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Regulation of Protein Kinase CKII by Direct Interaction with the C-Terminal Region of p47^{phox}

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Protein kinase CKII is a Ser/Thr kinase which is involved in many proliferation-related processes in the cell. p47^{phox} is a component of the leukocyte NADPH oxidase, which is an important element of host defense against microbial infection. In this study, we demonstrate that a truncated form of the p47^{phox} lacking its N-terminal region (p47^{phox}/SH3-C), but not a truncated form of the p47^{phox} lacking its C-terminal region (p47^{phox}/N-SH3), undergoes better phosphorylation by CKII in the presence of arachidonic acid. The yeast two-hybrid test and the glutathione *S*-transferase (GST) pull-down assay showed that p47^{phox} interacts specifically with the regulatory β subunit (CKIIβ), but not with the catalytic α subunit (CKIIα) of the tetrameric CKII holoenzyme. The binding of p47^{phox} to CKIIβ requires the C-terminal region of p47^{phox} and is completely abolished by addition of spermine, indicating that a highly basic region in the C-terminal region of p47^{phox} contributes to binding to CKIIβ. In addition, p47^{phox} stimulates the catalytic activity of CKII holoenzyme; this stimulation also requires the C-terminal region of p47^{phox}. Coimmunoprecipitation experiments showed that CKII holoenzyme interacts with p47^{phox} in human neutrophils. Taken together, the present data indicate that the C-terminal region of p47^{phox} plays a significant role in the arachidonic acid-dependent phosphorylation of p47^{phox} by CKII and that the same region of p47^{phox} associates directly with CKIIβ and can modulate the catalytic activity of CKII holoenzyme.

Protein kinase CKII (CKII, formerly known as casein kinase II) is a ubiquitous and highly conserved Ser/Thr kinase, which is found in all eukaryotes examined and in various subcellular compartments (1–3). It is a calcium-independent and cyclic nucleotide-independent enzyme which utilizes ATP as well as GTP as a phosphate donor. Negatively-charged compounds such as heparin have been shown to be inhibitors of CKII, whereas polybasic compounds such as polyamines and polylysine stimulate CKII activity (4). CKII holoenzyme is a heterotetramer of two catalytic (CKIIα and/or CKIIα') and two regulatory subunits (CKIIβ). CKIIα and CKIIα' are the products of distinct genes (5, 6); the acidic inhibitory compounds act on the catalytic subunit. The regulatory role of CKIIβ is complex. Upon binding to the CKIIβ subunit, CKIIα changes catalytic activity and substrate specificity (7, 8). The stimulation of CKII activity by polybasic compounds is also mediated by CKIIβ (9). The CKIIβ subunit mediates tetramer formation by both the β-β homodimerization and α-β heterodimerization (10).

CKII catalyzes the phosphorylation of a large number of both cytoplasmic and nuclear proteins including DNA binding proteins, nuclear oncoproteins, and transcription factors, and the phosphorylation modulates the activities of these proteins either positively or negatively (1–3). Genetic analysis has demonstrated that CKII is required for cell viability and progression of the cell cycle (11, 12). The overexpression of the catalytic α subunit of CKII leads to tumorigenesis in mice overexpressing myc (13). These observations suggest that CKII plays a critical role in cell growth and proliferation; however, its complete physiological role and regulatory mechanism remain obscure.

The NADPH oxidase of phagocytes, an important element of host defense against microbial infection, catalyzes the reduction of oxygen to O_2^- using NADPH as the electron donor (14). The oxidase is dormant in resting neutrophils, but acquires catalytic activity when cells are exposed to appropriate stimuli. Oxidase activity is located in the plasma membrane; however, it is known that in resting cells the oxidase components are distributed between a membrane fraction and the cytosol. When activation takes place either in intact cells or in a cell-free system, the oxidase components

Abbreviations used: CKII, protein kinase CKII (also called casein kinase II); CKIIα, α subunit of CKII; CKIIβ, β subunit of CKII; DTT, ditiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; SH3, Src homology 3.

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$p47^{phox}$ and $p67^{phox}$, which exist in the cytosol as a ~240-kDa complex (15–17), and small guanine nucleotide-binding protein Rac2, which also participates in oxidase activation (18, 19), migrate to the membrane, where the $p47^{phox}$-$p67^{phox}$ complex associates with the membrane cytochrome b_{558} to assemble the functioning oxidase (20–23).

Previously we have demonstrated that the $p47^{phox}$ protein can be phosphorylated by CKII, and that its phosphorylation is modulated by arachidonic acid, an activator of NADPH oxidase which induces conformational changes in $p47^{phox}$ (24). Here we show that the C-terminal region of $p47^{phox}$ plays a significant role in the conformation-dependent phosphorylation of $p47^{phox}$ by CKII in the presence of arachidonic acid. In addition, we describe that $p47^{phox}$ interacts with CKIIβ *in vitro* as well as *in vivo*, and that the highly basic C-terminal region of $p47^{phox}$ mediates this interaction as well as the stimulation of the catalytic activity of CKII holoenzyme.

MATERIALS AND METHODS

Materials. *Escherichia coli* DH5α was the transformation recipient for all plasmid construction. *E. coli* BL21 (DE3) was used as the host for the expression of recombinant proteins. *Saccharomyces cerevisiae* HF7c (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 URA3::GAL4*$_{17mers(x3)}$-*CyC1*$_{TATA}$-*lacZ*) was used for the yeast two-hybrid system. Polylysine (molecular weight 4000–15,000), spermine, Nonidet P-40, Tween 20, glutathione, glutathione-agarose beads, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Other protease inhibitors were from Roche Molecular Biochemicals. [γ-^{32}P]ATP, enhanced chemiluminescence (ECL) detection system, and protein A-sepharose were from Amersham Corp. Dulbecco's modified Eagle medium and fetal bovine serum were obtained from Gibco.

CKII peptide substrate (RRREEETEEE) was synthesized using an automatic synthesizer (Model 431A, Applied Biosystems) and purified by reverse phase chromatography. Polyclonal anti-CKIIα and anti-CKIIβ antibodies were obtained from Calbiochem. Polyclonal anti-$p47^{phox}$ antibodies were raised in rabbits against a recombinant GST-$p47^{phox}$ fusion protein.

Plasmid constructions. To insert the complete open reading frame of human $p47^{phox}$ into the vectors pGADGH, the entire coding sequence of $p47^{phox}$ was PCR amplified using the following sequences: 5′ primer, 5′-CAGAATTCGATGGGGGACACCTTCATCCGT-3′ and 3′ primer, 5′-ACCAGTCGACCTCAGACGGCAGACGCCAGCTT-3′. For construction of pGADGH-$p47^{phox}$/N-SH3, 5′ primer, 5′-CAGAATTCGATGGGGGACACCTTCATCCGT-3′ and 3′ primer, 5′-ACTGGTCGACTCACCCCGACTTTTGCAGGTA-3′ were used to amplify the N-SH3 fragment (amino acid residues 1–284) from $p47^{phox}$. The *Eco*RI and *Sal*I sites are underlined. The PCR incubations were carried out 25 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 2 min. The PCR products were digested with *Eco*RI and *Sal*I, purified, and cloned into the *Eco*RI and *Sal*I sites of pGADGH. To insert the C-terminal region of $p47^{phox}$ into the vectors pGEX-1λT, the fragment containing $p47^{phox}$ residues 285-390 was PCR amplified using the following sequences: 5′ primer, 5′-CGCGGATCCCAAGACGTGTCCCAGGCCCAA-3′ and 3′ primer, CGGAATTCTCAGACGGCAGACGCCAGCTT-3′. The *Bam*HI and *Eco*RI sites are underlined. The PCR products were digested with *Bam*HI and *Eco*RI and subcloned into the *Bam*HI and *Eco*RI sites of pGEX-1λT. A reading frame of hybrid plasmids was confirmed by nucleotide sequencing.

Purification of recombinant proteins. The transformed *E. coli* BL21 containing the pGEX-1λT plasmid with an insert of the full-length $p47^{phox}$ cDNA or the truncated $p47^{phox}$ cDNA were grown, and GST fusion proteins were purified by affinity chromatography on glutathione agarose beads as described elsewhere (17). The fusion proteins were cleaved by treatment with thrombin (10 U/ml) in an elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ for 2 h at room temperature. The full-length $p47^{phox}$ and $p47^{phox}$/SH3-C were then loaded on a CM-Sepharose column equilibrated with 5 mM phosphate buffer, pH 7.0, containing 0.1 mM PMSF and eluted with a 40 ml gradient of 0–0.4 M NaCl in the same buffer. $p47^{phox}$/N-SH3 was purified using GSH-agarose beads. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

Human CKII holoenzyme was purified to homogeneity via four chromatography steps using DEAE-cellulose, phosphocellulose, heparin-agarose, and gel filtration from bacteria expressed bicistronically both CKIIα and CKIIβ as described previously (25) with some modification. Human CKIIβ was also bacterially expressed and purified to homogeneity using DEAE-cellulose, hydroxyapatite, and poly-L-lysine agarose column chromatography. The purities of the CKII holoenzyme and CKIIβ were verified by Coomassie blue staining of SDS–polyacrylamide gels and by immunodetection using CKIIα- and CKIIβ-specific antibodies.

Phosphorylation of $p47^{phox}$ by CKII. Phosphorylation of recombinant $p47^{phox}$ by CKII was performed in a reaction mixture containing 20 mM Tris–HCl, pH 7.5, containing 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 100 μM [γ-^{32}P]ATP, and 6 μg of recombinant $p47^{phox}$ in a total volume of 30 μl. In some experiments, the reaction mixtures were supplemented with arachidonic acid (90 μM). The reactions were started by the addition of purified CKII and incubated for 15 min at 30°C. The samples were then separated on 15% SDS–polyacrylamide gel. The gel was stained with Coomassie blue, dried, and subjected to autoradiography.

Yeast two hybrid assay. The *in vivo* protein-protein interaction was monitored by the expression of the HIS3 reporter gene using the two-hybrid system. The reporter strain *S. cerevisiae* HF7C was co-transformed with various combinations of hybrid plasmids containing a DNA-binding domain or a transcriptional activation domain. Transformants were plated on synthetic media lacking tryptophan and leucine. After 4 days of growth, transformants were streaked to selective media lacking tryptophan, leucine, and histidine, and incubated for 3 days at 30°C (26).

GST pull-down assays. *In vitro* binding assays were performed by incubating glutathione agarose beads coated with 500 ng of GST-fusion proteins with CKIIβ in 200 μl of SP buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3). The reaction was allowed to proceed for 1 h, rocking at 4°C. After the beads were washed three times with SP buffer, the bound proteins were eluted with an elution buffer (50 mM Tris–HCl, pH 8.0, 5 mM glutathione), denatured in 4× SDS reducing protein gel loading buffer, and resolved by SDS–polyacrylamide gel. The eluted proteins were visualized by Western blotting with anti-CKIIβ antibody.

CKII activity assay. The standard assay for phosphotransferase activity of CKII was conducted in a reaction mixture containing 20 mM Tris–HCl, pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P] ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) in a total volume of 30 μl at 30°C. The reactions were started by the addition of purified CKII or immunoprecipitates and incubated for 15 min. In some experiments, $p47^{phox}$ protein, BSA, or poly-L-lysine were added to the reaction mixtures. The reaction was stopped by the addition of TCA to a final concentration of 10% and centrifuged, and 10 μl of supernatant was applied to P-81 paper.

The paper was washed in 100 mM phosphoric acid, and the radioactivity was measured by scintillation counting.

Preparation of neutrophil cytosol. The cytosol of mature neutrophils was prepared as described previously (27). Briefly, neutrophils were obtained from normal adult human subjects by dextran sedimentation and Ficoll–Hypaque fractionation of freshly drawn citrate-anticoagulated blood. The neutrophils were suspended at a concentration of 10^8 cells/ml in a modified relaxation buffer (10 mM Pipes buffer, pH 7.3, 3.5 mM $MgCl_2$, 100 mM KCl, 3 mM NaCl), and cytosol and plasma membrane were separated by nitrogen cavitation and centrifugation through Percoll. The cytosol fraction was divided into aliquots and stored at −70°C until use.

Preparation of HeLa cell extract. HeLa cells were grown in Dulbecco's modified Eagle media supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Approximately 1×10^6 cells in 100-mm dishes were washed with ice-cold phosphate buffered saline (PBS), collected by scraping with a rubber policeman, and lysed in 100 μl of ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) by sonication. The particulate debris was removed by centrifugation at 12,000*g*. The volumes of the supernatants were adjusted for equal protein concentration.

Immunoprecipitation. Four hundred micrograms of cell extracts were diluted in 500 μl of TSNE buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1% Nonidet P-40, 5 mM EDTA), and precleared twice with normal rabbit serum and protein A sepharose for 30 min on ice. Each extract was incubated with 5 μl of anti-$p47^{phox}$ antibody for 1 h on ice. For a control experiment preimmune rabbit serum was used in the precipitations. 30 μl of 10% (v/v) protein A-sepharose was added to each sample followed by constant agitation on a rocker for 1 h at 4°C. The pellets were collected by centrifugation and washed 3 times in TSNE buffer.

Immunoblotting. Electrophoresis was performed on 12 or 15% polyacrylamide gel in the presence of SDS. Proteins were transferred electrophoretically to nitrocellulose membrane, blocked with 5% skim milk in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 2 h, and then incubated with antibodies specific to CKIIβ or CKIIα at a 1:500 dilution in 1% skim milk for 1 h. The membrane was washed 3 times in TBST, and then treated with ECL system (Amersham Corp).

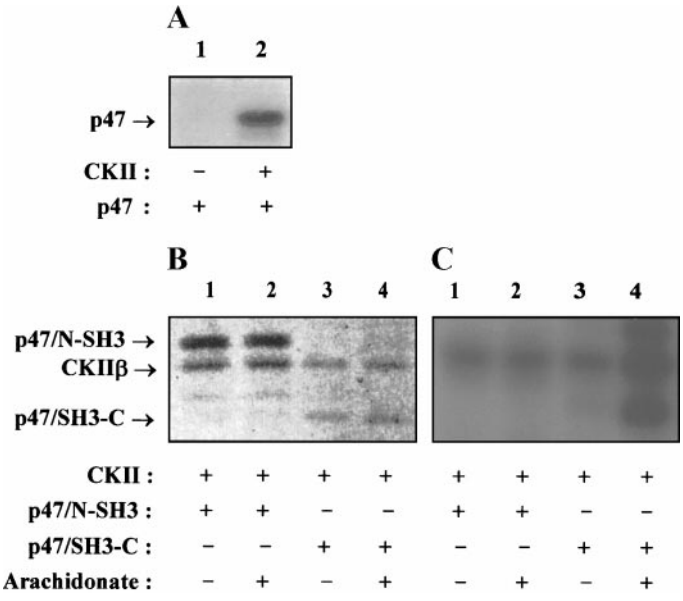

FIG. 1. Effect of the C-terminal region of $p47^{phox}$ on its conformation-dependent phosphorylation by CKII. (A) Full-length $p47^{phox}$ was incubated with [γ-^{32}P]ATP in the absence (lane 1) or presence (lane 2) of CKII under standard assay conditions as described under Materials and Methods. Reaction mixtures were boiled in a sample buffer for 5 min prior to loading on a 12% (w/v) SDS–polyacrylamide gel. ^{32}P incorporation was monitored by autoradiogram. (B and C) Two truncated forms of the $p47^{phox}$ lacking its N-terminal region ($p47^{phox}$/SH3-C) or its C-terminal region ($p47^{phox}$/N-SH3) were incubated with CKII and [γ-^{32}P]ATP under standard assay conditions. The reactions were carried out in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 90 μM arachidonic acid for 15 min, and radiolabeled proteins were then separated on a 15% (w/v) SDS–polyacrylamide gel. Coomassie blue staining (B) and autoradiography (C) are shown. The positions of $p47^{phox}$, $p47^{phox}$/N-SH3, $p47^{phox}$/SH3-C, and CKIIβ subunit are indicated on the left.

RESULTS

Conformation-Dependent Phosphorylation of $p47^{phox}$ by CKII

Recently we have demonstrated the phosphorylation of $p47^{phox}$ by CKII *in vitro* (also shown in Fig. 1A) and this phosphorylation is specifically enhanced by arachidonic acid (24). Dependence of phosphorylation on conformation of $p47^{phox}$ was supported by the finding that the wild-type of $p47^{phox}$ undergoes better phosphorylation by CKII in the presence of arachidonic acid, which induces conformational changes in $p47^{phox}$. In the present study, to investigate whether the N- or C-terminal region of $p47^{phox}$ is involved in the conformation-dependent phosphorylation of $p47^{phox}$ in the presence of arachidonic acid, two truncated proteins corresponding to amino acids 1–284 ($p47^{phox}$/N-SH3) and amino acids 151–390 ($p47^{phox}$/SH3-C) of $p47^{phox}$ were expressed and purified in *E. coli*. When the truncated proteins were incubated with CKII and [γ-^{32}P]ATP, $p47^{phox}$/N-SH3 was not phosphorylated by CKII in the presence or absence of arachidonic acid, indicating that the N-terminal region of $p47^{phox}$ plays no significant role in the conformation-dependent phosphorylation of $p47^{phox}$ by CKII. $p47^{phox}$/SH3-C was slightly phosphorylated by CKII in the absence of arachidonic acid, but this phosphorylation was strongly stimulated in the presence of arachidonic acid (Figs. 1B and 1C). Because arachidonic acid did not stimulate CKII activity itself (data not shown), the results indicated that arachidonic acid induces a conformational change in $p47^{phox}$/SH3-C, but not in $p47^{phox}$/N-SH3, and then results in unmasking the SH3 domain. In addition, the fact that $p47^{phox}$/N-SH3 is not phosphorylated by CKII in the presence or absence of arachidonic acid suggests that the C-terminal region of $p47^{phox}$ may be required for the interaction between $p47^{phox}$ and CKII.

Binding of the C-Terminal Region of $p47^{phox}$ to the β Subunit of CKII

To investigate whether $p47^{phox}$ would interact with the subunits of CKII using a yeast two-hybrid system,

the entire open reading frame of p47^{phox} was subcloned into pGADGH as described under Materials and Methods. pGBT9-CKIIα and pGBT9-CKIIβ are the yeast shuttle vector plasmids pGBT9 containing the complete open reading frame of the human CKIIα and CKIIβ subunits, respectively (28). The reporter strain *S. cerevisiae* HF7c was cotransformed with pGADGH-p47^{phox} and pGBT9-CKIIα or pGBT9-CKIIβ, and the protein-protein interactions were detected by the expression of the HIS3 reporter gene. All hybrid plasmids were also tested against the empty expression vectors pGBT9 and pGADGH in order to control for autonomous activation of the hybrid proteins. In those control transformants, expression of the reporter gene was not evident. In contrast to control transformants, when the full-length p47^{phox} (p47^{phox}/wt) and CKIIβ hybrid proteins were coexpressed in the reporter cells, the transformants were able to grow on selective media lacking tryptophan, leucine, and histidine. When p47^{phox} and CKIIα hybrid proteins were coexpressed in the reporter cells, the transformants were unable to grow on the selective media. These data indicated that p47^{phox} binds to CKIIβ, but not to CKIIα. To investigate the role of the C-terminal region of p47^{phox} on binding to CKIIβ, we constructed a deletion mutant form (p47^{phox}/N-SH3) of p47^{phox} that retained the amino acid residues 1–284. The pGADGH-p47^{phox}/N-SH3 construct failed to activate transcription in the presence of either pGBT9-CKIIα or pGBT9-CKIIβ. Thus, we concluded that the C-terminal region of p47^{phox} is necessary for its binding to CKIIβ (Fig. 2A).

Direct p47^{phox} interaction with the β subunit of CKII was further tested *in vitro* using the GST pull-down assay. GST-p47^{phox}/wt (amino acids 1–390), GST-p47^{phox}/N-SH3 (amino acids 1-284), GST-p47^{phox}/SH3-C (amino acids 151–390), and GST-p47^{phox}/C (amino acids 285–390) fusion proteins were immobilized on glutathione agarose beads and incubated with the CKIIβ protein. The beads were washed and the coprecipitated CKIIβ protein was visualized by Western blotting with anti-CKIIβ antibody. As shown in Fig. 2B, the CKIIβ subunit was capable of binding to beads containing GST-p47^{phox}/wt, GST-p47^{phox}/SH3-C, or GST-p47^{phox}/C, but not GST-p47^{phox}/N-SH3. These results confirmed that the β subunit of CKII interacts directly with the C-terminal region of p47^{phox}.

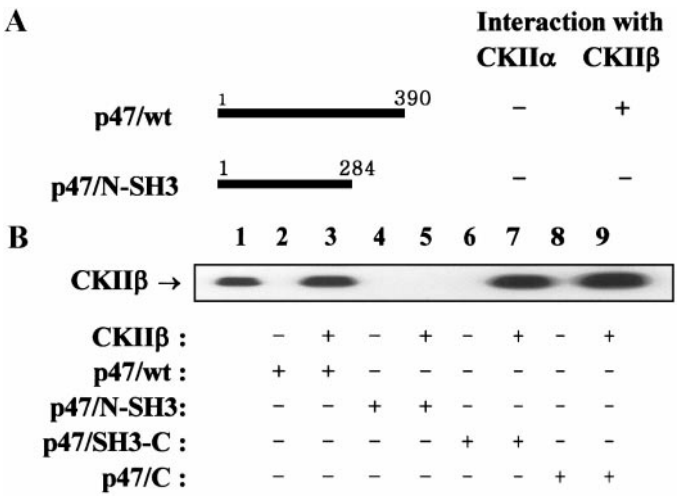

FIG. 2. Specific interaction of the C-terminal region of p47^{phox} with CKIIβ subunit. (A) The report strain was cotransformed with various combinations of hybrid plasmids encoding the respective GAL4 domains fused to truncated p47^{phox} and full-length CKIIα and CKIIβ subunits. Double transformants were patched for 4 days at 30°C on selective media lacking tryptophan, leucine, and histidine. Interactions between the hybrid proteins were tested for their ability to express the reporter genes, *HIS3*. All constructs were also screened against the empty expression vectors pGBT9 and pGADGH, in order to control for autonomous activation of the hybrid proteins. +, the reporter gene was activated; −, the reporter gene was not activated. (B) Glutathione agarose beads coated with GST-fusion p47^{phox}/wt (lanes 2 and 3), p47^{phox}/N-SH3 (lanes 4 and 5), p47^{phox}/SH3-C (lanes 6 and 7), or p47^{phox}/C (lanes 8 and 9) proteins were incubated in the absence (lanes 2, 4, 6, and 8) or presence (lanes 3, 5, 7, and 9) of CKIIβ. The reaction was allowed to proceed for 1 h, rocking at 4°C. After an extensive wash, the immobilized complexes were recovered by an elution step performed in the presence of 5 mM glutathione. The proteins were separated by SDS–polyacrylamide gel electrophoresis and visualized by Western blotting with anti-CKIIβ antibody. Lane 1, control CKIIβ protein.

Effect of Spermine on the Interaction of CKIIβ with p47^{phox} in Vitro

The C-terminal region of the p47^{phox} protein sequence is unusually rich in lysine and arginine (29). This suggests the possibility that the C-terminal region of the p47^{phox} protein binds to the cluster of acidic residues (amino acids 55 to 80) of CKIIβ which interacts with polybasic compounds. To address this possibility, we examined the effects of spermine on interaction between p47^{phox} and CKIIβ. GST-p47^{phox}/SH3-C fusion proteins were immobilized on glutathione agarose beads and incubated with CKIIβ in the presence or absence of spermine. As shown in Fig. 3, CKIIβ was coprecipitated with GST-p47^{phox}/SH3-C fusion protein in the absence of spermine, but this coprecipitation was not observed in the presence of spermine, indicating that spermine completely inhibited the interaction of p47^{phox}/SH3-C with CKIIβ. The results suggest that the C-terminal region of p47^{phox} and spermine compete for same binding site within CKIIβ (Fig. 3).

Stimulation of CKII Catalytic Activity by p47^{phox} in Vitro

Polybasic compounds such as polyamines and polylysine stimulate the catalytic activity of CKII through its interaction with CKIIβ (9). The finding that the C-terminal region of p47^{phox} is highly basic and mediates the interaction with CKIIβ suggests that p47^{phox} may stimulate CKII activity. To address this possibil-

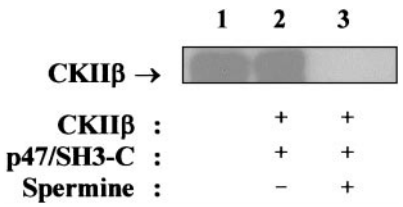

FIG. 3. Effect of spermine on the interaction between CKIIβ and the C-terminal region of p47^{phox}. CKIIβ was incubated with glutathione agarose beads coated with GST-fusion p47^{phox}/SH3-C protein in the absence (lane 2) or presence (lane 3) of 5 mM spermine for 1 h. After an extensive wash, the immobilized complexes were recovered by an elution step performed in the presence of 5 mM glutathione. The proteins were separated by SDS–polyacrylamide gel electrophoresis and visualized by Western blotting with anti-CKIIβ antibody. Lane 1, control CKIIβ protein.

ity, we examined the effects of the C-terminal region of p47^{phox} on CKII activity with the synthetic peptide (RRREEETEEE) as substrate under standard assay conditions as described under Materials and Methods. Polylysine, full-length p47^{phox}, p47^{phox}/SH3-C, and p47^{phox}/C stimulated CKII activity toward the synthetic peptide substrate RRREEETEEE, as shown in Fig. 4. Full-length p47^{phox}, p47^{phox}/SH3-C, and p47^{phox}/C were more effective than polylysine, generating a 2.5-fold increase in phosphotransferase activity of CKII under our standard assay conditions. Only slight increase (1.5 times) in CKII activity was observed with the same concentration (on a molar basis) of p47^{phox}/N-SH3, indicating that the C-terminal region of p47^{phox} plays a significant role in the stimulation of CKII activity. Taken together, these results indicate that p47^{phox} stimulates CKII activity by a physical interaction with CKIIβ (Fig. 4).

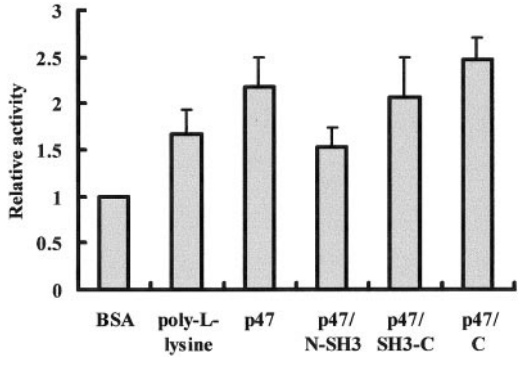

FIG. 4. Stimulation of CKII activity by p47^{phox}. The activity of CKII holoenzyme was monitored with the synthetic peptide substrate (RRREEETEEE) under standard assay conditions as described under Materials and Methods. BSA, poly-L-lysine, full-length p47^{phox}, p47^{phox}/N-SH3, p47^{phox}/SH3-C, or p47^{phox}/C were added to the reactions as indicated. Bars represent the relative activity of CKII holoenzyme determined by the ability to incorporate [^{32}P]phosphate into the peptide substrate. Error bars represent the range of duplicate experiments, each of which was analyzed twice by scintillation counting.

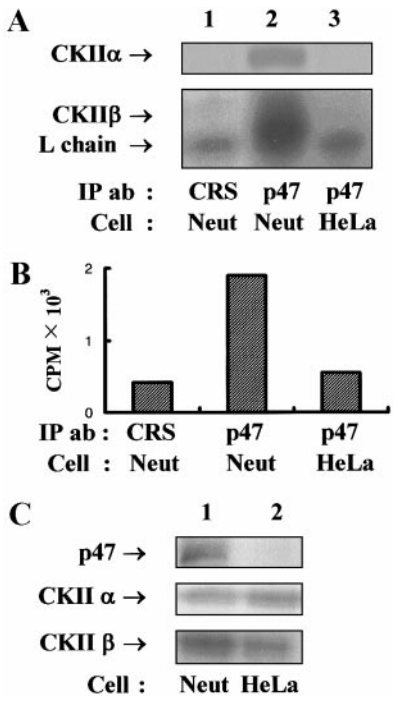

FIG. 5. CKII interacts with p47^{phox} *in vivo*. (A) 400 μg of human neutrophil cytosol (lanes 1 and 2) and HeLa cell lysates (lane 3) were subjected to immunoprecipitation (IP) with either anti-p47^{phox} antibody (ab, lanes 2 and 3) or control rabbit serum (CRS, lane 1). The precipitated proteins were further analyzed by Western blot using either anti-CKIIα (up) or anti-CKIIβ (bottom) antibodies. (B) p47^{phox} proteins were immunoprecipitated from neutrophils or HeLa cells. *In vitro* kinase assays were performed on the beads by adding [γ-^{32}P]ATP and the standard CKII substrate peptide (RRREEETEEE). The ^{32}P incorporation in the standard peptide was measured by scintillation counting. (C) 20 μg of neutrophil cytosol (lane 1) and HeLa cell lysate (lane 2) were analyzed by Western blot using anti-p47^{phox} (up), anti-CKIIα (middle), or anti-CKIIβ (bottom) antibodies. Neut, neutrophil; L chain, immunoglobulin light chain.

Interaction of CKII with p47^{phox} in Neutrophil

To examine whether CKII interacts with p47^{phox} in mammalian cells, we performed coimmunoprecipitation experiments. Human neutrophil cytosol and HeLa cell lysate were immunoprecipitated with anti-p47^{phox} antibody or control rabbit serum. Western blots of these immunoprecipitates probed anti-CKIIα and anti-CKIIβ antibodies showed that both CKIIα and CKIIβ were coimmunoprecipitated with p47^{phox} specifically in neutrophils (Fig. 5A). Using the synthetic peptide substrate RRREEETEEE, the CKII activity in p47^{phox} immunoprecipitates was also determined. The activity of CKII was detected in p47^{phox} immunoprecipitates from neutrophil, but not in control antibody immunoprecipitates from neutrophil or p47^{phox} immunoprecipitates from HeLa lysate (Fig. 5B). The expression patterns of p47^{phox}, CKIIα, and CKIIβ in human neutrophils and HeLa cells were examined by Western blotting. Although CKIIα and CKIIβ were detected in both neutrophils and HeLa cell lysates, p47^{phox} was not detected

in HeLa cell lysate (Fig. 5C). Taken together, the results of these experiments showed that the $p47^{phox}$ interacts with CKII holoenzyme in neutrophils.

DISCUSSION

Phosphorylation of $p47^{phox}$ is known to modulate its function (14). Recently we have demonstrated that CKII phosphorylates $p47^{phox}$ on Ser-208 and Ser-283 residues in the SH3 domain and on Ser-348 in the C-terminal region *in vitro,* and that phosphorylation in the SH3 domain is specifically enhanced by arachidonic acid. In addition, although it remains to be established the CKII target sites of $p47^{phox}$ *in vivo,* a CKII inhibitor, 5,6-dichloro-1-β-*o*-ribofuranosyl benzimidazole (DRB), potentiates formyl-Met-Leu-Phe-induced NADPH oxidase activity in dimethyl sulfoxide-differentiated HL-60 cells, suggesting that CKII may play a crucial role in regulating the NADPH oxidase activation/deactivation (24). An anionic amphiphile such as arachidonic acid or SDS has been known as an activator of NADPH oxidase which induces conformational changes in $p47^{phox}$ (30, 31). Sumimoto *et al.* (32) have shown that the SH3 domain of $p47^{phox}$, initially masked by a proline-rich stretch in the C-terminal region of the molecule, could be exposed by arachidonic acid. In the present study, we have demonstrated that $p47^{phox}$/SH3-C, but not $p47^{phox}$/N-SH3, undergoes better phosphorylation by CKII in the presence of arachidonic acid. Thus, the present results demonstrate a significant role of the C-terminal region of $p47^{phox}$ in the arachidonic acid-dependent phosphorylation of the molecule by CKII.

CKII is a tetrameric complex consisting of α, α', and β subunits and exists as an $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ structure. The CKIIα and CKIIα' subunits are the catalytic subunits, while the CKIIβ subunit has been known to be a regulatory subunit because it mediates tetramer formation (10), modulates catalytic activity (7, 8), and influences substrate recognition (9). The present yeast two-hybrid assay and GST pull-down assay demonstrated that $p47^{phox}$ interacts directly with CKIIβ and that the C-terminal region of $p47^{phox}$ mediates its binding to CKIIβ. Since CKII phosphorylates $p47^{phox}$, our current observation that $p47^{phox}$ protein interacts with CKIIβ provides strong evidence that the β subunit of CKII mediates the interaction of the catalytic subunit with the specific substrate. This is consistent with previous studies in which the CKIIβ subunit may determine the substrate specificity (7, 9).

CKII can interact with its substrates either in its holoenzyme form or as individual subunits, as illustrated by the interaction CKIIα with PP2A (33) and the interaction of CKIIβ with several proteins including ribosomal proteins L5 (28) and L41 (34), DNA topoisomerase IIα and IIβ (35), p53 (36), A-Raf (37, 38), Mos (39), SAG/CKBBP1 (40), and CD5 (41). Given the interaction of CKIIβ with $p47^{phox}$ in the two-hybrid system assay, we can conclude the holoenzyme form of CKII associates with $p47^{phox}$ in intact cells based on the coimmunoprecipitation of CKII with $p47^{phox}$ and the presence of CKII kinase activity in $p47^{phox}$ immunoprecipitates.

CKII activity is often regulated by binding to other proteins; for example, CKII activity has been shown to be regulated by interaction with ribosomal protein L41 or cell surface receptor CD5 (34, 41). The present study indicates that $p47^{phox}$ enhances the catalytic activity of CKII by physical interaction with the regulatory CKIIβ subunit, providing another example of modulation of CKII activity by other protein's binding. The N-terminal portion (amino acids 55 to 80) of CKIIβ contains clusters of acidic residues which are responsible for an intrinsic negative regulation of CKII activity and for interaction with the polybasic compounds (42, 43). Our current observation, which the basic C-terminal region of the $p47^{phox}$ protein sequence stimulates CKII activity and spermine completely inhibits the interaction between $p47^{phox}$ and CKIIβ, suggests that the acidic domain of CKIIβ is the most likely candidate for the binding of $p47^{phox}$ protein, and that electrostatic interaction may contribute to the association of CKIIβ to $p47^{phox}$.

Immunocytochemical studies have shown that CKII is mostly localized both in the cytoplasm and the nucleus of cells. However, recent studies have revealed that CKII tightly associates with highly purified plasma membrane preparations. Sarrouilhe *et al.* (44) have shown that the tight association of CKII to membrane components is mediated by a specific CKIIβ domain localized between residues 51 and 110. $p47^{phox}$, a component of NADPH oxidase, is localized in the plasma membrane of phagocytes, especially when cells are exposed to appropriate stimuli. Thus, our present study raises the possibility that $p47^{phox}$ may function as one of potential CKII anchoring proteins present in the plasma membrane of phagocytes.

In summary, this study is the first to demonstrate the activation of CKII by direct association with $p47^{phox}$. The findings presented here have the potential to expand the role of $p47^{phox}$ as a modulator of CKII activity in addition to the role of CKII as a regulator of $p47^{phox}$.

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