Continuous Translocation of Rac2 and the NADPH Oxidase Component p67\textsubscript{phox} during Phagocytosis*

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In this study, the translocation of the NADPH oxidase components p67\textsubscript{phox} and Rac2 was studied during phagocytosis in living cells. For this purpose, green fluorescent protein (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 by wild-type PLB-985 cells and PLB-985 X-CGD (chronic granulomatous disease) cells, which lack expression of flavocytochrome b\textsubscript{558}. Subsequently, these constructs were used for fluorescence recovery after photobleaching studies to elucidate the turnover of these proteins on the phagosomal membrane. The turnover of p67GFP and GFP-Rac2 proved to be very high, indicating a continuous exchange of flavocytochrome b\textsubscript{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 b\textsubscript{558}-bound p67GFP and GFP-Rac2 for cytosolic p67GFP and GFP-Rac2 was still intact in cytochalasin B-treated cells, indicating that the translocation of these proteins does not depend on a rearrangement of the actin cytoskeleton.

Phagocytes comprise a very important factor in the neutralization of infections by potentially harmful microorganisms (1). Their main task is to track down, phagocytose, and subject the microbes to an impressive array of antimicrobial proteins and products, a combination that ultimately leads to eradication of the infection (1). One of the most important, and best studied, antimicrobial systems is the phagocyte NADPH oxidase (2, 3). This enzyme produces superoxide in the phagosome by transferring electrons from NADPH over the phagosomal membrane to molecular oxygen (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 (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\textsuperscript+ 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 microorganism (6). 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 b\textsubscript{558} which consists of two integral membrane proteins, p22\textsubscript{phox} and gp91\textsubscript{phox} (phox: phagocyte oxidase). Flavocytochrome b\textsubscript{558} contains the FAD and two heme groups needed to transfer electrons from NADPH to oxygen (7). The activity of flavocytochrome b\textsubscript{558} is regulated by the interaction with three cytosolic proteins, p47\textsubscript{phox}, p67\textsubscript{phox}, and Rac2 (8–10). 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 gp91\textsubscript{phox} is induced after the translocation of the cytosolic proteins to the phagosomal membrane and their subsequent interaction with the flavocytochrome (2). The translocation of p47\textsubscript{phox}, p67\textsubscript{phox}, and Rac2 is driven by several changes in the conformation of these proteins, occurring after binding of chemokines or opsonins to phagocyte surface receptors (8, 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 terminus (10). Once attached to the membrane, Rac2 is able to bind to flavocytochrome b\textsubscript{558} and forms a binding partner for p67\textsubscript{phox} (10). The cytosolic proteins p47\textsubscript{phox} and p67\textsubscript{phox} can form a complex with another protein, p40\textsubscript{phox}, in the cytosol of the neutrophil (12). During activation, phosphorylation of p47\textsubscript{phox} at crucial serine residues leads to conformational changes in this protein, allowing the formation of the p47\textsubscript{phox}-p67\textsubscript{phox}-p40\textsubscript{phox} complex as well (12, 13). The outcome of these conformational changes is an increase in the capacity of these proteins to interact with the membrane, with Rac2, and with flavocytochrome b\textsubscript{558} three interactions that are crucial for translocation and subsequent activation of gp91\textsubscript{phox} (9, 10, 13, 14).

To study the translocation of p67\textsubscript{phox} and Rac2 during phagocytosis, enhanced GFP; FRAP, fluorescence recovery after photobleaching; CGD, chronic granulomatous disease.

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The abbreviations used are: ROS, reactive oxygen species; STZ, serum-treated zymosan; GFP, green fluorescent protein; EGFP, enhanced GFP; FRAP, fluorescence recovery after photobleaching; CGD, chronic granulomatous disease.

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Component p67

SERUM-TREATED ZYMOSAN; GFP, GREEN FLUORESCENT PROTEIN; EGFP, ENHANCED GFP; FRAP, FLUORESCENCE RECOVERY AFTER PHOTobleaching; CGD, CHRONIC GRANULOMATOUS DISEASE

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ocytosis 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) (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 5α-α cells were cultured in Iscove’s modified Dulbecco’s medium (In Vitrogen) supplemented with 10% fetal calf serum, penicillin (200 $\mu$g/ml), streptomycin (200 $\mu$g/ml), and L-glutamine (4 mM) in a 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, ApaI) to facilitate further cloning into the pEGFP vector (Clontech). The primers used were (5’ to 3’): gatcggaattccctaatcatgtccctggtggagg (sense) and (5’ to 3’): gatcctcgagttcaggccatcaagtgtgtg (antisense). The PCR reaction contained PWO polymerase (Roche Applied Science), 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 Qiagen PCR purification columns (Qiagen, Valencia, CA). The PCR product and the destination vector pEGFP were then digested with EcoRI and ApaI for 1.5 h 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 Qiagen columns (Qiagen). Fragments were ligated with the FastLink ligase kit (Epicerent Technologies, Madison, WI) and subsequently transformed into competent DH5α cells. Plasmids were examined for correct inserts by restriction digests and sequenced for detection of unwanted mutations. Analogously, 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’): gattcgcgttggaatcatgtccctggtggagg (sense) and (5’ to 3’): gattcgcgttggaatcatgtccctggtggagg (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 p67$^{phox}$, p47$^{phox}$, and gp91$^{phox}$ (16) were transiently transfected by electroporation (0.25 kV, capacitance 960 microfarads, 0 ohms of resistance) on a Bio-Rad Biopulser with wild-type p67$^{phox}$, p67GFP, or GFP-Rac2. Cells were grown for 48 h before analysis. GFP expression was assayed by flow cytometry on a BD Biosciences FacsStar.

**Hydrogen Peroxide Generation by K562 Cells—**Hydrogen peroxide production of K562 cells after phorbol myristate acetate (Sigma) activation was measured by the Amplex Red Assay (Molecular Probes, Eugene, OR) measured on a PerkinElmer Life Sciences plate reader.

Western Blotting of K562 Cells—For immunodetection, 10$^5$ cells were boiled in SDS sample buffer (125 mM$\times$1 liter Tris, pH 6.8, 20% w/v SDS, and 12.5% [v/v] $\beta$-mercaptoethanol) for 5 min and loaded on a 12.5% polyacrylamide gel, according to Laemmli (35), in a gel apparatus (Moldaenke). Western blotting was performed on a Trans-Blot cell, Bio-Rad) according to the manufacturer’s recommendations.

For the detection of p67GFP and GFP-Rac2, a polyclonal antibody against GFP (Clontech, catalog number 6372-1) 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 Biosciences).

**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 (mam-ampho) by calcium phosphate transfection (In Vitrogen). After selection of transfected cells by puromycin (1 $\mu$g/ml) (In Vitrogen), virus was harvested and used for retroviral transduction of PLB-985 and PLB-985 X-CGD cells with 10 $\mu$g/ml of N-1-(2,3-dioleyloxypropyl)-N,N,N-trimethylammonium chloride (Roche Applied Science). Transduced cells were sorted on a FacsStar (BD Biosciences) cell sorter.

**Confocal Imaging of PLB-985 Cells—**For in vivo imaging of p67GFP and GFP-Rac2 fusion proteins, 500 $\mu$l of a suspension of 2 $\times$ 10$^5$ PLB-985 cells transduced with these constructs were allowed to adhere to glass coverslips in complete Iscove’s modified Dulbecco’s medium at 37°C in a heat stage. 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 analyzed with LSM 5 software (Zeiss, Göttingen, Germany).

**Fluorescence Recovery after Photobleaching (FRAP)—**PLB-985 cells were allowed to adhere to glass coverslips in complete Iscove’s modified Dulbecco’s medium at 37°C in a heat stage. Cells were allowed to adhere for 5 min before STZ was added. Rectangles were bleached with the full intensity of a 488-nm laser beam, and 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$^{phox}$, p67$^{phox}$, and gp91$^{phox}$ were transfected with the p67GFP construct, wild-type p67$^{phox}$ or GFP. The superoxide-producing capacity of the cells transfected with the p67GFP construct was compared with K562 cells transfected with wild-type p67$^{phox}$ and with the GFP-transfected K562 cells that express all NADPH oxidase components except p67$^{phox}$. Upon phorbol myristate acetate stimulation, K562 cells transfected with wild-type p67$^{phox}$ were able to produce hydrogen peroxide, in contrast to GFP-transfected cells, which showed no NADPH oxidase activity (Table I). The K562 cells expressing the p67GFP construct showed similar superoxide-producing capacities as the wild-type p67$^{phox}$-transfected cells (Table I), proving that this construct is able to fulfill its role in the activation of the NADPH oxidase. To ensure that GFP was not cleaved from the C terminus of p67$^{phox}$, 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$^{phox}$-transfected K562 cells were taken. The fusion protein was detected after staining with anti-GFP at a molecular mass of ～95 kDa, in good agreement with its predicted molecular mass (94 kDa) (Fig. 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 (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$^{phox}$, p67$^{phox}$, and gp91$^{phox}$ (Table I). Furthermore, the GFP-
Rac2 fusion protein was detected at the expected molecular mass of 48 kDa by Western blotting (Fig. 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 gp91phox 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 (Fig. 2e). 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 (Fig. 2, a and b). In contrast, p67GFP was lost from the phagosome in the PLB-985 X-CGD cells after complete uptake of the STZ particles (Fig. 2, c and d). An identical pattern was observed for the localization of GFP-Rac2 (Fig. 2, e–h). Like p67GFP, this fusion protein is also located at the phagosome during and after complete internalization of the particle by PLB-985 cells, but loss of the protein from the phagosome was observed after internalization 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 b558 as an essential factor for correct translocation of p47phox, p67phox, and Rac2 (18–20). Furthermore, in PLB-985 X-CGD cells, the translocation of p47phox, p67phox, 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.

Photobleaching of p67GFP and GFP-Rac2 during NADPH Oxidase Activation—To investigate the turnover of p67phox and Rac2 on the phagosomal membrane, 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.

PLB-985 X-CGD cells were then fixed. Distribution of p67GFP was analyzed by confocal microscopy. Arrows indicate an STZ particle. Wild-type PLB-985 cells showed p67GFP staining on nascent and progressed phagosomes (a and b). In contrast, PLB-985 X-CGD cells lost staining of the p67GFP fusion protein as the phagosome progressed to more mature stages (c and d). GFP-Rac2 expressed in wild-type PLB-985 cells (e) or PLB-985 X-CGD cells (f) showed similar localization as p67GFP.
after the completion of phagocytosis. At the phagosome, the fluorescence of p67GFP at the site of photobleaching was rapidly restored, with a recovery time \( t_{1/2} \) 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, thus preventing a reliable estimation of the \( t_{1/2} \) for this protein (Fig. 3a). The translocation of GFP-Rac2 was also investigated by FRAP (Fig. 3b). This protein also displayed rapid recovery at the site of bleaching, again with a \( t_{1/2} \) that was lower than 700 ms. 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 (22, 23). F-actin is surrounding the particle during internalization, after which actin is lost again from the phagosome (24). Depolymerization of the actin cytoskeleton by actin-modifying compounds inhibits phagocytosis by preventing the internalization but not the binding of opsonized particles (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 (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 (28). Furthermore, p47\(_{phox}\) has been shown to bind directly to actin, and p47\(_{phox}\) and p67\(_{phox}\) have been shown to accumulate on nascent phagosomes together with F-actin (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 the 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 the 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 (Fig. 4, a–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 the translocation of p67GFP and GFP-Rac2 to phagosomes proved to be a continuous process, as concluded from the FRAP experiments, the question was raised of 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 the translocation of p67GFP and GFP-Rac2 during and after phagocytosis, cells were allowed to take up STZ for 5 min before cytochalasin B was added. Typically, the 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 with 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 (Fig. 5, a and b). Like untreated cells, cytochalasin B-treated cells showed a rapid recovery of fluorescence at the bleached spots of the phagosome (Figs. 5b and 6). In all cases, the \( t_{1/2} \) was too small to be reliably detected by confocal laser scanning microscopy. Furthermore, the use of actin-modifying agents allowed the bleaching of phagocytic cups because uptake of STZ particles was arrested after the 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).

**DISCUSSION**

The NADPH oxidase of phagocytic leukocytes consists of the membrane-bound flavocytochrome \( b_{558} \) and three essential cy-

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**Fig. 3.** FRAP of p67GFP and GFP-Rac2 in PLB-985 cells during phagocytosis. 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).

**Fig. 4.** Effect of pretreatment with cytochalasin B on p67GFP and GFP-Rac2 translocation. Untreated cells showed the translocation of p67GFP after binding of STZ particles (a). Arrows indicate STZ particle. In contrast, PLB-985 cells pretreated with 10 mM cytochalasin B for 10 min before the 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). Pretreatment of the cells with 10 mM cytochalasin B prohibited the translocation of GFP-Rac2 after attachment of STZ particles (d).
tosolic components, p47phox, p67phox, and Rac2. In this report, the localization of p67phox 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 p67phox 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 p47phox, but the p47GFP fusion protein was not able to restore superoxide production in K562 cells lacking p47phox (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 gp91phox, which is consistent with experiments performed previously with these cell lines as well as with granulocytes from CGD patients (18, 30).

During phagosome formation, the translocation of the cytosolic 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 cytosolic components p47phox, p67phox, and Rac2 may only transiently associate with flavocytochrome b_{558}. Since the 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 turnover 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} (31). Furthermore, Akard et al. (32) 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. 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, which 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 the translocation of p67phox and Rac2 during phagocytosis was investigated by the use of an actin-modifying agent. The effect of cytochalasin B, a potent actin depolymerizing agent, on the translocation behavior of p67GFP and GFP-Rac2 was tested. Pretreatment 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 the translocation of p67GFP and GFP-Rac2. It remains unclear why disruption of the actin cytoskeleton disturbs the primary translocation of the cytosolic factors. Rac2, p40phox, and p47phox have been shown to be able

**Fig. 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 µM 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).

**Fig. 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 s, 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.
to interact with the membrane through their prenylated C terminus or PX domains, respectively (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, the 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, the 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 (25–27). However, most of these experiments were performed in a cell-free system and did not address the 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 the addition of STZ. Among these agents were LY290042 (PI3-kinase inhibitor), SB20358 (an inhibitor of p38MAPK), and U0126 (an 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 the translocation of p67phox 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 p40phox and p47phox 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 p67phox 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 p67phox and Rac2 to the phagosomal membrane.

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