Built for the kill. Studies on the neutrophil NADPH oxidase
van Bruggen, R.

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Chapter 4

Continuous translocation of Rac2 and the NADPH oxidase component p67phox during phagocytosis.

Robin van Bruggen\textsuperscript{1,2}, Eloise Anthony\textsuperscript{1}, Mar Fernandez-Borja\textsuperscript{1} and Dirk Roos\textsuperscript{1}.

\textsuperscript{1} Sanquin Research, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

\textsuperscript{2} Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
Abstract
In this study, the translocation of the NADPH oxidase components p67\textsuperscript{phox} and Rac2 was studied during phagocytosis in living cells. For this purpose, GFP-tagged versions of these proteins were expressed in the myeloid cell-line PLB-985. First, the correct localization of p67GFP and GFP-Rac2 was shown during phagocytosis of serum-treated zymosan (STZ) by wild-type PLB-985 cells and PLB-985 X-CGD cells, which lack expression of flavocytochrome \textit{b}$_{558}$. Subsequently, these constructs were used for fluorescence recovery after photobleaching (FRAP) studies to elucidate the turn-over of these proteins on the phagosomal membrane. The turn-over of p67GFP and GFP-Rac2 proved to be very high, indicating a continuous exchange of flavocytochrome \textit{b}$_{558}$-bound p67GFP and GFP-Rac2 for cytosolic, free p67GFP and GFP-Rac2. Furthermore, the importance of an intact actin cytoskeleton for correct localization of these proteins was investigated by disrupting the actin cytoskeleton with cytochalasin B. However, cytochalasin B treatment of PLB-985 cells did not alter the localization of p67GFP and GFP-Rac2 once phagocytosis was initiated. In addition, the continuous exchange of flavocytochrome \textit{b}$_{558}$-bound p67GFP and GFP-Rac2 for cytosolic p67GFP and GFP-Rac2 was still intact in cytochalasin B-treated cells, indicating that translocation of these proteins does not depend on a rearrangement of the actin cytoskeleton.

Introduction
Phagocytes comprise a very important factor in the neutralization of infections by potentially harmful microorganisms\textsuperscript{1}. Their main task is to track down, phagocytose and subject the microbes to an impressive array of anti-microbial proteins and products, a combination that ultimately leads to eradication of the infection\textsuperscript{1}. One of the most important, and best studied, anti-microbial systems is the phagocyte NADPH oxidase\textsuperscript{2,3}. This enzyme produces superoxide in the phagosome by transferring electrons from NADPH in the cytosol over the phagosomal membrane to molecular oxygen\textsuperscript{4}. The superoxide generated by the phagocyte NADPH oxidase forms the basic compound from which other reactive oxygen species (ROS), such as hydrogen peroxide and hypochlorous acid, are formed. High concentrations of ROS in the phagosome are toxic, and ultimately lead to killing of the phagocytosed microbe\textsuperscript{2,5}. Furthermore, the transfer of electrons over the phagosomal membrane leads to changes in the membrane.
potential that are crucial for the influx of K⁺ ions into the phagosome, an event that liberates matrix-bound proteases to diffuse freely into the phagosome and contribute to the successful killing of the microorganism⁶. Thus, there is a central role for the phagocyte NADPH oxidase in the killing of phagocytosed microorganisms; a direct one in generating ROS and an indirect one in the dispersion of intracellular proteases in the phagosome.

Since ROS are potentially harmful to the host itself, the activity of the phagocyte NADPH oxidase is tightly regulated in space and time. The enzymatic core of the phagocyte NADPH oxidase is flavocytochrome b₅₅₈, which consists of two integral membrane proteins, p₂₂phox and gp₉₁phox (phox: phagocyte oxidase). Flavocytochrome b₅₅₈ contains the FAD and two heme groups needed to transfer electrons from NADPH to oxygen⁷. The activity of flavocytochrome b₅₅₈ is regulated by the interaction with three cytosolic proteins, p₄₇phox, p₆₇phox and Rac₂⁸⁻¹⁰. Mutations in any of these five subunits can lead to a severe immunodeficiency, called chronic granulomatous disease (CGD)¹¹. CGD is characterized by defective killing of phagocytosed pathogens, which leads to recurrent life-threatening infections.

Activation of gp₉₁phox is induced after translocation of the cytosolic proteins to the phagosomal membrane and their subsequent interaction with the flavocytochrome². Translocation of p₄₇phox, p₆₇phox and Rac₂ is driven by several changes in the conformation of these proteins, occurring after binding of chemokines or opsonins to phagocyte surface receptors⁸⁻¹⁰. The Rho GTPase Rac₂ is dissociated from its inhibitor RhoGDI after stimulation and is then able to interact with membranes via its prenylated C-terminus¹⁰. Once attached to the membrane, Rac₂ is able to bind to flavocytochrome b₅₅₈ and forms a binding partner for p₆₇phox¹⁰. The cytosolic proteins p₄₇phox and p₆₇phox can form a complex with another protein, p₄₀phox, in the cytosol of the neutrophil¹². During activation, phosphorylation of p₄₇phox at crucial serine residues leads to conformational changes in this protein, allowing the formation of the p₄₇phox-p₆₇phox-p₄₀phox complex as well¹²,¹³. The outcome of these conformational changes is an increase in capacity of these proteins to interact with the membrane, with Rac₂ and with flavocytochrome b₅₅₈, three interactions that are crucial for translocation and subsequent activation of gp₉₁phox⁹,¹₀,¹³,¹⁴.

To study the translocation of p₆₇phox and Rac₂ during phagocytosis in living cells, GFP-tagged fusions of these proteins were introduced in the myeloid cell line, PLB-985. The translocation kinetics of these proteins were studied during
phagocytosis by wild-type PLB-985 cells as well as by PLB-985 cells that do not express flavocytochrome b_{558}, due to targeted disruption of gp91^{phox} expression (PLB-985 X-CGD)\textsuperscript{15}. Furthermore, by means of fluorescence recovery after photobleaching (FRAP) experiments we found that the translocation of p67^{phox} and Rac2 is a cyclic process, in which flavocytochrome b_{558}-bound p67GFP and GFP-Rac2 are continuously exchanged for free p67GFP and GFP-Rac2. Finally, the effect of the actin-modifying compound cytochalasin B on the translocation of p67^{phox} and Rac2 during phagocytosis was investigated. The disruption of the actin cytoskeleton by this compound did not change the localization of p67GFP and GFP-Rac2 nor did it affect the cyclic translocation, indicating that the actin cytoskeleton is not important for the correct localization of these proteins during phagocytosis.

**Experimental Procedures**

**Culture of cell lines**

K562, PLB-985 and \( \phi \)nx-\( \alpha \) cells were cultured in IMDM (Gibco) supplemented with 10\% fetal calf serum, penicillin (200 \( \mu \)g/ml), streptomycin (200 \( \mu \)g/ml) and L-glutamine (4 mM) in a \( \text{CO}_2 \) incubator at 37\°C. PLB-985 cells were induced to differentiate to granulocytes by the addition of 0.5\% dimethylformamide. The cells were then cultured for 6 days and subsequently harvested.

**Generation of fluorescent constructs.**

For the generation of C-terminally GFP-tagged p67^{phox} the cDNA encoding p67^{phox} was amplified with primers designed to discard the stop codon of this gene. In addition, these primers contained restriction sites (EcoRI, Apal) to facilitate further cloning into the pEGFP vector (Clontech, Palo Alto, CA, USA). The primers used were (5' to 3') gatcgaattccatatgctcctgag gg (sense) and (5' to 3') gatcggggccggctctcctctgcgag gg (antisense). The PCR reaction contained PWO polymerase (Roche, Basel, Switzerland), buffer, dNTPs, 100 ng of plasmid DNA encoding p67 and 100 ng of each primer. The PCR conditions used were: 1 cycle 95\°C 5 min, 35 cycles 1\,min 95\°C, 1 min 56\°C and 2.5 min 68\°C and 1 cycle 5 min 68\°C. The PCR product was purified over QiaSpin PCR purification columns (Qiagen, Valencia, CA, USA). The PCR product and the destination vector pEGFP were then digested with EcoRI and Apal for 1.5 hrs at 37\°C. Both digests were run
on a low-melting point agarose gel (1% w/v) and stained with ethidium bromide. DNA fragments of the right size were cut out of the gel and purified with QiaQuick columns (Qiagen). Fragments were ligated with the FastLink Ligase Kit (Epicentre Technologies, Madison, WI, USA) and subsequently transformed into competent DH5α cells. Plasmids were examined for correct inserts by restriction digests and sequenced for detection of unwanted mutations. In analogy, GFP-Rac2 was generated by fusing eGFP N-terminally with Rac2. For the cloning of Rac2 in the eGFP vector the cDNA of Rac2 was amplified with primers containing XhoI and NotI restriction sites. The primers used were (5’ to 3’)
gatcctcagttcaggccataagttggtg (sense) and (5’ to 3’)
gagatcgcggcgctaggtcggcggc (antisense). The PCR reaction and subsequent cloning were carried out as described for the p67GFP construct.

**Transient expression of p67GFP and GFP-Rac2 in K562 cells**

K562 cells stably expressing p22phox, p47phox and gp91phox were transiently transfected by electroporation (0.25 kV, capacitance 960 μF, 0 Ω resistance) on a BioRad Biopulsar (Hercules, CA, USA), with GFP, wild-type p67phox, p67GFP or GFP-Rac2. Cells were grown for 48 hours before analysis. GFP expression was assayed by flow cytometry on a Becton Dickinson FacsStar (Palo Alto, CA, USA).

**Hydrogen-peroxide generation by K562 cells**

Hydrogen-peroxide production of K562 cells after phorbol-myristate acetate (PMA, Sigma) activation was measured by the Amplex Red Assay (Molecular Probes, Eugene, OR, USA) measured on a Perkin Elmer plate reader.

**Western blotting of K562 cells**

For immunodetection, 10⁵ cells were boiled in SDS sample buffer (125 mmol/L Tris, pH 6.8; 20% (w/v) SDS and 12.5% (v/v) β-mercaptoethanol) for 5 min and loaded on a 12.5% polyacrylamide gel, according to Laemmli, in a gel apparatus (Mini-Protean II, BioRad). Western blotting was performed (Mini Trans-Blot cell, BioRad) according to the manufacturer's recommendations. For the detection of p67GFP and GFP-Rac2 a polyclonal antibody against GFP (Clontech, #8372-1) was used. The secondary antibody was a swine-anti-rabbit-Ig horseradish-peroxidase-conjugated polyclonal antibody (DAKO, Glostrup, Denmark). Detection was performed with the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia, Uppsala, Sweden).
Stable expression of selected mutants in PLB-985 cells

PLB-985 and PLB-985 X-CGD cells were retrovirally transduced with p67GFP or GFP-Rac2. In brief, cDNA constructs encoding p67GFP and GFP-Rac2 were cloned from the pEGFP vector into the retroviral expression vector pLZRS. pLZRS constructs were then transfected into a retroviral packaging cell line (φnx-ampho) by calcium-phosphate transfection (Gibco). After selection of transfected cells by puromycin (1 μg/ml) (Gibco), virus was harvested and used for retroviral transduction of PLB-985 and PLB-985 X-CGD cells with 10 μg/ml of DOTAP (Roche, Basel, Switzerland). Transduced cells were sorted on a FacsStar (Becton Dickinson) cell sorter.

Confocal Imaging of PLB-985 cells

For in vivo imaging of p67GFP and GFP-Rac2 fusion proteins, 500 μl of a suspension of 2x10^6 PLB-985 cells transduced with these constructs were allowed to adhere to glass coverslips in complete IMDM medium at 37°C in a heatstage. Cells were allowed to adhere for 5 min before serum-treated zymosan (STZ) was added. Images were taken using a Zeiss Axiovert 100 confocal laser scanning microscope and analysed with LSM 5 software (Zeiss, Göttingen, Germany).

Fluorescence recovery after Photobleaching (FRAP)

PLB-985 cells were allowed to adhere to glass coverslips in complete IMDM medium at 37°C in a heatstage. Cells were allowed to adhere for 5 min before STZ was added. Rectangles were bleached with full intensity of a 488 nm laser beam, the recovery of fluorescence in the bleached spot was quantified with LSM 5 software (Zeiss). The experiments were performed several times on different days.

Results

Generation of p67GFP and GFP-Rac2 fusion proteins and expression in K562 cells

For live imaging of p67^phox translocation, the cDNA encoding this protein was fused C-terminally to GFP. Before the fusion of the cDNAs, the ATG of the GFP cDNA was deleted by site-directed mutagenesis, because free GFP was observed when several different fusion proteins were constructed with the parent vector
containing the start codon. K562 cells already expressing p22\textsuperscript{phox}, gp91\textsuperscript{phox} and p47\textsuperscript{phox} were transfected with the p67GFP construct, wild-type p67\textsuperscript{phox}, or GFP. The superoxide-producing capacity of the cells transfected with the p67GFP construct was compared with K562 cells transfected with wild-type p67\textsuperscript{phox} and with the GFP-transfected K562 cells that express all NADPH oxidase components except p67\textsuperscript{phox}. Upon PMA stimulation, K562 cells transfected with wild-type p67\textsuperscript{phox} were able to produce hydrogen peroxide, in contrast to GFP-transfected cells, which showed no NADPH oxidase activity (Table 1). The K562 cells expressing the p67GFP construct showed similar superoxide-producing capacities as the wild-type p67\textsuperscript{phox}-transfected cells (Table 1), proving that this construct is able to fulfill its role in the activation of the NADPH oxidase.

**Table 1. Hydrogen-peroxide production by K562 cells.**

<table>
<thead>
<tr>
<th>transfected with</th>
<th>RFU</th>
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<tr>
<td>empty vector</td>
<td>0</td>
</tr>
<tr>
<td>eGFP</td>
<td>0</td>
</tr>
<tr>
<td>wild-type p67\textsuperscript{phox}</td>
<td>10000</td>
</tr>
<tr>
<td>p67GFP</td>
<td>9500</td>
</tr>
<tr>
<td>wt p67\textsuperscript{phox} + Rac2GFP</td>
<td>10500</td>
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Hydrogen-peroxide-producing capacity of K562 cells transfected with empty vector, eGFP, wild-type p67\textsuperscript{phox} or p67GFP. In addition, the effect of GFP-Rac2 expression was tested in K562 cells that expressed all essential components of the NADPH oxidase. The number of cells tested for hydrogen peroxide production was corrected for the percentage of transfected cells of each individual transfection. Values are given as relative fluorescence units (RFUs), with wild-type p67\textsuperscript{phox}-transfected cells at 10,000 RFUs. The RFUs generated by untransfected cells were subtracted. A representative experiment from three experiments is shown.

To ensure that GFP was not cleaved from the C-terminus of p67\textsuperscript{phox}, a possibility that could not be excluded from the previous experiments, Western blot analysis was performed on total lysates of the transfected K562 cells. As controls, untransfected, GFP-transfected and wild-type p67\textsuperscript{phox}-transfected K562 cells were taken. The fusion protein was detected after staining with anti-GFP at a molecular mass of approximately 95 kD, in good agreement with its predicted molecular mass (94 kD) (Figure 1).

The GFP-Rac2 fusion was constructed by fusing eGFP N-terminally to Rac2. This approach has been used successfully for Rac1 and several other small GTPases\textsuperscript{17}. Due to the lack of Rac-negative K562 cells, it was impossible to prove
that the GFP-Rac2 fusion is able to perform the function of wild-type Rac2 in the activation of the NADPH oxidase. However, no inhibitory effect of this construct was observed on hydrogen-peroxide production in K562 cells stably transfected with p47\textsuperscript{phox}, p67\textsuperscript{phox} and gp91\textsuperscript{phox} (Table 1). Furthermore, the GFP-Rac2 fusion protein was detected at the expected molecular mass of 48 kD by Western blotting (Figure 1).

**Localization of p67GFP and GFP-Rac2 during NADPH oxidase activation**

The localization of p67GFP and GFP-Rac2 during NADPH oxidase activation was studied in PLB-985 cells. The constructs were retrovirally expressed in myeloid PLB-985 cells and in a derivative of this cell line in which the expression of gp91\textsuperscript{phox} has been disrupted, i.e. PLB-985 X-CGD cells. The cells were then induced to differentiate into granulocytes. The localization of the fusion proteins was studied during phagocytosis of STZ. Upon binding of STZ to both PLB-985 cell lines, p67GFP was rapidly recruited to the site of attachment (Figure 2a).

During particle internalization and the formation of a phagosome, p67GFP was located at the membrane of the phagosome in both cell lines. When the particle was completely taken up, p67GFP in the wild-type cells remained located around the phagosomes through time (Figure 2a,b). In contrast, p67GFP was lost from the phagosome in the PLB-985 X-CGD cells after complete uptake of the STZ particles (Figure 2c,d). An identical pattern was observed for the localization of GFP-Rac2 (Figure 2e,f,g,h). Like p67GFP, this fusion protein is also located at the phagosome during and after complete internalisation of the particle by PLB-985 cells, but loss of the protein from the phagosome was observed after internalisation of STZ in PLB-985 X-CGD cells. These findings are consistent with previous
studies that showed loss of cytosolic NADPH oxidase components from the phagosomal membrane in X-CGD neutrophils, identifying cytochrome b$_{558}$ as an essential factor for correct translocation of p47$^{\text{phox}}$, p67$^{\text{phox}}$ and Rac2$^{18-20}$. Furthermore, in PLB-985 X-CGD cells translocation of p47$^{\text{phox}}$, p67$^{\text{phox}}$ and Rac2 is known to be disturbed$^{21}$. Together with the observation that p67GFP and GFP-Rac2 localize correctly in both PLB-985 and PLB-985 X-CGD cells, this identifies the p67GFP and GFP-Rac2 expressing cell lines as suitable models to study the localization of these proteins in more detail.

![Image of Figure 2: Localization of p67GFP and GFP-Rac2 in PLB-985 cells.](image)

**Figure 2.** Localization of p67GFP and GFP-Rac2 in PLB-985 cells. PLB-985 cells, adhered to glass coverslips, were allowed to phagocytose STZ for 15 minutes and were then fixed. Distribution of p67GFP was analysed by confocal microscopy. Arrows indicate an STZ-particle. Wild-type PLB-985 cells showed p67GFP staining on nascent and progressed phagosomes. (a,b) In contrast, PLB-985 X-CGD cells lost staining of the p67GFP fusion protein as the phagosome progressed to more mature stages (c,d). GFP-Rac2 expressed in wild-type PLB-985 cells (e) or PLB-985 X-CGD cells (f) showed similar localization as p67GFP.

**Photobleaching of p67GFP and GFP-Rac2 during NADPH oxidase activation**

To investigate the turn-over of p67$^{\text{phox}}$ and Rac2 on the phagosomal membrane, fluorescence recovery after photobleaching (FRAP) experiments were performed with the p67GFP- and GFP-Rac2-expressing PLB-985 cells. First, the bleaching protocol (see Experimental Procedures) was tested on fixed PLB-985 cells expressing p67GFP or GFP-Rac2 to ensure that all fluorescence was bleached in
the target area with these specific settings. Indeed, the fluorescence in p67GFP and GFP-Rac2 expressing cells completely disappeared in the bleached area under these conditions (data not shown). PLB-985 cells were then allowed to phagocytose STZ and bleaching of phagosomes was performed after the completion of phagocytosis. At the phagosome, the fluorescence of p67GFP at the site of photobleaching was rapidly restored, with a recovery time ($t_{1/2}$: time in which 50% of the fluorescence is recovered in the bleached area) that was generally lower than the scan time (700 ms) needed for the generation of an image by confocal laser scanning microscopy (CLSM), thus preventing a reliable estimation of the $t_{1/2}$ for this protein (Figure 3a). The translocation of GFP-Rac2 was also investigated by FRAP (Figure 3b). This protein also displayed rapid recovery at the site of bleaching, again with a $t_{1/2}$ that was lower than 700 ms.

![Figure 3. FRAP of p67GFP and GFP-Rac2 in PLB-985 cells during phagocytosis.](image)

To study the redistribution of p67GFP during phagocytosis, single bleaching of small parts of phagosomes (indicated by the rectangle) was applied during real-time imaging. Rapid recovery of fluorescent signal was detected and a concomitant loss of fluorescent signal was observed in the rest of the cell (a). The same pattern of fluorescence recovery was observed for GFP-Rac2 (b).
Sequential bleaching of the same spot in individual PLB-985 cells expressing p67GFP or GFP-Rac2, led to a complete loss of fluorescence in the whole cell, illustrating the free diffusion of these proteins throughout the cell (data not shown).

Effects of actin-modifying compounds on translocation of p67GFP and GFP-Rac2

Phagocytosis is an actin-dependent event, and actin polymerization is rapidly induced upon binding of an opsonized particle to phagocyte surface receptors\textsuperscript{22,23}. F-actin is surrounding the particle during internalisation, after which actin is lost again from the phagosome\textsuperscript{24}. Depolymerization of the actin cytoskeleton by actin-modifying compounds inhibits phagocytosis by preventing the internalization but not the binding of opsonized particles\textsuperscript{23,24}. Several reports have indicated the importance of an intact actin cytoskeleton for NADPH oxidase activity, in the cell-free system as well as in living cells\textsuperscript{25-27}. During activation of the NADPH oxidase the cytosolic oxidase components bind to the cytoskeleton, as determined by analysis of the detergent-insoluble cytoskeletal fraction\textsuperscript{28}. Furthermore, p47\textsuperscript{phox} has been shown to bind directly to actin, and p47\textsuperscript{phox} and p67\textsuperscript{phox} have been shown to accumulate on nascent phagosomes together with F-actin\textsuperscript{29}. Whether correct p67GFP and GFP-Rac2 localization requires an intact and rearranging actin cytoskeleton during phagocytosis was tested in PLB-985 cells by treating the cells with the actin-modifying compound cytochalasin B. First, the importance of an intact actin cytoskeleton for translocation of p67GFP and GFP-Rac2 after binding of STZ particles to the cell surface was determined. PLB-985 cells were pretreated with cytochalasin B, a potent actin-depolymerizing agent, before addition of STZ. As expected, pretreatment with cytochalasin B inhibited the uptake of STZ after binding, and diminished the translocation of p67GFP and GFP-Rac2 to the site of STZ attachment (Figure 4a,b,c,d). The same result was obtained by pretreating PLB-985 cells with latrunculin A, a compound that, like cytochalasin B, depolymerizes existing actin filaments (not shown).

Since translocation of p67GFP and GFP-Rac2 to phagosomes proved to be a continuous process, as concluded from the FRAP experiments, the question was raised whether this process of continuous translocation to the phagosome requires an intact actin cytoskeleton. To determine the effect of disruption of the actin cytoskeleton on translocation of p67GFP and GFP-Rac2 during and after
Untreated cells showed translocation of p67GFP after binding of STZ particles, arrows indicate STZ particle (a). In contrast, PLB-985 cells pre-treated with 10 mM cytochalasin B for 10 min before addition of STZ, showed no translocation of p67GFP after binding of STZ particles (b). Like p67GFP, GFP-Rac2 showed translocation to the membrane after binding of STZ particles (c), pre-treatment of the cells with 10 mM cytochalasin B prohibited translocation of GFP-Rac2 after attachment of STZ particles (d).
phagocytosis, cells were allowed to take up STZ for 5 min before cytochalasin B was added. Typically, addition of this compound rapidly led to an arrest in phagocytosis and cell movement. However, the localization of p67GFP and GFP-Rac2 was not altered in cytochalasin B-treated cells in comparison to untreated cells (not shown). To determine whether the process of continuous translocation of p67GFP and GFP-Rac2 to the phagosome was still intact in PLB-985 cells with a disrupted actin cytoskeleton, FRAP experiments were performed on cytochalasin B-treated cells (Figure 5a,b). Like untreated cells, cytochalasin B-treated cells showed a rapid recovery of fluorescence at the bleached spots of the phagosome (Figure 5b,6). In all cases the $t_{1/2}$ was too small to be reliably detected by CLSM. Furthermore, the use of actin-modifying agents allowed the bleaching of

![pre-bleach bleach 10 s](image)

**Figure 5. Effect of cytochalasin B treatment on FRAP of p67GFP and GFP-Rac2.** For this experiment, PLB-985 cells were allowed to phagocytose STZ for 5 min before cytochalasin B was added. Real-time imaging of PLB-985 cells treated with 10 mM cytochalasin B revealed normal distribution of p67GFP (a) and GFP-Rac2 (b). To study the redistribution of p67GFP after treatment with cytochalasin B, single bleaching of small parts of phagosomes (indicated by the rectangle) was applied during real-time imaging (a). Rapid recovery of fluorescent signal was detected and a concomitant loss of fluorescent signal was observed in the rest of the cell (a). The same pattern of fluorescence recovery was observed for GFP-Rac2 (b).
phagocytic cups, because uptake of STZ particles was arrested after addition of either cytochalasin B or latrunculin A. FRAP experiments of phagocytic cups showed no differences in recovery between fluorescence at completely closed phagosomes and phagocytic cups (not shown).

![Graph](image)

**Figure 6. FRAP kinetics of p67GFP and GFP-Rac2 in PLB-985 cells.**

In this experiment, cells were allowed to phagocytose STZ for 5 min before cytochalasin B or latrunculin A was added. Single phagosomes were bleached for 1 sec and fluorescence recovery was subsequently measured. The recovery kinetics for untreated cells and for cells treated with the actin-modifying compounds cytochalasin B and latrunculin A were determined. Shown are representative curves for each condition. Relative fluorescence at a given time point is defined as the ratio of fluorescence of the bleached spot over the whole cell at a given time point divided by the ratio of fluorescence of the bleached spot over the whole cell at t=0.

**Discussion**

The NADPH oxidase of phagocytic leukocytes consists of the membrane-bound flavocytochrome \( b_{558} \) and three essential cytosolic components \( p47^{phox} \), \( p67^{phox} \) and Rac2. In this report, the localization of \( p67^{phox} \) and Rac2 during NADPH oxidase activation was studied by means of GFP fusions of these proteins. The p67GFP fusion protein was first tested for its ability to support superoxide production in K562 cells expressing all essential NADPH oxidase components but lacking \( p67^{phox} \) expression. Expression of the fusion protein was seen to restore the superoxide-producing capacity of the K562 cells. Initially, the same approach was taken for \( p47^{phox} \), but the p47GFP fusion protein was not able to restore superoxide production in K562 cells lacking \( p47^{phox} \) (data not shown); therefore this construct was not used in this study. The GFP-Rac2 could not be tested in a similar manner,
but the GFP-Rac2 fusion protein did not have deleterious effects on superoxide production by K562 cells. Expressed in a granulocytic cell line, p67GFP and GFP-Rac2 show the same intracellular localization as the endogenous proteins during STZ phagocytosis. Additionally, their localization was disturbed in later stages of phagocytosis in cells that lack gp91$^{phox}$, which is consistent with experiments performed previously with these cell lines as well as with granulocytes from CGD patients\textsuperscript{18,30}.

During phagosome formation, translocation of the cytoplasmic NADPH oxidase components and their subsequent interaction with flavocytochrome $b_{558}$ may result in the formation of a stable complex of these proteins. Alternatively, the cytoplasmic components p47$^{phox}$, p67$^{phox}$ and Rac2 may only transiently associate with flavocytochrome $b_{558}$. Since translocation of the cytosolic NADPH oxidase components is rapidly terminated in cells that lack flavocytochrome $b_{558}$, it is generally believed that flavocytochrome $b_{558}$ and the cytosolic proteins form a stable complex, resulting in the containment of the cytosolic proteins at the phagosomal membrane. To determine the turn-over of p67GFP and GFP-Rac2 on the phagosome, FRAP experiments were performed. The fluorescence recovery of both proteins was extremely rapid, indicating that there is continuous exchange of soluble p67GFP and GFP-Rac2 for flavocytochrome $b_{558}$-bound p67GFP and GFP-Rac2, even after complete internalization of zymosan particles. This is in accordance with a previous study in which evidence was obtained suggesting that a limited amount of cytosolic components can activate an excess of flavocytochrome $b_{558}$, which also argues against the formation of a stoichiometric complex between the cytosolic factors and flavocytochrome $b_{558}$\textsuperscript{31}. Furthermore, Akard et al. have provided evidence that sustained superoxide production is a result of continuous replenishment of a pool of active NADPH oxidase, which is also in line with the data obtained in this study\textsuperscript{32}. The presence of flavocytochrome $b_{558}$ in the membrane is crucial for the observed continuous translocation of the cytosolic proteins, as illustrated by the loss of p67GFP and GFP-Rac2 from the phagosomes of PLB-985 X-CGD cells. This is in sharp contrast to the translocation of the cytosolic proteins to nascent phagosomes, that clearly does not depend on the presence of flavocytochrome $b_{558}$, since p67GFP and GFP-Rac2 localize correctly during this stage of phagocytosis in PLB-985 X-CGD cells.

Furthermore, the involvement of the actin cytoskeleton in translocation of p67$^{phox}$ and Rac2 during phagocytosis was investigated by the use of an actin-modifying agent. The effect of cytochalasin B, a potent actin de-polymerizing
agent, on the translocation behaviour of p67GFP and GFP-Rac2 was tested. Pre-
treatment of PLB-985 cells with this compound prevented an increase in the
amount of p67GFP and GFP-Rac2 at the binding site of STZ, identifying the
importance of the actin cytoskeleton in the initiation of translocation of p67GFP
and GFP-Rac2. It remains obscure why disruption of the actin cytoskeleton
disturbs the primary translocation of the cytosolic factors. Rac2, p40phox and p47phox
have been shown to be able to interact with the membrane through their prenylated
C-terminus or PX domains, respectively\textsuperscript{14,33,34}. In cytochalasin B-treated cells, the
interaction of the PX domains of p40phox and p47phox with the phosphoinositides
formed in the membrane after binding of STZ particles to cell-surface receptors
was anticipated to still occur, as well as the interaction of Rac2 with the membrane.
However, translocation of p67GFP and GFP-Rac2 is absent in cytochalasin B-
treated cells upon binding of zymosan particles, which identifies the association of
these proteins with the actin cytoskeleton as a step preceding the interaction of
these proteins with the membrane and flavocytochrome $b_{558}$.

In contrast, cytochalasin B had no effect on the localization and continuous
exchange of p67GFP and GFP-Rac2 on the phagosomal membrane when
phagocytosis had already been initiated. According to our data, translocation of
cytosolic NADPH oxidase components is independent of the actin cytoskeleton
after the initiation of phagocytosis. Several earlier reports have indicated the
importance of actin polymerization for NADPH oxidase activity\textsuperscript{25-27}. However,
most of these experiments were performed in a cell-free system and did not address
translocation of the cytosolic factors to flavocytochrome $b_{558}$. Furthermore,
inhibition of NADPH oxidase activity in intact cells may well be due to the effects
of actin cytoskeleton disruption on primary translocation, which was not identified
in these studies.

Besides actin-modifying compounds, several other pharmacological agents
were tested in PLB-985 p67GFP and GFP-Rac2 cells. In an attempt to identify the
mechanism that drives the continuous translocation of p67phox and Rac2, the PLB-
985 cells were treated with several inhibitors after addition of STZ. Among these
agents were LY290042 (PI3-kinase inhibitor), staurosporine (general inhibitor of
protein kinase C), SB20358 (inhibitor of p38MAPK) and U0126 (inhibitor of
pErk). Unfortunately, no effect of any of these inhibitors was found on the
continuous translocation of p67GFP and GFP-Rac2. The lack of effect of these
inhibitors is probably due to simultaneous stimulation of several signal
transduction routes leading to activation of the NADPH oxidase.
Overall, these results indicate that translocation of p67\textsuperscript{phox} and Rac2 during phagocytosis can be divided into three stages. The first stage is the initiation of phagocytosis after binding of an opsonized particle, in which translocation is dependent on an intact actin cytoskeleton, and independent of flavocytochrome \(b_{558}\). The second stage is the formation of the phagosome, during which translocation seems to be independent of flavocytochrome \(b_{558}\) and the cytoskeleton, but may be dependent on the interaction of the PX domains of p40\textsuperscript{phox} and p47\textsuperscript{phox} and the prenylated C-terminus of Rac2 with the membrane. The last stage, after closure of the phagosome, requires the presence of flavocytochrome \(b_{558}\) for correct localization of p67\textsuperscript{phox} and Rac2. Further studies are needed to elucidate the role of the actin cytoskeleton in NADPH oxidase activity in living cells during phagocytosis as well as the mechanism that drives continuous translocation of p67\textsuperscript{phox} and Rac2 to the phagosomal membrane.

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**Reference List**


Chapter 4


