Ki-1/57 Interacts with RACK1 and Is a Substrate for the Phosphorylation by Phorbol 12-Myristate 13-Acetate-activated Protein Kinase C*

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Ki-1/57, the 57-kDa human protein antigen recognized by the CD30 antibody Ki-1, is a cytoplasmic and nuclear protein that is phosphorylated on serine and threonine residues. When isolated from the Hodgkin’s lymphoma analogous cell line L540 Ki-1/57 co-immunoprecipitated with a Thr/Ser protein kinase activity. It has been also found to interact with hyaluronic acid and has therefore been termed intracellular IHABP4 (hyaluronan-binding protein 4). Recent studies demonstrated, however, that Ki-1/57 engages in specific interaction with the chromo-helicase-DNA-binding domain protein 3, a nuclear protein involved in chromatin remodeling and transcription regulation. We used the yeast two-hybrid system to find proteins interacting with Ki-1/57 and identified the adaptor protein RACK1 (receptor of activated kinase 1). Next, we confirmed this interaction in vitro and in vivo, performed detailed mapping studies of the interaction sites of Ki-1/57 and RACK-1, and demonstrated that Ki-1/57 also co-precipitates with protein kinase C (PKC) when isolated from phorbol 12-myristate 13-acetate (PMA)-activated L540 tumor cells and is a substrate for PKC phosphorylation in vitro and in vivo. Interestingly, the interaction of Ki-1/57 with RACK1 is abolished upon activation of L540 cells with PMA, which results in the phosphorylation of Ki-1/57 and its exit from the nucleus. Taken together, our data suggest that Ki-1/57 forms a stable complex with RACK1 in unstimulated cells and upon PMA stimulation gets phosphorylated on threonine residues located at its extreme C terminus. These events associate Ki-1/57 with the RACK1/PKC pathway and may be important for the regulation of its cellular functions.

The first monoclonal antibody that specifically detected the malignant Hodgkin’s and Sternberg-Reed cells in Hodgkin’s lymphoma was called Ki-1 and binds to the 120-kDa lymphocyte co-stimulatory molecule CD30 (Ki-1/120) on the surface of the Hodgkin’s cells (1, 2). It has however been noticed early on that this antibody also cross-reacts with an intracellular phosphoprotein antigen of 57 kDa termed Ki-1/57 (3, 4). In vitro phosphorylation experiments performed with the Ki-1/57 antigen isolated from tumor cells demonstrated that it is associated with a serine/threonine protein kinase activity (5). Electron microscopic analysis showed that the Ki-1/57 antigen is located in the cytoplasm, at the nuclear pores, and in the nucleus, where it is frequently found in association with the nucleolus and other nuclear bodies (6). Tryptic digestion of the Ki-1/57 antigen resulted in the cloning of a partial cDNA encoding Ki-1/57 (7). The isolated contig of 1380 bp length encodes the C-terminal 60% of the Ki-1/57 protein. Later, another group cloned the full-length Ki-1/57 cDNA (8). Huang et al. (8) found that Ki-1/57 has a hyaluronan binding activity and gave it the second name, intracellular hyaluronan-binding protein 4 (IHABP4). They also found that IHABP4/Ki-1/57 binds to other negatively charged glycosaminoglycans like chondroitin sulfate, heparane sulfate, and RNA, although with lower affinity. The functional meaning of Ki-1/57 interaction with these macromolecules remains open.

When we were searching the sequence data bank for Ki-1/57 related molecules, we found the human protein CGI-55, which amino acid sequence has 40.7% identity and 67.4% similarity with that of Ki-1/57 (9). This high degree of similarity suggests that both proteins might be paralogues and may have related functions. CGI-55 has also been described to bind to the 3’-region of the mRNA encoding the plasminogen activator inhibitor (PAI) type 1 (10). Heaton et al. (10) have therefore termed CGI-55 as PAI RNA-binding protein 1 and suggested that it could be involved in the regulation of the stability of the PAI mRNA, although they do not provide experimental data to support this hypothesis.

We explored the yeast two-hybrid system to identify possible interacting proteins for both Ki-1/57 and CGI-55 and in this way obtain clues for the functional context of these proteins. Our analysis resulted in the identification of the human protein chromo-helicase-DNA-binding domain protein 3 (CHD3) as a partner for both proteins (9). The CHD proteins are members of the chromo domain family, a class of proteins that are involved in transcriptional regulation and chromatin remodeling (11–17). The binding of the proteins Ki-1/57 and CGI-55 to CHD3 might define them as a family of CHD3-binding proteins and suggested the possibility that they could be involved in nuclear functions associated with the remodeling of chromatin and the regulation of transcription. Whereas in the case of the...
CGI-55, 42% of the found interacting clones represented CHD3, only 4% of the clones interacting with Ki-1/57 represented CHD3 (9).

Here we report that the vast majority of clones (54%) found to interact with Ki-1/57 represent the scaffold and regulatory protein RACK-1 (receptor of activated kinase 1), a protein that we did not identify in the interaction screen of the putative Ki-1/57 parologue CGI-55. RACK1 has a molecular mass of 36 kDa and is composed of seven WD repeats (18, 19). Its overall structure resembles that of the subunit of G proteins (20, 21).

RACK1 has been reported to interact with PKCζ (22–24); Src (25); β-integrins (26); PDE4D5 (27); the β-subunit of the granulocyte-macrophage colony-stimulating factor, interleukin 3, and interleukin 5 receptors (28); 413) as a C-terminal fusion to Saccharomyces cerevisiae pBTM116-Ki-1/57(122–terminus of LexA DNA-binding domain peptide in pBTM-116 (40) vector was used to express a fragment of the protein Ki-1/57 linked to the C terminus of the β-galactosidase activity test, plasmid DNA extraction, and sequencing were performed as described previously (9).

The Yeast Two-hybrid Assay, Western Blot Analysis, and Cell Culture—GST or GST-Ki-1/57 fusion proteins were co-immunoprecipitated with His6-tagged and affinity-purified proteins (Protein A-Sepharose beads (Amersham Biosciences)) or Ni-NTA-Sepharose as described before (44).

Western blot Analysis—His6-RACK1, and deletion constructs of Ki-1/57 were phosphorylated in complete kinase buffer (25 mM Tris, pH 7.5, 157 mM NaCl, 2.7 mM KCl, 1% Triton X-100, protease inhibitors). The lysates were treated with DNase (Promega) and cleared at 14,000 x g for 30 min. Next 20 μl of protein A-Sepharose beads (Amersham Biosciences) were loaded with the indicated antibodies overnight at 4 °C, washed in buffer NaCl/Tris, and incubated with the L540 lysate overnight at 4 °C. After further three washes with the buffer NaCl/Tris/EDTA (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM NaCl), the beads were resuspended in SDS-PAGE loading buffer, boiled, and analyzed by SDS-PAGE and Western blot using the indicated antibodies. Western blots were developed by chemiluminescence as described previously (44).

The Yeast Two-hybrid Assay and Phosphoamino Acid Analysis—5 x 10^5 L540 cells were treated or not with PMA, collected, and lysed as described previously. Endogenous PKC was immunoprecipitated from these lysates with anti-phospho-PKC-Pan antibody (Cell Signaling) coupled to protein A-Sepharose beads. No these beads were incubated with PKC-GST-Ki-1/57, His6-RACK1, both, or GST in kinase buffer (25 mM Tris, pH 7.5, 1.32 mM CaCl2, 5 mM MgCl2, 1 mM EDTA, 1.25 EGTA, 1 mM dithiothreitol) containing 10 mM PMA, 5 μM ATP, and 0.5 μCi of [γ-32P]ATP, in a total volume of 25 μl for 30 min at 30 °C. Phosphorylated proteins were run out by SDS-PAGE. The gel was stained, dried, and exposed to x-ray film. In other experiments purified GST, GST-Ki-1/57, His6-RACK1, and deletion constructs of Ki-1/57 were phosphorylated in complete kinase buffer in a final volume of 50 μl at 30 °C with purified PKC-Pan, PKCζ, or PKCθ for 15 min. The PKCζ or PKCθ was human recombinant His-tagged and affinity-purified proteins (Promega). PKC-Pan was purified from rat brain and consists predominantly of the PKC isofoms α, β, and γ (Promega). Radioactively labeled proteins were visualized and dehydrated as described above.

Phosphoamino acid analysis was basically performed as described in Machado et al. (47). Briefly, the [32P]-radioabeled phosphorylated proteins were hydrolyzed with 6 N HCl for 60 min at 90 °C. The hydrolysates were lyophilized, dissolved in water, and spotted onto Sigma cell type 11000 paper and dried in a stream of warm air. The dried hydrolysates were sequenced by the software NetPhos 2.0 Prediction server available at the website of the Center for Biological Sequence Analysis (www.cbs.dtu.dk/services/NetPhos).

Mitochondrial Labeling, in Vivo Phosphorylation Assay, and Kinase Inhibitors—5 x 10^5 L540 cells were preincubated or not for 1 h with...
protein kinase inhibitors: Bs-32-0422 (28 nm), and staurosporine (0.7 nm) (Calbiochem). This inhibitor incubation was performed with phosphate-free L540 standard medium (the fetal calf serum in this medium had been dialyzed against a 150 mM NaCl solution). Next the cells were activated or not by the addition of 100 ng/ml of PMA for a second hour. In parallel to the PMA treatment, the cells were metabolically labeled by the addition of 0.4 mCi of radioactive 32P-labeled inorganic phosphate (Amersham Biosciences). After lysis Ki-1/57 was immunoprecipitated from the lysates of the metabolically labeled L540 cells with anti-Ki-1 antibody A26 coupled to protein A-Sepharose beads and analyzed by autoradiography and SDS-PAGE.

Preparation of Cytoplasmic and Nuclear Cell Fractions—L540 cells were harvested and incubated with 300 μl of hypotonic buffer A (10 mM Tris, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM dithiothreitol, 25% v/v glycerol) followed by incubation on ice for 30 min. After centrifugation, the fractions were incubated with the antibodies at 4°C on ice for 30 min. After centrifugation, the fractions were incubated with the antibodies at 4°C overnight. On the next day 20 μl of protein A-Sepharose were added for 2 h.

Immunofluorescence Analysis—HeLa cells grown on glass coverslips were stimulated or not with PMA for 4 h at 37°C. The cells were fixed with 100% methanol and immunostained with primary antibody monoclonal mouse Ki-1, mouse anti-RACK1, or rabbit anti-Phospho-PKC, and secondary antibody fluorescein anti-mouse or rhodamine anti-rabbit antibody. The cells were examined with a Nikon microscope. DAPI staining was used to show the positions of the nuclei. The cells were examined with Nikon fluorescence microscope. Immunolabeled proteins were presented with the respective color. Superimposing the two colors (merge) results in a yellow/orange signal.

RESULTS

Yeast Two-hybrid Screen—To identify Ki-1/57 interacting proteins, the yeast two-hybrid system (40–43) was employed, utilizing a human fetal brain cDNA library (Clontech). In a first screen we used a fragment of the Ki-1/57 cDNA that encodes its C-terminal 60% as a bait. 2.0 x 10^9 screened co-transformants yielded 250 clones positive for both His3 and LacZ reporter constructs. Library plasmids DNA of 80 clones transformants yielded 250 clones positive for both His3 and LacZ (Fig. 1A). The two hybrid screen Ki-1/57(1–150) and Ki-1/57(151–263) failed to bind to RACK1. Full-length Ki-1/57, the C-terminal construct used in the two hybrid screen Ki-1/57(122–413) as well as the C-terminal deletion Ki-1/57(264–413) all interacted with RACK1. Another protein identified was CHD3, which had already been described previously elsewhere (9) and represented 4% of the interacting clones. Other nuclear proteins involved in the regulation of transcription have also been identified but will be described elsewhere.

Mapping the Interaction Sites of Ki-1/57 and RACK1—Next, we mapped the Ki-1/57 region required for the interaction with RACK1 using the yeast two-hybrid method (Fig. 1). N- and C-terminal deletion constructs of the Ki-1/57 protein were fused to the LexA DNA-binding domain (Fig. 1A) and tested for their ability to bind full-length RACK1 (Fig. 1B). The two constructs that encompass the N-terminal and central regions: Ki-1/57(1–150) and Ki-1/57(151–263) failed to bind to RACK1. Full-length Ki-1/57, the C-terminal construct used in the two hybrid screen Ki-1/57(122–413) as well as the C-terminal deletion Ki-1/57(264–413) all interacted with RACK1. This suggests that the RACK1-binding site is located at the Ki-1/57 C terminus. The co-transformation of pBTM116-Ki-1/57 with several unrelated “bait” constructions, including pACT2-AUF1 (44) (not shown) and with empty pBTM116 vector (Fig. 1B), showed no interaction.

Furthermore, we mapped the RACK1 regions that are required for the interaction with Ki-1/57, N- and C-terminal deletion constructs of the RACK1 protein were fused to the Gal4 activation domain (vector pACT2; Fig. 1C) and tested for their ability to bind to full-length Ki-1/57 (Fig. 1D). None of the four different deletion constructs of RACK1 interacted with Ki-1/57. This shows that full-length RACK1 is required for an interaction with Ki-1/57.

In Vitro Confirmation of the Ki-1/57-RACK1 Interaction with Purified Fusion Proteins—To confirm the interaction between Ki-1/57 and RACK1 in vitro, we next performed in vitro pull-down assays with purified recombinant proteins that had been expressed in E. coli (GST, GST-Ki-1/57, and His6-RACK1) and purified by affinity chromatography. GST-Ki-1/57 bound specifically to RACK1, whereas the control protein GST did not (Fig. 2A, left panel). We controlled the equal loading of the glutathione beads with GST or GST-Ki-1/57 fusion protein by developing the same membrane with an anti-GST monoclonal antibody (Fig. 2A, right panel).

Co-precipitation of Ki-1/57 with His6-RACK1 from a Lysate of L540 Cells—When a lysate of L540 cells was incubated with His6-RACK1-loaded Ni-NTA-Sepharose beads, Ki-1/57 could be specifically pulled down (Fig. 2B, left panel). On the other hand, when Ni-NTA-Sepharose beads were loaded with control proteins such as the nonrelated protein His6-FEZ1 (49) or the Ki-1/57 homologue protein CGI-55 (9), no co-precipitated Ki-1/57 band could be detected in the Western blot. We obtained a corresponding result when we used glutathione-Sepharose control beads loaded with GST-Ki-1/57 (Fig. 2C, left panel). RACK1 was only pulled down from the lysate of L540 cells with the GST-Ki-1/57 but not with the GST-loaded beads. Both Western blot experiments were checked by detecting the input proteins. These were run out on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and visualized by anti-His6 monoclonal antibody or anti-GST antibody, depending on the nature of the fusion part (Fig. 2, right panels).

In Vitro Phosphorylation of Ki-1/57 by PKC Isolated from PMA-stimulated L540 Cells—Early experiments with the Ki-1/57 antigen had demonstrated that the Ki-1 antibody immunoprecipitated an serine/threonine protein kinase activity that had been initially attributed to the Ki-1/57 antigen (5). The cloning of the cDNA encoding Ki-1/57 revealed, however, that the Ki-1/57 sequence does not encode a kinase domain (7, 8). Further experiments with the full-length recombinant Ki-1/57 protein also did not reveal a kinase activity of the Ki-1/57 protein toward itself or other proteins (data not shown). Our finding that Ki-1/57 strongly interacts with the protein RACK1 in the yeast two-hybrid system immediately suggested a hypothesis for an alternative explanation for the co-immunoprecipitation of the kinase activity with Ki-1/57. RACK1 is an receptor for activated protein kinase C and interacts in vitro and in vivo with activated PKC. Therefore, the observed kinase activity in the Ki-1 immunoprecipitate might be PKC associated to RACK1 and/or to Ki-1/57. To test this hypothesis, we first analyzed whether Ki-1/57 can be a substrate for the phosphorylation by PKC immunoprecipitated from the lysate of nonstimulated and PMA-stimulated L540 cells (Fig. 3A, left panel). The GST-Ki-1/57 fusion protein used in this assay was only weakly phosphorylated by the PKC that had been immunoprecipitated from the lysate of nonstimulated cells by an anti-Phospho-Pan-PKC antibody (Fig. 3A, left panel, second lane). It was, however, strongly phosphorylated by the PKC isolated from the lysate of PMA-activated L540 cells (Fig. 3A, fifth lane). RACK1 itself did not suffer phosphorylation by PKC under these conditions (Fig. 3A, left panel, first and fourth lanes), nor did its presence influence the extend of phosphorylation of Ki-1/57 (Fig. 3A, left panel, third and sixth lanes). The right panel of Fig. 3A demonstrates the equal protein loading of the different lanes. GST control protein was not phosphorylated by PKC (Fig. 3B, upper panel, first lane).

In a similar approach we next wanted to know whether PKC isoforms have a differential phosphorylation activity toward Ki-1/57 and tested therefore a panel of monoclonal antibodies against different PKC subtypes isolated from PMA-activated...
L540 cells (Fig. 3B, upper panel). The PKCs had been immunoprecipitated by anti-phospho-PKC antibodies or as indicated in Fig. 3B. We found a strong phosphorylation of Ki-1/57 by PKCαβ, PKCδ, PKCα/γ, and especially by PKCθ, however not by PKCμ. These data show that Ki-1/57 can serve in principal as a substrate for a wide variety of different PKC isoforms but also that its phosphorylation is strongest with PKCθ. The equal loading of the different lanes is demonstrated by the control SDS-PAGE shown at the bottom panel of Fig. 3B.

We also performed phosphorylation experiments with commercial purified PKC-Pan and found a strong phosphorylation of Ki-1/57, which was neither promoted nor inhibited by the presence of His6-RACK1 protein in equal amounts or slight excess (Fig. 3C).

Ki-1/57 Interaction with RACK1 Is Abrogated by Its Phosphorylation or by the PMA Activation of the Cell in Vivo—When we performed the pull-down experiments of RACK1 with a GST-Ki-1/57 protein that we had previously submitted to in vitro phosphorylation with PKC-Pan, we observed a complete abrogation of the interaction (Fig. 4A, left panel, lane 3). Such a down-regulation of the interaction by the phosphorylation of Ki-1/57 might be functionally relevant and could serve to down-regulate the adaptor functions of RACK1 once that PKC has phosphorylated Ki-1/57 in vivo. Therefore we tested whether this effect can also be observed in vivo. We found that His6-RACK1 fusion protein coupled to Ni-NTA-Sepharose beads co-sediments an approximately three times smaller quantity of Ki-1/57 from lysates of PMA stimulated then from unstimulated L540 cells (not shown). This indicates that the phosphorylation of Ki-1/57 in vivo also diminishes its capacity to interact with external recombinant RACK1.

Ki-1/57 Is Only Phosphorylated on Its Extreme C Terminus (Residues 346–413)—We now expressed several deletion constructs of the Ki-1/57 protein and submitted the purified recombinant proteins to in vitro phosphorylation experiments to determine the regions of Ki-1/57 that are a target for the phosphorylation by PKC (Fig. 4B). From these studies it became clear that neither the N-terminal region (1–150) nor the middle region of Ki-1/57 (151–263) but only its C-terminal region (both 264–413 and 122–413) are phosphorylated by PKC-Pan (Fig. 4B, left panel). Recombinant PKCθ and PKCζ gave the same results as purified PKC-Pan (not shown). The loading
of the in vitro phosphorylation reaction with equal amounts of recombinant protein fragments is shown in Fig. 4B (right panel). These results suggested performance of a more detailed deletional analysis of the C terminus of Ki-1/57, because this region contains 15 Ser/Thr residues (Fig. 5A), all of which could be target residues of phosphorylation by PKC. Therefore, we generated the indicated subdeletions (Fig. 5A) of the fragment Ki-1/57/264–413) and expressed them in bacteria as GST fusions proteins for in vitro studies and in the yeast as LexA fusion proteins to be able to assess their capacity to still interact with RACK1. Two of the constructs, Ki-1/57/294–413) and Ki-1/57/346–413) were still able to interact with RACK1 (Fig. 5B). Most interestingly, these same two constructs, when expressed in fusion with GST and used as substrates in the in vitro phosphorylation assays with PKC-Pan, were the only two of the five tested subdeletions that could be phosphorylated (Fig. 5C, left panel). In vitro phosphorylations of these five fragments with PKCα and PKCζ gave the same result (not shown). Fig. 5C (right panel) shows equal loading of the reactions with proteins or control protein GST, which was not phosphorylated.

**Ki-1/57 Phosphorylation Can Be Blocked by Protein Kinase Inhibitors in Vitro and in Vivo**—To gather further evidence that the kinase that phosphorylates Ki-1/57 is PKC, we tested a series of protein kinase and PKC inhibitors for their potential to block Ki-1/57 phosphorylation in vitro (Fig. 4C) and in vivo (Fig. 4D). We found that the general kinase inhibitor staurosporine and the PKC-specific inhibitor Ro-32-0432 were the most effective inhibitors of the phosphorylation of His<sub>6</sub>-Ki-1/57 (264–413) by PKC-Pan (Fig. 4C, lanes 7 and 8), PKCζ, and PKCθ (not shown) in vitro. There was no difference in the inhibition profile for the three PKCs tested. We then tested the best two inhibitors in vitro and found that only Ro-32-0432 but not staurosporine (at the tested relative low concentration) can inhibit the phosphorylation of Ki-1/57 in vitro (Fig. 4D, lanes 3 and 4). These data support the hypothesis that Ki-1/57 is also a substrate for PKC phosphorylation in vivo. A comparison of lanes 1 and 2 of Fig. 4D demonstrates the increased phosphorylation of Ki-1/57 after the stimulation of the L540 cells with PMA and in the absence of inhibitor. The equal loading of the lanes with immunoprecipitated Ki-1/57 is shown in the lower panel of Fig. 4D.

**Ki-1/57 Is Phosphorylated at Two Threonine Residues Located at Its Extreme C Terminus (Residues 346–413)**—Our phoshoamino acid analysis confirmed previously published data (5) that Ki-1/57(264–413) is phosphorylated mainly on threonine residues. PKCζ phosphorylated Ki-1/57 (264–413) strongly on threonine but also on serine residues (Fig. 5D), whereas PKC-Pan phosphorylates this fragment on threonine only.
The extreme C-terminal fragment Ki-1/57(346–413) in fusion with GST is only phosphorylated on threonine using PKC-Pan. PKCθ and PKCζ also phosphorylated this fragment only on threonine, but the degree of phosphorylation was lower (not shown). This suggests that the two threonine residues present in this fragment (Thr354 and Thr375) might be the main target residues for phosphorylation by PKC in vitro. This also demonstrates that the phosphorylation of Ki-1/57 by PKC is highly specific, considering that there are 34 Ser/Thr residues in the whole amino acid sequence of Ki-1/57, and apparently only the two most C-terminal threonines are targets of phosphorylation in vitro. The reaction was controlled with free GST protein, which itself does not suffer phosphorylation by the three PKCs tested (not shown; see also Fig. 5). Ki-1/57 Interacts with RACK1 in Vivo Only before PMA Activation and with PKC Afterward—Next we wanted to test whether Ki-1/57 engages also in interaction with RACK1 and PKC in human cells. To assess the subcellular localization of the co-immunoprecipitated proteins, we analyzed the cytoplasmic and nuclear compartments of the L540 cells separately. When we immunoprecipitated RACK1 from the lysate of L540 cells, we detected the 57-kDa band of co-immunoprecipitated Ki-1/57 in the Western blot developed with A26 antibody only in the nuclear compartment of the cell but both before and after cell stimulation with PMA (Fig. 6, lanes 25 and 26). There was a slight decrease in the co-precipitated amount of Ki-1/57 after the addition of PMA.

When we immunoprecipitated Ki-1/57 we also co-immunoprecipitated RACK1 but only from the nucleus and in the absence of PMA (Fig. 6, lane 16). These results suggest that Ki-1/57 and RACK1 form a stable complex in human L540 cells, until the cells are activated by PMA. Interestingly, we also detected co-immunoprecipitated Ki-1/57 when we used antiphospho-PKCθ (and anti-phospho-PKCζ, not shown) antibody in the immunoprecipitation, in the nucleus and a little less in the cytoplasm, but only after PMA activation (Fig. 6, lanes 30 and 32). Anti-phospho-PKCaβII did not co-immunoprecipitate Ki-1/57, neither with or without PMA treatment (not shown).

Ki-1/57 Exits the Nucleus upon PMA Activation—Ki-1/57 is located in the cytoplasm and the nucleus of cells (6) and interacts with nuclear proteins involved in the regulation of transcription and the remodeling of chromatin such as CHD3 (9). Therefore, we were interested to know whether its localization to the nucleus or that of its interacting proteins RACK1 and PKC is affected by the cell stimulation with the PKC activator PMA and whether the interaction of RACK1 and Ki-1/57 is affected by PMA. First, we stimulated L540 cells with PMA or not and then fractionated the cellular lysates into cytoplasmic and nuclear compartments.
FIG. 4. The interaction between Ki-1/57 and RACK1 in vitro is abolished by the phosphorylation of Ki-1/57. A, left panel, GST-Ki-1/57 in vitro phosphorylated by purified PKC-Pan no longer co-precipitates RACK1 in vitro. Right panel, Western blot (WB) anti-GST for demonstration of equal protein loading of the lanes. B, in vitro phosphorylation of different deletion constructs of Ki-1/57 by recombinant PKCα. All of the indicated fragments of Ki-1/57 were expressed as fusion proteins with an N-terminal His6 tag and had been purified by affinity chromatography. Left panel, autoradiography of 32P-labeled proteins (AR). Right panel, control SDS-PAGE of loaded proteins. C, in vitro inhibition of His6-Ki-1/57 (264–413) phosphorylation by PKC-Pan by using a panel of different kinase inhibitors. Upper panel, autoradiography after incubation of PKC-Pan and GST-Ki-1/57 without or with the indicated protein kinase inhibitors: Bisindoylmaleimide I (lane B, 10 nM), calphostin C (lane Ca, 50 nM), chelerythrine chloride (lane C, 560 nM), Go 6976 (lane Go, 6.2 nM), inhibitor 19-27 (lane I9, 8 μM), Ro-32-0432 (lane Ro, 28 nM), and staurosporine (lane St, 0.7 nM). Lower panel, SDS-PAGE for demonstration of equal loading with protein. D, in vivo inhibition of the phosphorylation of Ki-1/57 in L540 cells treated or not with PMA for 1 h and incubated with or without the protein kinase inhibitors Ro-32-0432 (Ro) and staurosporine (St). Ki-1/57 was immunoprecipitated from the nuclear fraction. Upper panel, AR. Lower panel, control SDS-PAGE to demonstrate equal loading with immunoprecipitate.

and nuclear fractions, from which we then immunoprecipitated Ki-1/57, RACK1, and Phospho-PKC (Fig. 6). When immunoprecipitated by antibody A26, the nuclear fraction of Ki-1/57 is no longer detectable after 4 h of PMA stimulation of the cells (Fig. 6, lanes 35 and 36), whereas there was no alteration in the amounts of cytoplasmic Ki-1/57. When immunoprecipitated with antibody E203, this decrease of the amount of nuclear Ki-1/57 could also be noticed, albeit to a lesser degree in comparison with A26 (Fig. 6, lanes 39 and 40). There is slightly more RACK1 in the cytoplasmic fraction after PMA stimulation (Fig. 6, lanes 1 and 2), whereas the nuclear fraction is unaffected by the PMA stimulation (Fig. 6, lanes 3 and 4). The amount of RACK1 co-immunoprecipitated with phospho-PKC-Pan is increased both in the cytoplasm as well as in the nucleus, whereas RACK1 that co-immunoprecipitated with PKCαII was only detectable in the cytoplasm after PMA stimulation (Fig. 6, lane 10).

The fact that Ki-1/57 is only associated with RACK1 in the nucleus and in the absence of cell stimulator PMA (Fig. 6, lanes 15 and 16) suggests that the interaction of Ki-1/57 with RACK1 is only stable under unstimulated conditions but is abrogated after the activation with PMA. These experiments were controlled with an antibody against the nonrelated protein Ki-67 (46), which co-immunoprecipitates neither RACK1 (Fig. 6, lanes 17–20) nor Ki-1/57 (Fig. 6, lanes 41–44).

We next tested whether the observed disappearance of Ki-1/57 from the nucleus (Fig. 6, lanes 36 and 40) can be seen by immunofluorescence localization studies in human HeLa cells (Fig. 7). We observed that both RACK1 (Fig 7C) and Ki-1/57 (Fig 7, A and B) exit the nucleus upon PMA activation. The exit of Ki-1/57 from the nucleus is accompanied by that of phospho-PKCαII and phospho-PKCζ/λ (Fig. 7), and that of RACK1 is accompanied by the exit of phospho-PKCaβII. These experiments were repeated with L540 cells and essentially gave the same results (not shown).

DISCUSSION

To find a functional context for the protein Ki-1/57 we set out to perform a yeast two-hybrid screen to identify possible interacting protein partners. Screens of a human fetal brain cDNA library with both Ki-1/57 and with its homologue protein CGI-55 previously identified the chromatin remodeling factor CHD3 (9). This was the first report that described a specific protein-protein interaction for both CGI-55 and Ki-1/57 and could
define them as a new family of CHD3 interacting proteins. The majority (54%) of clones found to interact with Ki-1/57, however, represent the signaling adapter molecule RACK1.

The interactions between Ki-1/57 and RACK1 were confirmed \textit{in vitro} and \textit{in vivo} by co-precipitation assays from L540 Hodgkin’s disease analogous cells. Because RACK1 has been described previously to be an adapter protein for activated protein kinases C and helps to maintain PKC in an activated
state, we tested whether Ki-1/57 also interacts with cellular PKC and whether it represents a target molecule for PKC phosphorylation. Our phosphorylation assays with PKC show that Ki-1/57 is a substrate for PKC isolated from PMA-activated but not from nonactivated control L540 cells. Immuno-precipitation of PKC from PMA-stimulated but not from unstimulated L540 cells showed co-immunoprecipitated Ki-1/57 protein, thereby demonstrating that PKC interacts with Ki-1/57 after cell activation. The interaction of Ki-1/57 with RACK1, however, was abolished after PMA stimulation, suggesting that this interaction is regulated. Together these results might suggest a hypothesis for a sequential mode of interactions between the three molecules Ki-1/57, RACK1, and PKC: (i) before PMA activation Ki-1/57 is firmly attached to RACK1, this interaction occurs mainly in the nucleus; (ii) after PMA activation Ki-1/57 gets phosphorylated, this results in the abrogation of the interaction with RACK1; and (iii) the newly created phosphoamino acid groups in the C terminus of Ki-1/57 could now serve as docking sites for the interaction of kinases or other proteines with Ki-1/57.

It had been shown previously that different proteins that interact with RACK1 interact with different docking sites involving one or more of the seven individual blades of the putative propeller structure of RACK (18). In case of the protein Src, the smallest unit of RACK1 that was capable of an interaction consists of only a single WD repeat blade (50). The binding of the interferon receptor on the other hand involves five of the seven blades (30). Therefore, we set out to map the regions of RACK1 involved in the interaction with Ki-1/57 and found that none of our constructed deletions but only full-length RACK1 was able to engage in protein-protein interaction with Ki-1/57. This is corroborated by the fact that all of the clones we identified in the yeast two-hybrid screen contained the full-length RACK1 coding region. Ki-1/57 seems therefore to be the first protein found that only interacts with full-length RACK1. The mapping of the interaction site of Ki-1/57 on the other hand demonstrated that its extreme C terminus (amino acids 346–413) is fully capable to interact with RACK1. This suggests that Ki-1/57 might be a multi-domain protein with its C terminus containing a docking domain/motif for RACK1.

It was shown before that although RACK1 engages in protein interaction with several structurally and functionally different proteins (22–33), its interaction among proteins of the same family is highly specific (50, 27). Yarwood et al. (27) have for instance shown that RACK1 interacts with PDE4D5 but with none of the other PDE4 isoforms tested. This high degree of selectivity of the interaction holds true in the opposite direction, too. PDE4 does not interact with any of a series of WD repeat-containing proteins other than RACK1 (27). Our results confirm this trend, because none of the other proteins identified

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![Image](https://example.com/image.png)

**Fig. 6.** The nuclear association between Ki-1/57 and RACK1 is abolished upon PMA activation of the L540 cells. L540 cells were or were not stimulated for 4 h with PMA. The cytoplasmic (C) or nuclear (N) fractions of their cell lysates were immunoprecipitated (IP) with antibodies anti-RACK1, anti-phospho-PKC-Pan, anti-phospho-PKCaβ, anti-phospho-PKCθ, anti-Ki-1/57(A26 or E203), or the unrelated control antibody anti-Ki-67 (46). Ki-1/57 co-immunoprecipitates some protein bands of a molecular mass between 30 and 50 kDa, the molecular mass of which corresponds neither to Ki-1/57 (57 kDa) nor to PKC (~80 kDa) (see lanes 41–44). Co-immunoprecipitation of RACK1 (36 kDa) or Ki-1/57 (using antibody A26) was detected by specific Western blots (WB), as indicated. Black arrowheads indicate specific proteins (upper panels, RACK1; lower panels, Ki-1/57) identified by Western blot. L in the lower row indicates the loading control of the lysate to indicate the position of the input Ki-1/57 protein. A in the lower row indicates lanes that have only been loaded with antibody to demonstrate the position of the heavy and light chains of the antibody. Control experiments demonstrated that the bands marked with asterisks represent the heavy chain antibodies (~55 kDa), and the bands marked by open circles represent the light chain antibodies (~25 kDa) (not all data shown).
so far in our yeast two-hybrid analysis of the Ki-1/57 protein belongs to the WD repeat-containing family of proteins (not shown). RACK1 interacts neither in the yeast two-hybrid system nor in vitro with the Ki-1/57 homologue protein CGI-55 (not shown).

RACK1 has been described to have an activating influence...
on PKC activity and even a small peptide derived of amino acids 234–241 of WD blade 6 of RACK1 bound to PKC and activated it in vitro and in vivo (22, 46). However, in contradiction to this finding another report showed no influence of RACK1 on the kinase activity of PKC, CAMP-dependent protein kinase, or casein kinase II toward peptide substrates but an inhibitory effect of RACK1 on the autophosphorylation activity of Src and Yes and on the peptide phosphorylation activity of Src and Lck (25). These results demonstrate that it is not yet possible to conclude whether RACK1 has an overall stimulatory or inhibitory role on the kinase activity of different kinases but rather suggest that not only the type of kinase but most likely also the kind of substrate involved might be of importance. Our results with the phosphorylation of the PKC substrate Ki-1/57 in the presence of RACK1 did not show any influence of RACK1 on the outcome of the kinase reaction (Fig. 3C).

Ki-1/57 has been also described previously as an intracellular hyaluronan-binding protein (IHABP4), because of its capacity to interact with a series of negatively charged macromolecules, including hyaluronan, heparan sulfate, chondroitin sulfate, and RNA (8). According to Huang et al. (8), the binding of IHABP4/Ki-1/57 to hyaluronan depends on the presence of so-called hyaluronan binding motifs of the structure (R/K)X2(R/K). However, the majority of nuclear proteins are overproportionally rich in the positively charged amino acids Lys and Arg. Our analysis of several randomly selected nuclear proteins revealed that all of them contained several of such putative hyaluronan-binding motifs. However, some of them contained many of such putative hyaluronan-binding motifs: CHD3 (accession number NM_0012727.1) contains 49; Topors (accession number AF098300) contains 36; human polymob2 (accession number AF013956) contains 7; p53 (accession number AAH03596) contains 3; and c-Fos (accession number K00650) contains 2. This would suggest that the majority of Arg/Lys-rich nuclear proteins have the potential to interact with hyaluronan. Even Huang et al. (8) state that it remains open whether hyaluronan is indeed a natural ligand for IHABP4/Ki-1/57 (8). They speculate that because both hyaluronan and Ki-1/57 have been found in the nucleus and cytoplasm, Ki-1/57 might be involved in the regulation of hyaluronan functions (8).

Our recent studies point to other possible nuclear functions of Ki-1/57 as well as its homologue CGI-55 (9). We found that CGI-55 and Ki-1/57 interact with CHD3, a nuclear protein involved in the remodeling of chromatin and the regulation of transcription (9). Furthermore, both Ki-1/57 (6) and CGI-55 are localized in the nucleus, nucleolus, and other small nuclear bodies, and CGI-55 has been shown to co-localize to p80 coilin—localized in the nucleus, nucleolus, and other small nuclear bodies, and CGI-55 has been shown to co-localize to p80 coilin—localized in the nucleus, nucleolus, and other small nuclear bodies. These findings demonstrate that Ki-1/57 and CGI-55 have common interacting nuclear protein partners (CHD3, Daxx, and Topors) and as specific interaction partners like RACK1 for Ki-1/57 and hPc2 for CGI-55. They further point to the possibility that both Ki-1/57 and CGI-55 might be involved in nuclear functions such as the remodeling of chromatin and the regulation of transcription, like several of its interacting nuclear protein partners.

In this context our observation of the nuclear exit of Ki-1/57 after stimulation of the cells with PMA may be of functional relevance. It has been shown recently that the activity of the chromatin-remodeling factor HDAC7 is regulated by its PMA-induced export from the nucleus (51, 52). The combined PMA/ionomycin treatment mimics the T cell receptor activation, and the PMA-induced nuclear export of HDAC7 was accompanied by a drop in a HDAC7-dependent Nur77 promoter activity, which controls a luciferase reporter gene. This demonstrates how the regulated nuclear export of a protein can affect the transcriptional regulation of genes. Because Ki-1/57 has been shown to interact with CHD3, another factor involved in chromatin remodeling and transcriptional regulation, it is tempting to speculate that the PMA-dependent nuclear export of Ki-1/57 could have functional consequences for CHD3s activity. While this manuscript was in the review process we became aware of a recent publication by Ozaki et al. (53). This group had found that RACK1 interacts with the C terminus of the p53 homologue protein p73. Most interestingly, Ozaki et al. were able to demonstrate that RACK1 inhibits both p73-mediated transcription from a test promoter as well as p73-mediated apoptosis. Future experiments will address whether and how Ki-1/57 and CGI-55 are involved in the regulation of transcription and what are the exact functions of these interesting novel proteins.

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**Functional Association of Ki-1/57, RACK1, and PKC**

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Ki-1/57 Interacts with RACK1 and Is a Substrate for the Phosphorylation by Phorbol 12-Myristate 13-Acetate-activated Protein Kinase C
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