Human VRK2 (Vaccinia-related Kinase 2) Modulates Tumor Cell Invasion by Hyperactivation of NFAT1 and Expression of Cyclooxygenase-2*†§

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**Background:** Cellular invasion is regulated by expression of COX-2 gene.

**Results:** VRK2 directly phosphorylates NFAT1 and promotes expression of COX-2, facilitating cellular invasion.

**Conclusion:** VRK2 hyperactivates NFAT1, activates COX-2 expression, and increases cellular invasion by tumor cells.

**Implications:** VRK2 forms part of a novel pathway regulating cellular invasion that might be targeted in cancer and immunosuppression.

Cell invasion is one of the most clinically relevant biological characteristic of cancer cells. The nuclear factor of activated T cells (NFAT)3 pathway is functional in many cell types affecting different processes, such as metastasis, invasion (1, 2), proliferation (2–4), and angiogenesis. All of these processes are characteristic of the cancer phenotype (5).

Recently, a new subfamily of serine-threonine kinases was identified in the human kinome (6), known as vaccinia-related kinase (VRK) (7–9). These kinases have appeared late in evolution (10), and their target proteins are likely to be those that are already present in the cell and participate in different signaling pathways. Thus, the main role of these late kinases is probably to add an additional layer of control in complex organisms. VRK genes are expressed in many different cell types, and their protein levels are higher in proliferating cells (7, 11, 12). The best characterized member is VRK1 (13), which plays a role in the regulation of cell cycle initiation and behaves as an early response gene like c-myc and c-fos (13, 14). VRK1 is also involved in cellular stress and DNA damage responses mediated by p53 (8, 15–17), forming an autoregulatory loop (18, 19), and phosphorylates c-Jun (20), ATF-2 (21), and CREB1 (22).

The serine-threonine kinase VRK2 is correlated with high levels of estrogen and progesterone receptors, whereas it is inversely correlated with ERBB2 in human breast cancers (23). VRK2 has two isoforms: VRK2A (VRK2), composed of 508 amino acids and localized in the cytoplasm anchored to the endoplasmic reticulum (24), and VRK2B, a shorter isoform generated by alternative splicing, which can partially replace VRK1 in the nucleus (24). VRK2A can modulate signaling pathways that are assembled on the JIP1 scaffold protein by a direct and stable interaction, independently of its kinase activity (25, 26). VRK2A interaction with JIP1 is able to reduce the stress response to hypoxia (25) and to interleukin-1β (26). Also, VRK2A directly interacts with the KSRI scaffold protein and

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modulates the EGF-ERBB2-RAF-RAS signaling pathway (23, 27). Thus, VRK2 can alter the balance among the different pathways responding to a common stimulation.

NFAT1 is located in the cytosol in an inactive hyperphosphorylated state. Upon cell stimulation, there is an increase of intracellular calcium that activates the phosphatase calcineurin, which dephosphorylates NFAT1 (28–31). Then NFAT1 translocates to the nucleus (32), where it regulates gene transcription (33). Phosphorylation is a key process in the regulation of NFAT1 activity, so it is of interest to identify what kinases are implicated in the modulation of NFAT pathway. Calcineurin interacts with its inhibitor RCAN1 (MCIP1 or DSCR1) in the cytosol (34–36), and after stimulation RCAN1 can be phosphorylated by kinases, such as MEKK3 (34, 37). RCAN1 phosphorylation disrupts its interaction with calcineurin (34, 38) and the inhibitory effect on its phosphatase activity. In this way, kinases that phosphorylate RCAN1 indirectly regulate NFAT1 activation. The activated and dephosphorylated NFAT1 is translocated to the nucleus, but this activation can be further enhanced by additional phosphorylation in its N-terminal domain (39–41). Kinases that have been reported to mediate this phosphorylation are JNK (42), Cot/Tpl2 (39, 40), and PKC (43). Other kinases, such as CK1 (44), GSK3 (45), and DYRK (5), are involved in maintaining NFAT1 in the hyperphosphorylated state in the cytosol or rephosphorylating nuclear NFAT1 to inactivate it and export it to the cytoplasm.

The COX-2 gene promoter is regulated by NFAT1. In basal conditions, COX-2 expression is low in most cells, but it is induced by several stimuli. Distal and proximal NFAT1 response elements identified in the COX-2 promoter region are required for induction of COX-2 expression upon cell stimulation and suggest a role of NFAT1-induced COX-2 expression in tumor cells (46, 47). NFAT1 plays a significant role in promoting migration and invasion of breast and colon carcinoma cells (48, 49), and up-regulation of the COX-2, an important NFAT1 target gene, has been involved in cell invasion in a study of the gene expression profile of breast cancer cells expressing NFAT1 (50). It has been reported that invasion of breast cancer cells is associated with COX-2 expression at different stages of breast cancer development (51), and COX-2 is a marker of poorer prognosis (52). Also, COX-2 expression has been associated with breast cancer metastasis in bone and in the brain (5, 53, 54).

In this work, we have analyzed the role of VRK2 in the regulation of COX-2 expression in tumor cell lines, because VRK2 can identify two major groups in human breast cancer cells (23), and the contribution of this kinase to cell invasion potential. We have demonstrated that VRK2A induces COX-2 gene expression by a direct NFAT1 phosphorylation. Together, these results identified the first functional role of VRK2A as an active kinase by demonstrating its implication in regulation of cancer cell invasion through phosphorylation and activation of NFAT1-dependent COX-2 transcription. Therefore, these data detect a new regulatory component of the VRK2 pathway in higher eukaryotes and contribute to build the signaling network in which VRK2 participates.

EXPERIMENTAL PROCEDURES

Plasmids—HA-VRK2A, HA-VRK2B (24), and HA-VRK1 (16) were generated by cloning in pCEFL vector. GST-VRK2A wild type and the inactive kinase GST-VRK2A K169E, GST-VRK2B (24), and GST-VRK1 (8) were generated by cloning in pGEX4T1 vector for bacterial expression. The NFAT-Luc reporter plasmid containing three tandem copies of the distal NFAT1 site of the human IL-2 promoter fused to the minimal human IL-2 promoter and the full-length murine NFAT1-HA cloned in pEFBOS plasmid were kindly provided by Dr. J. M. Redondo (42). The GST-NFAT 4–57, GST-NFAT 4–68 and GST-NFAT 4–384 constructs were kindly provided by Dr. M López-Cabrera (43). The pCS2+MT-Myc-RCAN1 plasmid was used for eukaryotic expression, and the pCEFL-FLAG-MEK5-CA was kindly provided by Dr. A. Pandiella. The human COX-2 promoter constructs cloned in pXP2Luc reporter plasmid were kindly provided by Dr. M. Fresno (47).

Protein Expression and Purification—GST-VRK2, GST-VRK1, and GST-NFAT1 fusion proteins were expressed in Escherichia coli cells and purified using glutathione-Sepharose beads (GE Healthcare). Then fusion proteins were eluted with glutathione at 20 mM and stored at −20 °C (24, 55).

Cell Lines and Transfections—MDA-MB-435, MDA-MB-231, and HEK293T cells were growth in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum. All cell lines were obtained from the ATCC. Transfections were performed using JetPEI reagent (Polyplus, Illkirch, France) or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The total amount of DNA was kept constant by completion with the corresponding empty vector (25, 26).

Luciferase Assay—Transcriptional activity was measured by luciferase reporter gene assays in the transiently transfected MDA-MB-435 cell line. Cells were cotransfected with 1 μg of the correspondent reporter plasmid and the indicated plasmids in each experiment. After 24 h, cells were stimulated with 100 ng/ml PMA (Sigma) and 0.5 μM ionomycin (Io) (Sigma) or left without stimulation for an additional 16 h. Then cells were harvested and lysed. Luciferase activity was determined by using a luciferase assay kit (Promega) with a luminometer Lumat LB 9507 (Berthold Technologies). Luciferase experiments were performed in triplicate, and the data presented are the mean of the determinations in relative activity ± S.D. A representative immunoblot from each experiment is shown.

Immunoprecipitation and In Vitro Kinase Assay—HA-NFAT1 or Myc-RCAN1 plasmids were transfected in HEK293T cells, and proteins were immunoprecipitated using α-HA monoclonal antibody (Covance) and α-Myc polyclonal antibody (Upstate Biotechnology, Inc. (Lake Placid, NY)), respectively. Immunoprecipitates were washed and used for an in vitro kinase assay with 1 μg of GST-VRK2 in a final volume of 40 μl containing kinase buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.5 mM DTT, and 150 mM KCl), 5 μM ATP, and 5 μCi of [γ-32P]ATP. Purified GST-NFAT constructs and GST-VRK proteins were used for in vitro kinase assays in a final volume of 30 μl in the same conditions described previously (24, 55). The reactions were performed at 30 °C for 30 min and stopped by...
boiling in Laemmli buffer. The phosphorylated proteins were analyzed in 10% SDS-PAGE. Gels were stained with Coomassie Blue or transferred to Immobilon-P membrane (Millipore), and the incorporated radioactivity was detected.

Quantitative RT-PCR and Immunoblot—MDA-MB-231 and MDA-MB-435 cell lines were transfected with 200 nm specific siRNA to knock down VRK2 (siVRK2-06 (5'-GCAAGGUUC-UUGGAUGAUU-3'; Dharmacon) or siVRK2-M (5'-GAUAG-UUCCCAAUUGGGAA-3'; Mission series, Sigma)), and COX-2 mRNA and protein levels were determined. For quantitative RT-PCR analysis, cells were transfected with si-VRK2-06 or control siRNA, and after 3 days, cells were stimulated with 100 ng/ml PMA and 0.5 μM Io for 6 h or left without stimulation. Total RNA was extracted using the RNeasy extraction kit from Qiagen (Hilden, Germany). RNA was analyzed and quantified using the Bioanalyzer 2100 nanolab chip from Agilent Technologies (Germany), and 100 ng of total RNA were used in a one-step reverse transcription real-time PCR amplification reaction using the quantitative SYBR Green RT-PCR kit from Qiagen in an iCycler (Bio-Rad). The reaction was analyzed with iCycler software (Bio-Rad), and mRNA relative levels were represented as a mean of three independent experiments ± S.D. The following primers were used for human mRNA detection: human VRK2 (VRK2TA, 5'-AGTGAAGAGACGCTG-AGTCTT-3'; VRK2TB, 5'-CAAAAGGTCTTGAGACTCTTG-3'), human COX-2 (COX-2F, 5'-CAAAGCCTGGGA-AGCTTCTCTAA-3'; COX-2R, 5'-GCCCAAGCGGTGG-TGAAAG-3'), human NFAT1 (hNFAT1-F, 5'-TGCACTCTAA-CCCCATCGAGTG-3'; hNFAT1-R, 5'-TGAGGACTTCTTGG-CTGGCC-3'). Human GAPDH amplification was used as a housekeeping marker.

For Western blot analysis, cells were transfected with siVRK2 or control siRNA, and 3 days post-transfection, cells were stimulated with PMA plus ionomycin for 30 min and incubated for an additional 24 h. Cells were lysed with lysis buffer (50 mM HEPES, pH 7.5, 4 mM EDTA, 150 mM NaCl, 1.5 mM MgCl₂, 10 mM NaF, 1% Triton-X-100, 0.1% SDS, and 10% glycerol and protease and phosphatase inhibitors), and 30 μg were loaded in a 7.5% or 10% PAGE. Proteins were transferred to an Immobilon-P membrane (Millipore), blocked in TBS-T buffer with 5% nonfat milk, and then incubated with the indicated specific antibody. Bands were detected using a chemiluminescence ECL kit (GE Healthcare).

Antibodies—VRK2 was detected with a rabbit polyclonal antibody (24), COX-2 with a monoclonal antibody from R&D Systems (Minneapolis, MN) and NFAT1 with a monoclonal antibody from BD Biosciences. Myc epitope was detected with monoclonal or polyclonal antibodies from Upstate Biotechnology, Inc. FLAG epitope was detected with polyclonal or monoclonal antibodies from Sigma. HA epitope was detected with a monoclonal antibody from Covance (Emeryville, CA), and β-actin was detected with a monoclonal antibody from Sigma.

Wound Healing and Matrigel Invasion Assays—Wound healing assays were performed with MDA-MB-231 cells transfected in 60 dishes with control siRNA or siVRK2-06. After 3 days, each dish was divided into 6-well plates, and cells were allowed to reach confluent monolayers. Cells were stimulated with PMA and Io for 30 min, and then a straight scratch was made in individual wells with a yellow pipette tip. Cells were washed with PBS, and then fresh medium was placed. Cells were allowed to grow, and wound size was photographed (×10 magnification) under the microscope at several time points using a digital camera (Canon PowerShot A640) connected to an inverted microscope (Zeiss Axiovert 25). Measurements of wound width in each point of time were made in three different experiments, and the results were represented in graphs.

Invasion assays were performed in transwell chambers with 8-μm pore filters (BD Biosciences) coated with 1 mg/ml Matrigel (BD Biosciences). Cells were transfected with RNA interference for 3 days and were then stimulated or not for 30 min with PMA/ionomycin. Cells were harvested, and 50,000 cells were resuspended in serum-free medium to the upper chamber of the transwell. Medium with 10% FBS was added in the lower chamber, and cells were allowed to invade the Matrigel-coated filters at 37 °C. After 42 h, cells that had not invaded were removed with a cotton swab, and invasive cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Invasive cells were photographed using a digital camera (Canon PowerShot A640) connected to an inverted microscope (Zeiss Axiovert 25) and counted using ImageJ version 1.45 (Wayne Rasband, National Institutes of Health). They were represented as the percentage of invasive cells mean ± S.D. of three independent experiments.

RESULTS

**VRK2A Enhanced NFAT1 Transcriptional Activity after PMA/Io Stimulation in MDA-MB-435 Cells**—Cell invasion is a malignant characteristic common to different types of carcinomas (56–58), lymphomas, or melanomas (50, 59). Tumor cell invasion is associated with COX-2 expression that is regulated by the NFAT1 transcription factor (50). Therefore, we studied if VRK2A might be regulating the NFAT1 transcriptional activity. For this aim, we determined the effect of VRK2A on the NFAT transcriptional response, performing a luciferase reporter assay in the MDA-MB-435 cell line. VRK2A increased NFAT transcriptional activity in a dose-dependent manner, but this effect was only detected in cells stimulated with PMA/ionomycin (Fig. 1A), indicating that the activation is secondary to the PMA/Io stimulation. Next, we determined if other members of the VRK family could also induce the same effect. NFAT1 activity increased with cytosolic VRK2A, whereas there was a lower effect with the free VRK2B isoform that is located in both nucleus and cytosol. The nuclear VRK1 has no effect on NFAT1 transcriptional activity. In the case of both VRK2 isoforms, the effect on NFAT1 activity required PMA/Io stimulation (Fig. 1B).

VRK2 also potentiated NFAT1 activity following PMA and Io stimulation in Jurkat cells (supplemental Fig. S1). This result suggests that the effect of VRK2A on NFAT1 activity is independent of cell type.

**VRK2 in Vitro Phosphorylation of NFAT1 N-terminal Transactivation Domain on Serine 32**—To determine if VRK2 was acting by either phosphorylation or interaction with NFAT1, we first performed in vitro kinase assays with GST-VRK2A and
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GST-VRK2B fusion proteins were used for an in vitro kinase assay with several GST-NFAT1(4–68) mutants as substrate to determine the residue phosphorylated by VRK2A. As observed in Fig. 2C, only serine 32 mutation of NFAT1 showed a lower phosphorylation by VRK2A, whereas serines 53, 54, and 56 mutations did not show differences in phosphorylation. Serines 53, 54, and 56 had been reported as phosphorylation sites enhancing NFAT1 transactivation (43), but there are no previous data about serine 32. Luciferase assays showed that only the two VRK2 isoforms enhanced NFAT1 activity. We decided to determine if VRK2 was the only VRK member able to phosphorylate NFAT1. GST-VRK2A, GST-VRK2B, and GST-VRK1 fusion proteins were used for an in vitro kinase assay with the shortest NFAT1 construct (NFAT1 4–57) as substrate. As we expected, both VRK2 isoforms, but not VRK1, phosphorylated the NFAT1 construct (Fig. 2D). These results suggest that VRK2A could increase NFAT1 activity by a direct phosphorylation mechanism.

**Interaction between VRK Proteins and NFAT1**—Next, it was determined if there was a direct interaction between VRK and NFAT1 proteins. For this aim, we performed a pull-down assay with GST-VRK and HA-NFAT1 proteins overexpressed in HEK293T cells. VRK2A and VRK2B, but not VRK1, were able to directly interact with NFAT1 (Fig. 3A). This interaction was also determined by immunoprecipitation of HA-NFAT1, and only VRK2A and VRK2B interacted with NFAT1 (Fig. 3B). Moreover, different VRK2A constructs fused to GST and HA-NFAT1 were used in a pull-down assay to determine the region of VRK2 interacting with NFAT1. The N-terminal domain of VRK2 between residues 1 and 320 interacted with NFAT1 (Fig. 3C), consistent with the interaction also seen in the case of VRK2B, because the two VRK2 isoforms have an identical sequence up to amino acid 364 (24). In addition, the region of NFAT1 interacting with endogenous VRK2A was mapped in an in vitro pull-down assay using NFAT1 constructs fused to GST. This NFAT1 interaction region was located between residues 68 and 384 (Fig. 3D).

**VRK2A Did Not Increase NFAT1 Activity by an Indirect Mechanism Involving RCAN1 Phosphorylation**—RCAN1 is a calcineurin-interacting protein that has an inhibitory role in calcineurin activity (36). It has been reported that RCAN1 phosphorylation by MAPK proteins disrupts its interaction with calcineurin, increasing calcineurin activity and NFAT1 activation (34). We tested if VRK2A might also indirectly contribute to regulate NFAT1 activity through a similar mechanism. First, we performed an in vitro kinase assay with recombinant GST-VRK2A fusion protein and immunoprecipitated

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**FIGURE 1. VRK2A potentiated NFAT1-dependent transcription after PMA and ionomycin stimulation in MDA-MB-435 cells.** A, melanoma MDA-MB-435 cells were transfected with NFAT-Luc reporter plasmid and increasing amounts of HA-VRK2A. After 24 h, cells were treated with 100 ng/µl PMA plus 0.5 µM Ilo or left without stimulation, and luciferase activity was measured after 16 h. B, MDA-MB-435 cells were transfected with NFAT-Luc reporter plasmid and each of the three VRK proteins, HA-VRK2A, HA-VRK2B, and HA-VRK1. After 24 h, cells were treated with 100 ng/µl PMA plus 0.5 µM Ilo for 16 h or left without stimulation, and luciferase activity was measured. Results are the mean of triplicate measurements of at least three independent experiments. The values were compared with the mock control. **, *p < 0.005; ***p < 0.0005. The expression of the transfected VRK proteins detected in Western blots is shown at the bottom. Error bars, S.D.
Myc-RCAN1 from HEK293T cells as substrate. Immunoprecipitated HA-NFAT1 was used as positive control. As shown in Fig. 4A, VRK2A did not phosphorylate RCAN1, but it phosphorylated NFAT1. Furthermore, to rule out an indirect effect of VRK2A on the NFAT1 pathway through RCAN1, we performed luciferase assays with the reporter plasmid NFAT-Luc in MDA-MB-435 cells. RCAN1 overexpression inhibited NFAT1 activation induced by PMA/Io, and VRK2A overexpression enhanced NFAT activity, as we expected. But when both plasmids were transfected together, VRK2A was not able to counteract the inhibitory effect of RCAN1 (Fig. 4B, top). However, MEK5 CA (constitutively active) counteracted the inhibitory effect of RCAN1, and NFAT activation was observed in this positive control. Therefore, phospho-RCAN1 presented different electrophoretic mobility and was detected by Western blot. Phospho-RCAN1 levels were not affected in the presence of VRK2A, but phospho-RCAN1 levels increased in the presence of MEK5 CA (Fig. 4B, bottom).

VRK2 Silencing Decreased COX-2 Gene Expression—We decided to study the effect of VRK2A down-regulation with a specific siRNA on COX-2 mRNA levels after PMA plus Io stimulation by performing quantitative real-time PCR. COX-2 is an inducible gene, whose mRNA level is very low in non-stimulated cells, but after cell stimulation with PMA plus Io, COX-2 mRNA levels are increased. We transfected breast cancer MDA-MB-231 cells, which have high levels of endogenous NFAT1 and are an invasive cell line (50), with VRK2-specific siRNA, siVRK2-06, for 3 days, and we observed a reduction of COX-2 mRNA levels after PMA plus Io stimulation (Fig. 5A). The same results were observed in the MDA-MB-435 cell line (Fig. 5B). VRK2 knockdown had no effect on NFAT1 mRNA.
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Levels in either of these two cell lines (Fig. 5C). COX-2 mRNA was also reduced using a different siRNA for VRK2 (siVRK2-M) either in MDA-MB-231 cells (supplemental Fig. S2A) or in MDA-MB-435 cells (supplemental Fig. S2B).

We also determined COX-2 protein levels in MDA-MB-231 cells stimulated or not with PMA/Io after VRK2 knockdown by siRNA transfection. In basal conditions, there was no detectable COX-2 protein expression, but after PMA/Io stimulation, there was an increase in COX-2 protein expression. When VRK2 was down-regulated, COX-2 protein levels decreased, but NFAT1 protein levels were not affected (Fig. 5D).

VRK2A Up-regulated COX-2 Transcription through NFAT Response Elements in the COX-2 Promoter—Because VRK2A increased NFAT1 transactivation activity, we tested if NFAT1 activation might be able to mediate NFAT1-dependent transcription in MDA-MB-435 cells. COX-2 is an NFAT1 target gene, so, we analyzed COX-2 promoter activity. For this aim, we performed luciferase assays with the reporter plasmid p2–274,
which contains two NFAT1 binding sites: distal (dNFAT) and proximal (pNFAT) (47). VRK2A overexpression enhanced the transcriptional activity of the COX-2 gene promoter. This effect was not observed with either inactive kinase VRK2A(K169E) or VRK1 overexpression (Fig. 6A). Also, the increase in COX-2 promoter transcription induced by VRK2A was reduced in cells treated with the calcineurin inhibitor cyclosporin A (Fig. 6B), CsA. Cyclosporin A is a compound that inhibits calcineurin activity and consequently inhibits NFAT1 activation. We also carried out luciferase assays with the reporter plasmid p2–274, containing one or both NFAT binding sites mutated. As shown in Fig. 6C, VRK2A-increased transcription was significantly reduced when only the proximal NFAT1 binding site was mutated. Together, these results indicate that the effect of VRK2A on COX-2 transcription is NFAT1-dependent. Furthermore, we decided to perform the same experiments in a colon cancer cell line, because an increase in COX-2 promoter activity through activation of NFAT1 has been reported (46), and it is also known that COX-2 is a gene with relevant implications in colon cancer progression. In the SW-620 colon carcinoma cell line, we observed results similar to those in the MDA-MB-435 cell line (supplemental Fig. S3), indicating again that the effect of VRK2A on NFAT1-dependent COX-2 transcription was independent of cell type. Moreover, we performed in this SW-620 cell line luciferase-reporter experiments down-regulating VRK2 expression. Results showed that VRK2 overexpression enhanced COX-2 promoter activity, whereas VRK2 down-regulation reduced COX-2 promoter activity (supplemental Fig. S3).

**VRK2 Down-regulation Reduced Cell Invasion**—To evaluate the role of VRK2A in COX-2-induced cell invasion through NFAT1 activation, we performed a Matrigel invasion assay in the MDA-MB-231 cell line. As shown in Fig. 7A, cells were more invasive after PMA/ionomycin stimulation, but in the case of VRK2 down-regulation with siVRK2-06, cell invasion decreased significantly. The same result was obtained in the MDA-MB-231 cell line. This effect was also detected using another siRNA (siVRK2-M) for VRK2 in this cell line and in MDA-MB-231 cells (supplemental Fig. S4). The difference in invasiveness between these two cell lines can be explained
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FIGURE 5. VRK2 silencing decreased COX-2 mRNA levels and COX-2 expression. A, total RNA from MDA-MB-231 breast cancer cells transfected with control siRNA (siC) or VRK2-specific siRNA (siVRK2-06) and stimulated or not with 100 ng/ml PMA and 0.5 μM Io was used for quantitative RT-PCR analysis to measure COX-2 and VRK2 mRNA levels. Data were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, and results are the mean of three independent experiments. **, p < 0.005; ***, p < 0.0005. B, total RNA from MDA-MB-435 melanoma cells treated as in A. ***, p < 0.0005. C, VRK2 knockdown by specific siRNA transfection did not affect NFAT1 mRNA levels in MDA-MB-435 and MDA-MB-231 cell lines. D, COX-2 protein expression decreased in MDA-MB-231 cells with reduced VRK2 protein levels. NFAT1 protein levels were not affected by VRK2 silencing. At the right is shown the quantification of protein levels. Error bars, S.D.

because MDA-MB-435 cells have a much lower level of NFAT1 (Fig. 7B), and thus the potential for activation of COX-2 expression is more limited. Therefore, we determined the effect of VRK2 down-regulation on cell migration, performing a wound healing assay. Control cells closed the wound area after 30 h, but cells with reduced VRK2 levels migrated more slowly than control cells and were not able to completely close the wound after 30 h (supplemental Fig. S5).

DISCUSSION

NFAT1 phosphorylation represents the first target identified for VRK2 as an active kinase because other VRK2 effects that have been reported are only mediated by protein-protein interactions (23, 25–27). Thus, this finding extends the significance of the biological role of VRK2 in cell signaling and cancer biology, where it has been mainly studied. Phosphorylation plays three different roles in NFAT1 regulation. One of them implicates an indirect mechanism by RCAN1 phosphorylation, which releases calcineurin from RCAN1 interaction and inhibition. Another effect is a direct phosphorylation in the NFAT1 N-terminal domain that enhances NFAT1 transcriptional activity. These additional activations might affect the interaction with other transcriptional factors or coactivators. Third, nuclear NFAT1 phosphorylation leads to inactivation and export of NFAT1 to the cytosol. Also, there are kinases that phosphorylate NFAT1 to maintain it in the cytosol in the inactive hyperphosphorylated state. Thus, NFAT1 phosphorylation in the cytosol has two different roles; one is to retain this transcription factor in the cytoplasm, and the other is to enhance the activity of activated NFAT1. This positive effect may also facilitate selection of other factors or interacting proteins. The N-terminal transactivation domain is the most important domain in the regulation of NFAT1 transactivation function. It has been reported that Cot kinase and PKC phosphorylate this domain in serine 53 and 56, increasing NFAT1 activity (41, 42, 60). In this report, we show that VRK2A is a new NFAT kinase and phosphorylates the NFAT1 transactivation domain in another residue, serine 32, that has not been previously reported, enhancing NFAT1 transcriptional activity. This effect occurs in the cytosol because it is mediated by the VRK2A isoform, which is anchored to the endoplasmic reticulum, but it might also occur in the nucleus in cells that also express the VRK2B isoform, as is the case with p53 (24). Thus, VRK2 can be incorporated in the complex regulation mediated by phosphorylation of NFAT1 (Fig. 8).

The role of COX-2 in cancer has been mainly studied in the context of colorectal carcinoma (61), but it is likely that it plays a role in other cancers, such as breast carcinomas or melanomas. Also, COX-2 has been associated with bone and brain metastasis (52, 53, 62, 63). The requirement of NFAT1 phosphorylation for the regulation of COX-2 gene expression suggests that kinases might play a significant role. The increased NFAT1 activity by VRK2A is selective because it is dependent on NFAT1 response elements in the COX-2 gene promoter. The existence of two NFAT1 binding sites in this promoter might represent the possibility to respond to different input signals by the formation of alternative transcriptional complexes. COX-2 expression requires NFAT1 activation, and different levels of activated NFAT1 might have different functional effects.
VRK2 inhibits the mitogenic signal mediated by AP1 transcription factor (23) but promotes survival signals (23) and dissemination (this report). In tumors, VRK2 might alter the balance between these two properties. VRK2 might inhibit the proliferative signal and facilitate the invasive potential in breast cancer. Human breast cancer can be classified in different types, and VRK2 is positively associated with estrogen and progesterone receptor expression and inversely correlated with ERBB2 expression. This is explained by the fact that VRK2A has an inhibitory effect on the mitogenic signal mediated by the ERBB2-RAS-RAF-ERK pathway without affecting the survival pathway (23).

Targeting kinases is currently proving to be a useful therapeutic strategy in cancer (64, 65). The different phenotypes of breast carcinomas have different biological characteristics. Estrogen receptor-positive patients, in which VRK2 is expressed at high levels, can be controlled by antiestrogenic therapy, but if stimulatory signals are received, tumors might increase their invasive potential due to the action of VRK2 on NFAT1 activity. In breast cancer, NFAT1 expression and activation are relatively little characterized, but it is known that it plays a role in tumor dissemination. This might be associated with the inflammatory response triggered by interleukins present in the tumor environment as a consequence of COX-2 expression (54, 66). In this way, COX-2 expression might facilitate tumor invasion (67). Perhaps combined therapy of COX-2 and VRK2 inhibitors might be a feasible strategy in the future to reduce the invasiveness of tumor cells in breast cancer patients. Currently there are no inhibitors for VRK2, and this kinase family is very insensitive to current inhibitors due to their structural characteristics (55, 68).

In this work, we have identified the first reported role of VRK2 as an active kinase phosphorylating NFAT1 protein in a previously unidentified residue, serine 32, and enhancing the activity of this transcription factor. Also, we have demonstrated that VRK2 participates in the activation of NFAT1-dependent

**FIGURE 6.** NFAT1 binding sites in the COX-2 promoter were required for increased COX-2 transcription induced by VRK2A. A, HA-VRK2A overexpression, but not HA-VRK1, increased COX-2 promoter transcription in MDA-MB-435 cells after 100 ng/µl PMA and 0.5 µM ionomycin stimulation for 16 h. VRK2A(K169E) and VRK2A(K189E) are kinase-dead proteins. Western blot with the level of the transfected proteins is shown to the right. B, effect of 10 µM cyclosporin A (CsA) treatment for 1 h before PMA/Io stimulation on the NFAT1 activation induced by VRK2A. C, effect of VRK2A on the transcriptional activity of the reporter p2–274 mutated in the proximal (p) or both distal and proximal (d&p) NFAT binding sites. Levels of transfected HA-VRK2A are shown in the blot at the bottom. Graphs show the mean of the relative -fold increase in luciferase activity values of triplicate measurements of three independent experiments. *, p < 0.05; **, p < 0.005; ***, p < 0.0005. Error bars, S.D.
VRK2 Phosphorylates NFAT1 and Activates COX-2 Expression

A

MDA-MB-231 cell line

Treatment: Control  PMA/Io

siControl

siVRK2-06

B

FIGURE 7. Effect of VRK2 down-regulation on invasion of breast cancer MDA-MB-231 cells. A, MDA-MB-231 cells were transfected in p60 dishes with control siRNA (siControl) or VRK2-specific siRNA (siVRK2-06). After 3 days, cells were treated as control or stimulated with PMA/Io for 30 min; afterward, 50,000 cells were placed in serum-free medium in the upper chamber of a Transwell coated with Matrigel (1 mg/ml). Medium with 10% FBS was added in the lower chamber. The invasion assay was performed for 42 h. Invasive cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet dye. The microscopic image of the invasive cells is shown to the left. The mean values from duplicate samples of three independent experiments are shown to the right. *** p < 0.0005. At the bottom is shown the level of COX-2 protein (left) and the level of VRK2 mRNA by quantitative RT-PCR (right). B, protein levels of VRK2 and NFAT1 in human cell lines of different origins. Jurkat (T-cells), SW-620 (colon carcinoma), MDA-MB-435 (melanoma), and MDA-MB-231 (breast cancer). The antibodies used are indicated under “Experimental Procedures.” Error bars, S.D.

FIGURE 8. Model of the regulation of NFAT1 activation and participation of different kinases.
VRK2 Phosphorylates NFAT1 and Activates COX-2 Expression

COX-2 transcription, and this effect on COX-2 expression by VRK2 can modulate the invasive properties of tumor cells. Thus, in the future, it will be necessary to study these proteins on human primary tumors in order to identify breast cancer subgroups, in which eventually an anti-VRK2 therapy might be used when available.

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