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Plk3 Interacts with and Specifically Phosphorylates VRK1 in Ser^{342}, a Downstream Target in a Pathway That Induces Golgi Fragmentation▽†

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Golgi fragmentation is a process that is necessary to allow its redistribution into daughter cells during mitosis, a process controlled by serine-threonine kinases. This Golgi fragmentation is activated by MEK1 and Plk3. Plk3 is a kinase that is a downstream target in the Golgi fragmentation pathway induced by MEK1 or by nocodazole. In this work, we have identified that Plk3 and VRK1 are two consecutive steps in this signaling pathway. Plk3 interacts with VRK1, forming a stable complex detected by reciprocal immunoprecipitations and pull-down assays; VRK1 colocalizes with giantin in the Golgi apparatus, as Plk3 also does, forming clearly detectable granules. VRK1 does not phosphorylate Plk3, but Plk3 phosphorylates the C-terminal region of VRK1 in Ser342. VRK1 with substitutions in S342 is catalytically active but blocks Golgi fragmentation, indicating that its specific phosphorylation is necessary for this process. The induction of Golgi fragmentation by MEK1 and Plk3 can be inhibited by kinase-dead VRK1, the knockdown of VRK1 by siVRK1, kinase-dead Plk3, or PD98059, a MEK1 inhibitor. The Plk3-VRK1 kinase module might represent two consecutive steps of a signaling cascade that participates in the regulation of Golgi fragmentation.

The Golgi apparatus in mammalian cells is formed by cistern stacks, tubules, and small vesicles, which undergo extensive and sequential fragmentation in mitosis (33). The reorganization of the Golgi apparatus, involving fragmentation, dispersal, and reassembly, is tightly regulated during mitosis (1, 27, 30), and reversible phosphorylation plays a critical role (1, 21), although the components and their sequential organization in the context of the initiation or execution of the signal required for Golgi fragmentation are only partially known.

Many signaling pathways are composed of consecutive kinases. Characterization of new signaling pathways requires the identification of their components, the connections between them, and the order in which they are organized. Human VRK1 is a novel serine-threonine kinase that phosphorylates several proteins implicated in cellular responses to stress and DNA damage, such as p53 (5, 20, 40), c-Jun (31), and ATF2 (32), as well as proteins needed for nuclear envelope assembly required at the end of mitosis, such as Baf (25). In addition, VRK1 kinase activity is inhibited by interaction with RanGDP, and this inhibition is relieved by RanGTP, suggesting an asymmetric distribution of its activity within the nucleus and in mitosis (29). These properties suggest that the *VRK1* gene plays a role in the regulation of cell cycle initiation and/or progression, consistent with its requirement for entry into the cell cycle, where it behaves as an immediate-early response gene like c-*MYC* and *FOS* (36). The loss of VRK1 by use of small interfering RNA (siRNA) induces an early G_1 block, before cyclin D1 expression (36), which is accompanied by a reduction in the phospho-retinoblastoma level and an accumulation of cycle inhibitors, such as p27 (36), resulting in a stop in cell cycle progression (36, 40).

Several kinases are implicated in the control of cell proliferation and in different mitotic checkpoints; among them are the polo-like kinase (Plk) family, which is a group composed of four proteins (14, 39, 46). One of them, Plk3, contributes as a mediator of DNA damage checkpoint responses, since its kinase activity increases after oxidative stress (43) and induction of DNA damage by ionizing radiomimetic drugs (45). Plk3 physically interacts with and phosphorylates p53 in Ser20, and this interaction increases in response to DNA damage and induces either cell cycle arrest or apoptosis (44) so that genetic stability can be maintained by the prevention of the accumulation of genetic damage. Furthermore, Plk3 interacts with Chk2 (2, 45), an important mediator of DNA damage responses (6, 16), and there is a functional connection between them since Plk3 phosphorylates Chk2 in Ser62 and Ser73, which are necessary for full Chk2 activation by ATM (4). In mitotic cells, Plk3 is localized associated with the spindle poles and mitotic spindles, and deregulated expression of Plk3 induces cell cycle arrest and apoptosis by the perturbation of microtubule integrity (41). In addition, Plk3 expression is induced after mitogenic stimulation, and it is required for mitotic (28) and S-phase (48) entry. Plk3 also regulates Cdc25C (3, 23, 26) and the NF-κB signaling pathway (19). VRK1 phosphorylates p53 in Thr18 (20, 40), a residue phosphorylated in response to taxol, an inhibitor of microtubule polymerization (34).

There is a possibility that VRK1 and Plk3 might be connected in some way, since subpopulations of both VRK1 (37) and Plk3 (28) have been detected in the Golgi apparatus near the centrosome, where they colocalize with Golgi markers such as giantin or GM130 (33). Golgi fragmentation can be induced by MEK1 (1, 15), and this signal is partly mediated by Plk3 (28,

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42). Moreover, Golgi fragmentation is a required step during mitosis, occurring late in the G_2/M phase of the cell cycle (11), and MEK1 is implicated in the activation of this process (1, 15).

The common biological aspects of VRK1 and Plk3 proteins and the association of VRK1 and Plk3 subpopulations in the Golgi apparatus led us to think that there might be a functional connection between these two kinases and thus that they might be components in a common signaling pathway. In this work, we explored the possible connection between VRK1 and Plk3 and determined if they were functionally related in a biological process, Golgi fragmentation, in which one of them, Plk3, is already known to participate. This work demonstrates that Plk3 and VRK1 are consecutive components in the signaling pathway that induces Golgi fragmentation in mitosis.

MATERIALS AND METHODS

Plasmids. The *Escherichia coli* expression constructs GST-Plk3 and GST-Plk3^{K52R} were obtained from P. Stambrook (2). The pCR253-Plk3 and pCR253-Plk3^{K52R} constructs were obtained from W. Dai (26), and the Plk3 inserts were taken out of these plasmids and subcloned into the EcoRI-NotI cloning sites in vectors pCEFL-GST, pCEFL-HA, and pCEFL-Flag for mammalian expression with different epitopes. The amino acid substitution Ser342A was introduced into human VRK1 by site-directed mutagenesis using the QuikChange mutagenesis system (Stratagene, San Diego, CA), with plasmids pGEX4T-VRK1^{K179E}, pCEFL-HA-VRK1, and pCEFL-HA-VRK1^{K179E} as substrates (40). The following oligonucleotides were used to introduce the S342A substitution into these plasmids by site-directed mutagenesis: forward, 5′-GGCAAATTGGACCTCGC TGTTGTGGAGAATG-3′; and reverse, 5′-CATTCTCCACAACAGCGAGGT CCAATTTGCC-3′. VRK1-FL-myc (full-length VRK1), VRK1-NL-myc (VRK1 residues 1 to 332), and VRK1-NS-myc (VRK1 residues 1 to 267) were cloned into pCDNA3-myc. Plasmid pFC-MEK1, expressing constitutively active MEK1$^{(S218/222E,\ \Delta 32–51)}$ protein, was purchased from Stratagene (San Diego, CA). The glutathione *S*-transferase (GST)–p53 fusion protein FP221 containing the N terminus of murine p53 (residues 1 to 85) was a gift of D. Meek (University of Dundee).

Reagents. PD98059 (a MEK1 inhibitor), okadaic acid, and brefeldin A were obtained from Calbiochem (San Diego, CA). Nocodazole was obtained from Sigma (St. Louis, MO). In some experiments, cells grown in chamber slides were treated with PD98059 (20 μM), nocodazole (1 μg/ml for 150 min), okadaic acid (1 μM for 1 h), or brefeldin A (5 μg/ml for 90 min) to induce Golgi fragmentation by different mechanisms.

Antibodies. Human VRK1 was detected with a rabbit polyclonal antibody (40) or with murine monoclonal antibody 1F6 (37). Human Plk3 protein was detected with a monoclonal antibody (clone B37-2) (BD Pharmingen, San Diego, CA) or a polyclonal antibody (10977-1AP) (ProteinTech Group Inc., Chicago, IL). Giantin was detected with a polyclonal antibody (Covance, Berkeley, CA). GM130 was detected with monoclonal antibody clone 35 (BD Transduction Laboratories). An antihemagglutinin (anti-HA) monoclonal antibody (immunoglobulin G1 [IgG1] isotype) was obtained from Covance. GST was detected with monoclonal antibody B14 (Santa Cruz). Actin was detected with monoclonal antibody AC-15 from Sigma (St. Louis, MO). The Flag epitope was detected with either a polyclonal or a monoclonal anti-Flag antibody from Sigma (St. Louis, MO). FluorolinkCy2-conjugated anti-rabbit IgG, FluorolinkCy3-conjugated anti-rabbit IgG, FluorolinkCy2-conjugated anti-mouse IgG, and FluorolinkCy3-conjugated anti-mouse IgG were purchased from GE Healthcare.

Kinase activity assay. Kinase activity was determined by assaying protein phosphorylation in a kinase buffer specific for VRK1 or Plk3. To test the VRK1 activity, the following kinase buffer was used: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 mM KCl, and 5 μM ATP (20). To test the Plk3 activity, the following kinase buffer was used: 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 mM dithiothreitol. The kinase reaction was initiated by the addition of ATP (5 μCi of 5 μM [γ-^{32}P]ATP). The reactions in both assays were performed at 30°C for 30 min.

Cell lines, transfections, and immunoblots. HEK293T human embryo kidney and HeLa human cervical adenocarcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin at 37°C in a 5% CO_2 humidified atmosphere. A549 human lung carcinoma cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Transfections were performed using either the calcium phosphate method, JetPEI reagent (Polytransfection, Illkirch, France), or Lipofectamine 2000 reagent (Invitrogen) according to manufacturer recommendations. The total amount of DNA within the experiments was kept constant by adding the respective empty vector plasmid DNA to the transfection mixtures. For immunoblot analysis, cells were harvested at 36 h posttransfection and lysed with a specific lysis buffer for each experiment. Twenty-five to 50 μg of total protein lysate was fractionated in a 10% sodium dodecyl sulfate-polyacrylamide gel and analyzed by Western blotting to identify the proteins present. Western blots were performed and developed by chemiluminescence techniques, using the ECL detection system and horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare Pharmacia) or anti-rabbit antibody (Sigma, St. Louis, MO) as previously reported (8, 9).

Pull-down and immunoprecipitation assays. For pull-down experiments, HEK293T cells were grown in 100-mm dishes and were transfected with different fusion proteins in mammalian expression vectors. The amount and type of specific plasmid used are indicated in each individual experiment. Whole-cell extracts prepared 36 h after transfection were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 10% (vol/vol) glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). To bring down the fusion protein with its associated proteins, the extract was mixed with glutathione-Sepharose beads (GE Healthcare Pharmacia) for 12 h at 4°C with gentle shaking. The beads were extensively washed with lysis buffer and subjected to electrophoresis followed by immunoblot analysis with the corresponding antibodies indicated for each experiment.

For immunoprecipitation experiments, HEK293T cells were grown in 100-mm dishes and were transfected with different plasmids tagged with the Flag or HA epitope. The amount and type of specific plasmid used are indicated in each individual experiment. Whole-cell extracts prepared 36 h after transfection were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, and the following protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Extracts were incubated on ice for 20 min and precleared by centrifugation at 14,000 rpm for 20 min at 4°C. Extracts were then immunoprecipitated with the specific antibody for each experiment. Immune complexes were recovered with γ-Bind G Sepharose (GE Healthcare Pharmacia). Beads were washed five times with lysis buffer and subjected to electrophoresis followed by immunoblot analysis with the corresponding antibodies indicated for each experiment.

Immunofluorescence and confocal microscopy. HeLa, A549, and HEK293T cells were grown on uncoated glass coverslips introduced into 60-mm plates (150,000 cells/plate). Transfections were performed using JetPEI (Polytransfection) or Lipofectamine 2000 reagent (Invitrogen) for the siRNA experiments (36, 40). Cells were collected, fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, treated with 0.1 M glycine for 10 min at room temperature, and then permeabilized with PBS–0.2% Triton X-100 for 30 min at room temperature. The cells were blocked by incubation with PBS containing 1% bovine serum albumin for 30 min at room temperature, followed by double immunostaining with the corresponding antibodies. Finally, cells were stained with DAPI (4′,6-diamidino-2-phenylindole) (Sigma) diluted 1:1,000 in PBS for 10 min at room temperature, the cells were washed with PBS, and slides were mounted with Gelvatol (Monsanto). Fluorescence images were captured with a Zeiss LSM 510 confocal microscope. Fluorochromes were excited using an ^{18}Ar laser (488-nm excitation wavelength) for Cy2 and a ^{2}He/^{10}Ne laser (543-nm excitation wavelength) for Cy3.

VRK1 knockdown by siRNA interference or by short hairpin RNA (shRNA). For siRNA experiments, synthetic SMART-selected siRNA duplexes were purchased from Dharmacon RNA Technologies (Lafayette, CO). The targeted sequence for VRK1 (GenBank accession number NM_003384) was CAAGGA ACCTGGTGTTGAA (duplex siVRK1-02) from Dharmacon. The functional siControl nontargeting siRNA pool from Dharmacon was used as a negative control, and fluorescently labeled siGLO lamin A/C siRNA was used as a silencing and transfection efficiency control. Transfection of siRNA duplexes at a final concentration of 80 nM was carried out using Lipofectamine 2000 reagent (Invitrogen) in HeLa cells following the manufacturer's instructions. Depending on the experiment, 5 days later cells were retransfected with 3 μg of MEK1$^{(S218/222E,\ \Delta 32–51)}$ or 3 μg of Plk3 for 15 h or 13 h, using Lipofectamine 2000 reagent (Invitrogen). After transfection, cells were processed for Western blotting, immunofluorescence, or microscopy, as indicated.

For shRNA experiments, empty pSUPER vector or pSUPER-VRK1 was used. The targeted sequence for VRK1 was GAAAGAGAGTCCAGAAGTA. HeLa

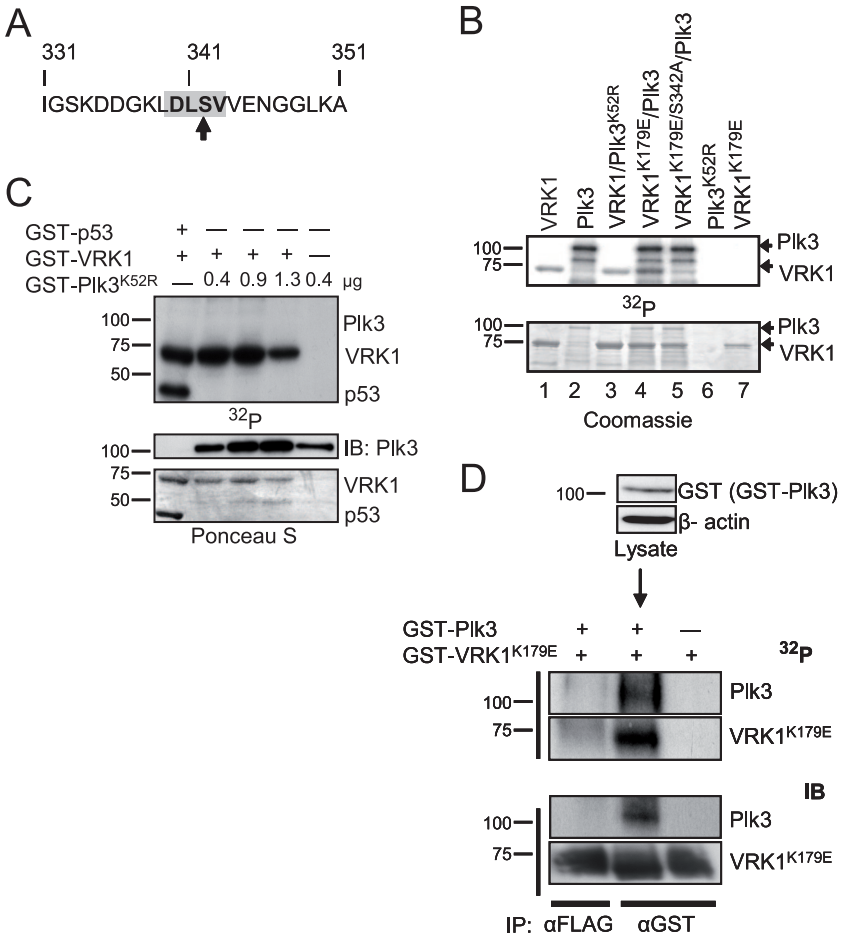

FIG. 1. VRK1 is phosphorylated in Ser342 by Plk3. (A) Sequence in the C-terminal region of VRK1 containing a conserved sequence that is a potential target for Plks. (B) In vitro kinase assay to determine the phosphorylation order between Plk3 and VRK1 and the residue phosphorylated. Plk3 and human VRK1 were expressed in *E. coli* and purified for the in vitro kinase assay. KE, K179E substitution in VRK1 that is kinase dead. The phosphorylated proteins were detected by autoradiography (top), and the proteins present in the assay are shown by Coomassie blue staining (bottom). (C) In vitro kinase assay to demonstrate that VRK1 does not phosphorylate kinase-dead Plk3. pGEX-GST-VRK1, pGEX-GST-p53 (1-82), and pGEX-GST-Plk3^{K52R} were expressed in *E. coli.* The assay was carried out with a constant amount of GST-VRK1 and increasing amounts (as indicated) of GST-Plk3. GST-p53 was used as a positive control. The phosphorylated proteins were detected by autoradiography (top), and the proteins present in the assay are shown by immunoblotting (middle) or Ponceau staining (bottom). (D) Phosphorylation of VRK1 by transfected Plk3. HEK293T cells were transfected with pCEFL-GST-Plk3 (5 μg), and cell extracts were used for immunoprecipitation with monoclonal anti-Flag antibody (control) or monoclonal anti-GST antibody (Plk3). The immunoprecipitates were used for kinase assays using pGEX-GST-VRK1^{K179E} as a substrate.

cells were transfected with 6 μg of empty vector as a control or with the specific shRNA-expressing plasmid. The total DNA was mixed with 12 μl of JetPEI transfection reagent. Depending on the experiment, 5 days later cells were retransfected with 3 μg of MEK1$^{(S218/222E,\ \Delta 32-51)}$ or 3 μg of Plk3, using Lipofectamine 2000 reagent (Invitrogen). Next, 15 h or 13 h after transfection, cells were processed for Western blotting, immunofluorescence, or microscopy, as indicated.

Protein interaction accession number. The protein interactions in this publication have been submitted to the IMEx (http://imex.sf.net) consortium through IntAct (pmid 17145710) and assigned the identifier IM-9274.

RESULTS

VRK1 is phosphorylated in Ser342 by Plk3. The amino acid sequence of human VRK1 has a consensus target sequence for phosphorylation by PLK proteins in its C-terminal region; this predicted target sequence is located in residues 340 to 343, based on sequence analysis with the Eukaryotic Linear Motif program (EMBL). In this sequence, serine 342 is the candidate residue to be phosphorylated by Plk3 (Fig. 1A), because it is located within the predicted regulatory region and outside the kinase domain (20). If this observation is confirmed, it would indicate that Plk3 and VRK1 might represent two consecutive steps of a novel signaling pathway.

Both Plk3 (2, 44, 45) and VRK1 (20, 31, 32, 40) are serine-threonine kinases. Therefore, our initial approach aimed to determine if the VRK1 and Plk3 proteins are able to phosphorylate each other and in which order they are organized. For this aim, in vitro kinase assays were performed using different combinations of these two proteins in kinase-active and -inactive forms. For these experiments, active and kinase-dead (K52R) Plk3 constructs were used for kinase assay using human active or kinase-dead (K179E) VRK1. Both kinases Plk3 and VRK1 have autophosphorylation activity, as detected by

the strong phosphorylation of the corresponding bands (Fig. 1B, lanes 1 and 2). Active VRK1 did not phosphorylate target Plk3^{K52R} (Fig. 1B, lane 3), but active Plk3 was able to phosphorylate the inactive VRK1^{K179E} protein (Fig. 1B, lane 4). The lack of activity of both kinase-dead proteins is shown as negative controls (Fig. 1B, lanes 6 and 7). These data situate Plk3 as an upstream kinase with respect to VRK1. The next step was to identify the residue phosphorylated on the VRK1 molecule. The candidate serine was mutated to alanine (S342A) and introduced into both active and kinase-dead forms of VRK1, both in GST fusion protein constructs and in mammalian expression vectors. When GST-VRK1$^{K179E/S342A}$ was used as a substrate for Plk3 activity, there was no phosphorylation signal for kinase-dead VRK1$^{K179E/S342A}$ (Fig. 1B, lane 5), suggesting that Ser342 is the target residue. To specifically demonstrate that VRK1 does not phosphorylate Plk3, a specific kinase assay was performed in which a fixed amount of active VRK1 was used to phosphorylate increasing amounts of inactive Plk3^{K52R}, using p53 as a positive control. VRK1 was autophosphorylated and phosphorylated p53 (20, 40) but not the inactive Plk3^{K52R} protein (Fig. 1C, top panel), although nonphosphorylated Plk3 was readily detected by immunoblot analysis (Fig. 1C, bottom panel).

To further confirm that VRK1 was indeed a substrate of Plk3, HEK293T cells were transfected with a plasmid expressing GST-Plk3, the cell extracts were immunoprecipitated with an anti-GST antibody, and the immunoprecipitate was used for an in vitro kinase assay with inactive VRK1^{K179E} as the substrate (Fig. 1D). Inactive VRK1^{K179E} was phosphorylated when Plk3 was present (Fig. 1D, lane 2) but not in the control (Fig. 1D, lanes 1 and 3).

The other VRK proteins also have potential target sequences for Plk3. VRK2A and VRK2B have a consensus target sequence located in residues 367 to 370, and VRK3 has a target sequence in residues 85 to 91. The VRK2 proteins in their kinase-dead form and nonactive VRK3 were used as substrates of active Plk3 in kinase assays performed in vitro. None of them was phosphorylated by Plk3 (see Fig. S1 in the supplemental material). Therefore, it can be concluded that only VRK1 is a specific phosphorylation target of Plk3.

Expression, interaction, and intracellular colocalization of endogenous VRK1 and Plk3. Initially, we determined the levels of expression of endogenous VRK1 and Plk3 proteins in a panel of mammalian cell lines (Fig. 2A). In nonsynchronized cultures, most cell lines (H1299, A549, HEK293T, MCF7, and WS1) expressed similar levels of both proteins, with the exceptions of U2OS cells, in which the protein level of Plk3 was higher than that of VRK1, and HeLa, Jurkat, and Cos1 (monkey) cells, where there was more VRK1 than Plk3 protein. The cell lysate from HEK293T cells was used to determine if the two endogenous proteins can interact in a stable manner. For this aim, the cell lysate was used for immunoprecipitation of endogenous VRK1, and the immunoprecipitate was analyzed with antibodies specific for Plk3 or VRK1. In the VRK1 immunoprecipitate, the Plk3 protein was detectable (Fig. 2B).

Since a fraction of VRK1 and Plk3 proteins can be immunoprecipitated, it is very likely that they form intracellular complexes that might be detected as colocalizing signals within the cell. The determination of the possible colocalization of endogenous VRK1 and Plk3 was studied using confocal microscopy of HEK293T and A549 cells. The location of endogenous VRK1 was detected with a specific polyclonal or monoclonal antibody (37, 40) and analyzed for the potential of overlapping fluorescence signals with transfected Plk3 that was tagged with either the HA or Flag epitope. In the HEK293T cell line, VRK1 had a particulate perinuclear, nuclear, and fine granular cytosolic aspect and colocalized with Plk3 in the perinuclear granules (Fig. 2C). Both VRK1 and Plk3 also colocalized in cytosolic granules in A549 cells (Fig. 2D), although their appearance was slightly different from that in HEK293T cells. Endogenous VRK1 is mostly located within the nucleus (24, 40), but in some cell types a subpopulation is also detected in the cytosol (7, 37) or in cytosolic vesicles and the Golgi apparatus (38). These data suggested that at least a subpopulation of endogenous VRK1 and Plk3 proteins might form an intracellular particulate complex.

This interaction between VRK1 and Plk3 was also detected by proteomic analysis of proteins associated with VRK1 in HEK293T cells (29). The proteomic identification of Plk3 by mass spectrometry is shown in Fig. S2 in the supplemental material.

VRK1 and Plk3 can interact to form a stable complex. Many kinases implicated in cell signaling are able to form stable complexes. Therefore, it was tested if Plk3 and VRK1 were able to form such a stable complex, as suggested by previous colocalization experiments. To detect their interaction in live cells, three different approaches were used. The initial approach consisted of reciprocal immunoprecipitations to detect the interaction between transfected proteins. The tagged HA-VRK1 and Flag-Plk3 proteins were correctly expressed in cell lysates (Fig. 3A, top panels). These lysates were used for immunoprecipitation with either an anti-HA (Fig. 3A, bottom left panel) or anti-Flag (Fig. 3A, bottom right panel) antibody, and the immunoprecipitates were used for immunoblotting with the reciprocal antibody. In both cases, the VRK1-Plk3 interaction was detected (Fig. 3A, right and left bottom panels).

Next, a different approach was used, based on the pulldown of GST fusion proteins. In this approach, HEK293T cells were transfected with pCEFL-HA-VRK1 or kinase-dead pCEFL-HA-VRK1^{K179E} (40), with and without the mutant substitution (S342A) in the residue targeted by Plk3, in combination with the mammalian expression construct pCEFL-GST-Plk3 or its kinase-dead form, pCEFL-GST-Plk3^{K52R}, as a fusion protein. These extracts were used to pull down GST-Plk3 and its associated proteins. These were analyzed by immunoblotting for the presence of VRK1 (Fig. 3B, top panels). Their correct expression was determined in a Western blot of the cell lysate (Fig. 3B, bottom panels). VRK1 interacted with active and inactive Plk3, while the inactive VRK1^{K179E} protein interacted more stably with inactive Plk3^{K52R}. The interaction was lost if VRK1 had the S342A substitution, indicating that the intact target sequence in VRK1 was necessary in order to form a stable complex with Plk3 (Fig. 3B, top panels).

To further confirm this interaction, a third approach was used, consisting of the pulldown of the endogenous VRK1 or Plk3 protein with a transfected fusion protein, either GST-Plk3 (Fig. 3C) or GST-VRK1 (Fig. 3D), respectively. First the endogenous VRK1 was brought down from cells transfected with either pCEFL-GST-Plk3 or pCEFL-GST-Plk3^{K52R}. In the

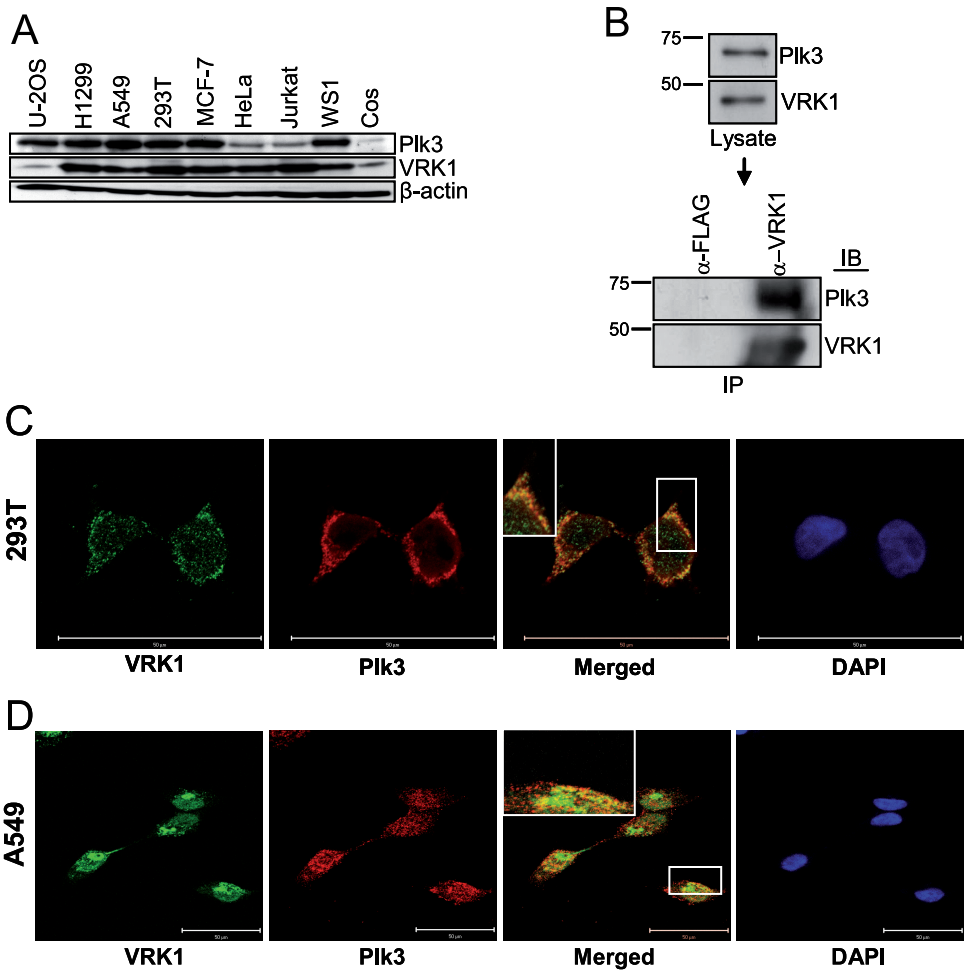

FIG. 2. Expression, interaction, and colocalization of endogenous human VRK1 and Plk3 proteins. (A) The expression of endogenous VRK1 and Plk3 proteins in different cell lines was determined by immunoblotting. Cell lysate (20 μg) from each cell line was fractionated in a sodium dodecyl sulfate-(10%) polyacrylamide gel and transferred to an Immobilon-P membrane. VRK1 was detected with the VC1 polyclonal antibody, and Plk3 was detected with a monoclonal antibody. (B) Interaction of endogenous VRK1 and Plk3 proteins in HEK293T cells. One milligram of cellular extract was used for immunoprecipitation of the endogenous VRK1 protein with a monoclonal antibody (1F6) or with a control antibody (monoclonal anti-Flag). The endogenous Plk3 immunoprecipitated was detected with a specific polyclonal antibody for Plk3. IP, immunoprecipitation; IB, immunoblot. (C). Colocalization in HEK293T cells. The endogenous VRK1 protein was detected with a specific polyclonal antibody (VE1). HEK293T cells were transfected with 3 μg of Plk3 tagged with an HA epitope and detected with a mouse monoclonal antibody specific for the HA tag. Nuclei were identified by DAPI staining. (D). Colocalization in A549 cells. The endogenous VRK1 protein was detected with a specific monoclonal antibody (1F6). A549 cells were transfected with 3 μg of Plk3 tagged with a Flag epitope and detected with a polyclonal antibody for the Flag epitope. Nuclei were stained with DAPI. Bar, 50 μm.

pull-down assay, VRK1 was detected associated with Plk3 but not with GST (Fig. 3C). Reciprocally, the endogenous Plk3 protein was detected bound to VRK1, either active or kinase dead, but not to GST, in the extracts of cells transfected with pCEFL-GST-VRK1 or pCEFL-GST-VRK1^{K179E} (Fig. 3D).

All of these data confirmed that VRK1 and Plk3 are able to form a stable complex that can be detected by different experimental approaches and that their interaction is independent of their kinase activity.

Mapping of the Plk3-VRK1 interaction. To identify the region of the VRK1 protein implicated in the interaction with Plk3, two constructs of VRK1, the full length (GST-VRK1-FL) and its carboxy-terminal region, residues 267 to 396 (GST-VRK1-C), were used for a pull-down assay of Plk3. The full-length but not the C-terminal region of VRK1 was able to interact with Plk3 (Fig. 4A). Next, an inverse experiment was performed, using GST-Plk3 to detect constructs spanning different regions of VRK1, either the full length (FL), residues 1 to 332 (NL), or residues 1 to 267 (NS). Plk3 was able to bind to all of them (Fig. 4B). The common region of VRK1 that interacts with Plk3 is located in the region comprised of residues 1 to 267 (Fig. 4C).

Endogenous VRK1 in the Golgi apparatus is fragmented in mitosis and by treatment with nocodazole, okadaic acid, or brefeldin A. A subpopulation of VRK1, detected with the 1F6 monoclonal antibody, is located in the Golgi apparatus, where it colocalizes with giantin, a Golgi protein (37). The Golgi apparatus is fragmented in the G_2/M cell cycle phase, and in this fragmentation process Plk3 is also redistributed, dispersed into smaller vesicles throughout the cytoplasm, and colocalized

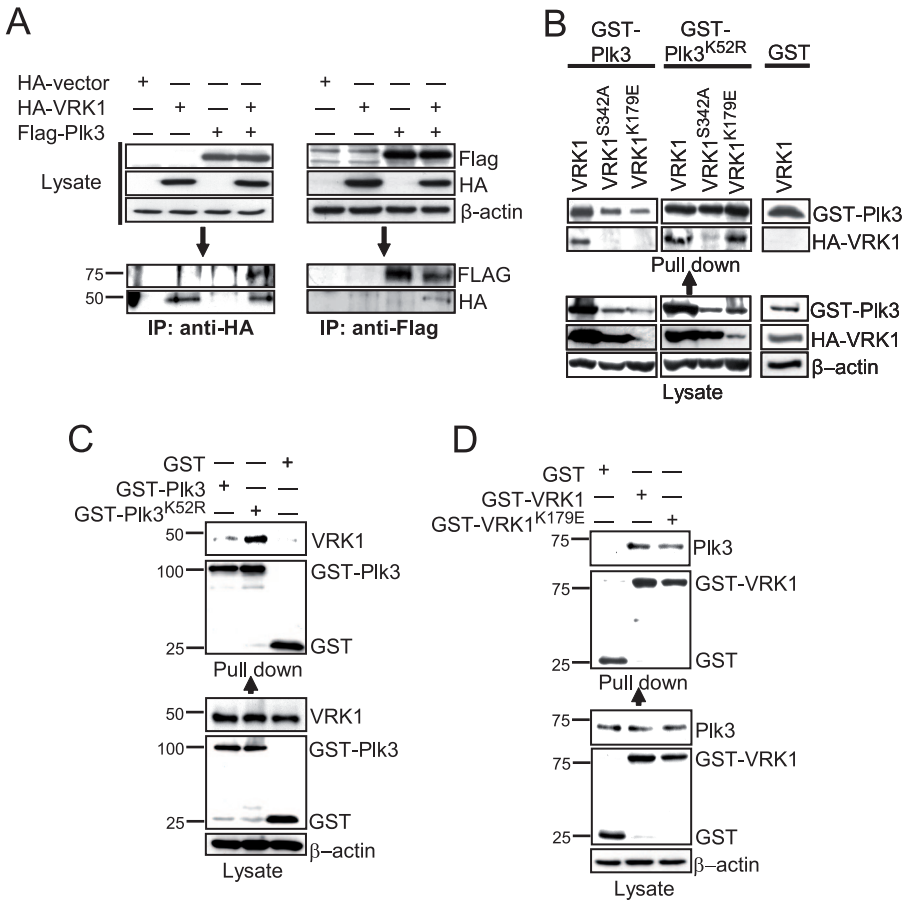

FIG. 3. Stable interaction between Plk3 and VRK1. (A) Reciprocal immunoprecipitation of transfected proteins. HEK293T cells were transfected with empty vector (pCEFL-HA) or plasmid pCEFL-HA-VRK1 (5 μg) in combination with pCEFL-Flag-Plk3 (5 μg). The cell lysate (top) was used for immunoprecipitation of proteins that bind to either VRK1 or Plk3 with an antibody specific for the corresponding epitope. (Bottom) Detection of the reciprocal proteins was determined by immunoblotting. (B) In vivo interaction of transfected VRK1 and Plk3 proteins. HEK293T cells were transfected with plasmids expressing active (pCEFL-GST-Plk3) and kinase-dead (pCEFL-GST-Plk3^{K52R}) Plk3 in combination with VRK1, either active (pCEFL-HA-VRK1), inactive (pCEFL-HA-VRK1^{K179E}), or nonphosphorylatable by Plk3 (pCEFL-HA-VRK1^{S342A}). Their correct expression was checked in cell lysates (bottom), which were used for a pull-down assay of proteins associated with the GST-Plk3 constructs. (C) Detection of the interaction between endogenous VRK1 and transfected Plk3. HEK293T cells were transfected with plasmid pCEFL-GST-Plk3 (6 μg), pCEFL-GST-Plk3^{K52R} (6 μg), or pCEFL-GST (2 μg) as a control. The expression of the proteins was determined by Western blotting (bottom). The different lysates were used for pull-down assay with glutathione-Sepharose to bring down the GST-Plk3 proteins. The pull-down proteins were detected with anti-VRK1 (1F6) monoclonal antibody. (D) Detection of the interaction between endogenous Plk3 and transfected VRK1. HEK293T cells were transfected with plasmid pCEFL-GST-VRK1 (8 μg), pCEFL-GST-VRK1^{K179E} (8 μg), or pCEFL-GST (2 μg) as a control. The expression of the proteins was determined by Western blotting (bottom). The different lysates were used for a pull-down assay with glutathione-Sepharose to bring down the GST-VRK1 proteins. In the pull-down assay, the endogenous Plk3 protein was detected with a specific antibody.

with the distribution of the giantin marker (28, 42), where it is implicated in regulating the cell cycle (17, 48). We first determined how the VRK1 subpopulation in the Golgi apparatus was redistributed during mitosis (Fig. 5). In interphase and late telophase cells, VRK1 and giantin colocalized in a compact body, but in intermediate steps along the progression of mitosis, they retained their colocalization in smaller vesicles (Fig. 5).

Next, we determined if giantin and VRK1 proteins continued to colocalize in cells in which Golgi fragmentation was induced by different types of treatment, with either nocodazole, which causes microtubule depolymerization leading to dispersion of the Golgi apparatus from the perinuclear region, okadaic acid, a phosphatase inhibitor, or brefeldin A, which also induces Golgi fragmentation by the collapse of the Golgi apparatus into the endoplasmic reticulum. VRK1 and giantin colocalized in the Golgi apparatus (Fig. 6, control) and also in the fragmented Golgi apparatus following treatment with nocodazole, okadaic acid, or brefeldin A (Fig. 6), suggesting that they form part of a very stable complex in both assembled and fragmented Golgi complexes.

VRK1 can block the Golgi apparatus fragmentation phenotype induced by MEK1 and Plk3. There is a subpopulation of VRK1 localized in the Golgi apparatus (37), an organelle whose structure is tightly controlled, among other kinases, by Plk3 (28) as a downstream target of MEK1 (42). MEK1 is known to contribute to Golgi fragmentation in G_2/M phase (15). Therefore, we tested if VRK1 or its mutant forms could

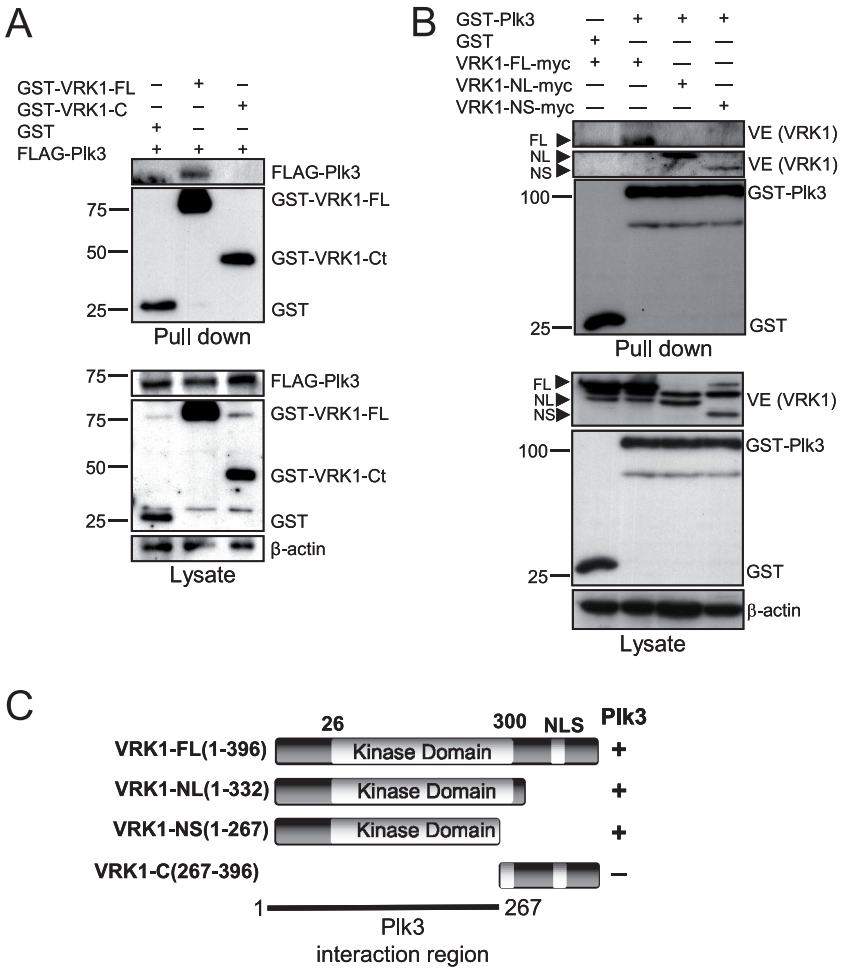

FIG. 4. Mapping of the region of VRK1 interacting with Plk3. (A) HEK293T cells were transfected with plasmids expressing mammalian GST-VRK1 (FL, full-length; C, C-terminal region) and Plk3 tagged with a Flag epitope. HEK293T cells were cotransfected with constructs of VRK1-FL (8 μg) and VRK1-C (6 μg) fused to GST and with Flag-Plk3 (3 μg). The correct expression levels of proteins were checked in the lysates. The lysates were used for a pull-down assay with glutathione-Sepharose beads, and the associated proteins were detected in Western blots (top). VRK1 constructs were detected with an anti-GST antibody. Plk3 was detected with an anti-Flag antibody. (B) HEK293T cells were transfected with plasmids expressing different regions of human VRK1 tagged with myc and a mammalian construct expressing GST-Plk3. HEK293T cells were cotransfected with constructs of VRK1 fused to myc expressing different regions of the VRK1 protein (pCDNA-myc-VRK1-FL [5 μg], pCDNA-myc-VRK1-NL [5 μg], and pCDNA-myc-VRK1-NS [9 μg]) and with Plk3 (6 μg) fused to GST. The lysates were used for a pull-down assay with glutathione-Sepharose beads, and the associated proteins were detected in Western blots (top). GST empty vector was used as a control. Plk3 was detected with an anti-GST antibody. VRK1 was detected with a specific rabbit polyclonal antibody (VE1). (C) Potential region of interaction between VRK1 and Plk3.

interfere with this MEK1-Plk3 Golgi fragmentation induction pathway, detected using giantin as a marker. First, we tested if the MEK1 effect was indeed inducible in HeLa cells (Fig. 7A, panel b) and could be blocked by the MEK1 inhibitor PD98059 (Fig. 7A, panel c). The effect was clearly detectable in this cell line (Fig. 7A, panels a to c), which thus could be used for studying the effects of either Plk3 or VRK1. Both kinase-dead $Plk3^{K52R}$ (Fig. 7A, panel d) and $VRK1^{K179E}$ (Fig. 7A, panel e) were able to block the induction of Golgi fragmentation by MEK1, indicating that their kinase activities were also required for the process. Thus, the kinases Plk3 and VRK1 are downstream of MEK1 in this signaling pathway.

Next, we tested if Plk3 induction of fragmentation (Fig. 7B, panel a) required the participation of VRK1. VRK1 by itself without activation does not induce Golgi fragmentation (Fig. 7B, panel b), but it does transmit the signal in the presence of Plk3 (Fig. 7B, panel c), suggesting that the activation of VRK1 by a specific signal is necessary to cause this induction. Moreover, both kinase-dead $VRK1^{K179E}$ and the active kinase $VRK1^{S342A}$ (Fig. 7B, panel e) blocked the fragmentation induced by Plk3 (Fig. 7B, panel d), confirming the implication of VRK1 downstream of Plk3. The quantification of Golgi fragmentation depending on the transfected proteins is summarized at the bottom of Fig. 7.

The knockdown of VRK1 prevents the Golgi fragmentation phenotype. To confirm that VRK1 is indeed a component of the pathway leading to Golgi fragmentation, experiments were performed using both specific siVRK1 and shVRK1. The knockdown of the VRK1 protein level induced by siVRK1-02 resulted in a loss of VRK1 protein in HeLa cells (Fig. 8A). This

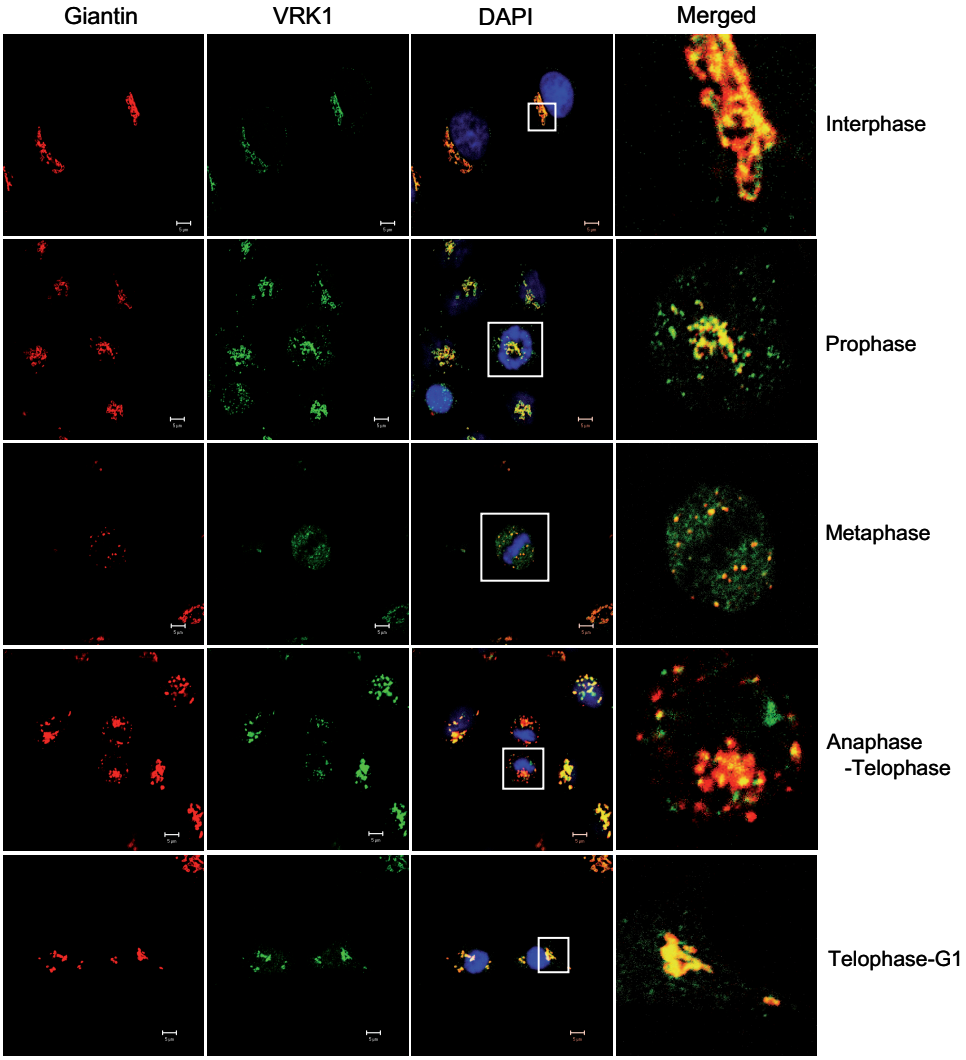

FIG. 5. VRK1 distribution during Golgi fragmentation in different phases of mitosis. Colocalization in HeLa cells of endogenous VRK1 with giantin is shown for different stages of mitosis. Endogenous VRK1 was detected with the monoclonal antibody 1F6, specific for VRK1. The Golgi apparatus was detected with a polyclonal antibody specific for giantin. Bar, 5 μm.

loss is similar to that detected in other cell lines, such as HCT116 lung carcinoma cells (40) or WS1 fibroblasts (36). The need for a long time for detection is a consequence of the high stability of the VRK1 protein (36). The loss of VRK1 in HeLa cells also resulted in a decrease in cell number (Fig. 8B) because it blocked cell cycle progression early in G_1 (36). To determine the effect of knocking down VRK1 on the Golgi fragmentation phenotype, HeLa cells were treated with specific siVRK1. In the controls, there were more cells (Fig. 8C, first and second rows) and Golgi fragmentation was induced by MEK1 (Fig. 8C, second row). The specific siVRK1 prevented the fragmentation induced by MEK1 (Fig. 8C, third row), and the endogenous VRK1 signal was lost as expected. Plk3 also induced fragmentation in Plk3-transfected cells treated with siControl (Fig. 8C, fourth row), which was also blocked in cells treated with the specific siVRK1 (Fig. 8C, fifth row). The average number of intracellular particles was analyzed using the ImageJ program (http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). This analysis confirms in a quantitative way the effect of VRK1 knockdown (Fig. 8C, bar graph). Identical results were obtained when the experiments where performed using a plasmid for specific shVRK1 instead of specific siVRK1 (see Fig. S3 in the supplemental material). These data confirm that VRK1 is a downstream target in the pathway leading to Golgi fragmentation induced by MEK1 and Plk3.

DISCUSSION

In general, signaling pathways are described as a series of consecutive steps, which appear to be similar in all cell types. However, very often the cellular response to the same type of stimulation varies depending on cell type. To account for this differential response, signaling has to be considered as a complex network in which there are many divergent modulatory steps along the different levels of the main route, and these can condition the specificity of the effect as well as the relative distribution of a signal that has more than one response path-

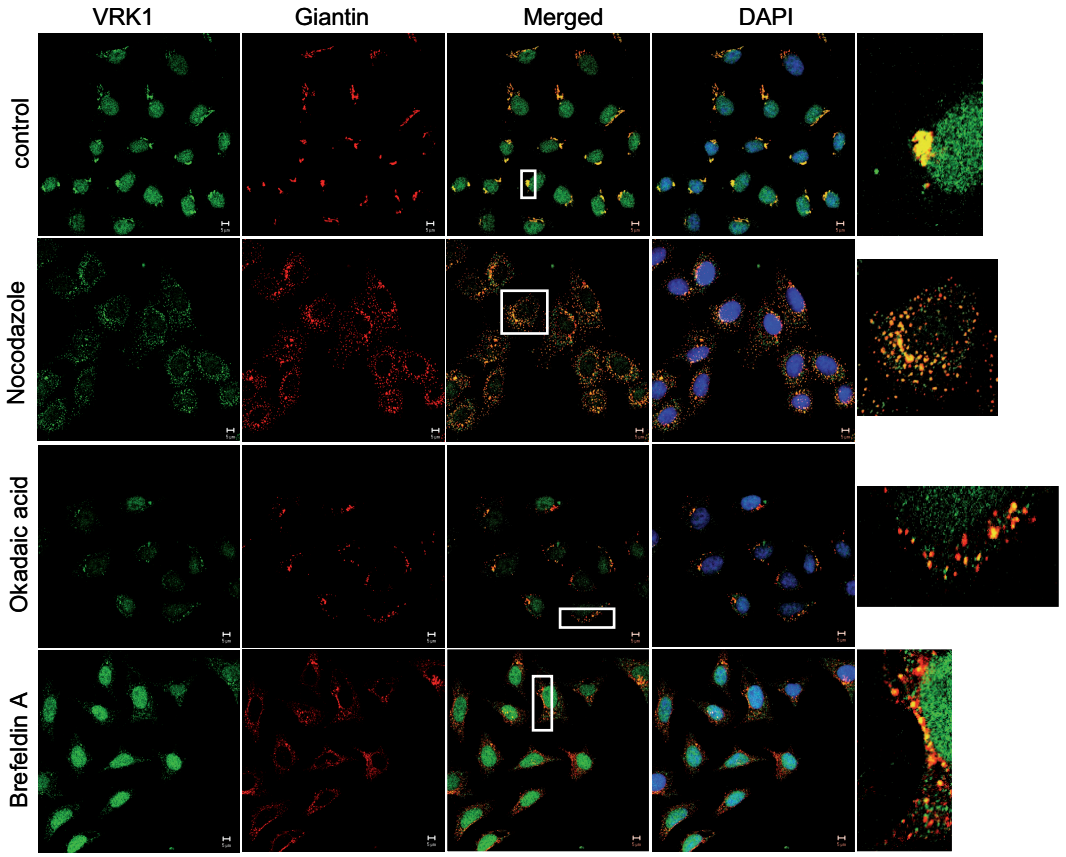

FIG. 6. VRK1's Golgi redistribution phenotype after treatment with nocodazole, brefeldin A, and okadaic acid in HeLa cells. The VRK1 distribution patterns after treatment with agents that disrupt this organelle were similar to that of giantin. VRK1 was detected with monoclonal antibody 1F6 (green). Giantin was detected with a rabbit polyclonal antibody (red). DNA was stained with DAPI (blue). Golgi fragmentation was induced by treatment with okadaic acid (OA; 1 μM for 1 h), brefeldin A (BFA; 5 μg/ml for 90 min) or nocodazole (1 μg/ml for 150 min), and the colocalization of VRK1 with Golgi markers was determined. Representative images are shown. Bar, 5 μm.

way. In this context, protein-protein interactions are likely to play a major role in signal magnitude and distribution within the cell that may also be manifested as an internal and functional compartmentalization. The specificity of cell signaling, an aspect that has received relatively little attention but which will increasingly be more important, refers to the internal compartmentalization of the different components in the pathway, which can also be associated with specific and even alternative functional roles. This intracellular organization of signaling proteins is well known in the assembly of mitogen-activated protein kinase modules, which are composed of three consecutive kinases (13, 22, 35) and a group of alternative scaffold proteins, such as the JIP family (47). But the assembly of other signaling complexes is practically unknown in the context of other processes, and in this context the identification of a stable Plk3-VRK1 complex represents an important step in the organization of a specific signaling process related to Golgi fragmentation.

The redistribution of cellular organelles into two daughter cells is a necessary step in cell division. Golgi apparatus reorganization during mitosis is partially characterized by the signals implicated in its regulation, which are necessary to redistribute it into daughter cells. Some of the components have been identified as individual participants, but the connection between them is only partially known. Golgi fragmentation requires its disassembly, which is a controlled process associated with centriole dynamics that occurs at the onset of mitosis. The signaling pathways implicated in Golgi fragmentation are relatively little known. One of the first mitogenic signal transducers is represented by MEK1, a mediator of mitogenic signals, and its signal is mediated at least partially by cdc2 (18) and a subpopulation of Plk3 proteins (28, 42). MEK1 induces Golgi fragmentation, probably as a consequence of its effect on proliferation, but there is part of the signal that must diverge since it does not reach extracellular signal-regulated kinase 1/2 (1), suggesting the existence of an alternative pathway with a different function, and this fragmentation signal is blocked by kinase-dead Plk3 (42). MEK1 has been detected colocalizing with the giantin Golgi marker (1, 12). In this context, a subpopulation of Plk3 proteins was identified in the Golgi apparatus colocalized with giantin (28), and its loss of function resulted in prevention of Golgi fragmentation. This Plk3 effect was postulated to be mediated by an indirect effect on microtubule dynamics, since nocodazole, a microtubule-disrupting drug, induces Golgi fragmentation which is blocked by kinase-dead Plk3; thus, Plk3 must be located downstream of microtubule disruption (42).

Stable kinase complexes containing Plk3-VRK1 represent a

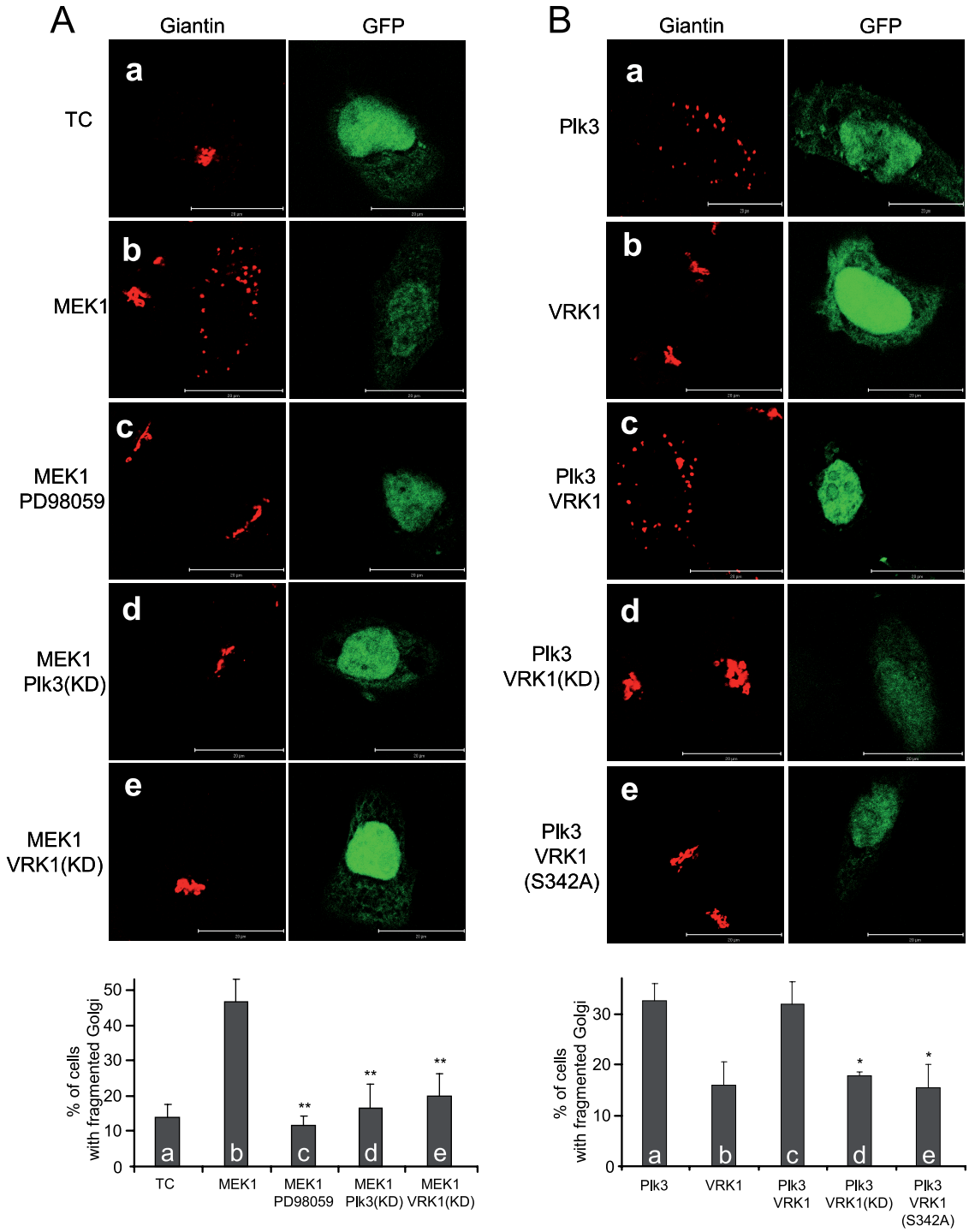

FIG. 7. Effect of VRK1 on MEK1-Plk3 fragmentation pathway phenotype. (A) Effects on Golgi fragmentation induced by MEK1. At the bottom is shown the quantification of the effect. (B) Effects on Golgi fragmentation induced by Plk3. HeLa cells were transfected with constructs expressing the indicated proteins. The MEK1 protein is constitutively active. The transfected cells were identified by the use of green fluorescent protein (GFP) as a fluorescent tracer protein or of an antibody against the tag in the transfected protein. The Golgi apparatus was detected with a polyclonal antibody specific for giantin. Bar, 20 μm. TC, transfection control with empty vector pEGFP-N1. The quantification of the effects in panels A and B are shown at the bottom of the corresponding column. At least 100 cells from three independent experiments were counted for each type of transfection, and the proportion of cells with fragmented Golgi complexes was determined. In the left column, the reference value is the fragmentation induced by MEK1 (b). In the right column, the reference value is the fragmentation induced by Plk3 (a). The mean values with their standard deviations are represented in the bar graph. *, $P < 0.05$; **, $P < 0.005$.

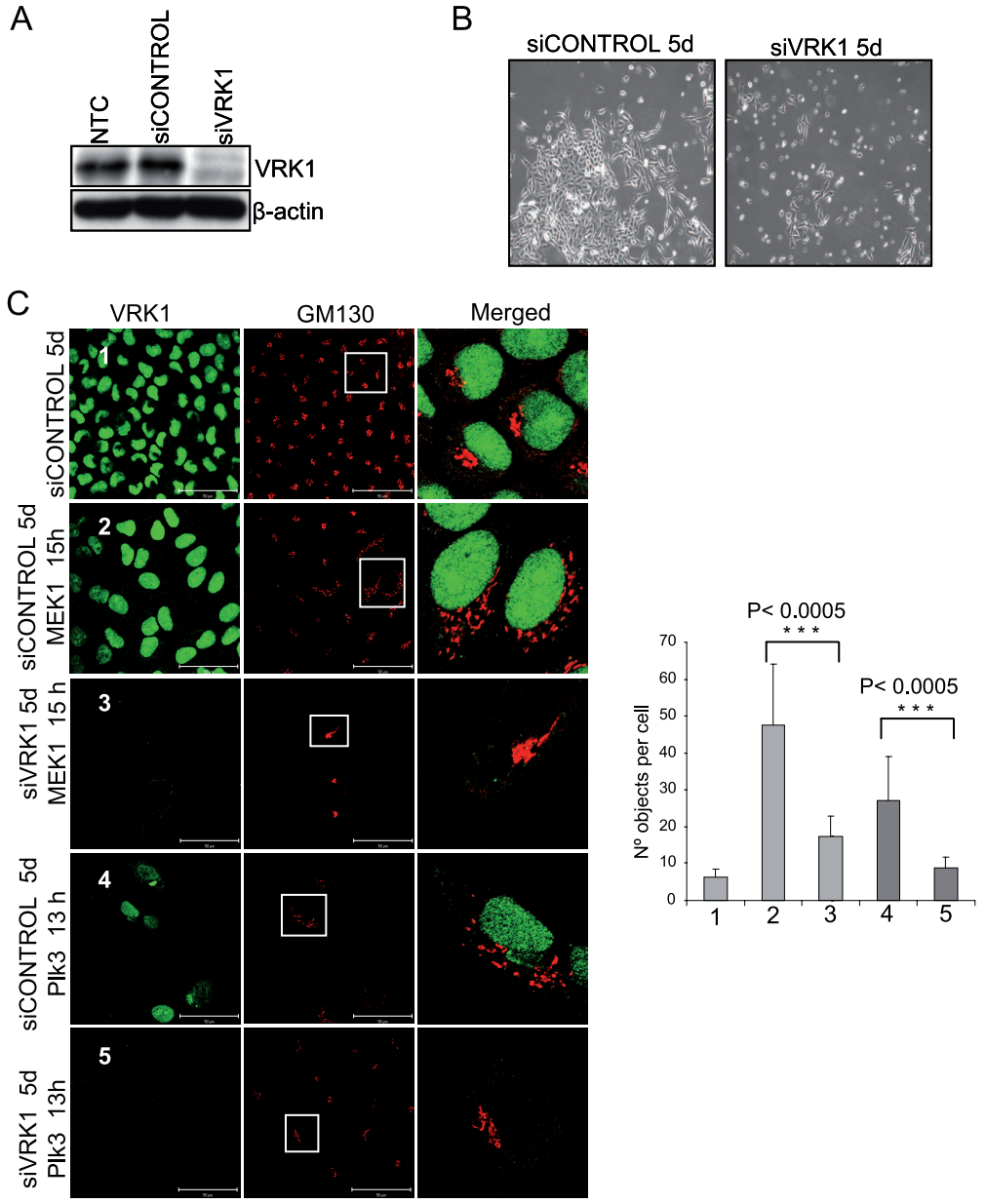

FIG. 8. Knockdown of VRK1 blocks the Golgi fragmentation phenotype induced by either MEK1 or Plk3. (A) Levels of VRK1 endogenous protein in nontransfected cells or cells transfected with siRNA control or with siRNA specific for VRK1 were determined after 5 days. NTC, nontransfected cells. (B) Photograph of HeLa cells transfected with siControl (left) or siVRK1 (right) for 5 days. (C) Immunofluorescence of endogenous VRK1 protein detected with VC polyclonal antibody. The Golgi marker GM130 was used to detect the fragmentation of the Golgi apparatus. Cells were treated with the indicated siControl or specific siVRK1 as well as with MEK1 or Plk3 as an inducer of Golgi fragmentation, as indicated in each row. Five days after siRNA treatment, cells were retransfected with 3 μg of constitutively active $MEK1^{(S218/222E,\ \Delta 32-51)}$ or 3 μg of Plk3. Fifteen or 13 h after retransfection, cells were fixed and stained with polyclonal VC1 antibody to VRK1 (green) and a specific monoclonal antibody to GM130 (red). Bar, 50 μm. Transfected cells were identified by GFP or by an anti-tag antibody. The number of intracellular particles (objects) in transfected cells was analyzed by counting them using the ImageJ program (http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The mean values with their standard deviations are shown in the bar graph. A minimum of 20 cells were counted in each case.

subpopulation of the intracellular pool of each individual kinase that is partly localized in the Golgi apparatus. Golgi fragmentation is a stage in the process of cell division that requires protein phosphorylation during G_2/M (10), and this effect is partly mediated by Plk3 (28, 42) and contributes to Golgi fragmentation in G_2/M (15). VRK1 is also located in the Golgi apparatus, colocalizing with giantin (37), like a subpopulation of MEK1 and Plk3. VRK1 by itself does not induce Golgi fragmentation; it needs activation by either MEK1 or Plk3, and its effect on Golgi fragmentation was lost when the residue in VRK1 targeted by Plk3, Ser342, was mutated to alanine, indicating that the effect is signal specific and that the

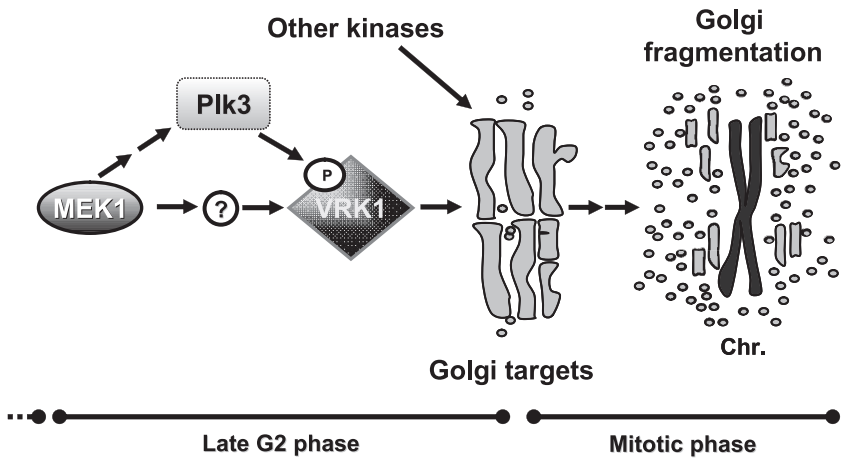

FIG. 9. Diagram illustrating the situation of VRK1 in the pathway controlling Golgi fragmentation.

substrate specificity of VRK1 can be regulated in a specific manner.

A next step in the characterization of this pathway might be the identification of VRK1 targets in the Golgi fragmentation process, considering that VRK1 represents a new step in this cascade. Among the candidates are likely to be components of the microtubule system, but this possibility will need to be tested. The loss of VRK1 blocks fragmentation induced by MEK1 and Plk3, thus functionally placing it in the same signaling route as Plk3 (15, 18). The requirement of VRK1 for fragmentation in the G_2/M phase indicates that VRK1 loss will also contribute to the regulation of cell cycle progression, indicating that VRK1 has additional roles in the cell cycle apart from those already known, such as its requirement for entry into the G_1 phase and its being an early gene such as c-*MYC* and *FOS* (36). In addition, its knockdown by siRNA results in a block of proliferation (36, 40). VRK1 is located at the end of a signaling pathway formed by MEK-Plk3-VRK1, but the connection with its activation signals and downstream targets inducing fragmentation are issues that will require further studies.

In this work, it was shown that VRK1, already known to be located partly in the Golgi apparatus, colocalized with giantin (37), is able to participate in the process of Golgi fragmentation by interacting with Plk3, for which it is a downstream phosphorylation target that is required to induce the fragmentation of the Golgi apparatus (Fig. 9). VRK1 was detected as being regulated in the cell cycle, where it plays a role early in G_0 exit-G_1 (36), but its requirement for progression of cell division suggested that it might have additional roles late in G_2/M. This contribution of VRK1 to a step required in cell division, in addition to its requirement for cell cycle progression, further strengthens the possibility of its use as a potential target for tumor therapies based on inhibition of cell proliferation.

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