the interacting region of VRK2. Therefore, an alternative experimental approach was used to detect interactions of endogenous proteins. First we examined whether endogenous VRK2A can associate with transfected HA-TAK1a. For this aim, HeLa cells were transfected with empty vector of HA-TAK1a and stimulated or not with DFO at different times, after which cells were lysed and used for immunoprecipitations with an anti-HA antibody, followed by immunoblotting with an anti-VRK2 antibody (Fig. 6C). The endogenous VRK2A stably interacts with the transfected HA-TAK1a protein under normoxic conditions (Fig. 6C, second lane), and this association was not affected by the DFO exposure of VRK2 protein (Fig. 6C, third and fourth lanes). Next, to detect the interaction in a reciprocal way, a pull-down approach was followed. HeLa cells were transfected with plasmids carrying either GST or GST-VRK2A and then treated as described before. The endogenous TAK1 protein was brought down by GST-VRK2A (Fig. 6D, second lane) under normoxic conditions, and this interaction was not affected by DFO (Fig. 6D, third and fourth lanes).

From these data, it can be concluded that both VRK2A and TAK1 can interact with each other and with JIP1 (Blanco et al., submitted) by different regions to form a stable interaction; thus, VRK2A, by interacting with TAK1 and JIP1, or VRK2B, by interacting only with JIP1 (Blanco et al., submitted), might modulate the signaling complex formed by TAK1, JNK, and JIP1, which responds to stress signals such as DFO or hypoxia.

DISCUSSION

The level of activation of signaling routes by specific stimuli determines the biological response. Thus, low-level activation is related to cell survival, while a strong activation triggers an apoptotic response (26). Moreover, the activation of specific routes depends on several factors, such as the duration of the stimulus, the molecular context, or the cellular environment. For instance, the association of MAPK with JIP scaffold proteins, in addition to its modularity, permits the assembly of signaling complexes that can channel signal transmission to induce specific effects. There are a large number of scaffold proteins implicated, such as the JIP proteins, and several kinases at each of the three docking sites (10, 30). This permits a large number of combinations that no doubt determines the biological specificity of the effect. The complexes formed and the different complexes present in a cell at a given time are going to condition the biological response. These complexes might also be modulated by additional protein-protein interactions or covalent modification of their components, thus explaining the often different responses found among different cell types despite receiving the same stimulation. JIP1 has been implicated in the cell response to oxidative stress (47, 55). Also, in different cell types, particularly those of neural origin, JIP1 has been associated with the regulation of apoptosis (11, 40,

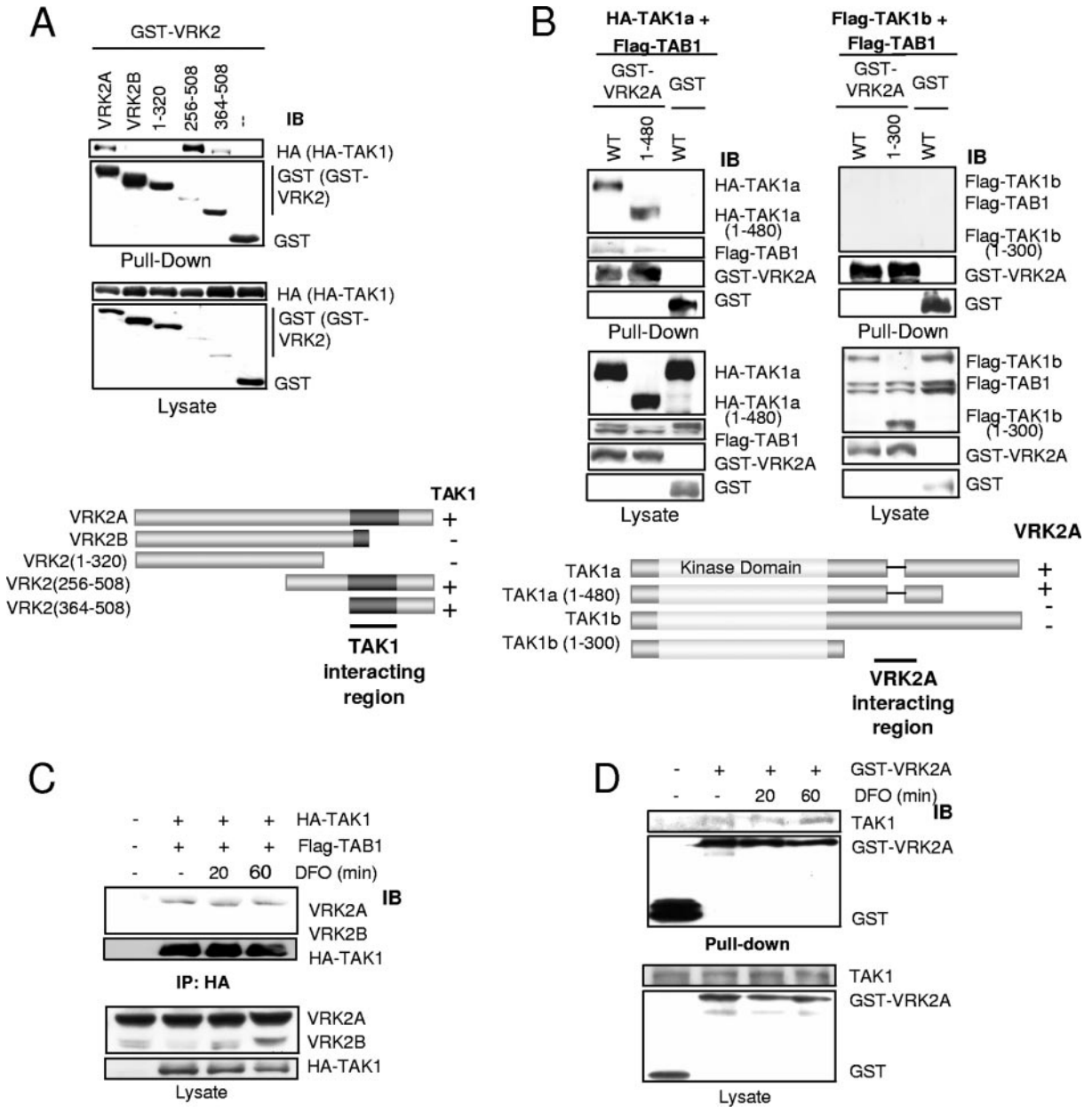

FIG. 6. Interaction between VRK2 and TAK1. (A) Mapping the region of VRK2 that interacts with TAK1. Cos1 cells were transfected with plasmids pHA-TAK1 and pFlag-TAB1 and with constructs of VRK2 fused to GST expressing different regions of the VRK2 proteins. The expression of the proteins was determined by Western blotting (left panel). The different lysates were pulled down with glutathione-Sepharose to bring down the GST-VRK2 proteins. The pull-down proteins were detected with antibodies that recognize the HA epitope in TAK1 and VRK2 (right panel). A schematic representation of the VRK2A interacting region with TAK1 is shown (bottom panel). (B) Mapping the TAK1 region that interacts with VRK2A. Cos1 cells were cotransfected with constructs of GST-VRK2A or GST as control and with 50 ng of plasmids expressing different regions of HA-TAK1 (variant a) (left) and Flag-TAK1 (variant b) (right) and pFlag-TAB1 (50 ng). Then, whole-cell extracts were used for a pull-down assay of GST proteins. The diagram shows the region of TAK1 required for interaction with VRK2A (bottom). (C) Interaction between endogenous VRK2A and HA-TAK1. HeLa cells were transfected with plasmids expressing HA-TAK1 and Flag-TAB1 or with empty vector as control. Twenty-four hours after transfection, serum was removed, and 24 h later, cells were lysed. Some plates were treated with 300 μM of DFO for different times. The expression of the proteins was determined by Western blotting (bottom panel). The different lysates were immunoprecipitated with anti-HA antibody, and the precipitated proteins were detected with polyclonal anti-VRK2 or anti-HA antibodies. (D) Interaction between endogenous TAK1 and transfected GST-VRK2A. HeLa cells were transfected with plasmid pCEFL-GST-VRK2A or pCEFL-GST as control and then were treated as described before. The TAK1 protein in the pull-down assay was detected with a specific monoclonal antibody.

60). However, the MAPKKKs, which interact and are involved in these signaling routes, are still undefined. Furthermore, the molecular mechanisms of the rapid and transient activation of the atypical MAPKKK TAK1 by stress signals are still unclear. TAK1, it has been suggested, is autophosphorylated in its activation loop and, at least in some systems, is dependent on the interaction of additional cofactors, such as TAB1 and TAB2 or its homolog TAB3, as is the case in the responses to interleukin

1 (17). However, in other responses, these cofactors are not necessary (45). In this study, we have identified two novel protein interactions of TAK1, namely, that with the scaffold JIP1 and another with a new regulatory protein, VRK2. We also have analyzed how VRK2 can alter signaling by MAPKs and affect the response to hypoxia induced by low oxygen pressure or by the chelation of iron with DFO.

TAK1 is able to stably interact with JIP1, as the MLK protein family does (31), which is closely related to TAK1 according to the human kinome (29). This interaction takes place without the need to be assembled with other kinases of the complex or TAB cofactors, and this interaction is a step that potentiates the response to hypoxia via JNK (Fig. 1). The interaction of TAK1 occurs by a JIP1 central region (residues 283 to 471) and is likely to lock JIP1 in a conformation that increases the binding of JNK to JIP1, making the transduction of the signal, through the JIP1-JNK module, more efficient. However, TAK1 also interacts with VRK2A, and because the regions of interaction are different, it can simultaneously form a complex with both JIP1 and VRK2A (Blanco et al., submitted). Nevertheless the VRK2A interaction with the TAK1-JIP1 complex has important functional consequences, since it downregulates the response to hypoxia by disrupting only the signaling through the TAK1-JIP1-JNK complex and therefore switching the balance among the different pathways in response to a common stimulation and determining cell fate. It has been shown in the cell response to interleukin 1 that VRK2A binding to JIP1 reduces the incorporation of JNK and thus reduces the activation of c-Jun-dependent transcription (Blanco et al., submitted).

The stress response to hypoxia has been studied mainly in the context of cardiac or neural biology (11, 60), but the contributions of JNK to apoptosis or survival after oxidative stress are still conflicting. In cardiomyocytes, apoptosis is reduced by inhibition of the JNK pathways (5, 48). On the other hand, the activation of the JNK pathway under moderate hypoxia (5% O_2) also contributes to the expression of proangiogenic genes, such as VEGF (4), where there is cooperation between c-Jun and Hif1 for this activation (1). These observations are very important in the context of a hypoxic tumor mass that might trigger an angiogenic response; in human gliomas, overexpression of VEGF is dependent on JNK activation (59). Also, this response promotes the expression of MDR genes that can make the cell more resistant to chemotherapy (8).

Interestingly this JNK activation induced by hypoxia appears to be initiated at the mitochondria (12), where part of VRK2A resides (6). Furthermore, calreticulin, a molecule that colocalizes with VRK2A, also affects cardio protection that is dependent on JNK activation (27). VRK2A also interacts with the BHRF1 protein of Epstein-Barr virus, which is a Bcl2 homolog, and this interaction appears to stimulate protection against apoptosis (25). In the context of hypoxia, the upstream events that result in the activation of TAK1 by the low oxygen level shown in this work remain to be identified. Here we report that the intracellular level of VRK2 conditions the magnitude of the stress response to hypoxia. Therefore, it is possible that, depending on the expression levels of VRK2, each tissue might have different responses to hypoxia and that in pathological situations such as cancer, tumors might have higher levels of VRK2 (34) that will permit survival under hypoxic conditions, a common stress in the tumor mass as it grows. For instance, in developing brain neurons, transient hypoxia and reoxygenation after hypoxia trigger apoptosis through the JNK signaling pathway (7), whereas in cardiomyocytes, which have relatively high levels of VRK2 (34), the activation of JNK, by reoxygenation induces survival (13). Hence, the modulation of MAPK pathways by additional proteins is a central process for the determination of the type of cellular reaction to specific stimulation; the levels of these other proteins might explain the differences found among different cell types to identical stimulation, and JIP1 and VRK2 appear to be some of them. Thus, the levels of VRK2 may modulate different responses where the assembly of JIP1 complexes is required. In this context, it will be very interesting to know how, in tumor biopsy samples, VRK2 is distributed in relation to the distance of a tumor cell from vessels.

In conclusion, in this work we have shown that hypoxia activates TAK1 and results in the activation of c-Jun-dependent transcription. The magnitude of this response can be modulated by the formation of a complex in which TAK1 directly interacts with JIP1 and VRK2A.

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