# The assembly of neutrophil NADPH oxidase: effects of mastoparan and its synthetic analogues

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Detergent-mediated activation of the phagocyte superoxide-generating NADPH oxidase requires the participation of at least four proteins: the membrane-bound heterodimeric cytochrome $b_{558}$ and three cytosolic components, p47-phox, p67-phox and a Rac1/Rac2 protein. Peptides corresponding to sequences of different subunits of NADPH oxidase have been used as probes of the mechanism and sequence of assembly of the active complex. In the present study effects of mastoparans on activation of NADPH oxidase were investigated. Mastoparans are wasp venom cationic amphiphilic tetradecapeptides capable of modulation of various cellular activities. Natural mastoparans, as well as several synthetic mastoparan analogues, unrelated to oxidase components, blocked activation of the oxidase in the cell-free system ($EC_{50}$ = 1.5 $\mu$M) and in guanosine 5′-[$\gamma$-thio]triphosphate (GTP[S])/ATP-stimulated neutrophils permeabilized with streptolysin O. In the cell-free system the effect was not relieved by raising the detergent concentration and could not be ascribed to changes in critical micellar concentration values of the activating SDS or arachidonate. Chromatography of neutrophil cytosol on an immobilized mastoparan column suggested interaction of cytosolic p47-phox and p67-phox with the peptide. In spite of this interaction mastoparan did not interfere with translocation of p47-phox and p67-phox to the cell membranes.

## INTRODUCTION

The phagocyte superoxide-generating NADPH oxidase plays a major role in the killing of invading micro-organisms. The active multicomponent complex is assembled in cell membranes of appropriately stimulated phagocytes [1,2]. In a detergent-induced (e.g. arachidonate, SDS) cell-free system the assembly of an active NADPH oxidase requires the participation of at least four proteins: the membrane-bound heterodimeric cytochrome $b_{558}$ and three soluble proteins, p67-phox, p47-phox and a Rac1 or Rac2 small G-protein [3,4]. In view of the previously demonstrated translocation of cytosolic subunits of the oxidase to the membrane [5–8], the assembly of the enzymic complex represents an intriguing example of a protein–protein interaction. The mechanism of this process has not been elucidated. Peptides corresponding to sequences of different subunits of NADPH oxidase have been used as probes of the mechanism and sequence of assembly of the active complex [9–11].

Mastoparan (MP) is a cationic amphiphilic tetradecapeptide isolated from wasp venom and initially described as a mast cell secretagogue [12]. In subsequent studies the peptide was shown to activate phospholipases $A_2$ and C [13,14] and to inhibit calmodulin [15]. MPs perturb membranes and modulate activities of heterotrimeric and low-molecular-mass G-proteins [16,17].

The high reactivity of MP with apparently unrelated systems prompted us to delineate effects of the peptide on activation of neutrophil NADPH oxidase in a detergent-mediated cell-free system. In the present study we report inhibition of NADPH oxidase activation by low concentrations of MP and its synthetic analogues. Our data suggest that this inhibition is caused by a high-affinity association of MPs with cytosolic subunits of the oxidase.

## MATERIALS AND METHODS

### Materials

Mastoparans (MPs) of vespula and polistes were purchased from Sigma; the synthetic analogues were synthesized as described [18] and were kindly provided by Dr. E. A. Sausville and Dr. P. Worland (NIH, Bethesda, MD, U.S.A.). All other materials were from Sigma. Goat antisera to p47-phox and p67-phox were a kind gift from Dr. H. L. Malech and Dr. T. L. Leto (NIH, Bethesda, MD, U.S.A.).

### Fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by standard procedures [19]. Cells ($10^8$/ml) in 10 mM potassium phosphate-buffered saline (pH 7.0) (PBS) containing 1 mM EGTA, 3.5 mM PMSF and leupeptin (15 $\mu$g/ml) were disrupted by sonication and fractionated into cytosolic and light membrane fractions as described elsewhere [19].

### Cell-free activation of NADPH oxidase

Optimal concentrations of SDS or arachidonate were determined for each series of experiments. The SDS-reaction mixture (0.8 ml) consisted of 10 mM Hepes-buffered saline (HBS), pH 6.7/10 $\mu$M flavin adenine dinucleotide/80 $\mu$M cytochrome $c$/1 mM EGTA. Arachidonate-supported activation was carried out in 10 mM PBS, pH 7.0/0.34 M sucrose/1 mM EGTA/1 mM $MgCl_2$. Neutrophil membranes and cytosol at $2 \times 10^6$ and $4 \times 10^6$ cell equivalents/ml respectively (corresponding to approx. 10 $\mu$g and 120 $\mu$g of membrane and cytosolic protein) were preincubated with the detergents for 5 min at 30 °C. NADPH (0.12 mM) was then added and superoxide dismutase-inhibitable reduction of

Abbreviations used: CMC, critical micellar concentration; ECL, enhanced chemiluminescence; GTP[S], guanosine 5′-[$\gamma$-thio]triphosphate; KRP, Krebs–Ringer phosphate solution; LDH, lactate dehydrogenase; MP, mastoparan; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leucocyte; SLO, streptolysin O.

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cytochrome *c* was followed at 550 nm. In several experiments membranes ($2 \times 10^8$ cell equivalents/ml) were solubilized by incubation on ice (30 min) with 40 mM octylglucoside [20]. The extract obtained by centrifugation (45 min, 200000 *g*) replaced membranes in the NADPH oxidase assay.

### Translocation of cytosolic p47-phox and p67-phox to the membrane

Cell membranes used in the experiments were washed by centrifugation at 200000 *g*. Cytosol was precleared by ultracentrifugation. Membranes and cytosol ($8 \times 10^6$ and $1.1 \times 10^7$ cell equivalents respectively) were preincubated at 28 °C for 2 min in 8 mM potassium phosphate buffer, pH 7.0, 131 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$ with 10 $\mu$M guanosine 5'-[$\gamma$-thio]-triphosphate (GTP[S]) in the presence or absence of vespula MP (3.1 $\mu$g/ml) in a total volume of 1.6 ml and stimulated with 63 $\mu$M arachidonic acid. After 10 min at 28 °C, the mixture was loaded on 4 ml of 15% (w/v) buffered sucrose and centrifuged for 60 min at 200000 *g* [21]. Supernatants were carefully removed and pellets were solubilized in SDS/PAGE sample buffer and analysed by immunoblotting with specific goat antisera to p47-phox and p67-phox. The proteins were detected by enhanced chemiluminescence (ECL). In some experiments pre-activated pellets were resuspended in the buffer and their superoxide generation was determined separately [19].

### SDS/PAGE

This was performed essentially as described by Laemmli [22].

### Preparation of MP affinity column

Vespula MP (0.5 mg in 0.5 ml of sodium bicarbonate buffer, pH 8.0) was coupled to 50 mg (dry weight) of CH-Sepharose (Pharmacia) according to the instructions of the manufacturer. The resin (in 20 mM Tris/HCl, pH 8.0) was loaded on to a minicolumn to which 0.2 ml of neutrophil cytosol was applied. The flowthrough of the column was rechromatographed twice. The resin-treated cytosol (the last flowthrough of the column) was used in the NADPH oxidase activation assay and analysed by Western blotting using equal amounts (in protein) of untreated cytosol as a control. An aliquot of the cytosol-treated resin was washed with the column equilibration buffer, combined with 25 $\mu$l of SDS/PAGE sample buffer and analysed by immunoblotting with antibodies to p47-phox and p67-phox [19].

### Estimation of critical micellar concentration (CMC)

A 0.25 $\mu$M solution of diphenylhexatriene in NADPH oxidase activation buffer was titrated with SDS or arachidonate [23]. The effect of MP on the fluorescence enhancement of the solution at 430 nm was measured in a Perkin–Elmer 44B recording spectrofluorimeter. Excitation wavelength was set at 358 nm.

### Secondary structure prediction

PeptideStructure and PlotStructure computer programs of Jameson and Wolf were employed [24].

### Two-step permeabilization of polymorphonuclear leucocytes (PMNs) [25,26]

In the first step, $5 \times 10^7$ PMN in Krebs–Ringer phosphate solution (KRP) containing 1 mM $MgCl_2$/1 mM EGTA were incubated on ice (15 min) with streptolysin O (SLO) (Wellcome Diagnostics; Dartford, U.K.) at 1.65 units/ml and washed by centrifugation with 4 vol. of the ice-cold buffer. For permeabilization the SLO-treated cells were resuspended in lysis buffer (KRP containing 1 mM EGTA, 5 mM $MgCl_2$ and 0.7 mM $CaCl_2$) and transferred to 37 °C. The process was followed by monitoring the release of lactic dehydrogenase (LDH), which was completed in 5–10 min [26]. For stimulation of permeabilized cells, the incubation at 37 °C was carried out in the presence of 80 $\mu$M cytochrome *c*, 100 $\mu$M NADPH, 1 mM ATP and one of the following stimulants: GTP[S] (10 $\mu$M), formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) or phorbol 12-myristate 13-acetate (PMA) ($10^{-7}$ M) [26]. MP was added prior to the addition of the stimulants.

### Protein concentration

This was determined with Bradford reagent (Bio-Rad) using BSA as a standard [27].

## RESULTS

### Inhibition of NADPH oxidase activation by vespula and polistes MP

The presence of MP interfered with SDS-mediated activation of NADPH oxidase. In Figure 1(a) dose–response curves of the inhibition by the peptides are shown. Half-maximal inhibition required approx. 1.5 $\mu$M peptide. Inhibition was also apparent when arachidonic acid substituted for SDS as the oxidase-activating detergent in the presence or absence of GTP[S] (Figure 1b). Addition of MP to the NADPH oxidase assay mixture after completion of the activation had no effect on rates of superoxide

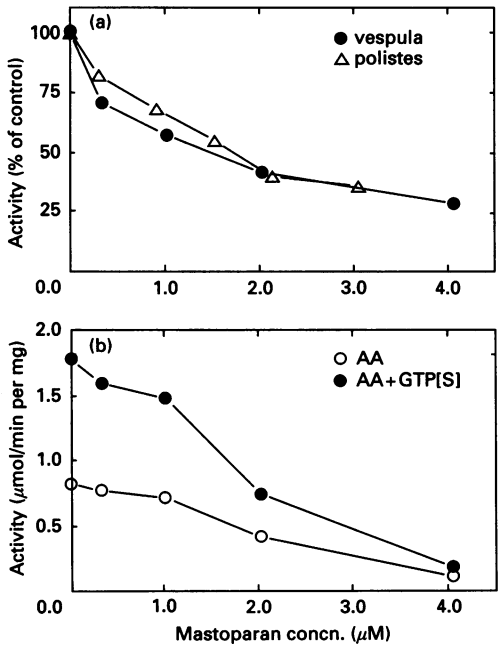


**Figure 1 Dose dependency of the inhibition of NADPH oxidase activation by mastoparans**

Activating detergents: (**a**) SDS; (**b**) arachidonate (AA). Each curve summarizes a representative experiment performed in duplicate and repeated at least three times. In SDS-activation, the absolute rates of superoxide release in the presence of MP vespula and polistes respectively were 384 and 480 nmol/mg of membrane protein respectively.

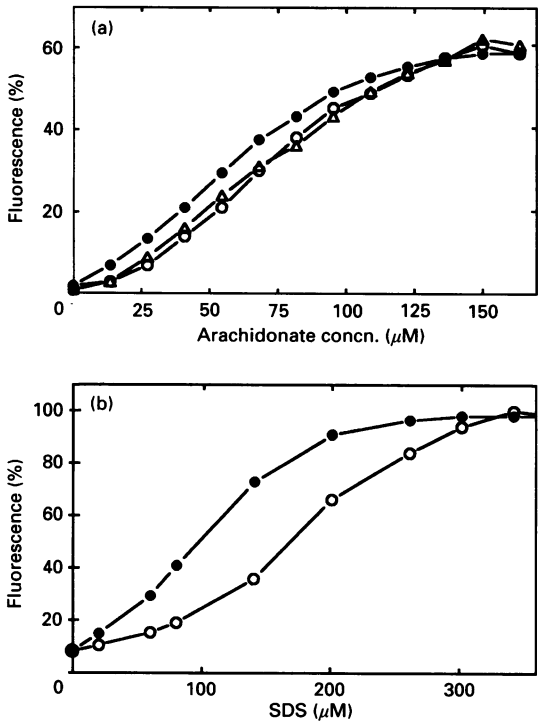


**Figure 2 Determination of CMC values for (a) arachidonate and (b) SDS (●, △) in the presence and (○) absence of MP (3.45 μM)**

●, MP vespula; △, MP-18. Fluorescence (430 nm) titration curves of 0.25 μM DPH solution.

production, implying participation of the peptide in the activation process and not in the catalytic step (results not shown).

Since MP is a cationic amphiphile, it might interact with oxidase-activating anionic detergents (arachidonate or SDS) and thus reduce their effects. Indeed MP slightly lowered the CMC value of SDS (Figure 2); SDS, however, activated oxidase at submicellar concentrations (50 μM) in the presence and absence of MP and the inhibition by MP was not relieved by increasing the concentration of the activating detergent (results not shown).

The inhibitory effect of MP was retained when octylglucoside extracts of neutrophil membranes [20] were employed as a source for NADPH oxidase cytochrome $b_{558}$, indicating that the effect was not related to the membrane-perturbing activity of the peptide (results not shown).

### The effect of MP on the $K_m$ of NADPH oxidase for NADPH

The dependence of superoxide generation rates on the concentration of the reduced pyridine nucleotide was estimated in the presence and absence of polistes or vespula MP. $K_m$ values evaluated for the uninhibited and for MP vespula- and MP polistes-containing activation mixtures were $38.8 \pm 7.8$, $24.7 \pm 5.0$ and $24.6 \pm 3.4$ μM respectively.

### Comparison of effectiveness of MP analogues in the activation of NADPH oxidase

In Table 1 sequences of several different MP analogues are aligned and their effects on NADPH oxidase activation are summarized. MP-9, MP-14, MP-17, MP-18 and MP-24 are synthetic analogues which exert different effects in other experimental systems [18]. In NADPH oxidase activation, all

**Table 1 Amino acid sequences of MP analogues and their inhibitory effects**

The C-terminal residues of all the analogues are amidinated. Results are from a representative experiment performed in duplicate.

| Peptide | Sequence | Net charge | Inhibition (%) 2 μg | 5 μg |
|---|---|---|---|---|
| MP vespula | INLKALAALAKKIL | +4.0 | 44.3 | 70.3 |
| MP-9 | IRALRRRSQLARRIL | +7.0 | 35.3 | 65.5 |
| MP-14 | INLGALAALAKKIL | +3.0 | 32.1 | 56.7 |
| MP-17 | IKKKALAALAKKIL | +6.0 | 40.0 | 61.0 |
| MP-18 | ISSKASSASSKKSS | +4.0 | 2.0 | 1.0 |
| MP-24 | AVLAKLAKLAKKILKILK | +7.0 | 42.8 | 81.2 |
| MP polistes | VDWKKIGQHILSVL | +3.5 | 38.0 | 64.2 |

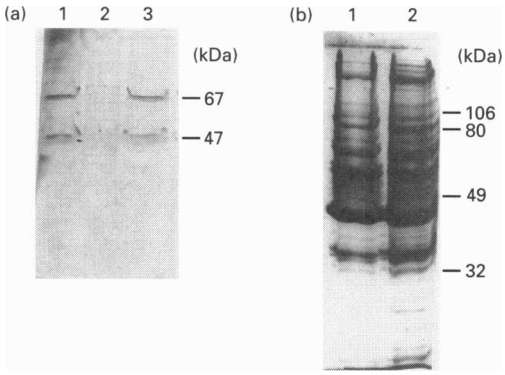


**Figure 3 Chromatography of neutrophil cytosol on a column of immobilized MP**

(**a**) Western blots developed with antibodies to p47-phox and p67-phox: lane 1, untreated cytosol; lane 2, column flowthrough; lane 3, affinity column resin (25% of the total volume of the minicolumn) eluted with SDS/PAGE sample buffer. (**b**) Coomassie Blue staining of SDS/PAGE gel of: lane 1, untreated cytosol; lane 2, affinity column flowthrough.

analogues, with the exception of MP-18, displayed inhibitory activity, with the highest inhibition exerted by MP-24 (Table 1). Lack of inhibition by MP-18 suggests that a positive net charge of the peptide was insufficient for effective blockade of oxidase assembly. Correspondingly, inhibition of oxidase activation by $(Lys)_4$ required significantly higher concentrations of the tetrapeptide ($EC_{50} = 100$ μM; results not shown).

### Chromatography of cytosol on an MP affinity column

The possibility of interaction of MP with a cytosolic component/s of the oxidase was tested. For this purpose neutrophil cytosol was chromatographed on a column of immobilized MP prepared as described in the Materials and methods section. The flowthrough of the column was subjected to SDS/PAGE, stained with Coomassie Blue, immunoblotted for p47-phox and p67-phox and tested for activity in the oxidase activation assay. The profile of Coomassie Blue-stained proteins of the flowthrough was very similar to that of untreated cytosol, indicating that most cytosolic proteins were not retained by the resin (Figure 3b). Chromatography of cytosol on an MP affinity column depleted it of the oxidase-activating activity (results not shown); moreover, p47-phox and p67-phox were missing in Western blots of the flowthrough (Figure 3a). SDS/PAGE sample buffer eluted the

**Table 2 Inhibition of NADPH oxidase activity in SLO-permeabilized cells**

At zero time SLO-pretreated cells were stimulated with 1 mM ATP and 10 $\mu$M GTP[S]. Superoxide dismutase-inhibitable reduction of cytochrome *c* was followed for 5 min at $3 \times 10^6$ PMNs/ml as described in the Materials and methods section (39.8 nmol of superoxide/5 min in uninhibited control). Results are from a representative experiment performed in duplicate and repeated with three preparations of cells.

| Additions | $O_2^-$ production (%) |
|---|---|
| None | 100.0 |
| MP vespula | |
| (3.5 $\mu$g) | 61.3 |
| (8.0 $\mu$g) | 40.2 |
| MP-24 | |
| (3.5 $\mu$g) | 76.9 |
| (8.0 $\mu$g) | 69.2 |
| MP-18 | |
| (3.5 $\mu$g) | 105.7 |
| (8.0 $\mu$g) | 111.0 |

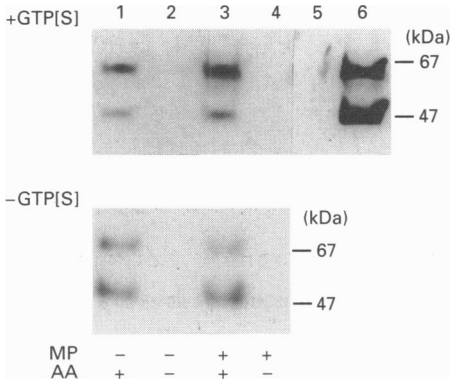


**Figure 4 The effect of MP on the translocation of cytosolic p47-phox and p67-phox to neutrophil cell membranes in a cell-free system**

Activation mixtures consisted of cytosol and membranes preincubated with (lanes 1 and 3) and without arachidonate (AA) (lanes 2 and 4) in the presence and absence of GTP[S]. Membranes were re-isolated by centrifugation of the activation mixtures through sucrose layer and analysed by Western blotting as described in the text. The mixture depicted in lane 5 contained only cytosol and arachidonate as a control for a possible detergent-induced aggregation of cytosolic proteins. In lane 6 neutrophil cytosol (3 $\mu$l) was loaded on the gel.

missing oxidase subunits from the resin as shown by Western blots in Figure 3(a).

### The effect of MPs on oxidase activation in SLO-permeabilized cells

Lu and Grinstein described activation of the NADPH oxidase in electropermeabilized neutrophils by the combined action of GTP[S] and ATP [28]. Both nucleotides were also shown to activate the enzyme in SLO-permeabilized cells [26]. If the inhibitory effect of MP is mediated through its interaction with the cytosolic components of the oxidase, inhibition of activation in permeabilized cells should be expected. Indeed, as shown in Table 2, MP interfered with superoxide release by SLO-pretreated PMNs. MP-18, inactive in the cell-free system, was also ineffective in permeabilized cells.

### The effect of MP on translocation of p47-phox and p67-phox to the membrane

For detection of translocated proteins we adopted arachidonate-mediated activation of the oxidase by the experimental procedure of Sawai et al. [21]. Neutrophil membranes were re-isolated from activation mixtures by centrifugation through a sucrose layer and their superoxide release was determined. In the experiment summarized in Figure 4 rates of cytochrome *c* reduction by the re-isolated activated control membranes and membranes activated in the presence of MP were 6 nmol/min and 0.2 nmol/min respectively. Contrary to other protocols [5,7], translocation of a fraction of cytosolic p47-phox and p67-phox to the membranes isolated by the procedure of Sawai required the simultaneous presence of arachidonate and membranes during the activation (Figure 4, lane 1) and did not detect non-specific, arachidonate-mediated aggregation of cytosolic proteins (Figure 4, lane 5). Unexpectedly, blockade of activation by MP did not affect translocation (Figure 4, lane 3). It is also of note that, contrary to previous reports [29,30], in our experimental system translocation of p47-phox and p67-phox was independent of the presence of GTP[S].

## DISCUSSION

The molecular basis of assembly of the NADPH oxidase in the cell-free system is not well understood. Reconstitution experiments indicated participation of at least four proteins, essential also in the intact cell: the membrane-bound heterodimeric cytohrome $b_{558}$ and cytosolic p47-phox, p67-phox and Rac1/Rac2 proteins [3,4]. Additional enzymes (e.g. kinases) known to participate in signal transduction processes *in vivo* were not implicated.

MPs have been shown to interact with cell membrane components. In our system, however, the blocking effect of MPs was not affected by the use of octylglucoside extracts of neutrophil membranes, suggesting that their mechanism of action was not related to membrane perturbation. The most prominent feature of all the blocking analogues was their positive net charge (Table 1). Since MP-18, however, in spite of its positive charge, was inactive, basicity was not sufficient for effective inhibition. Consistent with this was the finding that tetralysine required almost two orders of magnitude higher concentrations for inhibition. Predictions of secondary structure [24] suggest that, with the exception of MP-18 and MP-9, the peptides listed in Table 1 may fold into an amphiphilic $\alpha$ helix. The capacity to adopt a helical fold was not a prerequisite for inhibition by MPs, since the non-helical MP-9 also blocked activation (Table 1).

The amphiphilic structure of MPs might in principle interfere with oxidase activation by interacting with the negatively charged detergent (SDS or arachidonate) and by reducing its effective concentration. Attempts to relieve inhibition by raising the detergent concentration were, however, unsuccessful. Likewise CMC values of the activating detergents determined on the basis of diphenylhexatriene fluorescence [23] were not altered sufficiently by MP to account for the inhibition observed (Figure 2): although an apparent shift of titration curves to lower concentrations of SDS was observed, optimal doses for oxidase activation in both cases were in the submicellar range. Furthermore, the finding that MPs interfere also with activation of the NADPH oxidase in SLO-permeabilized cells stimulated with GTP[S] and ATP suggests that arachidonate or SDS were not involved in the reaction.

Results of chromatography on immobilized MP implicate cytosolic components of the oxidase in the interaction with MP

which abrogates the superoxide-releasing potential of the cytosol. Coomassie Blue staining of MP-treated cytosol (Figure 4) indicates that only a small fraction of cytosolic proteins was retained on the column, implying specificity of the reaction. Since both p47-phox and p67-phox, believed to form a complex in the cytosol of neutrophils [29,30], were eluted from the extensively washed affinity matrix (Figure 3a), interaction of each of them with MP might, in principle, be responsible for the association of the entire complex with the column. This association interfered with subsequent activation of NADPH oxidase without blocking translocation of p47-phox and p67-phox to the membrane. It follows that translocation to the membrane does not necessarily reflect formation of a catalytically active enzymic complex.

In several studies of oxidase activation (*in vivo* or *in vitro*) synthetic peptides corresponding to partial sequences of oxidase subunits were employed as probes of the mechanism and progress of assembly [9–11]. Peptides derived from cytoplasmic domains of the large and small subunits of cytochrome $b_{558}$ were shown to block activation both in the cell-free system and in electropermeabilized neutrophils [9]. The arginine-rich C-terminal sequence of p47-phox (residues 324–332) inhibited phosphorylation, translocation and superoxide generation in broken cell preparations [10] and competition of the peptide with p47-phox for the binding site on the cytoplasmic domain of cytochrome $b_{558}$ was suggested. Similarly, the 178–188 sequence of Rac1 was shown to inhibit cell-free activation competitively with Rac1 [11], implying a specific interaction of the peptide with the target of Rac1, possibly cytochrome $b_{558}$. This specificity was recently questioned by the demonstration of Joseph et al. [31] that activation of the Rac2-dependent human NADPH oxidase was blocked by the C-terminal peptide of Rac1 but not by the Rac2 peptide. The authors ascribed inhibition by Rac1 peptide to the action of its polybasic domain [31]. This resembles the effect of defensin, a highly charged cationic polypeptide constituent of azurophilic granules of PMNs, previously shown to interfere with cell-free activation [32].

Most inhibitory MP analogues employed in this study are devoid of polybasic domains of five/six basic amino acids (Table 1). Moreover, they are unrelated to oxidase components. Nevertheless, all the analogues, with the exception of MP-18, blocked activation in the micromolar range of concentrations, exhibiting an apparent affinity for their target(s) higher than or equal to the affinity of oxidase-derived sequences [9–11]. These findings imply that, as far as specificity is concerned, data obtained in peptide inhibition studies should be interpreted with great caution. A similar conclusion was reached recently by Fensome et al. who demonstrated inhibition of ARF (ADP-ribosylation factor)-dependent and ARF-independent cellular activities by ARF peptides and MPs [33].

Contrary to the helical fold characterizing peptides active in the study of Fensome et al. [33], helical structure was not essential for effective inhibition of oxidase activity by MP analogues. In view of this we suggest that motifs XKKX or XRRX, in which X symbolizes a hydrophobic amino acid, might represent a minimal inhibitory sequence in NADPH oxidase activation.

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