Phosphorylation of the leucocyte NADPH oxidase subunit p47^{phox} by casein kinase 2: conformation-dependent phosphorylation and modulation of oxidase activity

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The leucocyte NADPH oxidase of neutrophils is a membrane-bound enzyme that catalyses the reduction of oxygen to O₂^{•−} at the expense of NADPH. The enzyme is dormant in resting neutrophils but becomes active when the cells are exposed to the appropriate stimuli. During oxidase activation, the highly basic cytosolic oxidase component p47^{phox} becomes phosphorylated on several serines and migrates to the plasma membrane. Protein kinase CK2 is an essential serine/threonine kinase present in all eukaryotic organisms. The leucocyte NADPH oxidase subunit p47^{phox} has several putative CK2 phosphorylation sites. In the present study, we report that CK2 is able to catalyse the phosphorylation of p47^{phox} *in vitro*. Phosphoamino acid analysis of phosphorylated p47^{phox} by CK2 indicated that the phosphorylation occurs on serine residues. CNBr mapping and phosphorylation of peptides containing the putative site of CK2

indicated that the main phosphorylated residues are Ser-208 and Ser-283 in the Src homology 3 (SH3) domains, and Ser-348 in the C-terminal domain of p47^{phox}. Dependence of phosphorylation on the conformation of p47^{phox} is supported by the finding that p47^{phox} undergoes better phosphorylation by CK2 in the presence of arachidonic acid, a known activator of NADPH oxidase which induces conformational changes in p47^{phox}. In addition, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole, a CK2 inhibitor, potentiates formyl-Met-Leu-Phe-induced NADPH oxidase activity in DMSO-differentiated HL-60 cells. Taken together, we propose that CK2 is the p47^{phox} kinase, and that phosphorylation of p47^{phox} by CK2 regulates the deactivation of NADPH oxidase.

Key words: conformational change, deactivation, neutrophil NADPH oxidase, SH3 domain.

INTRODUCTION

The NADPH oxidase of phagocytes, an important element of host defence against microbial infection, catalyses the reduction of oxygen to O₂^{•−} using NADPH as the electron donor [1]. 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, but 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 p47^{phox} and p67^{phox}, which exist as a ≈ 240 kDa complex [2–4], and the small G-protein Rac2, which also participates in oxidase activation [5,6], migrate to the membrane. Here the p47^{phox}–p67^{phox} complex associates with a phagocyte-specific membrane-integrated flavocytochrome b₅₅₈, a heterodimer of p22^{phox} and gp91^{phox}, to assemble the functioning oxidase [7–12].

In whole cells, and under certain circumstances in the cell-free system, phosphorylation of p47^{phox} appears to play an important role in the activation of oxidase and the translocation of the p47^{phox}–p67^{phox} complex from the cytosol to the membrane [9,13,14]. The cytosolic oxidase component p47^{phox} is a basic protein that becomes extensively phosphorylated when the oxidase is activated [15,16]. The phosphorylation target is known to be a group of serines (residues 303–379) in the highly basic C-terminal quarter of the peptide [17,18]. It has been suggested

that a number of kinases [19–22] are potentially responsible for the phosphorylation of p47^{phox}.

Protein kinase CK2 is a ubiquitous and highly conserved serine/threonine kinase, which is found in all eukaryotes examined and in various subcellular compartments [23,24]. CK2 is a calcium-independent and cyclic-nucleotide-independent enzyme that utilizes GTP as well as ATP as a phosphate donor. CK2 is a heterodimer of two catalytic (α and/or α′) and two regulatory (β) subunits. It has been shown that the α and α′ subunits are different gene products and contain the catalytic activity of the enzyme. The β subunit is a regulatory subunit that modulates the catalytic activity of the α subunit and mediates the tetramer formation and substrate recognition [25–27]. Whereas the catalytic activity of CK2 is inhibited strongly by heparin, the enzyme is stimulated by polybasic compounds [28]. CK2 is known to phosphorylate a broad spectrum of substrates that are involved in cell growth and proliferation, including DNA binding proteins, nuclear oncoproteins and transcription factors; however, its precise physiological role and regulatory mechanism remain largely unknown.

In this study, we have examined purified CK2 for its ability to phosphorylate p47^{phox} *in vitro*. We have found that p47^{phox} can be phosphorylated by CK2, and its phosphorylation was modulated by arachidonate, a known activator of NADPH oxidase which induces conformational changes in p47^{phox}. In addition we show that the classic CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) enhances the formyl-Met-Leu-Phe

Abbreviations used: DRB, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; fMLP, formyl-Met-Leu-Phe; SH3, Src homology 3; dHL-60, differentiated HL-60; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; MAPK, mitogen-activated protein kinase.

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(fMLP)-induced NADPH oxidase activity of DMSO-differentiated HL-60 (dHL-60) cells. We suggest that CK2 plays a role in the regulation of the NADPH oxidase deactivation processes.

EXPERIMENTAL

Materials

Chemicals, enzymes and molecular-biology reagents were obtained from the following sources: pGEX-1 λ T vector from Pharmacia (Uppsala, Sweden); GSH, GSH-agarose, CM-Sepharose, human plasma thrombin, PMSF, phosphoserine, phosphothreonine, phosphotyrosine, arachidonic acid, DRB, DMSO, β -casein and fMLP were from Sigma (St. Louis, MO, U.S.A.); [γ - 32 P]ATP was from DuPont NEN Life Science Products. Electrophoresis reagents and the protein assay kit were from Bio-Rad (Hercules, CA, U.S.A.). The synthetic peptides encompassing the consensus sequence of the CK2 phosphorylation site and the synthetic peptide substrate for CK2 were synthesized, purified and confirmed by the Korean Collection for Type Cultures (Taejon, Korea).

Preparation of recombinant p47^{phox} protein

The transformed *Escherichia coli* containing the pGEX-1 λ T plasmid, with an insert of the p47^{phox} cDNA, was grown, and fusion protein was purified by affinity chromatography on GSH-agarose as described elsewhere [4]. The fusion protein was cleaved by treatment with thrombin (10 units/ml) in elution buffer containing 150 mM NaCl and 2.5 mM CaCl₂ for 2 h at room temperature. The protein mixture of recombinant p47^{phox} was further purified 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. The concentration of proteins was determined with the Bio-Rad assay kit using BSA as a standard.

Purification of CK2

Human CK2 holoenzyme was purified to homogeneity via four chromatography steps using DEAE-cellulose, phosphocellulose, heparin-agarose and gel filtration from the lysate of *E. coli* that expressed both α and β subunits of CK2 [29].

Cells

HL-60 cells were obtained from the A. T. C. C. (Rockville, MD, U.S.A.) and grown in RPMI medium with glutamine supplemented with 20 % fetal calf serum (Hyclone Laboratories). HL-60 cells were differentiated into neutrophil-like cells by growth in a medium containing 1.3 % DMSO [30] and harvested for assays after 6 days.

Phosphorylation of p47^{phox} by CK2

Phosphorylation of recombinant p47^{phox} by CK2 was performed in a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA, 0.1 mM ATP, 5 μ Ci of [γ - 32 P]ATP and 1 μ g of p47^{phox} in the absence and presence of 90 μ M arachidonate in a total volume of 30 μ l, unless otherwise indicated. The reactions were started by the addition of purified CK2 and incubated for 15 min at 30 °C. After terminating the phosphorylation reactions by the addition of 10 μ l of 4 $\times$ SDS sample buffer, the samples were subjected to SDS/PAGE using an 8 % running gel, according to the method of Laemmli [31]. The labelled proteins were visualized by autoradiography, and the 32 P was quantified by excising the

bands from the dried gel and measuring their radioactivity using Cerenkov counting. A piece of gel of similar size, excised from a 32 P-free portion of the gel, was also counted. This background value was subtracted from the other measurements.

Phosphoamino acid analysis

Phosphoamino acid analysis of p47^{phox} was performed using one-dimensional analysis by the method of Boyle et al. [32]. The PVDF membrane containing the radiolabelled p47^{phox} band was located by autoradiography, excised and transferred to a micro-centrifuge tube. Protein was then hydrolysed in 0.25 ml of 5.7 M HCl for 1 h at 110 °C. The sample was dried using a Speed-Vac (Savant) concentrator, and then resuspended in 10 μ l of a buffer composed of 10:1:189 (v/v) acetic acid/pyridine/water. The sample was spotted on a thin-layer cellulose plate (Merck) and subjected to electrophoresis at 1100 V for 45 min with water cooling. Phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml) were used as markers. The plate was then dried and sprayed with ninhydrin to localize the phosphoamino acid standards. 32 P-Labelled proteins on the dried plates were detected by autoradiography.

CNBr cleavage and Tris/Tricine SDS/PAGE

Samples of 32 P-labelled p47^{phox} were cleaved at methionine residues by incubation with 12.5 mg/ml CNBr in 0.15 ml of 70 % (v/v) formic acid for 16 h at room temperature in the dark. The reaction mixtures were then quenched with an equal volume of water and lyophilized in a Speed-Vac. The cleavage products were separated by Tris/Tricine SDS/PAGE [33] using a 16.5 % running gel followed by autoradiography. The separated peptides were transferred electrophoretically on to a nitrocellulose sheet [34], and the C-terminal fragment of p47^{phox} was visualized using a 1:10000 dilution of antibody against the C-terminal decapeptide of p47^{phox}, and finally detected with a 1:2000 dilution of alkaline phosphatase-labelled goat anti-rabbit Ig antibody (CALTAG) using 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium as substrate (Bio-Rad).

Phosphorylation of peptide

Synthetic peptides (40 μ M each) containing the consensus sequence of the CK2 phosphorylation site were incubated with CK2 in the same way as described for CK2 phosphorylation of recombinant p47^{phox} and assayed for bound radioactivity on reversed-phase TLC on a C₁₈ silica plate [35]. The plate was allowed to dry and was exposed to X-ray film. After development, the film was used to identify the boundaries of the radioactive spot at the origin, and the silica in the radioactive circle surrounding the origin was scraped off the plate into a scintillation vial. Each sample was measured for radioactivity by scintillation counting. A circle of similar size was also scraped off from a 32 P-free portion of plate and counted. This background value was subtracted from the other measurements.

CK2 activity assay

The standard assay for phosphotransferase activity of CK2 was conducted in a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 120 mM KCl, 10 mM MgCl₂ and 100 μ M [γ - 32 P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) or 5 mg/ml dephosphorylated β -casein in a total volume of 30 μ l at 30 °C. The reactions were started by the addition of the enzyme (25 nM) and incubated for 15 min. In some experiments, arachidonic acid (90 μ M) was added to the reaction mixture. When CK2 was assayed with β -casein, the

reaction was terminated by spotting 10 μ l of the reaction mixture on to P-81 phosphocellulose paper. When CK2 was assayed with the synthetic peptide substrate, the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10% and centrifuged, and 10 μ l 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.

O₂⁻ assays with cells

O₂⁻ formation by dHL-60 cells was assessed by the superoxide dismutase-sensitive reduction of cytochrome *c*. Cells were suspended at 2×10^7 /ml in Krebs–Ringer Hepes buffer with 5.5 mM glucose (KRHG), and 50–100 μ l of cells was added to a cuvette containing 100 μ M cytochrome *c* with or without 300 μ g/ml superoxide dismutase in 700–750 μ l of KRHG. The cells were incubated at 37 °C for 5 min while a stable baseline at $\lambda = 550$ nm was obtained. O₂⁻ formation was initiated by the addition of 1 μ M fMLP in the presence of 5 μ g/ml cytochalasin B. Cytochrome *c* reduction was continuously monitored at 550 nm, and the maximal rate was calculated.

Translocation of p47^{phox} from cytosol to plasma membrane

dHL-60 cells were harvested, sonicated on ice (2×10 s) in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM Pipes, 1 mM EGTA, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin and 0.5 mM PMSF) and centrifuged (600 *g* for 10 min at 4 °C) to remove nuclei and unbroken cells; the supernatant was then ultracentrifuged (100 000 *g* for 30 min at 4 °C). Pellets were washed in relaxation buffer, dissolved in Laemmli sample buffer and the proteins resolved by SDS/PAGE (10% gel). The separated proteins were then transferred electrophoretically to nitrocellulose, and the p47^{phox} bands were visualized by immunoblotting with anti-p47^{phox} antibody as described above.

Fluorescence assays

Phosphorylation of the recombinant p47^{phox} by CK2 was performed in the same manner as above, except that the radioactive ATP was excluded from the reaction mixture. After adding 0.27 ml of 50 mM Tris/HCl, pH 7.4, fluorescence measurements were taken. Steady-state fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at 22 °C. A 150 W xenon source was used. The slit width was fixed at 5 nm for excitation and emission. Unless otherwise stated, samples were excited at 280 nm, and the emission was monitored between 300 and 400 nm. Each recorded spectrum was an average of three separate scans. Background emission was eliminated by subtracting the signal from the buffer containing arachidonate and/or the appropriate quantity of effectors for CK2. Quenching studies were carried out and were analysed according to the Stern–Volmer relationship as described previously [36].

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

RESULTS

To examine whether CK2 catalyses the phosphorylation of p47^{phox}, we purified recombinant p47^{phox} and CK2. As shown in Figure 1 (top panel), when p47^{phox} was incubated with CK2 in

Figure 1 *In vitro* phosphorylation of recombinant p47^{phox} by CK2

(Top panel) Recombinant p47^{phox} (1 μ g) was incubated with [γ -³²P]ATP in the presence (lanes 1) or absence (lanes 2) of CK2. Reaction mixtures were boiled in sample buffer for 5 min prior to loading on the gel. Proteins were visualized with Coomassie Brilliant Blue stain (left-hand panel) and ³²P incorporation was monitored by autoradiography (right-hand panel). (Middle panel) Phosphoamino acid analysis of p47^{phox} phosphorylated by CK2. After phosphorylation by CK2 with [γ -³²P]ATP, p47^{phox} was separated by SDS/PAGE and transferred to a PVDF membrane. The p47^{phox} band was excised from the membrane. After hydrolysis of protein in concentrated HCl, phosphoamino acids were analysed by one-dimensional high-voltage electrophoresis on a TLC cellulose plate and autoradiography. pS, pT and pY denote the positions of phosphoserine, phosphothreonine and phosphotyrosine, respectively, as determined ninhydrin-stained standards. (Bottom panel) Phosphopeptides produced by CNBr cleavage of p47^{phox} phosphorylated by CK2. p47^{phox} phosphorylated by CK2 with [γ -³²P]ATP was cleaved with CNBr and the products were separated on Tris/Tricine SDS/PAGE as described in the text. ³²P-Labelled peptides were detected by autoradiography, and the C-terminal fragment of p47^{phox} was detected with the antipeptide antibody. Left-hand panel, autoradiogram; right-hand panel, immunoblot.

the presence of [γ -³²P]ATP, a single heavily phosphorylated band with an apparent molecular mass of 47 000 Da was observed (Figure 1, top panel, lanes 1). However, no phosphorylation of p47^{phox} was observed when CK2 was omitted from the reaction mixture (Figure 1, top panel, lanes 2). To determine the nature of the phosphorylated residues, we undertook phosphoamino acid analysis of *in vitro*-phosphorylated p47^{phox}. The amino acid residue modified by CK2 was identified by electrophoresis of acid hydrolysate of p47^{phox} that had been incubated with

Table 1 Synthetic peptides containing the putative CK2 phosphorylation site

Residues	Sequence	CK2 site	Domain
205–214	IPAS S FLEPLD	Ser ²⁰⁸	(SH3) ₂
212–221	PLD S PDETED	Ser ²¹⁵	(SH3) ₂
235–246	DEV S LLEGEA	Ser ²³⁸	(SH3) ₂
280–289	LQK S GQDVQS	Ser ²⁸³	(SH3) ₂
345–354	SPG S PLEER	Ser ³⁴⁸	C-terminal

Table 2 Phosphorylation of peptides that contain target serines

Synthetic peptides (40 μ M) containing a putative CK2 phosphorylation site were phosphorylated by CK2 with [γ -³²P]ATP, as in the Experimental section, and assayed for bound radioactivity by reversed-phase TLC on a C₁₈ silica plate. Values are means $\pm$ S.D. from three independent assays.

Peptide	Bound radioactivity (³² P, c.p.m.)
205–214	2038 $\pm$ 294
212–221	11 $\pm$ 9
235–246	17 $\pm$ 12
280–289	3419 $\pm$ 215
345–354	5128 $\pm$ 104

[γ -³²P]ATP and the kinase *in vitro* (Figure 1, middle panel). By comparing the positions of the phosphoamino acid standards stained with ninhydrin with the profile obtained after autoradiography, only phosphoserine residues are clearly present in the phosphorylated p47^{phox}. No phosphothreonine or phosphotyrosine was observed, even upon overexposure of the autoradiogram.

To determine the location of the phosphorylated serines, p47^{phox} was phosphorylated *in vitro* with [γ -³²P]ATP and CK2, digested with CNBr, and the resulting peptides separated by Tris/Tricine gel electrophoresis. The two fragments (9.6 kDa and 13.4 kDa) were exhibited as a target for phosphorylation, as shown in Figure 1 (bottom panel). The fact that two bands were observed after CNBr cleavage indicated the presence of multiple phosphorylation sites. The CNBr fragment of 13.4 kDa is the C-terminal region of p47^{phox}, corresponding to Tyr²⁷⁹–Val³⁹⁰, which was confirmed by the antibody against the C-terminal decapeptide of p47^{phox}. The radioactive peptide of 9.6 kDa represents Lys¹⁹⁹–Met²⁷⁸ encompassing the major part of Src homology 3 (SH3) domains, which was confirmed by the N-terminal sequencing. CK2 preferentially phosphorylates Ser/Thr residues followed by a stretch of acidic residues on the immediate C-terminal side. Among these acidic residues, the third position after the phosphoreceptor is the most important determinant [23]. In this sense, the fragment corresponding to the C-terminal of p47^{phox} has one possible serine at position 348 that could be phosphorylated by CK2, and the 9.6 kDa fragment has four possible serines at 208, 215, 246 and 283 that could be phosphorylated by CK2. In order to localize the targets of p47^{phox} for CK2 phosphorylation, we have examined five synthetic decapeptides (Table 1) that contain the consensus sequence of the CK2 phosphorylation site. As shown in Table 2, substantial incorporation of ³²P was only observed with synthetic peptides corresponding to the amino acid residues of 205–214, 280–289 and 345–354 by CK2 phosphorylation with γ -³²P. These results indicate that Ser²⁰⁸ and Ser²⁸³ in the SH3 domains and Ser³⁴⁸ in

Figure 2 Effect of arachidonate on the phosphorylation of p47^{phox} by CK2

(Top panels) Effect of arachidonate (Ara) on the phosphorylation of p47^{phox} by CK2 with [γ -³²P]ATP. The labelled proteins were separated by SDS/PAGE. The labelled bands were located by autoradiography, and the bands corresponding p47^{phox} were excised and counted. (Bottom panel) The labelled proteins, with or without arachidonate, were cleaved with CNBr, and the resulting peptides were separated by Tris/Tricine SDS/PAGE. The labelled bands were located by autoradiography, and the bands corresponding to the peptides Lys¹⁹⁹–Met²⁷⁸ and Tyr²⁷⁹–Val³⁹⁰ were excised and counted. Values correspond to means $\pm$ S.D. from three independent assays.

Table 3 Effect of arachidonate on CK2 activity

The CK2 activity was assayed as described in the Experimental section with either 1 mM synthetic peptide substrate (RRREEETEE) or 5 mg/ml β -casein as a substrate in the presence and absence of 90 μ M arachidonate. The incorporation of ³²P into a substrate was quantified by scintillation counting. Results are expressed as means $\pm$ range from two experiments.

Substrate	Arachidonate	Activity (³² P, c.p.m.)
Peptide	—	26398 $\pm$ 1334
Peptide	+	19904 $\pm$ 99
β -Casein	—	35423 $\pm$ 476
β -Casein	+	33009 $\pm$ 253

Figure 3 Effect of DRB on fMLP-induced superoxide formation in dHL-60 cells

(Top panel) dHL-60 cells were stimulated with 1 μ M fMLP with or without 5 μ M DRB pretreatment for 15 min. O_2^- production was determined by superoxide assays as described in the Experimental section. The values shown are relative to the control (set at 100%) and are means $\pm$ S.D. from triplicates determinations. (Middle panel) Reaction progress curves of 1 μ M fMLP-treated dHL-60 (■) and 5 μ M DRB and 1 μ M fMLP-treated dHL-60 (●). O_2^- formation was determined as cytochrome *c* reduction at 550 nm (see the Experimental section). Data are representative of three experiments. (Bottom panel) Translocation of p47^{phox} in fMLP-activated dHL-60 cells with or without DRB. The treated cells were sonicated and fractionated. Cytosolic and membrane fractions were analysed by SDS/PAGE and blotting. Immunoblots were performed as described in the Experimental section using the 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium detection system to visualize the blots. Data are representative of five experiments.

the C-terminal region of p47^{phox} are, presumably, targets for CK2 phosphorylation.

Dependence of phosphorylation on the conformation of p47^{phox} is supported by the finding that p47^{phox} undergoes better

Table 4 Changes in the intrinsic fluorescence of p47^{phox} and KI quenching parameter by arachidonate and phosphorylation with CK2

Fluorescence emission spectra of p47^{phox} samples (3 μ g/300 μ l) were dissolved in 50 mM Tris/HCl, pH 7.4. Spectra were obtained using an excitation wavelength of 278 nm. Changes in the protein total fluorescence intensity (ΔF) were determined at 338 nm. The Stern–Volmer constant (K_{sv}) was estimated graphically as the initial slope from the Stern–Volmer plots. ΔF results are means $\pm$ S.D. from three separate experiments. Each emission maximum (λ_{max}) was identical for three determinations.

Protein	ΔF (%)	λ_{max} (nm)	K_{sv} (M ⁻¹)
p47 ^{phox}	0	338	10.8
p47 ^{phox} + arachidonate	38.7 $\pm$ 0.8	343	12.7
p47 ^{phox} + arachidonate + CK2	34.1 $\pm$ 1.1*	341	15.1

* $P < 0.01$ compared with p47^{phox} treated with arachidonate.

phosphorylation by CK2 in the presence of arachidonate, an activator of NADPH oxidase that has been known to induce conformational changes in p47^{phox} (Figure 2, top panels). Arachidonate did not change the activity of CK2 (Table 3). Using CNBr cleavage and Tris/Tricine SDS/PAGE of p47^{phox} phosphorylated by CK2 in the presence and absence of arachidonate, we demonstrated that the enhancement of phosphorylation by arachidonate was due to the phosphorylation sites in SH3 domains. In the presence of arachidonate, the ³²P incorporation of a small fragment was significantly increased, whereas the phosphorylation of a large fragment was not changed (Figure 2, bottom panel).

Because p47^{phox} is an essential component for the activation of leucocyte NADPH oxidase, this study investigated whether or not CK2 modulates the activity of oxidase in dHL-60 cells. The HL-60 cell line consists predominantly of promyelocytes and proliferates continuously in suspension culture. This cell line, when induced by DMSO, differentiates into a neutrophil-like cell and develops a fully activated NADPH oxidase [30]. As shown in Figure 3 (top panel), pre-incubating dHL-60 cells with 5 μ M DRB, a classic CK2 inhibitor, for 15 min significantly increased fMLP (1 μ M)-induced oxidase activity 1.7-fold ($P < 0.01$). The enhancement of oxidase activity by the inhibition of CK2 indicated that CK2 phosphorylation negatively regulates the NADPH oxidase activity. The CK2 inhibitor not only enhanced, but also prolonged the respiratory burst provoked by fMLP (Figure 3, middle panel). Since p47^{phox} translocation to the plasma membrane is a key event in NADPH oxidase activation, we investigated the effect of DRB on this process by using Western-blot analysis of the membrane fractions. When HL-60 cells were stimulated with fMLP, pretreatment with 5 μ M DRB enhanced membrane translocation of p47^{phox} (Figure 3, bottom panel). Western blots were scanned, and the combined densitometry data indicated that in the presence of DRB the level of p47^{phox} translocation was $154 \pm 21\%$ of the control (means $\pm$ S.D., $n = 5$; $P < 0.01$).

To investigate the effects of CK2 phosphorylation on p47^{phox} conformation, a spectroscopic method was used to reliably monitor the structural modification. Recently, it has been reported that both amphiphile and protein kinase C (PKC) phosphorylation cause similar changes to the inherent tryptophan fluorescence of p47^{phox} [36,37]. The corrected steady-state fluorescence emission spectra between 300 and 400 nm of truncated p47^{phox} following excitation at 278 nm were obtained. As shown in Table 4, the intrinsic fluorescence derived from tryptophan residues of p47^{phox} was significantly quenched by the addition of 90 μ M arachidonate, and additional changes to

quantum yield, emission maximum and the Stern–Volmer constant (K_{sv}) were observed with CK2 phosphorylation.

DISCUSSION

It has been known for many years that p47^{phox}, one of the cytosolic subunits of the leucocyte NADPH oxidase, becomes heavily phosphorylated on serines when the oxidase is activated [15,16]. A number of kinases, including PKC [19], cAMP-dependent protein kinase (PKA) [38], mitogen-activated protein kinases (MAPKs), the p21^{rac/cdc42}-activated kinase [20] and other kinases yet to be characterized [21,22], may be potentially responsible for the phosphorylation of p47^{phox}. Phosphorylation is required for conformational changes in p47^{phox}, and it allows translocation and binding of p47^{phox} to membrane-associated cytochrome b_{558} . Although the identities of the kinases that phosphorylate p47^{phox} *in vivo* have not been established clearly, PKC is at least one of the enzymes capable of converting p47^{phox} into a functionally active molecule in intact neutrophil cells [19,39,40] as well as in the cell-free system [14]. However, little is known about the possible role of other kinases in the regulation of NADPH oxidase activation. Phosphorylation of p47^{phox} was shown to occur upon addition of dibutyl cAMP to neutrophil cytoplasts or cytosol, suggesting that p47^{phox} is also a substrate for PKA *in vivo*. Dibutyl cAMP did not induce O_2^- production, which indicates that phosphorylation by PKA alone is not sufficient to activate the oxidase. It is possible that the phosphorylation of p47^{phox} by PKA could be responsible for the negative regulation of oxidase activation, since the elevation of neutrophil cAMP inhibits O_2^- production [40]. It has been shown recently that MAPK is activated when neutrophils are treated with any of several activating agents, including fMLP, C5a and PMA [41,42], the time course of kinase activation bearing a remarkable resemblance to the time course of O_2^- production in response to the same stimuli. In addition, the same stimuli can activate MAPK kinase, another enzyme in the MAPK signal-transduction cascade [42]. However, it has been reported that, while MAPK activation coincides with polymorphonuclear leucocyte activation, it can be dissociated from the oxidative burst [43].

CK2 is a ubiquitous serine/threonine kinase that is found in both the nucleus and the cytoplasm, and it phosphorylates a number of signalling proteins in both compartments (e.g. Jun, Myc, Myb, Rb and p53) [44] at consensus phosphorylation sites composed of Ser/Thr-Xaa-Xaa-Glu/Asp [45]. Although there has been much work performed on the nature and activity of CK2, a definite role in signal transduction is still somewhat obscure [46]. In the present study, we have demonstrated that human p47^{phox} can be phosphorylated by CK2 *in vitro*. Phospho-amino acid analysis, CNBr mapping and phosphorylation of synthetic peptides containing putative CK2 target sites have allowed us to positively identify three CK2 phosphorylation sites and to localize them to Ser²⁰⁸ and Ser²⁸³ in the SH3 domains, and to Ser³⁴⁸ in the C-terminal region adjacent to SH3 domains of p47^{phox}. None of the potential acceptor sites in the N-terminal region of the protein are modified by CK2.

Other than many kinases responsible for the p47^{phox} phosphorylation, CK2 is not known to respond to any second-messenger molecules. This feature makes it difficult to study exactly what physiological roles CK2 may have and how the activity of this enzyme is regulated in NADPH oxidase activation. Therefore, it can be postulated that the mechanism, other than signal-transduction pathways, may involve the regulation of CK2 activity. An interaction between an SH3 domain in p47^{phox} and a proline-rich region in the protein is eliminated during

oxidase activation, exposing a region that is concealed in the resting protein. Conformational changes of p47^{phox} accompanied by the oxidase activation are induced by phosphorylation of multiple serine sites in the C-terminal region or by an anionic amphiphile, such as arachidonic acid or SDS [36,37]. It has been shown that arachidonate disrupts SH3-domain polyproline interaction in p47^{phox} [47]. The present study demonstrates the significant change in the p47^{phox} phosphorylation pattern by CK2 when performed in the presence of arachidonate. While the p47^{phox} phosphorylation by CK2 is conformation-dependent, the activity of CK2 itself is not altered by arachidonate. Arachidonate enhanced the incorporation of ³²P into p47^{phox} by CK2, and the increase in phosphorylation was observed in the Ser sites in SH3 domains, whereas phosphorylation of Ser³⁴⁸ was not significantly changed by arachidonate.

On the basis of our observations, we hypothesize that CK2 may play a crucial role in regulating the NADPH oxidase activation/deactivation. In order to investigate the possible role of CK2 phosphorylation of p47^{phox} in the NADPH oxidase activity, we treated dHL-60 cells with DRB, a classical CK2 inhibitor. It has been reported that DRB inhibits CK2 [48], but not PKA, PKC, tyrosine kinases [49] or MAPK [50]. DRB significantly enhanced fMLP-induced O_2^- production in dHL-60 cells, indicating that oxidase activity is modulated by CK2 *in vivo*. The conformation-dependent phosphorylation of p47^{phox} by CK2 and the enhancing effect of DRB on the activity of oxidase suggest that CK2 plays a role in NADPH oxidase deactivation.

Although the activation mechanism of NADPH oxidase has been investigated extensively, little information is available about the deactivation mechanism for the activated NADPH oxidase. Human NADPH oxidase is a very complex enzyme. The most likely reason for this complexity is the toxicity of superoxide and its derivatives produced by the active NADPH oxidase. These reactive species are not only bactericidal, but also toxic to the surrounding host cells. Therefore, improper activation of the NADPH oxidase must be prevented at all costs and, when the infection has been cleared, a rapid deactivation mechanism is imperative [51]. Several lines of circumstantial evidence for the existence of an efficient intracellular deactivation mechanism of neutrophil NADPH oxidase was provided in earlier studies. For example, NADPH oxidase activity does not persist in its activated state, and O_2^- production stimulated with fMLP is especially transient [52]. It has been proposed that lipid thiobis ester [53], the small GTPase Rac with the exchange of GTP to GDP [54], or cytosolic protein phosphatase [52], play an important role in the regulation of deactivation. Recently, it has been reported that the addition of DNase I, an F-actin-depolymerizing protein, caused an accelerated deactivation of the oxidase, and treatment of the cell-free mixture with a chemical cross-linker prevented the deactivation [55]. However, it is not clear that any of these mechanisms or all the mechanisms in concert may be responsible for the deactivation.

In view of the increased fMLP-induced NADPH oxidase activity and the higher level of transferred p47^{phox} to the plasma membrane in dHL-60 cells treated with DRB, it can be inferred that CK2 inhibitor prolongs the lifetime of the oxidase. Although the detailed deactivation mechanism for NADPH oxidase by phosphorylation of p47^{phox} by CK2 is not clarified in this study, it can be assumed that the phosphorylation of p47^{phox} by CK2 induces conformational changes in p47^{phox} and that these changes alter the ability of p47^{phox} to associate with p22^{phox} of cytochrome b_{558} and reduce interaction between p47^{phox} and p22^{phox} of cytochrome b_{558} . It has been suggested that deactivation of NADPH oxidase resulted in the release of p47^{phox} from the

activated oxidase complex [52]. Recently, DeLeo et al. [56] reported that the termination of oxidase activity correlates with the loss of p47^{phox} from cytochrome *b*-enriched phagosomes and the additional phosphorylation of membrane-associated p47^{phox}. However, the kinase responsible for such an event is not known. Spectroscopic data from the phosphorylated p47^{phox} by CK2 in the presence of arachidonate suggest that CK2 induces additional conformational change after the activation of NADPH oxidase through the conformational change of p47^{phox} caused by arachidonate.

In conclusion, the concept that emerges from this work is that conformation-dependent phosphorylation of p47^{phox} by CK2 is one of the mechanisms that regulates NADPH oxidase deactivation. Further work on phosphorylation of p47^{phox} by CK2 *in vivo* will be important for understanding the more detailed mechanism of deactivation of the NADPH oxidase.

This work was supported by a Basic Sciences Research Grant (BSRI-98-4403) from the Ministry of Education of Korea. We thank Dr Bernard M. Babior (Scripps Research Institute, La Jolla, CA, U.S.A.) for the p47^{phox} antibody.

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Received 3 April 2001/18 June 2001; accepted 19 July 2001