A Cys-Gly-Cys triad in the dehydrogenase region of Nox2 plays a key role in the interaction with p67phox

Iris Dahan,* Susan M. E. Smith,† and Edgar Pick∗,†

†The Julius Friedrich Cohnheim Laboratory of Phagocyte Research, Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel Aviv University, Tel Aviv; and †Department of Biology and Physics, College of Science and Mathematics, Kennesaw State University, Kennesaw, Georgia, USA

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ABSTRACT

p67phox is the paramount cytosolic regulator of the superoxide-generating Nox of phagocytes, by controlling the conformation of the catalytic component, Nox2. The initiating event of this process is a protein-protein interaction between p67phox and the part of Nox2 protruding into the cytosol, known as the dehydrogenase region. The aim of this study was to identify and characterize region(s) in Nox2 acting as binding site(s) for p67phox. For this purpose, we measured the binding of recombinant p67phox to an array of 91 overlapping synthetic pentadecapeptides covering the length of the dehydrogenase region (residues 288–570). We found that: 1) p67phox binds to a site corresponding to residues 357–383, represented by a cluster of 5 peptides (Nos. 24–28); 2) maximal binding corresponds to residues 357–383, represented by the 2 regions interacting with distinct partner sites in p67phox. J. Leukoc. Biol. 98: 859–874; 2015.

Introduction

Among the mediators of the killing of pathogenic microorganisms by phagocytes, ROS occupy a central position. All ROS produced by phagocytes originate from O2–, which is generated by the 1 electron reduction of O2 by a complex of proteins, known as the NADPH oxidase complex, with NADPH serving as the electron donor (reviewed in refs. [1, 2]). The NADPH oxidase complex consists of a membrane-localized protein, flavocytochrome b558, and 5 cytosolic proteins: p47phox, p67phox, p40phox, the small GTPase Rac(1 or 2), and Rho GDP dissociation inhibitor. Flavocytochrome b558 is a heterodimer of a 91 kDa glycosylated flavoprotein, known as Nox2, which is associated with a second protein of 22 kDa (p22phox). O2– generation is catalyzed by Nox2; all of the other components of the complex have exclusively regulatory functions.

Nox2 contains 570 residues and comprises 6 transmembrane α-helices linked by 3 outside-facing loops and 2 cytosol-facing loops and a cytosolic segment, which extends from residues 288 to 570, also known as the dehydrogenase region (DHR). As the only catalytic component, Nox2 contains 3 redox stations carrying the electrons from NADPH to O2. These are an NADPH-binding site and noncovalently bound FAD, present in the DHR, and 2 nonidentical helices, linked to the third and fifth transmembrane helices. From an evolutionary perspective, the DHR is homologous to the prokaryotic protein ferredoxin-NADP+ reductase, which also possesses NADP- and FAD-binding domains [3].

O2– production by the NAPDH oxidase is rigorously regulated; it does not occur in the resting phagocyte and is initiated by the interaction of membrane receptors with a variety of stimulatory ligands, typified by phagocytosed microorganisms. Thus, in the resting state, there is no electron flow along the redox centers in Nox2, this being initiated by a conformational change in Nox2, consequent to its interaction with the cytosolic components that translocate to the membrane. The process, known as NADPH oxidase assembly, generates a functional NADPH-consuming, O2–-generating, membrane-anchored complex (reviewed in ref. [4]). Under physiologic conditions, p47phox, p67phox, and Rac are all required for the induction of O2– production, but whether direct interaction of all components with the Nox2 DHR is required is a yet-unsettled question. The predominant view is that the key

Abbreviations: AD = activation domain, CGC = Cys-Gly-Cys triad, CGD = chronic granulomatous disease, DHR = dehydrogenase region, EAD = extension of the activation domain, FAD = flavin adenine dinucleotide, GRAVY = grand average of hydropathicity, MBP = maltose binding protein, NMP = 1-methyl-2-pyrrolidone, Nox = NADPH oxidase, O2 = molecular oxygen, O2– = superoxide anion, (continued on next page)
component responsible for the remodeling of Nox2 is p67phox, in conjunction with Rac-GTP [5–7].

Therefore, the identification of the region(s) in Nox2 DHR and p67phox, participating in the interaction between the two is essential for understanding the molecular basis of Nox assembly. Paradoxically, whereas progress was made in the description of binding sites in Nox2 DHR for p47phox [8] and Rac [9], there is a surprising lack of information on region(s) in Nox2 DHR participating in binding of p67phox. More is known about regions in p67phox involved in oxidase activation, in general, and in interaction with Nox2, in particular. An activation domain (AD), comprising residues 199–210 [10], or a wider region, extending from residue 190 to 208 [11] in p67phox, was found to be essential for oxidase activation but not for the actual p67phox–Nox2 interaction. Experimental data related to the latter are scarce and merely suggest that the binding site for Nox2 in p67phox is located N terminal to residue 199 [12, 13].

In the present work, we intended to identify the region(s) in Nox2 DHR engaged in interaction with p67phox by the use of a peptide array methodology (reviewed in refs. [14, 15]). We applied this successfully in the past to detect the binding sites in p47phox for p67phox [16], in p22phox for p47phox [17], and for characterizing the epitope in p22phox recognized by a mAb [18].

Ninety-one overlapping 15-mer peptides, corresponding to the DHR of Nox2 and spanning residues 288–570, were used (overlap of 12 residues; offset of 3 residues). The peptides were attached to 96-well plates and reacted with recombinant p67phox with a N-terminal 6His tag (r6His-p67phox) in the fluid phase, and peptide-bound p67phox was detected by an antipolyhistidine antibody. The mapping of Nox2 residues shared by clusters of peptides binding p67phox led to the identification of several potential p67phox-binding determinants. Intrinsic to the peptide array methodology is the possible occurrence of nonspecific results. A considerable effort was made to distinguish these from sequence-specific binding. This led to the identification of an area in the Nox2 DHR, extending from residue 357 to 383, which “lighted up” by virtue of the binding of p67phox to a cluster of 5 peptides (numbered 24–28), the first (peptide 24) and the last (peptide 28) exhibiting maximal binding ability. All 5 peptides shared a Cys-Gly-Cys (CCG) triad (residues 369–371) located at the C terminus and N terminus of peptides 24 and 28, respectively. Our results reveal an essential role for the CCG triad in Nox2 in the binding of p67phox, seconded by an additional CGG-independent interaction region, comprising residues C terminal to the CCG triad.

MATERIALS AND METHODS

Synthetic peptides

Two categories of synthetic peptides were used. The first comprised 91 overlapping, 15-residue peptides (PepSets), spanning the DHR of Nox2 from amino acids 288 to 570, with an overlap of 12 residues and an offset of 3 residues. The peptides had a biotin tag at the N terminus, attached by a Ser-Gly-Ser-Gly spacer, and a C-terminal amide. These peptides were synthesized by the multipin synthesis method [19] by Mimotopes (VIC, Australia) and were not purified and served for screening purposes only. The purity of the peptides ranged from 60 to 70% (based on random sampling by the manufacturer) but was not known at the level of individual peptides. The peptides, numbered from 1 to 91, their individual amino acid sequences, and their position in the Nox2 DHR protein sequence are listed in Fig. 1. The second category consisted of individual peptides of a purity of ≥70%, selected on the basis of the preliminary screening with the unpurified peptides. These were also 15-residues long, with a biotin tag at the N terminus (attached by a Ser-Gly-Ser-Gly spacer) and a C-terminal amide. Some peptides were synthesized with a biotin tag at the C terminus (attached by a Gly-Ser-Gly-Lys or an aminooxythiol ethoxy acetic acid-Lys spacer) and an acetyl at the N terminus. The purified peptides were synthesized by Mimotopes or Bachem (Bubendorf, Switzerland). Peptide purity was checked by reversed-phase chromatography and the molecular mass confirmed by MALDI-mass spectroscopy, both performed by the peptide manufacturers. In specific peptides, a Cys residue was replaced by Arg or Ser in the course of the synthesis. The absence of the cysteine residue was confirmed by quantification of the sulfhydryl groups by the method of Ellman [20]. Peptides were dissolved in a mixture of 75 parts 1-methyl-2-pyrrolidone (NMP) and 25 parts water (v/v) to a concentration of 1.5 mM to serve as stock solutions for further dilution. The peptide solutions were subjected to sonication by use of 5, 10 s pulses in a VCX 400 W ultrasonic processor equipped with a cup horn filled with an ice-water mixture (Sonnics & Materials, Newtown, CT, USA). The peptide stock solutions were divided into 190 µl aliquots and kept frozen at −75°C. Working solutions were freshly prepared on the day of performing the experiments.

Chemicals and reagents

Common laboratory chemicals, at the highest purity available, were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck KGaA (Darmstadt, Germany). Tween 20 (Product No. P1379), casein sodium salt (Product No. C8654), and imidazole (Product No. 10250) were purchased from Sigma-Aldrich. Mouse antipolyhistidine mAb, peroxidase conjugate (Clone HIS-1; product no. A7058) was obtained from Sigma-Aldrich.

Anti-p67phox C-19 (goat polyclonal, raised against a peptide mapping within residues 476–526 of human p67phox), p67phox H-300 (rabbit polyclonal, raised against residues 1–300 of human p67phox), and anti-p67phox N-19 (goat polyclonal, raised against a peptide within residues 2–52 of human p67phox) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Second anti-got IgG and anti-rabbit IgG antibodies, conjugated with peroxidase, were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Tetramethyl benzidine + substrate chromogen reagent (Reference S1599) was obtained from DakoCytomation (Glostrup, Denmark).

Preparation of recombinant proteins

Recombinant p67phox (rp67phox), full-length (residues 1–526), as well as truncations p67phox(1–212), (1–198), and (1–186) with an N-terminal 6His tag were expressed in Escherichia coli and purified on nickel sepharose, as described before [21]. The proteins were purified further by fast protein liquid chromatography gel filtration on a HiLoad 16/60 Superdex 200 prep-grade column for p67phox(1–526) and a HiLoad 16/60 Superdex 75 prep-grade column for the truncated p67phox proteins (both columns were from GE Healthcare Bio-Sciences AB, Uppsala, Sweden), as described before [21], with one modification concerning the running buffer, which was PBS (consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2PO4, and 1.4 mM KH2PO4, pH 7.3). p47phox and Rac1 (Q61L mutant), both with an N-terminal 6His tag, were expressed in E. coli and purified on nickel sepharose, including the gel-filtration step on Superdex 75, as carried out with p67phox. The protein concentration of the recombinant proteins was measured by the method of Bradford [22], with bovine y-globulin as a standard. The level of purity of the recombinant proteins was assessed by SDS-PAGE analysis. The purified
proteins were stored, dissolved in PBS, supplemented with 30% v/v glycerol, divided into small aliquots, and frozen at -27°C. Recombinant maltose binding periplasmic protein (MBP) from *E. coli* with a 6His N-terminal tag was constructed by use of the expression vector pET-30a (Novagen; EMD Chemicals, Merck KGaA, Darmstadt, Germany), carrying cDNA and encoding residues 29–392 of MBP, preceded by the sequence MHHHHHSGKT and followed by residues NSSS, and introduced into *E. coli* Rosetta 2(DE3)pLysS cells (Novagen). The protein was expressed and purified as done with p67phox.

Measuring peptide–protein binding by a kinetic ELISA

This assay was used to assess the binding of p67phox and of unrelated proteins in solution to surface-attached synthetic Nox2 peptides. The procedure is a thoroughly modified version of that described before [16, 17]. The original assay was an end-point procedure, which even under the most optimal conditions, was shown to be "semiquantitative." This is a result of the fact that the relationship between absorbance and the amount of bound enzyme is not linear over time, and thus, absorbance read at end point might be a plateau value, identical for reactions with different slopes. For the present study, we developed a modification of the kinetic ELISA [23, 24], in which data are expressed by the linear segment of the change in absorbance curve over time. A detailed description of the modified procedure is presented here. All binding experiments were performed with streptavidin-coated, 96-well plates (BioPlate Assembly, streptavidin-coated, Cat. No. 95029263; Thermo Electron, Vantaa, Finland). These plates have a streptavidin-coated area, corresponding to a volume of 200 μl/well, and a binding capacity of 25 pmol biotin/well. All of the solutions used in the binding assay were filtered through 0.45 μm filters (Supor 450 membrane filters; Pall, Ann Arbor, MI, USA). To the wells was added 300 μl/well PBS, supplemented with 0.1% v/v Tween 20 and 1% w/v casein (PBS-T-Cas), and the plates were kept at room temperature for 1 h to prevent nonspecific attachment of peptides and proteins to the well surface. The wells were washed 4 times with 300 μl/well PBS-T, with the use of an automatic microplate washer (Wellwash Ascent; Labsystems Oy, Helsinki, Finland) fitted with a "cell wash head" (Code N10802), found, in preliminary experiments, to be optimal for not disrupting low-affinity protein–protein interactions. Synthetic Nox2 peptides, with a biotin tag at the N or C terminus, were first diluted from the original 1.5 mM concentration in 75% NMP/25% water to a concentration of 100 μM in PBS, followed by further dilution to a concentration of 1 μM in PBS supplemented with 1% casein (PBS-Cas), on the day of the performance of the experiment. Volumes (200 μl/well; corresponding to 200 pmol/well, representing an 8-fold excess over the biotin-binding capacity of the wells) were added, the plates were incubated for 1 h at room temperature, and the well contents were mixed on an orbital shaker. After removing the unattached proteins were dissolved, stored in PBS, supplemented with 30% v/v glycerol, divided into small aliquots, and frozen at ~75°C. Reconstituent maltose binding periplasmic protein (MBP) from *E. coli* with a 6His N-terminal tag was constructed by use of the expression vector pET-30a (Novagen; EMD Chemicals, Merck KGaA, Darmstadt, Germany), carrying cDNA and encoding residues 29–392 of MBP, preceded by the sequence MHHHHHSGKT and followed by residues NSSS, and introduced into *E. coli* Rosetta 2(DE3)pLysS cells (Novagen). The protein was expressed and purified as done with p67phox.

Proteins were measured by a kinetic ELISA
peptide by 4 washes with 300 µl/well PBS-T, the wells were filled with 200 µl vol p67phox (or control proteins) at a concentration of 1.5 µM in PBS-Cas (corresponding to 300 pmol/well) and the plates kept for 16–18 h at 4°C, with mixing on an orbital shaker. After removal of unattached p67phox by 4 washes with 300 µl/well PBS-T, 200 µl/well of a 1/3000 dilution of peroxidase-conjugated antipolyhistidine antibody in PBS-T-Cas was added and the plate incubated for 1 h at room temperature, with mixing on an orbital shaker. Unbound antibody was removed by 4 washes with 300 µl/well PBS-T, followed by 2 washes with PBS. Bound, peroxidase-conjugated antibody was quantified by adding 200 µl/well TMB + substrate chromogen reagent and measuring the increase in absorbance at 650 nm over time for 10 min in a SpectraMax 340 microplate reader, fitted with SoftMax Pro 5.2 software (Molecular Devices, Sunnyvale, CA, USA) in the kinetic mode. Forty-one readings were made at 15 s intervals, and the content of the wells was mixed for 30 s before the start of reading and for 3 s between readings to compensate for the consumption of the peroxidase substrate in the fluid phase closest to the well surface. Results were expressed as increase in milliabsorbance units at 650 nm/min (absorbance at 650 nm × 1000/min),—the calculation based on the linear segment of the curve. A schematic representation of the kinetic ELISA is shown in Fig. 2. In a limited number of experiments, a 2-step antibody detection system was used. This consisted of incubation with anti-p67phox antibody C-19, or another anti-p67phox antibody (1/1000 dilution in PBS-T-Cas, 200 µl/well) for 1 h, followed by 4 washes with PBS-T and incubation with peroxidase-conjugated anti-IgG (1/5000 dilution in PBS-T-Cas, 200 µl/well) for 1 h. From this point on, the procedure was identical to that described above for peroxidase-conjugated antipolyhistidine antibody.

Graph plotting and statistics

The plotting of graphs and kinetic analyses was executed by use of GraphPad Prism Version 6.05 (GraphPad Software, San Diego, CA, USA) and SigmaPlot Version 2000 (SPSS, Chicago, IL, USA). Statistical analysis of comparing the means and sem of 2 groups was performed by unpaired t test.

RESULTS

Detection of a binding site for p67phox in the DHR of Nox2 by a peptide array assay—methodological aspects

Peptide arrays were used successfully in the past for the identification of sequences involved in protein–protein interactions essential for the assembly of the NADPH oxidase complex [16, 17]. We now applied this method to identify and characterize the region(s) in the DHR of Nox2 responsible for interacting with p67phox. Ninety-one overlapping, unpurified Nox2 DHR 15-mer peptides, with a biotin tag at the N terminus, were attached to streptavidin-coated, 96-well plates and allowed
to react with r6His-p67phox(1–526). The methodology differed from that used before by the following: 1) All recombinant proteins had a 6His tag at the N terminus; 2) detection of binding of the proteins to the peptides was based on the use of a single antipolyhistidine mAb peroxidase conjugate, able to react with full-length and truncated forms of p67phox and with the control proteins, with no need for a second antibody; and 3) the conventional ELISA was replaced by a kinetic assay, with an improved quantification capability. The choice of the antipolyhistidine antibody was the consequence of extensive preliminary experiments, in which we tested several anti-p67phox antibodies (listed under Materials and Methods), followed by peroxidase-conjugated anti-IgG antibody. With the exception of anti-p67phox C-19, reacting with a C-terminal epitope, none of the other antibodies was suitable for detection, as a result of a number of reasons, comprising lack of recognition of truncated forms of p67phox, cross-reaction with sequences in Nox2 DHR, or the involvement of the anti-p67phox epitope in binding of p67phox to the Nox2 peptides.

**p67phox** binds to several peptide clusters, corresponding to specific regions in Nox2 DHR, some of which are shared with p47phox

As apparent in Fig. 3A, full-length p67phox(1–526) binds to 5 clusters of peptides, composed of peptides 3–12, 24–28, 46–48, 53–54, and 72–73. A positive cluster was defined by the majority of the peptides in the cluster, binding p67phox at a level at least twice the mean value of binding of p67phox to wells not coated.

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Figure 3. The binding of p67phox(1–526) and control proteins to Nox2 DHR peptides. The binding of p67phox(1–526) (A), p47phox(1–390) (B), and MBP (C) in the fluid phase to surface-attached, unpurified Nox2 peptides. The concentration or reagents, method of binding, and expression of results were identical for the 3 proteins and are expounded in detail in Materials and Methods. The bold numbers above the bars indicate the number of the peptide(s) binding the proteins, as appearing in the list in Fig. 1. The numbers in brackets below the peptide number indicate the N- and C-terminal residues of the peptide clusters. The horizontal line in each graph represents the mean value of the level of protein binding in the absence of surface-attached peptides. (A, inset) The binding of p67phox(1–526) to purified Nox2 DHR peptides 24–28. The binding of p67phox to peptides 24 and 28, compared with binding to wells in the absence of peptides, was statistically significant (P < 0.020 for unpurified and < 0.016 for purified peptides). The binding of p67phox to purified peptides 25 and 27 (but not to peptide 26) was also statistically significant (P ≤ 0.016). Results in all 3 panels represent means ± SEM of 3 experiments.
with the peptides (appearing as a horizontal line along the x-axis). The residues forming the clusters are indicated in Fig. 1 by highlighting in yellow. The specificity of p67phox binding was explored by measuring the binding to Nox2 peptides of the cystolic component p47phox (1–390) and that of the unrelated MBP, both expressed in E. coli as recombinant proteins with an N-terminal 6His tag. The 2 proteins have relative molecular masses, smaller by ~2 kDa than that of p67phox, and exhibit no significant sequence similarity with p67phox. p47phox contains 3 protein–protein interaction modules, also present in p67phox (2 SH3 domains and a PRR), and has a GRAVY of −0.689, which is similar to that of p67phox (−0.519), but a higher pI (9.12) than that of p67phox (5.88). MBP has a pI similar to p67phox (5.48) but is more hydrophobic than p67phox (GRAVY = −0.398).

p47phox bound to clusters 3–12, 46–48, and 72–73 at a level resembling that of p67phox; no significant binding to clusters 24–28 and 53–54 was detected (Fig. 3B). No binding of MBP to Nox2 DHR peptides was found (Fig. 3C).

These findings focused the binding of p67phox to clusters 24–28 and 53–54. Whereas the majority of clusters consisted of a central “peak” peptide, shouldered by peptides of lesser binding capacity on both sides, cluster 24–28 comprised 2 peptides (24 and 28) with higher binding capacity and a core of 3 peptides (25–27) exhibiting lower binding. The fact that the set of 91 peptides was more hydrophobic than p67phox (GRAVY = −0.398), p47phox bound to clusters 3–12, 46–48, and 72–73 at a level resembling that of p67phox; no significant binding to clusters 24–28 and 53–54 was detected (Fig. 3B). No binding of MBP to Nox2 DHR peptides was found (Fig. 3C).

The binding of p67phox to DHR of Nox2 peptides

We next investigated the characteristics of the specific and nonspecific bonds between p67phox (1–526) and Nox DHR peptides. We first plotted the charge of the peptides in relation to the binding of p67phox. As apparent in Fig. 4A, upper, clusters 3–12, 46–48, and 72–73 consisted of positively charged peptides; clusters 24–28 and 53–54 of negatively charged peptides. As p67phox (1–526) has a pI of 5.88, its binding to clusters 24–28 and 53–54 cannot be based on electrostatic interaction. The binding of p67phox to clusters 3–12, 46–48, and 72–73 might involve electrostatic attraction, although the finding that p47phox (1–390), possessing a pI of 9.12, also binds to the same clusters does not support this.

To clarify this issue, we examined the effect of raising the ionic strength of the buffer, in which the p67phox–peptide interaction was assessed by increasing the concentration of NaCl from 137 to 500 mM. Considering the variability of absolute binding values (see possible reason for this in Discussion), it was essential to run experiments in which binding under various buffer conditions was compared in parallel, by use of the same batches and dilutions of peptides, p67phox, antibody, and other reagents, as well as the same experimental conditions. The increase of the ionic strength conserved the binding of p67phox to cluster 24–28 but eliminated binding to clusters 3–12 and 72–73 and reduced binding to clusters 46–48 and 53–54 (Fig. 4B compared with Fig. 4A, lower). We noticed an increase in binding to the individual peptide 28 at high ionic strength, a finding that was confirmed with purified peptide 28 (result not shown) and is referred to in Discussion.

Yet another possible reason for nonspecific binding of p67phox was an interaction between the polyhistidine tag on the recombinant protein and Nox2 DHR peptides. Thus, we assessed the binding of p67phox (1–526) to Nox2 DHR peptides in the presence of 200 mM imidazole, a known competitive inhibitor of histidine, by virtue of the presence of an imidazole side chain in histidine [25]. As seen in Fig. 4C, in the presence of 200 mM imidazole, the binding of p67phox to cluster 24–48 was undisturbed, whereas binding to clusters 3–12, 46–48, and 72–73 was eliminated and that to cluster 53–54 reduced.

As shown in Fig. 3B, p47phox also binds to Nox2 peptide clusters 3–12, 46–48, and 72–73. In the presence of 500 mM NaCl or 200 mM imidazole, the binding of p47phox to all 3 clusters was eliminated, mimicking the effect on binding of p67phox (result not shown). The very binding of p47phox to the 3 clusters of positively charged peptides is unexpected, in light of the overall positive charge of p47phox (1–390) (pI = 9.12), and the inhibitory effect of high salt and imidazole suggests that binding to these clusters might involve a mechanism distinct from electrostatic interaction.

These results support the hypothesis that some residues in the peptides in cluster 24–28 represent a specific interaction site for p67phox and that binding does not involve electrostatic or polyhistidine tag-dependent mechanisms.

The binding of p67phox to the DHR of Nox2 is centered on 2 Nox2-specific vicinal cysteines

An unexpected finding was the atypical pattern of p67phox binding to cluster 24–28, characterized by maximal binding to peptides 24 and 28 and bidirectional decrease in binding toward peptide 26 (Fig. 3A, inset). A look at the peptides arranged in an overlapping fashion revealed that all 5 peptides share a CGC triad, located at the C terminus in peptide 24 and at the N terminus in peptide 28 (Fig. 5A). In the least-reactive peptide 26, the CGC is located at the center, whereas in peptides 25 and 27, the CGC is located 3 residues from the C and N terminus, respectively. The alignment of the Nox2 sequence, extending from the N terminus of peptide 24 to the C terminus of peptide 28, with the corresponding region of Nox1, -3, -4, and -5, based on Kawahara et al. [27], demonstrated that the CGC triad was present only in Nox2 (Fig. 5B). The alignment of the CGC triad in human Nox2 with the corresponding region in other species, based on Kawahara et al. [27] and Davis et al. [28], showed that the CGC triad is highly conserved in Nox2 of mammals, birds, and amphibians, with a CGG triad, in a position corresponding to the CGC triad, found in fishes (Fig. 5C). It might also be of interest that a Leu-Gly-Cys triad “reappears” in the same position in the Nox2 equivalent (NoxB) of some filamentous fungi (Podostora anserina, Fusarium graminearum, Magnaporthe grisea; data not shown).

A schematic representation of the location of CGC in peptides 24–28, attached via an N-terminal biotin tag to the streptavidin-coated well surfaces, is shown in Fig. 5D. The modeling of
peptide 24 with the biotin tag linked to the N terminus via a 4-residues spacer [26] reveals an α-helical core from residues D to A, with an unstructured CGC triad at the C terminus (Fig. 5E). The most likely interpretation of the maximal binding of p67phox to peptides 24 and 28, derived from these data, is that binding is to a residue(s) in the CGC triad and that optimal binding correlates with the exposure of CGC to the fluid phase containing p67phox.

In light of these results and of the fact that a vicinal cysteine-containing sequence (85CysCys86) is also found in the cytosolic loop B of Nox2, we performed p67phox-binding experiments with the purified loop B peptide 85CCSTRVRRQ194, also reported to serve as a binding site for p47phox [8]. No binding was detected (result not shown).

Effect of C-terminal truncation of p67phox on binding to Nox2 DHR peptides

To get an idea about the partner structures in p67phox participating in interaction with Nox2 DHR peptides, we constructed 3 C-terminally truncated forms of p67phox (Fig. 6C). Truncation at residue 212 generates a protein comprising the 4 TPRs, the canonical AD (residues 199–210 [10]), and the extension of the AD (EAD) (residues 187–193 [29]). Truncation at residue 198 removes the canonical AD, and truncation at residue 186 removes the canonical AD and the EAD.

From this point on, all work was done with purified peptides. As only peptides 24 and 28 and variations of these 2 were used, there was no need to avoid nonspecific binding, and thus, binding experiments were performed in PBS.
As apparent in Fig. 6A, full-length p67phox (1–526) and all 3 truncated forms were bound with similar affinity to Nox2 peptide, but there was a tendency of enhanced binding of p67phox truncated at residues 198 and 186, as also apparent in other groups of experiments (see Figs. 7A and 9A). It should be noted here that p67phox (1–526) has a pI of 5.88, whereas the pI of the truncated forms ranges from 8.86 to 9.0, yet another indication for the lack of involvement of an electrostatic mechanism in binding.

The binding to Nox2 peptide 28, however, exhibited a different pattern, with all 3 truncations binding with lesser affinity compared with the full-length protein (Fig. 6B). This is a first indication that in addition to the shared CGC triad, other residues, which are different in Nox2 peptides 28 and 24, have a role in binding. On the p67phox aspect of the Nox2–p67phox interaction, it appears that all residues participating in binding to peptide 24 are present in the N-terminal 1–186 sequence, whereas binding to peptide 28 involves residues (or additional residues) present in the C-terminal 213–526 sequence.

These results are to be seen in the context of earlier findings that p67phox (1–210) was as capable as p67phox (1–526) of activating the Nox in a cell-free system, but p67phox (1–198) was inactive [10]. As opposed to this, p67phox truncated at residues 210 or 199 were both capable of binding to purified cytochrome b558.
an indication that the p67phox–Nox2 interaction is required but not sufficient for oxidase activation.

The replacement of 369Cys by Arg or Ser in Nox2 DHR peptides abolishes binding of p67phox to peptide 24 but not to peptide 28

A 369Cys-to-Arg (C369R) mutation in Nox2 in humans causes chronic granulomatous disease (CGD) of the 391+ form, with normal expression of a nonfunctional Nox2 [30]. O2− production was impaired in whole cells and in a cell-free system. The introduction of a C369R mutation in wild-type Nox2 DNA, followed by transfection into 391° CGD PLB-985 cells, led to the generation of cells mimicking the defect in Nox found in phagocytes of patients with 391+ CGD [31]. Translocation of p67phox and p47phox from the cytosol to the membrane was reported to be severely impaired in the phorbol ester-stimulated neutrophils of the C369R mutant CGD patient and in a cell-free system derived from the patient’s neutrophils [30]. Translocation of both cytosolic components to the phagosomal membranes of PBL-985 cells, transfected with C369R mutant DNA and stimulated with latex particles, was also defective [31].

Nox2 peptides 24 and 28 were synthesized, in which 369Cys was replaced by Arg (Fig. 7C). Otherwise, the mutant peptides were identical to the native peptides, containing an N-terminal biotin tag and a SGSG spacer. We confirmed the absence of 1 cysteine by measuring the SH groups in the mutant peptide compared with the native peptide by the method of Ellman [20].

The replacement of 369Cys by Arg in peptide 24 was dictated by...
the intention to mimic the in vivo mutation causing CGD [30]. However, Arg differs markedly from Cys by both a positive charge, versus the neutral character of Cys, and by a different GRAVY. Thus, a peptide 24 was also synthesized in which 369Cys was replaced by Ser, an amino acid more similar to Cys by its charge, versus the neutral character of Cys, and by a different GRAVY. The binding of p67<sub>phox</sub> to the mutant peptide 24 C369S was also markedly reduced, demonstrating that the lack of binding to the C369R mutant was not a result of the specific replacement of Cys by Arg (result not shown).

In contrast, the binding of p67<sub>phox</sub> to mutant peptide 28 C369R was similar to that to the unmodified peptide (Fig. 7B). The binding of the truncated forms of p67<sub>phox</sub> was low to unmodified and mutant peptide 28, as expected, based on the results shown in Fig. 6B. The different effect of the C369R “mutation,” when introduced in peptide 24, as opposed to peptide 28, was also assessed by measuring binding of p67<sub>phox</sub> to the peptides over a wide range of p67<sub>phox</sub> concentrations, from 9 to 600 pmol/well. As apparent in Fig. 8A and B, the binding of p67<sub>phox</sub> to the unmodified peptides 24 and 28 exhibited a linear relation to the concentration of p67<sub>phox</sub>. The binding of p67<sub>phox</sub> to mutant peptide 24 C369R was markedly decreased over the whole concentration range of p67<sub>phox</sub>, whereas the binding to mutant peptide 28 C369R overlapped the binding curve to the unmodified peptide.

These results indicate that the CGC triad represents the main site responsible for the binding of full-length and truncated forms of p67<sub>phox</sub> to peptide 24 but that in peptide 28, residues other than the CGC triad also participate in the binding of full-length p67<sub>phox</sub>. This interpretation of the data is also supported by peptide-scrambling experiments. Thus, peptide 24, in which the location of cysteines in the CGC triad was scrambled by replacing CGC by CCG, did not bind p67<sub>phox</sub> (result not shown). Furthermore, peptide 28, in which the native sequence CGCDKQEFQDAWKLP was scrambled to CCGEQAKPGQFDWL, did not bind p67<sub>phox</sub> (result not shown).

p67<sub>phox</sub> does not bind to Nox2 DHR peptides 24, in which the Nox2 sequence was replaced by Nox4 or Nox1, and to peptide 28, in which the Nox2 sequence was replaced by Nox4

The rationale behind the idea of measuring binding of p67<sub>phox</sub> to the Nox4 and Nox1 analogs of Nox2 peptides included: 1) that Nox4 is constitutively active, not requiring interaction with cytosolic activators, including p67<sub>phox</sub>; 2) the absence of the CGC triad in Nox4 and Nox1 (Fig. 5B); 3) that Nox1 activity, although not fully dependent on cytosolic activators, is enhanced by interaction with the p47<sub>phox</sub> and p67<sub>phox</sub> analogs NOXO1 and NOXA1 and in certain situations, with p47<sub>phox</sub> and p67<sub>phox</sub> [32].

Two peptides were synthesized, comprising sequences derived from Nox4 and Nox1, corresponding by alignment to Nox2 peptide 24 and 1 peptide, consisting of the Nox4 sequence, corresponding to Nox2 peptide 28. The alignments were based on Kawahara et al. [27]. The Nox4 and Nox1 peptides and their alignment with Nox2 peptides 24 and 28 are illustrated in Fig. 9C. Nox4 peptide 24 possesses 47% identity, and Nox1 peptide 24 possesses 33% identity with Nox2 peptide 24; Nox4 peptide 28 possesses 27% identity with Nox2 peptide 28. As apparent in Fig. 9A, Nox4 and Nox1 peptides 24 were incapable of binding the full-length p67<sub>phox</sub> or any of the
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Figure 8. A dose-response study of the binding of p67phox to native and C369R mutant Nox2 DHR peptides 24 and 28. (A) p67phox (1–526) exhibits lesser binding to C369R mutant peptide 24 compared with native peptide 24 at all concentrations of p67phox (9.37–600 μM); the difference in binding of p67phox to native versus mutant peptide 24 was statistically significant over the concentration range 150–600 nM p67phox (P ≤ 0.019). (B) p67phox (1–526) binds equally to native peptide 28 and to C369R mutant peptide 28 at all concentrations of p67phox (9.37–600 μM); the difference in binding of p67phox to native versus mutant peptide 28 was not statistically significant over the concentration range 150–600 nM p67phox (P ≥ 0.212). Results represent means ± sem of 3 experiments for binding to each of the 2 pairs of peptides.

The binding of p67phox to surface-attached Nox2 DHR peptides is governed by the exposure of the CGC triad to the fluid phase

The results described so far were all obtained with Nox2 peptides (unpurified and purified), attached via an N-terminal biotin tag to streptavidin-coated, 96-well plates. By examining peptide cluster 24–28, it became apparent that the location of the biotin tag affects the degree of exposure of the CGC triad to the fluid phase containing p67phox (Fig. 5D). In the particular case of peptide 24, although its precise conformation is unknown, modeling suggests the association of optimal binding of p67phox with the location of the CGC triad at C terminus, thus at the opposite end of the surface-attached biotin tag (Fig. 5E).

To test this hypothesis further, peptides 24 and 28 were synthesized with a C-terminal biotin tag attached via linkers of a similar length to the one used for attaching biotin to the N terminus. A schematic view of the effect of inverting the location of the biotin tag on the location of the CGC triad in peptides 24 and 28 appears in Fig. 10C. The binding of full-length p67phox and of p67phox truncated at residue 212 to peptide 24 with C-terminal biotin was markedly reduced (Fig. 10A). The placement of biotin at the C terminus of peptide 28 had the opposite effect; the binding of full-length p67phox was moderately enhanced, and even the minor level of binding of p67phox truncated at residue 212 got a certain boost (Fig. 10B).

The modeling of peptide 28 with C-terminal biotin (Fig. 10D) shows an image similar to that of peptide 24 with N-terminal biotin (Fig. 5E), exhibiting a central α-helix, followed by an unstructured segment containing the CGC triad. Results shown in Fig. 7B indicated that binding of p67phox to peptide 28 with N-terminal biotin was predominantly CGC independent, involving residues C terminal to the CGC triad. The fact that placing biotin at the C terminus leads to some enhancement of binding is probably a result of the better exposure of the CGC triad to p67phox. This suggests that binding to peptide 28 comprises CGC-independent and CGC-dependent elements, their relative participation dictated by their respective exposure to p67phox.

One of the arguments supporting a CGC-independent mechanism for binding of p67phox to peptide 28 with N-terminal biotin was the persistence of binding to the mutant peptide 28 C369R (Fig. 7B). Thus, we repeated the experiments shown in Fig. 7 by comparing binding with peptides 24 and 28 with biotin at the N or C terminus. The binding of p67phox (1–526) and p67phox (1–212) to the mutant peptide 24 C369R was impaired, independently of the location of biotin (Figs. 7A and 11A). In sharp contrast, the binding of p67phox (1–526) and p67phox (1–212) to the mutant peptide 28 C369R was dependent on the location of the biotin tag. The similar binding to the native and mutant peptide 28, when the biotin was attached to the N terminus, shown in Figs. 7B and 11B, was replaced by lack of binding to the mutant peptide, when biotin was attached to the C terminus (Fig. 11B).

DISCUSSION

There is multifaceted evidence for a central role of p67phox in the activation of the NADPH oxidase, culminating in O2– generation. This appears to be the result of a direct interaction of p67phox with the only catalytic component, Nox2. In this conceptual framework, p47phox and Rac function principally to facilitate the p67phox–Nox2 interaction, although this is not limited to a passive carrier role. Thus, in addition to the “mechanical” assistance in targeting p67phox to the membrane environment of flavocytochrome b558, Rac molds p67phox for optimal interaction with Nox2. The first indication for such a course of events came from cell-free activation studies,
Figure 9. p67\textsuperscript{phox} does not bind to Nox4 and Nox1 peptides corresponding to Nox2 DHR peptide 24 and to a Nox1 peptide corresponding to Nox2 peptide 28. Full-length p67\textsuperscript{phox} and all truncated forms do not bind to the Nox4 and Nox1 equivalents of Nox2 peptide 24 (A) and to the Nox1 equivalent of peptide 28 (B). The differences in binding of full-length p67\textsuperscript{phox} and all truncations to peptide 24 Nox2 versus both peptides 24 Nox4 and 24 Nox1 were statistically significant (\(P < 0.007\) for Nox2 vs. Nox4 and \(\leq 0.016\) for Nox2 vs. Nox1). The differences in binding of full-length p67\textsuperscript{phox} and all truncations to peptide 28 Nox2 versus peptide 28 Nox4 were also statistically significant (\(P < 0.002\)). Results represent means \(\pm\) SEM of 3–5 experiments of binding to each of the groups of peptides tested. (C) Alignment of sequence regions corresponding to peptide 24 in Nox2, Nox4, and Nox1 and to peptide 28 in Nox2 and Nox4. Residues in black font in peptides 24 Nox2 and Nox1 are shared by peptide 24 Nox2; residues in red font are different. Residues in black font in peptide 28 Nox4 are shared by peptide 28 Nox2; residues in red font are different.

<table>
<thead>
<tr>
<th>Peptide Number</th>
<th>Sequence in Nox</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 in Nox2</td>
<td>357-IVGDWTEGFLPACGC-371</td>
</tr>
<tr>
<td>24 in Nox4</td>
<td>357-IVGDWTEKRPDLILFP-367</td>
</tr>
<tr>
<td>24 in Nox1</td>
<td>357-AGAGDWTENLIRAPEQ-371</td>
</tr>
<tr>
<td>28 in Nox2</td>
<td>369-CGCDQEXRPQDQWKLDP-383</td>
</tr>
<tr>
<td>28 in Nox4</td>
<td>369-DLLDPSSS-QDSELILDP-397</td>
</tr>
</tbody>
</table>

Demonstrating the redundancy of p47\textsuperscript{phox}, as opposed to the absolute requirement for p67\textsuperscript{phox} (assisted by Rac) [33, 34]. This was followed by experiments showing that Rac-mediated targeting of p67\textsuperscript{phox} to the membrane was sufficient for Nox activation in vitro [5, 6] and by similar evidence derived from work with p67\textsuperscript{phox}-Rac chimeric constructs [35–38]. Finally, imaging of flavocytochrome b\textsubscript{58} by atomic force microscopy also revealed a paramount role for p67\textsuperscript{phox} in Nox activation [39].

Therefore, it is surprising that no information is available on the region(s) in Nox2 interacting with p67\textsuperscript{phox} except for the assumption that this is likely to be located in the DHR (residues 288–570) or in the cystolic loops B and D, bound to be accessible to p67\textsuperscript{phox}. We approached this question by assessing the binding of full-length and truncated forms of 6His-tagged p67\textsuperscript{phox} and all truncations to peptide 28. The interaction was monitored in a system in which the Nox2 peptides were biotin tagged and immobilized to the surface of streptavidin-coated, 96-well plates, and p67\textsuperscript{phox} was present in a solution covering the wells. The binding of p67\textsuperscript{phox} to the Nox2 peptides was quantified by a kinetic ELISA, based on the use of a peroxidase-conjugated, antipolyhistidine mAb.

This led to the discovery of a p67\textsuperscript{phox}-binding region, represented by peptides 24–28, in a solution covering the well, p67\textsuperscript{phox} was present in a solution covering the wells. The binding of p67\textsuperscript{phox} to the Nox2 peptides was quantified by a kinetic ELISA, based on the use of a peroxidase-conjugated, antipolyhistidine mAb.

To the best of our knowledge, this is the first description of a well-defined binding site for p67\textsuperscript{phox} in the DHR of Nox2.

Subjecting this basic finding to detailed analysis led to the following conclusions. 1) The binding is p67\textsuperscript{phox} specific, as shown by the lack of binding of p47\textsuperscript{phox} and MBP. 2) The binding is unrelated to charge, as both the peptides and p67\textsuperscript{phox} (1–526) have a negative charge expected to lead to repulsion, and the increase in the ionic strength, expected to counter electrostatic attraction, does not interfere with binding. 3) The CGC triad is present in Nox2 but absent in Nox1, -3, -4, and -5. It is well conserved in Nox2 of mammals, birds, and amphibians. 4) Optimal binding of p67\textsuperscript{phox} is associated with the exposure of the CGC triad to the fluid phase, containing p67\textsuperscript{phox}. As a result of the geometry of surface-attached peptides, exposure is best in peptide 24 (CGC at C terminus), when surface attached by N-terminal biotin, and in peptide 28 (CGC at N terminus), when surface attached by C-terminal biotin. 5) A clear distinction between peptides 24 and 28 is evident when binding of various truncated forms of p67\textsuperscript{phox} is compared: C-terminally truncated forms of p67\textsuperscript{phox}, at residues 212, 198, and 186, are bound to peptide 24, whereas only full-length p67\textsuperscript{phox} is bound to peptide 28. 6) The replacement of 306Cys by Arg or Ser in peptide 24 prevents binding of p67\textsuperscript{phox}, whereas the same replacement in peptide 28 has no effect on binding. 7) The reversal of the geometry of peptide 24 with a 366Cys-to-Arg replacement, by C-terminal attachment to the well, which exposes the CGC triad to the fluid phase, results in lack of p67\textsuperscript{phox} binding. 8) The sequence specificity of binding is also demonstrated by the lack of binding of p67\textsuperscript{phox} to peptide 24 with scrambled cysteines and to peptide 28 with random residue scrambling. 9) Peptides 24 and 28, in which the Nox2 sequence was replaced by Nox4, and peptide 24, in which the Nox2 sequence was replaced by Nox1, do not bind p67\textsuperscript{phox}.

These results suggest that binding of p67\textsuperscript{phox} to peptide 24 is predominantly or fully dependent on the CGC triad. It also appears
that the required reciprocal binding site(s) for peptide 24 on p67$^{phox}$ are located N terminal to residue 186. The binding of p67$^{phox}$ to peptide 28 is predominantly CGC independent and probably involves Nox2 residues 372–383; in the p67$^{phox}$ partner, it probably requires the participation of residues C terminal to residue 212.

Sequence analysis of Nox2 DHR indicates that the region corresponding to peptides 24 and 28 is an insertion in Nox compared with other dehydrogenases that do not require regulatory proteins for activity, making this region a likely candidate-binding site for Nox modulatory proteins. Three-dimensional modeling of the Nox2 DHR [40] suggests that the region centered on $^{369}$Cys is surface exposed and well suited for functioning as a docking site for p67$^{phox}$ [30, 31]. Molecular modeling also indicates that this region is situated at a critical site in the Nox2 DHR, with peptide 24 partially overlapping the ribityl chain-binding subdomain of the FAD-binding domain [28, 41] and peptide 28 adjacent to or part of the “strap” connecting the FAD- and NADPH-binding domains. The involvement of part
Results obtained so far indicate that binding of p67phox involves the establishment of disulfide bonds between cysteine(s) in peptide 24 and cysteine(s) in p67phox and that the introduction of an intramolecular disulfide bond between 369Cys and 371Cys in the peptide markedly enhances binding (ref. [42] and [unpublished results]). It is likely that the variability in absolute binding values, from experiment to experiment, evident in our results, is a result of unavoidable variations in the level of oxidation of cysteine in the Nox2 peptides and/or p67phox in the course of their preparation, storage, or use in experiments [43].

An important, unsolved question is the identity of the particular cysteine(s) in p67phox engaged in binding to Nox2. The finding that 3 truncated forms of p67phox (at residues 212, 198, and 186), all of which contain 4 cysteines, were bound to peptide 24 with an affinity equal or superior to that of full-length p67phox suggests that cysteine(s) located N terminal to residue 186 were involved.

Another only partially solved problem is the nature of the CGC-independent interaction between peptide 28 and full-length p67phox. Our results indicate that no cysteines are involved, although under specific experimental conditions, such as “forced” exposure of the CGC triad to p67phox, they do contribute to binding. The finding that binding of p67phox to peptide 28 is augmented at a high NaCl concentration (Fig. 4B) also suggests a distinct mechanism of binding, probably involving hydrophobic residues in the C-terminal half of the peptide. Also unknown remains the precise identity of the region(s) in p67phox participating in the CGC-independent interaction.

The lack of binding of p67phox to the Nox1 analog of peptide 24 should be considered in the context of the fact that Nox1 is subject to regulation by the p67phox analog NOXA1 and to a certain degree, by p67phox. The absence of binding might be explained by the Nox1–NOXA1 interaction being mediated by a CGC-independent mechanism, involving residues in Nox1 distinct from those present in peptide 24, or by differences between p67phox and NOXA1 (only 28% sequence identity, the lack of an N-terminal SH3 domain in NOXA1, and the constitutive presence of NOXA1 in the plasma membrane, thus in close vicinity to Nox1).

Nox2 peptides 24 and 28 were not found to be inhibitory when tested in an earlier study for an effect on NADPH oxidase activation in vitro [44]. As in that work, unpurified Nox2 peptides were screened at a concentration of 10 μM, we reexamined the ability of purified peptides 24 and 28 to inhibit NADPH oxidase activation in a cell-free system over a concentration range of 1.25–40 μM. We found percent inhibition of NADPH oxidase activity values for peptide 24 of 1.11 ± 2.02 at 10 μM peptide and 25.28 ± 3.90 at 40 μM peptide (means ± SEM of 7 experiments). For peptide 28, the values were 2.64 ± 2.45 at 10 μM peptide and 22.11 ± 3.84 at 40 μM peptide (means ± SEM of 3 experiments). However, the significance of these results was questionable because of the absence of typical sigmoidal dose-response curves and the fact that the mutant peptide 24 C369R exhibited an inhibitory potency similar to that of the native peptide 24. We do not have a ready explanation for this negative result, but both methodological and conceptual parameters might be relevant.

Thus, binding studies were performed in a setup, in which the Nox2 peptides were present in the solid phase and p67phox in the

Figure 11. The impairment in the binding of p67phox to Nox2 DHR peptides 24 and 28 with a C369R mutation is most evident when the peptides are attached to the wells in a position assuring exposure of the CGC triad to the fluid phase. (A) The binding of p67phox (1–526) and p67phox (1–212) to peptide 24, in which C369 was replaced by R (C369R), is markedly reduced compared with binding to the native peptide, whether the biotin tag is attached to the N or C terminus. The lesser binding of p67phox to native peptide 24 with a C-terminal biotin tag is also evident (as also seen in Fig. 10A). (B) The binding of p67phox (1–526) to peptide 28 with an N-terminal biotin tag is not influenced by replacing C369 by R (as also seen in Fig. 7B). However, the replacement of C369 by R in peptide 28 with a C-terminal biotin tag markedly reduces binding of p67phox (1–526); the same is true for binding of p67phox (1–212), although the extent of binding to native peptide 28 is low (as also seen in Fig. 10B). The difference in binding to the native and mutant peptides 28 with C-terminal biotin was statistically significant for both p67phox (1–526) and p67phox (1–212) (P ≤ 0.021). Results represent means ± SEM of 3 experiments for binding to each of the 2 pairs of peptides tested.

of the region corresponding to peptide 24 in binding of FAD is in good agreement with the reported impairment of FAD incorporation in membranes of PLB-985 cells transfected with C369R mutant DNA [31].
fluid phase, and interaction proceeded for 16–18 h at 4°C, whereas in the inhibition experiments, all components were in solution, and interaction took place for minutes at room temperature. Furthermore, cooperative interactions among the components present in the cell-free system might hinder binding of the Nox2 peptide to p67phox and therefore, prevent it from inhibiting.

Mimicking protein–protein interaction by replacing one of the partners by an array of overlapping peptides has many advantages at the methodological level (reviewed in refs. [14, 15]). It has, however, a number of limitations, such as the inability to detect non-contiguous binding regions, possible non-native conformations of synthetic peptides, and the effect of immobilization on the dynamics of interactions.

An important issue that could not be addressed in the present study was the possibility that binding of p67phox to Nox 2 is preceded by a change in p67phox conformation. p67phox was characterized as a protein with multiple intrinsically disordered regions [45, 46]. Intermolecular interactions involving disordered regions in proteins rest on 2 mechanisms [47, 48]. In the first, a conformational change precedes binding; in the second, the conformational change takes place upon binding. Should the first mechanism regulate binding of p67phox to Nox2 peptides, it might not be fully revealed in our experiments. We offered evidence for a Rac-induced conformational change in p67phox [37], and preliminary experiments indeed indicate enhanced binding to Nox2 peptides of a p67phox-Rac(GTP) chimeric protein [unpublished results].

Finally, the coexistence of CGC-dependent and CGC-independent Nox2–p67phox interactions may be best explained by a two-stage interaction, in which p67phox first binds to the Nox2 region corresponding to peptide 28, with subsequent disulfide bond formation between cysteine(s) in the CGC triad and in p67phox, leading to the stabilization of the complex.

These results will have to be supported by protein–protein binding experiments involving native and mutated p67phox and Nox2 DHR; such an approach is in progress [42, 49].

AUTHORSHIP

I.D. designed, performed, and analyzed the experiments and was involved in the writing of the paper. S.M.E.S. performed the structural modeling. E.P. was responsible for the conceptual planning of the project, contributed to the design and analysis of the experiments, and wrote the final version of the paper.

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DISCLOSURES

The authors declare no conflict of interest.

REFERENCES


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p67phox binds to a Cys-Gly-Cys triad in Nox2

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**KEY WORDS:** NADPH oxidase · superoxide · flavocytochrome b558 · peptide arrays · chronic granulomatous disease
A Cys-Gly-Cys triad in the dehydrogenase region of Nox2 plays a key role in the interaction with p67

Iris Dahan, Susan M. E. Smith and Edgar Pick

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