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Biochemical Pharmacology 61 (2001) 1063–1071

Biochemical Pharmacology

Structure-function relationship in the interaction of mastoparan analogs with neutrophil NADPH oxidase

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Received 15 May 2000; accepted 6 September 2000

Abstract

Mastoparan, an amphiphilic cationic tetradecapeptide was previously shown to block activation of the NADPH oxidase in the cell-free system presumably by association with a cytosolic component/s of the enzyme. Blockade of oxidase activation was now demonstrated in the semirecombinant NADPH oxidase system. The structural basis of the inhibitory effect of MP on oxidase assembly was explored employing a variety of truncated and specifically substituted synthetic peptide analogs. The data indicated that an α helical fold, positive net charge, hydrophobicity and amphiphilicity were essential for the inhibitory potency and that peptide analogs below eleven residues were inactive. To identify the MP-binding oxidase subunit three different binding assays were carried out utilizing free or immobilized recombinant p47-phox, p67-phox, p40-phox and Rac1 in conjunction with immobilized MP or soluble ^{125}I-tyr-MP, respectively. The data implicated p67-phox as the main MP-binding component. The binding site on the p67-phox was localized to the 1-238 aminoterminal fragment of the molecule. NADPH oxidase activation supported by this fragment was inhibitable by MP. In addition, SH3 domains of p47-phox and p40-phox and the carboxyterminal SH3 domain of p67-phox exhibited a low affinity towards MP.

Keywords: Mastoparan; Neutrophil; NADPH oxidase; Superoxide; Amphiphilic peptide; SH3 domain

1. Introduction

Assembly at the cell membrane of the superoxide-generating multicomponent NADPH oxidase of neutrophils requires participation of at least four proteins: the catalytically active transmembrane heterodimeric cytochrome b558, cytosolic p67-phox, p47-phox and a Rac1/Rac2 small G protein [reviewed in Ref. 1–3]. Whereas the *in vivo* activation of the enzyme involves diverse signal transducing pathways, the activation of the oxidase in a cell-free system may be induced by anionic detergents (e.g. arachidonate, SDS). In both cases the enzymatic complex is assembled in a process that comprises multiple protein-protein interactions and conformational changes. Mutagenesis, [4,5], phage-display libraries [6,7], the two-hybrid system [4,8] and synthetic peptides [9–13] were used as research tools for structural and functional characterization of the assembly of NADPH oxidase and identification of the participating sequences.

We have recently reported [14] a very potent blockade of cell-free activation of the NADPH oxidase by mastoparan (MP) and several synthetic analogs. MP vespula (INLKA LAALAKKIL) is an amphiphilic cationic tetradecapeptide of the wasp venom recognized for its capacity to modulate a variety of biological activities [15–20] including activation of heterotrimeric Go/Gi [17] as well as low molecular weight G proteins [18], inhibition of Na^+/K^+-ATPase [19] and Ca^{+2}-ATPase [20] and perturbation or pore formation in the membrane [21,22]. It was suggested that the α helical fold adopted by MP in the presence of membrane phospholipids [23] enables the peptide to mimic activated heptahelical receptors and bind to the α subunit of Go/Gi [17,24].

In the case of the NADPH oxidase we demonstrated association of MP with cytosolic oxidase subunits p47-

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Abbreviations: MP, mastoparan; SLO, Streptolysin O; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N′-tetraacetic acid; SH3, *src* homology 3; acid; GST, Glutathione S-transferase; CGD, Chronic Granulomatous Disease

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PII: S0006-2952(01)00561-5

phox/p67-phox and suggested that this interaction might account for the potent inhibition exerted by the peptide in the cell-free oxidase activation system [14]. MP blocked activation of the oxidase also in neutrophils permeabilized with Streptolysin O (SLO) implying a more general effect on the assembly of the oxidase and not an artifact of the cell-free system. The current study was aimed at a more detailed characterization of structural determinants necessary for the functional interaction between MP and NADPH oxidase components to permit identification of novel recognition domains on subunits of the NADPH oxidase complex and to pave a way for the design of inhibitory peptides to be utilized as therapeutic antiinflammatory agents. To establish the structure-function relationship, potencies of a variety of synthetic peptide analogs were compared. Identification of the oxidase subunit associating with MP was assessed by binding experiments utilizing neutrophil cytosol and recombinant cytosolic oxidase proteins or their fragments [25]. The data implicated p67-phox as the main MP-binding subunit. In addition, four out of five SH3 domains derived from cytosolic oxidase subunits were shown to engage in a weak interaction with the peptide.

2. Materials and methods

2.1. Materials

Protected amino acids, coupling agents and the polymer (Rink Amide) utilized for peptide synthesis were obtained from Nova Biochemical. Synthesis-grade solvents were obtained from Labscan. CH-Sepharose was purchased from Pharmacia. All other chemicals were from Sigma. Goat antisera to p67-phox and p47-phox were a kind gift of Dr. T.L. Leto. Cytosols of CGD patients were provided by Dr. P. Heyworth.

2.2. Peptide synthesis

Peptides were prepared by conventional solid-phase synthesis, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer. The Fmoc-strategy was used throughout peptide chain assembly, following the company's commercial protocols. The synthesis was initiated by coupling the C-terminal Fmoc-amino acid to the polymer. Composition of the peptides was confirmed by amino acid analysis and their purity was tested by HPLC (RP-18, 125 × 4 mμ). Molecular weights of peptides were ascertained by mass spectroscopy [26].

2.3. Isolation and fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by standard procedures of dextran sedimentation, gradient centrifugation on Lymphoprep (Nycomed) and hypotonic lysis of red blood cells [14]. Cells (10^8/ml) were broken by sonication and fractionated by centrifugation as described [27]. Fractions were stored at −70°.

2.4. Cell-free activation of the NADPH oxidase in neutrophil fractions

Superoxide-dependent and superoxide dismutase-inhibitable ferricytochrome c reduction was monitored in 96-well microplates at 550 nm in Thermomax Reader (Molecular Devices) at 30° [14]. Activation was induced by SDS (60 μM) utilizing neutrophil cytosol (4×10^6 cell equivalents) and membranes (2×10^6 cell equivalents). MP analogs dissolved in DMSO (to give 1 mM peptide stock solution) and subsequently diluted in assay buffer were added to the activation mixture prior to the addition of SDS.

2.5. Production of p47-phox, p67-phox and Rac1 recombinant proteins

Glutathione-S-transferase (GST)-fusion p40-phox, p47-phox, p67-phox, Rac-1, and their fragments subcloned into the bacterial expression vector pGEX2T were expressed in E. coli, induced with IPTG (0.1–0.4 mM) purified on glutathione-agarose beads and eluted with 10 mM glutathione [25]. Rac-1 was cleaved from the beads with thrombin (1 u/300 μg). In experiments testing the interaction of MP with immobilized subunits the glutathione elution step was omitted. Cytosolic subunits p47-phox and p67-phox were also produced in baculovirus-infected Sf9 cells [29] and purified on S-Sepharose and Q-Sepharose columns respectively. Baculovires carrying cDNA for p47-phox and p67-phox were kind gifts of Dr. T.L. Leto.

2.6. Semirecombinant cell-free activation of the NADPH oxidase

Recombinant p47-phox (50–120 nM), p67-phox (50–120 nM), and GTPγS-loaded Rac1 (140 nM) combined with solubilized membranes (8.4 nM in cytochrome b558) were preincubated in the 96-well microplate reader (Thermomax, Molecular Devices) for 1.5 min with SDS (60 μM) in 0.1 mL K,Na phosphate 0.13 M pH 7.0 buffer (containing 4 mM NaN_3, 2 mM $MgCl_2$, 2 mM EGTA, 20 μM FAD and 0.21 mM cytochrome *c*) at room temperature. The kinetics of cytochrome c reduction at 550 nm were monitored after the addition of 0.12 mM NADPH.

2.7. Preparation of MP-sepharose resin

MP (0.5 mg in 0.5 mL of sodium bicarbonate buffer pH 8.0) was coupled to 50 mg (dry weight) CH-Sepharose [14], aliquoted into four Eppendorf tubes and washed with 20 mM Tris-HCl pH 8, 150 mM NaCl (resin buffer) by a 5-sec centrifugation in an Eppendorf microfuge. The resin suspended in a 0.5 mL volume of the above buffer was incubated for 1 hour at room temperature with 15 μL of neu-

trophil cytosol or 1.5 μg of recombinant p40-phox, p47-phox or p67-phox, centrifuged and washed with resin buffer followed by 0.5 M NaCl solution in the buffer. The treated resin and unbound proteins in the supernatant and in washes were precipitated with trichloroacetic 10%, subjected to SDS-PAGE, and analyzed by immunoblotting with specific antibodies to the recombinant proteins [27].

2.8. Radioiodination of Tyr-MP

Iodogen (10 μg) and 50 μg of MP in 200 μL of 100 mM Hepes buffer, pH 7.5, were incubated 10 min with 0.2 mCi of $Na^{125}I$ and chromatographed on a Sephadex G-10 mini-column. The specific activity of the peptide was 10,000 cpm/μg.

2.9. Secondary structure prediction

PeptideStructure and PlotStructure computer programs of Jameson and Wolf were employed [30]. Molecular models of MP analogs were constructed employing the Hyperchem software (Hypercube, Inc.).

3. Results

In the first part of the study the interrelationship between the structure of synthetic analogs of MP and their potencies to block NADPH oxidase was investigated. In the second part identification of the MP-binding oxidase subunit was attempted.

3.1. Truncation or elongation of the aminoterminus

Analogs of MP vespula truncated at the aminoterminus (4–14 residues long) and retaining the carboxyterminal motif −AKKIL were synthesized and tested in the cell-free NADPH oxidase activation system. As shown in Fig. 1, truncation of the amino terminus progressively reduced the inhibitory potency of the peptides. The undecapeptide with an aminoterminal lysine (lysine 4 in the original peptide) exhibited a weak residual inhibitory power which was abolished by substitution of Lys 1 by Ala (not shown). The ineffectiveness of shorter peptides (N ≤ 10) indicated that truncation and deletion of the positively charged lysine 4 of the parent MP disrupted the interaction with the relevant oxidase component/s. The presence of the −AKKIL terminus in the inactive analogs implied that it could only in part account for the inhibitory activity of MP [14].

In contrast to the loss of inhibitory activity observed in truncated analogs, extension of the aminoterminal portion of the chain by five leucine residues did not reduce potency of the inhibition (Table 1). Assuming that MP interferes with the correct assembly of an active NADPH oxidase by interaction with the enzyme subunit/s [14], preservation of blockade by the extended peptide suggests an intersubunit location of the added residues in the assembling complex.

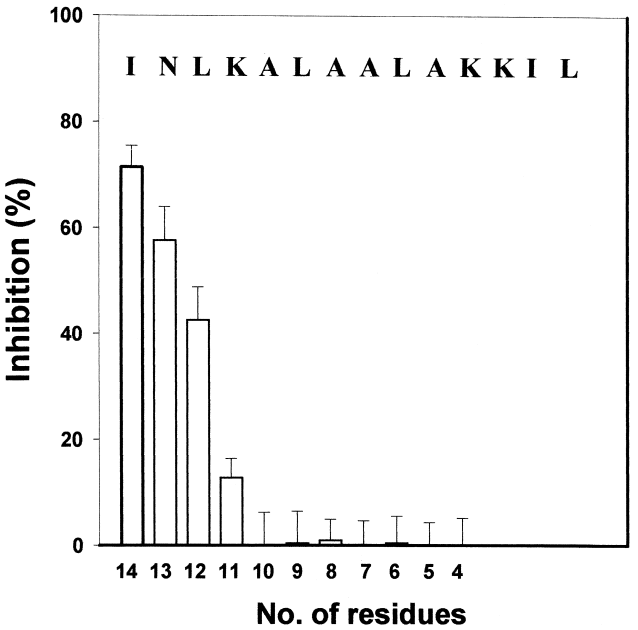

Fig. 1. The effect of truncation on the potency of MP analogs (6 μM) to block NADPH oxidase activation in the cell-free system. Mean values ± SD (N = 12).

3.2. Amphiphilicity of the peptides

In the α helical fold adopted by MP in the presence of phospholipids [23,31] or certain proteins [24] the three lysines (numbered 4, 11, and 12) point towards the same face of the amphiphilic helix. The significance of this feature is borne out by loss of the biological activity that followed introduction of an additional lysine in position 6 in MP-17 [Table 1 and Refs. 24,32]. Because lysine 6 in MP-17 resides on the hydrophobic face of the amphiphilic α helix, amphicilicity seems to be essential for the biological effects of MP. Consistent with this conclusion MP B [33], a hydrophilic analog of MP retaining lysines 4, 11, and 12 exerted a considerable inhibitory effect in the oxidase system (Table 1); it follows that that helix and positive charges are important determinants of MP biological activity.

Table 1
Inhibition of cell-free activation of the NADPH oxidase by diverse MP analogs

Analog (6 μM)		Inhibition (%)
MP vespula	INLKALAALAKKIL	68.7 ± 5.5
Extended MP	LLLLLINLKALAALAKKIL	44.6 ± 8.3
Reversed MP	LIKKALAALAKLNI	26.1 ± 9.3
MP-COO^-	INLKALAALAKKIL	0.7 ± 1.1
MP-17	INLKAKAALAKKIL	6.4 ± 5.6
MP-B	IKLKSLVSWAKKVL	68.1 ± 6.5

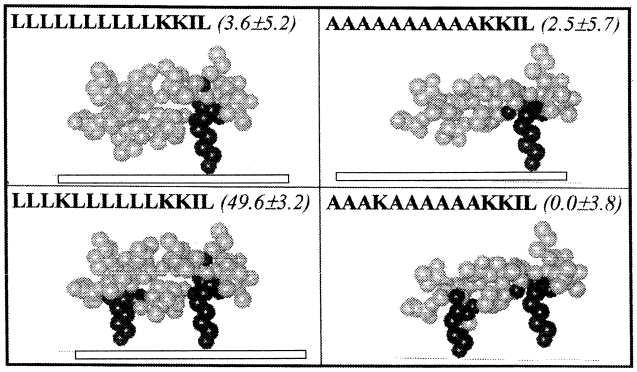

Fig. 2. α-helical representation of MP analogs A_{10}-KKIL or L_{10}-KKIL: the effect of the side chain and of lysine 4. The percentage of inhibition of superoxide production by 6 μM peptides is shown in parenthesis.

3.3. Substitutions in the aminoterminus

In another series of experiments tetradecapeptide peptide analogs retaining the carboxyterminal −KKIL motif were studied. Substitution of residues 1–10 of MP by leucines or alanines to give L_{10}-KKIL or A_{10}-KKIL abrogated their capacity to block oxidase activity (Fig. 2) corroborating the previous conclusion that the −KKIL sequence is insufficient for an effective interaction with the oxidase. Introduction of a lysine at position 4 to give L_3-KL_6-KKIL or A_3-KA_6-KKIL, restored the capacity to block NADPH oxidase activation to the leucine- but not to the alanine-containing peptide. This result indicated that the presence of a positive charge at position 4 was necessary but not sufficient. Fig. 2 illustrates the possible contributions of lysine 4 and leucine side-chains to the molecular structure of α helical MP analogs. If this fold, indeed, participates in the interaction with the oxidase subunit/s, the bulky and hydrophobic leucyl side-chains appear fundamental for formation of stable contacts that block the correct assembly of the enzyme.

3.4. Modifications in the carboxyterminus

In another group of peptides permutations and substitutions in the carboxyterminal −KKIL fragment were studied. Fig. 3 summarizes effects of the different peptides on the NADPH oxidase-mediated superoxide production. Replacement of lysine 12 or lysine 13 by an arginine did not affect potency of the inhibition. The capacity to block oxidase activation was however eliminated by substitution of one lysine by a leucyl residue. Interestingly, substitution of a lysine by alanine slightly reduced extent of the inhibition without eliminating it. A similar observation was reported by Higashijima *et al*. [31] in studies of MP-mediated activation of G proteins. Replacement of the two carboxyterminal residues, −IL, by −AA to give −KKAA abolished the capacity of the analog to interfere with oxidase activation whereas their replacement by −IA or −LI did not affect their interaction with the system. Hydrophobicity and/or bulkiness of the penultimate residue seem thus of importance for the biological effects of MP.

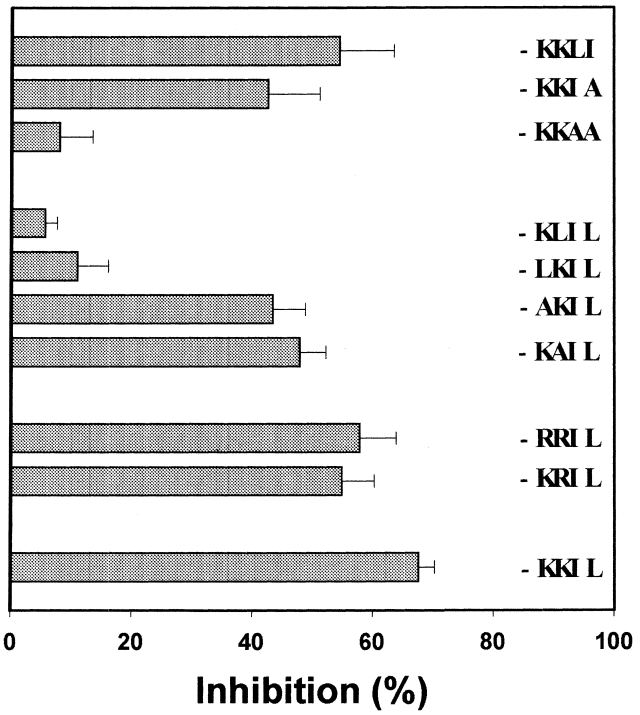

Fig. 3. Effects of permutations in the carboxyterminus of MP analogs (6 μM) on superoxide production in the NADPH oxidase cell-free system. Mean values ± SD (N = 8).

It is well established that the carboxyterminal residue of bioactive peptides is amidated. In agreement with this rule, the nonamidated MP-COO^- was unable to block oxidase activation (Table 1), suggesting that amidation was necessary also for MP-mediated blockade of the NADPH oxidase.

3.5. Orientation and steric configuration

An amidated peptide of inverted sequence lost a significant fraction of its NADPH oxidase blocking power (Table 1) in spite of its capacity to adopt an amphiphilic α helical configuration [30]; this finding implies that composition, secondary structure and amphiphilicity are not the sole determinants of the interaction of MP analogs with oxidase and that orientation of the peptide chain contributes to its biological activity.

Interestingly, three analogs of MP singly substituted by a D residue at lysine 4, lysine 12 or leucine 14 retained their capacity to block activation of the oxidase (Table 2).

3.6. Blockade of oxidase by synthetic copolymers

In view of the possible contribution of the helical fold to the blockade of oxidase activity by MP, effects of synthetic

Table 2
The contribution of D amino acids to the blockade of the NADPH oxidase activation by MP

	Inhibition (%)
INLKALAALAK$_D$KIL	61.7 ± 4.6
INLKALAALAKKIL$_D$	54.3 ± 5.1
INLK$_D$ALAALAKKIL	73.0 ± 2.9

copolymers of leucine and lysine capable of α helix formation were investigated in the cell-free system. As shown in Table 3 copolymers containing 1–2 lysines had no effect on activation of the NADPH oxidase whereas the addition of a third lysine endowed the peptide with an inhibitory power. The magnitude of the inhibition depended on the location of the positive charges: in the most potent analog the distribution of lysines at positions 4, 11, and 12 resembled the original MP molecule.

3.7. MP blocks activation of the NADPH oxidase in a semirecombinant cell-free system

In the previous study [14] crude cytosol and membranes were used as sources for the NADPH oxidase subunits. The direct functional interaction of MP with the subunits was now confirmed by demonstration of MP-mediated blockade of superoxide production in a semirecombinant cell-free system, consisting of baculovirus-derived recombinant p47-phox/p67-phox and bacterial Rac1 combined with solubilized neutrophil membranes. In the semirecombinant cell-free system EC_{50} of 3.0 μM was determined compared to 3.6 μM in neutrophil cytosol and membranes. This similarity in potency corroborated our initial hypothesis that interaction of MP with one of the oxidase components is responsible for inhibition of oxidase activation.

3.8. Cytosolic oxidase subunits of p47-phox, p67-phox, and p40-phox bind to MP-sepharose

In the previous communication cytosolic p47-phox and p67-phox were shown to interact with MP coupled to CH-Sepharose [14]. Since in the cytosol of neutrophils the existence of an intermolecular complex consisting of p47-phox, p67-phox and p40-phox has been documented [34] association of p40-phox with MP-Sepharose was also tested. The resin was preincubated with neutrophil cytosol, subjected to SDS-PAGE followed by electrotransfer and probed with anti-p40-phox, anti-p47-phox and anti-p67-phox. As shown in Fig. 4A all three oxidase subunits (p47-phox, p67-phox as well as p40-phox) were detected on the blots. This finding could be ascribed either to the presence of a single peptide-binding site on the ternary cytosolic oxidase complex or to a separate binding of the individual proteins to the immobilized MP.

When neutrophil cytosol was replaced by cytosol of CGD patients deficient in either p47-phox or p67-phox [35], p67-phox, or p47-phox, respectively, were detected on the Western blots of the resin-bound components (Fig. 4A, lanes 6 and 9) indicating an independent binding of each subunit to MP-beads. Consistent with the previously reported low levels of p40-phox in p67-phox-deficient cells [36], p40-phox was detected in resin preincubated with p47-phox-deficient (Fig. 4A, lane 6) but not with p67-phox-deficient cytosol (Fig. 4A, lane 9).

Interaction of the individual subunits of the oxidase with MP-Sepharose was studied utilizing recombinant p47-phox, p67-phox, p40-phox and Rac1. As shown in Fig. 4B p47-phox, p67-phox and p40-phox associated with the resin independently of each other, implying that each might possess a binding site for the peptide whereas Rac1 was detected in the supernatant of the resin (Fig. 4B, lanes 10–12). Egg lysozyme also did not bind to MP-Sepharose (data not shown). Two other negative controls were performed: in the first, recombinant p47-phox, p67-phox or p40-phox were incubated with unloaded and deactivated resin: p67-phox and p40-phox did not associate with the unloaded beads whereas a weak association of p47-phox was detected (data not shown). In the second type of control, MP-17 and A_3KA_6-KKIL, peptides inactive in the NADPH oxidase inhibition (Table 1 and Table 2, respectively) were covalently attached to the resin and incubated with each subunit. Also in this case no evidence for the association of p67-phox with the resins was obtained whereas a weak and apparently nonspecific interaction of p47-phox was observed (data not shown).

Table 3
Effects of synthetic copolymers of leucine and lysine on the blockade of the NADPH oxidase activation

	Inhibition (%)
LLLLLLLLLL**K**LLL	0.0 ± 0.4
LLL**K**LLLLLLL**K**LL	0.2 ± 1.1
LLL**K**LLLLLL**K**LLL	0.0 ± 0.2
LL**KK**LLLLLL**K**LLL	28.3 ± 9.6
LLL**K**LLL**K**LLL**K**LL	21.9 ± 5.9
LL**K**LLLLL**K**LL**K**LL	38.2 ± 6.7
LLL**K**LLLLLL**KK**LL	48.5 ± 4.9

3.9. Binding of ^{125}I-tyr-MP to immobilized GST-oxidase subunits

The described above experiments (Fig. 4) tested association of soluble oxidase subunits with immobilized MP. A reciprocal type of assay, examining interaction of soluble MP with glutathione-agarose-immobilized GST-oxidase subunits (p47-phox, p67-phox, p40-phox or Rac1) was next carried out. Glutathione-agarose beads carrying one of the GST-oxidase subunits (25 μg protein) were preincubated with the iodinated pentadecapeptide ^{125}I-tyr-MP (40,000 cpm), washed and counted in a gamma counter. It is of note

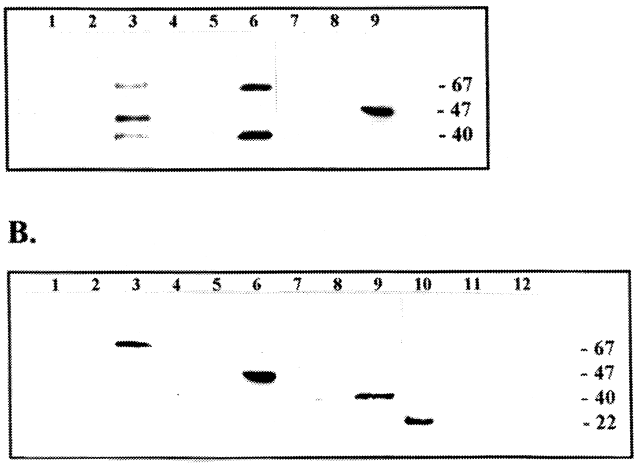

Fig. 4. Western blots analyzing the association of cytosolic oxidase proteins with MP-Sepharose. Normal neutrophil cytosol, CGD cytosol and recombinant cytosolic proteins were loaded on MP-Sepharose, washed and eluted with SDS-PAGE sample buffer. Lanes 3, 6, 9, 12: resin-bound proteins; lanes 1, 2, 4, 5, 7, 8, 10, 11: supernatants and washes. (A) Normal neutrophil cytosol (lanes 1–3) and CGD cytosol deficient in p47-phox (lanes 4–6) or p67-phox (lanes 7–9) probed with antisera to p40-phox, p47-phox and p67-phox. (B) Recombinant p67-phox (lanes 1–3), p47-phox (lanes 4–6), p40-phox (lanes 7–9) and Rac1 (lanes 10–12) probed with the corresponding antisera.

that the addition of a tyrosine residue at the aminoterminus of MP did not affect its capacity to block activation of the NADPH oxidase (71.5% ± 3.8 and 79.4% ± 2.9 inhibition estimated at 6 μM MP and tyr-MP, respectively). GST- or GST-Rac1-bound glutathione-agarose beads were used as negative controls. As shown in Fig. 5, the most prominent binding of ^{125}I-tyr-MP was exhibited by p67-phox. The association of p40-phox and p47-phox was significantly lower.

3.10. Detection of protein-bound ^{125}I-tyr-MP by gel filtration

In a third type of binding assay soluble GST-fusion NADPH oxidase subunits (25 μg) were preincubated with ^{125}I-tyr-MP (40,000 cpm in 0.1 mL) and loaded on a prespun 1 mL of Sephadex G-25-containing syringe. The syringe was spun, the eluate was collected and the procedure was repeated several times: each time 0.1 mL of elution buffer was loaded on the minicolumn. Fig. 6 compares sums of radioactivities in the first two eluates of the minicolumn. In the absence of any protein present during the preincubation ^{125}I-tyr-MP was not detected in the combined two eluates; the radioactivity of the peptide started to appear in the fifth eluate of the syringe (not shown). When GST-fusion p67-phox, p47-phox or p40-phox were present during the preincubation, the combined first two eluates contained residual ^{125}I-tyr-MP which represented fraction of the protein-peptide complex that did not separate by gel filtration on the minicolumn. The highest residual binding was observed in the case of GST-p67-phox (Fig. 6A). Since the counts in the first two eluates increased linearly with the concentration of the binding protein (data not shown), they were normalized for the initial molar concentration of each protein. This procedure increased the apparent binding capacity of p67-phox relatively to p47-phox and p40-phox (Fig. 6A). GST, BSA and Rac1 used as negative controls of interaction with ^{125}I-tyr-MP yielded low counts. The highest radioactivity was observed in the case of calmodulin, known to bind MP with an exceptionally high affinity [16].

Since the three cytosolic components, p67-phox, p47-phox and p40-phox contain SH3 domain/s, the possibility that the association of ^{125}I-tyr-MP with the oxidase subunits was mediated by these domains was tested. First, SH3-deleted p67-phox, p47-phox and p40-phox expressed as GST-fusion proteins were used in the gel filtration binding experiments. The data in Fig. 6B clearly indicate that deletion of SH3 domains, eliminated most of MP binding to p47-phox or p40-phox; in agreement with this finding isolated GST-SH3 domains of p47-phox and p40-phox exhibited interaction with ^{125}I-tyr-MP comparable to the interaction observed in the case of the intact protein (Fig. 6C). Contrary to p47-phox and p40-phox, SH3-deleted p67-phox retained affinity for ^{125}I-tyr-MP implicating a non-SH3 binding site in the interaction (Fig. 6B). The carboxyterminal SH3 domain of p67-phox contributed to the interaction with the peptide whereas the contribution of the aminoterminal SH3 was negligible (Fig. 6C). Interestingly, the highest affinity for MP was exhibited by the truncated aminoterminal (1–238) fragment of p67-phox (Fig. 6B).

3.11. Effect of MP on the assembly of truncated p67-phox

The functional effects of MP on NADPH oxidase activity were determined in a system in which SH3-deleted GST-fusion p67-phox proteins [25] were substituted for p67-phox (Fig. 7). Deletion of one or both SH3 domains did not diminish the capacity of MP to block oxidase activity. The 1–238 aminoterminal fragment of p67-phox, previously

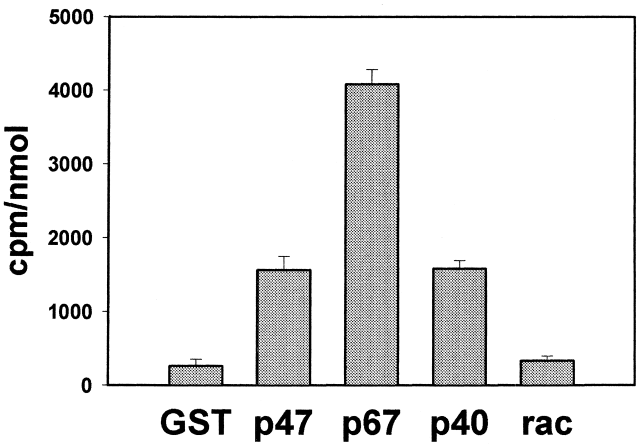

Fig. 5. Binding of ^{125}I-tyr-MP to GST-p47-phox/GST-p67-phox immobilized on glutathione-agarose beads. Mean values ± SD (N = 5).

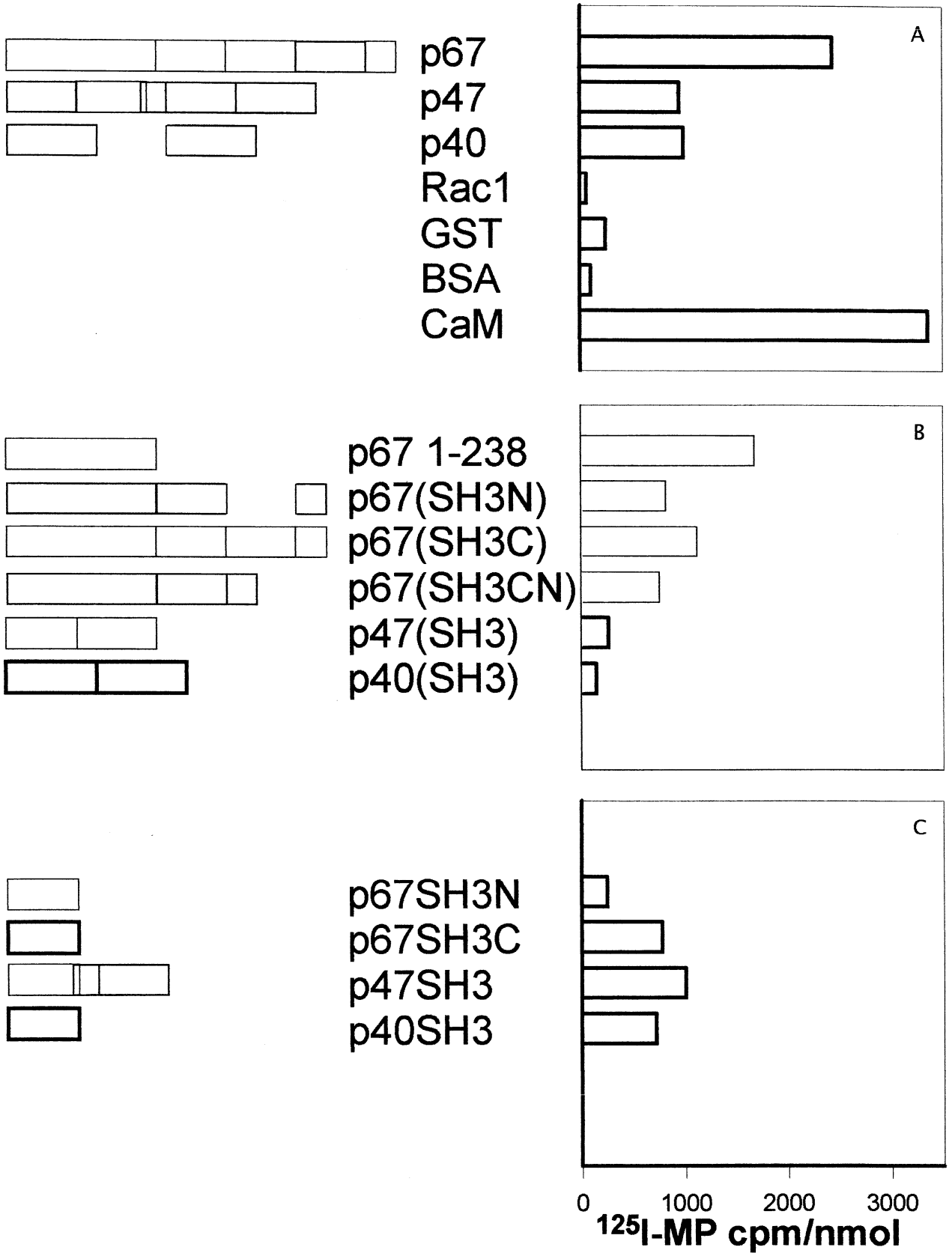

Fig. 6. Binding of ^{125}I-tyr-MP to oxidase subunits or their truncated forms followed by gel filtration on spin Sephadex G-25 columns. A. full length proteins B. deleted oxidase subunits. The brackets indicate deleted sequences C. N,C: the amino and carboxyterminal SH3 domains of oxidase subunits. CaM-calmodulin.

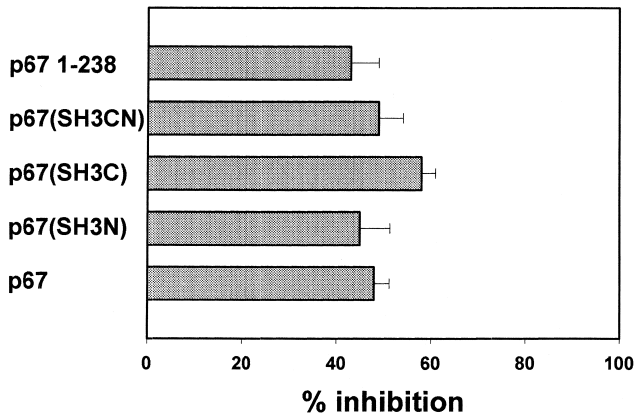

Fig. 7. Inhibition by MP of NADPH oxidase activation supported by SH3-deleted p67-phox and its 1–238 fragment. Mean values ± SD (N = 6).

shown to participate in the assembly of the active NADPH oxidase [37,38] also exhibited susceptibility towards inhibition by MP.

4. Discussion

Molecular recognition in biological systems is based on interactions among short amino acid sequences; this principle constitutes the basis of the extensive use of synthetic peptides as research tools in studies of protein-protein interactions. Peptide fragments employed for investigation of the superoxide-generating phagocyte NADPH oxidase were derived from the subunits of the NADPH oxidase (9–13).

In a previous study we showed that MP, an unrelated to the NADPH oxidase amphiphilic tetradecapeptide, interacted with the components of the enzyme and interfered with its activation [14]. The assumption that characterization of peptide features essential for a functional interaction with the system may allow identification of novel recognition domains on subunits of the NADPH oxidase complex and to help to develop antiinflammatory peptides capable of prevention of excessive oxygen radical production, prompted us to compare effects of a large number of synthetic MP analogs on the activation of the NADPH oxidase. The current study implies that similarly to other biological systems [23,24,31], the functional interaction of the peptides with the NADPH oxidase depended on the presence of an amphiphilic α-helical fold. Evidence implicating lysine 4 in the interaction with the NADPH oxidase system (Table 1) and abrogation of the biological activity by introduction of a positive charge on the hydrophobic face of the helix in MP-17 emphasize the significance of the amphiphilic structure of MP. A similar conclusion was reached in studies of the involvement of MP in G protein-mediated signaling pathways [32].

This conclusion is in an apparent contradiction with our previous hypothesis attributing the inhibitory action of MP to the carboxyterminal −AKKIL sequence of the peptide [14]. This hypothesis was based on the powerful inhibition of the NADPH oxidase activation by the strongly basic analog MP-9, incapable of adopting the α helical fold [30]. The discrepancy between our earlier and current conclusions may be ascribed to the highly polybasic character of MP-9 (six arginines instead of three lysines) which may have rendered the peptide unsuitable for comparison with the original peptide. Polybasic peptides were recently shown to interfere with oxidase activation [12].

The significance of the size and/or the hydrophobicity of the side chains was explored by comparison of A_3-KA_6-KKIL and L_3-KL_6-KKIL (Fig. 2). Both peptides share a similar α-helical fold and distribution of positive charges. The alanine-containing peptide lost its ability to interfere with oxidase activation whereas the corresponding leucine-containing peptide preserved this property. This finding indicates that the bulky side chains contribute to the interaction of the peptide with the NADPH oxidase. With respect to the length of the peptide, our data showing that truncation of the 1–4 amino terminal residues progressively abrogated effect on the NADPH oxidase activity (Fig. 1) suggest that the shortest segment capable of interaction with oxidase consists of 11 residues. Such a peptide is equivalent to about two and a half turns of an amphiphilic α helix.

MP-mediated blockade of activation of the oxidase in a semirecombinant cell-free assay utilizing recombinant cytosolic subunits and partially purified solubilized cytochrome b558, implied that one of the oxidase subunits was the target of MP. This hypothesis was substantiated by binding experiments demonstrating that immobilized MP bound recombinant p67-phox/p47-phox/p40-phox (Fig. 4) whereas immobilized peptide analogs inactive in the functional oxidase tests (e.g. MP-17, A_3-KA_6-KKIL) did not exhibit binding. In a reciprocal assay ^{125}I-tyr-MP was shown to associate with immobilized oxidase subunits (Fig. 5).

In gel filtration experiments employing either full length or SH3-deleted p47-phox/p67-phox/p40-phox, the highest binding capacity was exhibited by p67-phox (Fig. 6). The observation that SH3 domains, known to interact with polyproline sequences [39,40], associate, albeit weakly, with ^{125}I-tyr-MP was unexpected and further studies will be required to define the orientation of the interacting MP and SH3 in comparison with the well characterized polyproline-SH3 complex. Interestingly, the carboxyterminal SH3 of p67-phox was the only SH3 domain of p47-phox, p67-phox and p40-phox that did not bind MP (Fig. 6C). This finding suggested a certain extent of selectivity in the interaction with the peptide.

It was shown elsewhere [37,38] and confirmed by us (Fig. 7) that the 1–238 fragment of p67-phox fulfills the requirements for a productive NADPH oxidase assembly in the cell-free system. The correlation between MP-mediated blockade of the NADPH oxidase activity and binding of MP analogs to truncated forms of p67-phox suggests that the

functional interaction between p67-phox and the peptide is mediated by the aminoterminal fragment of the protein. It is noteworthy that this part of p67-phox participates also in the interaction with Rac [41] and contains four tetratricopeptide motifs, essential for activity and characterized by a highly ordered α-helical structure [42]. It should also be kept in mind that our binding experiments probed the affinity of MP towards the resting e.g. unassembled cytosolic oxidase components whereas activity assays contain contributions of the active enzyme and its activation complex as well. Furthermore, the finding that MP did not inhibit the preactivated enzyme [14], implies that the site on p67-phox that interacts with the amphiphilic α helix of MP blocking activation of the NADPH oxidase became inaccessible or inert in the active oxidase complex.

Acknowledgment

This work was supported in part by The Ela Kodesh Institute for Cancer Research.

References

[1] Segal AW, Shatwell KP. Ann NY Acad Sci 1997;832:215–22.
[2] Babior BM. Blood 1999;93:1464–76.
[3] Clark RA. J Infect Dis 1999;179(Suppl 2):S309–17.
[4] Nakamura R, Sumimoto H, Mizuki K, Hata K, Ago T, Kitajima S, Takeshige K, Sakaki Y, Ito T. Eur J Biochem 1998;251:583–9.
[5] Biberstine-Kinkade KJ, Yu LX, Dinauer MC. J Biol Chem 1999;274: 10451–7.
[6] DeLeo FR, Yu L, Burritt JB, Loetterle LR, Bond CW, Jesaitis AJ, Quinn MT. Proc Natl Acad Sci USA 1995;92:7110–4.
[7] Burritt JB, Quinn MT, Jutila MA, Bond CW, Jesaitis AJ. J Biol Chem 1995;270:16974–80.
[8] Fuchs A, Dagher MC, Vignais PV. J Biol Chem 1995;270:5695–7.
[9] Nakanishi A, Imajoh-Ohmi S, Fujinawa T, Kikuchi H, Kanegasaki S. J Biol Chem 1992;267:19072–4.
[10] Nauseef WM, McCormick S, Renee J, Leidal KG, Clark RA. J Biol Chem 1993;268:23646–51.
[11] Kreck ML, Uhlinger DJ, Tyagi SR, Inge KL, Lambeth JD. J Biol Chem 1994;269:4161–8.
[12] Joseph G, Gorzalczany Y, Koshkin V, Pick E. J Biol Chem 1994; 269:29024–31.
[13] Zhen L, Yu LX, Dinauer MC. J Biol Chem 1998;273:6575–81.
[14] Tisch D, Sharoni Y, Danilenko M, Aviram I. Biochem J 1995;310: 715–9.
[15] Hirai Y, Kuwada M, Yasuhara T, Yoshida H, Nakajima T. Chem Pharm Bull 1979;27:1945–6.
[16] Malencik DA, Anderson SR. Biochem Biophys Res Commun 1983; 114:50–6.
[17] Higashijima T, Sonoko U, Nakajima T, Ross EM. J Biol Chem 1988;263:6491–4.
[18] Koch G, Haberman B, Mohr C, Just I, Aktories K. FEBS Lett 1991;291:336–40.
[19] Raynor RL, Zheng B, Kuo JF. J Biol Chem 1991;266:2753–8.
[20] Longland CL, Mezna M, Michelangeli F. J Biol Chem 1999;274: 14799–805.
[21] Danilenko M, Worland P, Carlston B, Sausville EA, Sharoni Y. Biochem Biophys Res Comm 1993;196:1296–302.
[22] Suh D-C, Song S-K, Kim Y-K, Kim K-T. J Biol Chem 1996;271: 32753–9.
[23] Wakamatsu K, Okada A, Miyazawa T, Ohya M, Higashijima T. Biochemistry 1992;31:5654–60.
[24] Kusunoki H, Wakamatsu K, Sato K, Miyazawa T, Kohno T. Biochemistry 1998;37:4782–90.
[25] Wientjes FB, Panayotou G, Reeves E, Segal AW. Biochem J 1996; 317:919–24.
[26] Blank M, Shoenfeld Y, Cabilly S, Heldman Y, Fridkin M, Katchalski-Katzir E. Proc Natl Acad Sci USA 1999;96:5164–8.
[27] Klinger E, Sharabani M, Aviram I. Biochem J 1993;295:565–70.
[28] Kwong CH, Malech HL, Rotrosen D, Leto TL. Biochemistry 1993; 32:5711–7.
[29] Leto TL, Garrett MC, Fujii H, Nunoi H. J Biol Chem 1991;266: 19812–8.
[30] Jameson BA, Wolf H. Comput Appl Biosci 1988;4:181–6.
[31] Higashijima T, Burnier J, Ross EM. J Biol Chem 1990;265:14176–86.
[32] Ross EM, Higashijima T. Methods in Enzymology 1994;237:26–37.
[33] Song D-L, Chang G-D, Ho C-L, Chang C-H. Eur J Pharmacol 1993;247:283–8.
[34] Park JW, El Benna J, Scott KE, Christensen BL, Chanock SJ, Babior BM. Biochemistry 1994;33:2907–11.
[35] Cross AR, Curnutte JT, Heyworth PG. Blood Cells Mol Dis 1996; 22:268–70.
[36] Wientjes FB, Segal AW. Semin Cell Biol 1995;6:357–65.
[37] Hata K, Takeshige K, Sumimoto H. Biochem Biophys Res Commun 1997;241:226–31.
[38] Han C-H, Freeman JLR, Lee T, Motalebi SA, Lambeth JD. J Biol Chem 1998;273:16663–8.
[39] Ren R, Mayer BJ, Cichetti P, Baltimore D. Science 1993;259:1157–61.
[40] Finan P, Koga H, Zvelebil MJ, Waterfield MD, Kellie S. J Mol Biol 1996;261:173–80.
[41] Ahmed S, Prigmore E, Govind S, Veryard C, Kozma R, Wientjes FB, Segal AW, Lim LJ. Biol Chem 1998;273:15693–701.
[42] Koga H, Terasawa H, Nunoi H, Takeshige K, Inagaki F, Sumimoto HJ. J Biol Chem 1999;274:25051–60.