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Transducible form of p47^{phox} and p67^{phox} compensate for defective NADPH oxidase activity in neutrophils of patients with chronic granulomatous disease

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ABSTRACT

Protein delivery to primary cells by protein transduction domain (PTD) serves as a novel measure for manipulation of the cells for biological study and for the treatment of various human conditions. Although the method has been employed to modulate cellular function *in vitro*, only limited reports are available on its application in the replacement of deficient signaling molecules into primary cells. We examined the potential of recombinant proteins to compensate for defective cytosolic components of the NADPH oxidase complex in chronic granulomatous disease (CGD) neutrophils in both p47^{phox} and p67^{phox} deficiency. The p47^{phox} or p67^{phox} protein linked to Hph-1 PTD was effectively expressed in soluble form and transduced into human neutrophils efficiently without eliciting unwanted signal transduction or apoptosis. The delivered protein was stable for more than 24 h, expressed in the cytoplasm, translocated to the membrane fraction upon activation, and, most importantly able to restored reactive oxygen species (ROS) production. Although research on human primary neutrophils using the protein delivery system is still limited, our data show that the protein transduction approach for neutrophils may be applicable to the control of local infections in CGD patients by direct delivery of the protein product.

1. Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency that affects phagocytes of the innate immune system and is characterized by recurrent life-threatening bacterial and fungal infections. The disease is caused by the lack of a component of NADPH oxidase complex [1]. NADPH oxidase is a multicomponent enzyme that is critical in non-mitochondrial ROS production, and is composed of a flavocytochrome b558 (gp91^{phox} and p22^{phox}), cytosolic components (p40^{phox}, p47^{phox}, and p67^{phox}) and a small GTP-binding protein (Rac1 or Rac2) [2]. About 60% of CGD cases are caused by mutations in the gene encoding gp91^{phox} located on X chromosome. Mutation in *NCF1* (encoding p47^{phox}) causes the most common autosomal recessive form of CGD accounting for approximately 20% of all CGD cases. Mutation in *NCF2* (encoding p67^{phox}) accounts for about 5% of all CGD cases [1,3]. Hematopoietic cell transplantation is currently the only proven curative therapy, but is often associated with transplant-related mortality [1,4]. There has been no effective therapeutic method to modulate neutrophil function or to reconstitute the functional defects in CGD neutrophils.

The intracellular delivery of proteins or peptides, has been difficult to achieve until recently, primarily due to plasma membrane barrier restrictions on the uptake of macromolecules. Cell penetrating peptide or protein transduction domain (PTD) is a short peptide of generally fewer than 30 amino acids that can cross biological membranes in a receptor- and cell-cycle-independent manner [5–7]. PTD is especially useful for delivery of large molecules into transfection-resistant cells, and can be incorporated into virtually any types of cells [5,8,9].

The protein transduction technique has most commonly been employed for modulation of specific protein–protein interactions with target transcription factors, signal transduction proteins, and cell cycle mediators [10]. Specific proteins and peptides for therapeutic targeting of oncogenes have been developed and tested *in vitro* and with *in vivo* animal models [10,11]. The protein transduction approach has also been used for delivery of active enzymes or other functional molecules in neurodegenerative disorders and metabolic diseases. PTD enzyme replacement *in vitro* has been successfully demonstrated in many previous publications [12–14]; however, their potential advantage as a method for intracellular replacement therapy *in vivo* is still largely unknown.

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Replacement of functional signaling molecules by PTD techniques in cells is more technically demanding than delivery of protein–protein interaction modulators or active enzymes. This is because, in PTD, the delivered protein should be expressed at a physiological level, targeted to the specific cellular location, associated with other molecules, modified and translocated upon stimulation, and biologically active as long as the endogenous molecule.

The objective of this study was to compensate neutrophil dysfunction in CGD lacking a cytosolic component of the NADPH oxidase complex by the protein delivery system using Hph-1, an 11-amino acid long unique peptide of human origin, as PTD [15,16]. To achieve this, the transduced protein should function as its endogenous counterpart inside the cells. Activation of the complex is tightly controlled by plasma membrane targeting and/or phosphorylation of the cytosolic components [2,17]. Upon priming signal that activates PI3K, Rac2 released from GDP-dissociation inhibitor translocates to the membrane. During activation, p47 is phosphorylated on multiple Serines by PKC, leading to the translocation of the p47/p67/(p40) complex to the membrane [2,17,18]. The cytosolic components should locate in the cytoplasm in resting state, receive modification upon activation, associate with other molecules, and translocate to the membrane.

We also investigated possibility of the protein transduction to activate, or induce apoptosis in, neutrophils. Neutrophils have a short lifespan in the periphery and *ex vivo*, and the cells are quickly responsive and sensitive to the external stimuli, all factors that render cell manipulation even more difficult. Neutrophils sense microbes through various receptors, engulf foreign bodies, and are destined to undergo apoptosis after production of reactive oxygen species (ROS) and releasing neutrophil extracellular traps [19,20]. It has been postulated that PTD-mediated delivery of macromolecules does not elicit the innate immune response or cytotoxicity [9,10,13], but, to our knowledge, the cellular reactions elicited by protein delivery has not been formally addressed in human neutrophils.

We show here evidence that PTD-based protein delivery does not elicit non-specific activation or apoptosis of human neutrophils. We show that transduced recombinant p47 or p67 protein linked to Hph-1 distributes to a physiological location (i.e., moving to the plasma membrane) and restores normal ROS production in CGD neutrophils deficient in p47 or p67, respectively.

2. Results

2.1. Transduction efficacy of recombinant protein into human neutrophils and its effect on neutrophil activation and apoptosis

We first generated a construct for Hph-1-EGFP as a control protein for assessing expression kinetics, activation, and cellular apoptosis in neutrophils following protein transduction (Fig. 2A). The EGFP recombinant was expressed in bacteria, purified, treated with polymyxin B, and incubated with 1×10^6 purified human neutrophils with various concentrations and for various time periods. The recombinant was similarly incubated with human activated T-cells (CD3+ ≥95%) and with human B-cell line (i.e., Daudi cells).

The kinetics study monitored by flow cytometer showed the expression level at 10 min was higher in neutrophils compared to that in activated T-cells and Daudi cells. In neutrophils, the expression level reached a maximum by 30 min; and ⩾95% of the recombinant protein was expressed, while in activated T-cells the level peaked at 60 min (Fig. 3A).

The transduced protein was easily detectable in the cytoplasm at 1 μM by confocal fluorescence microscopy (CFM) as well as by flow cytometer, and the expression was increased in a dose dependent fashion (Fig. 3B and C). The dose–response was similar to that in Daudi cells; but significantly more EGFP protein was detected in neutrophils than in activated T-cells. The expression was detected at least up until 24 h post-Hph-1-EGFP transduction, suggesting that no major biodegradation of incorporated protein occurred (Fig. 3D).

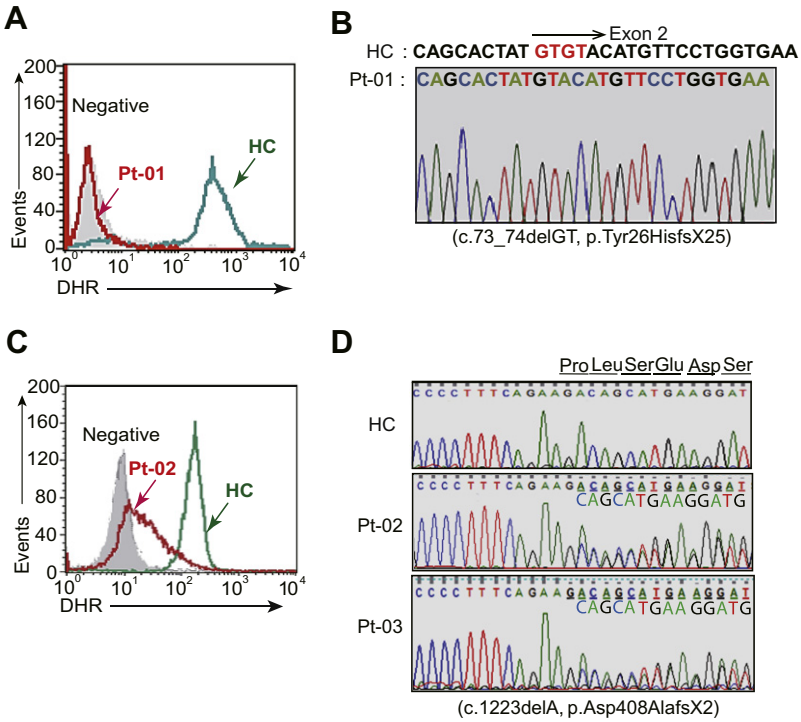
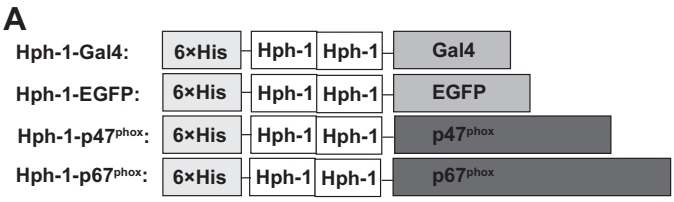


Fig. 1. Diagnosis of CGD in patients 1, 2, and 3 by assessment of ROS production and by sequencing analysis. (A and C) A representative FACS histogram for assessment of ROS production, measured by DHR123 fluorescence in purified neutrophils from healthy control (HC), p47-deficiency patient (Pt-01) (A) and from p67 deficiency (Pt-02) (C). (B and D) A result of sequencing analysis of *NCF1* in Pt-01 (B) and *NCF2* in Pt-02 and Pt-03 (D).



B

Primer	Sequence	Restriction enzyme
Gal4	5'-*GAATTC*AAGCTACTGTCTTCTATC-3'	*EcoR I*
	5'-*AAGCTT*CGGCGATACAGTCAA-3'	*Hind III*
EGFP	5'-*GAATTC*GTGAGCAAGGGCGAGGAG-3'	*EcoR I*
	5'-*AAGCTT*TCCTTGTACAGCTCGTCCATG-3'	*Hind III*
p47phox	5'-*GAATTC*GGGGACACCTTCATCCGTC-3'	*EcoR I*
	5'-*AAGCTT*GACGGCAGACGCCAGCTTCCGC-3'	*Hind III*
p67phox	5'-*GAATTC*TCCCTGGTGGAGGCCATCAGCCT-3'	*EcoR I*
	5'-*AAGCTT*CTAGACTTCTCTCCGAGTGCTTTCT-3'	*Hind III*

Fig. 2. Schematic diagram of Hph-1-recombinant constructs. (A) Schematic diagram of Hph-1-Gal4, Hph-1-EGFP, Hph-1-p47^{phox}, and Hph-1-p67^{phox}. (B) Primer sequences for construction of the indicated Hph-1-recombinant protein.

We next examined whether the transduction of foreign protein *per se* activates neutrophils or potentially elicits cellular damage leading to augmented or earlier apoptosis. Fig. 3D shows that the proportion of apoptotic cells does not increase in neutrophils transduced with Hph-1-EGFP compared to untreated neutrophils. Induction of Hph-1-EGFP did not induce significant activation of tyrosine kinases, activation of Akt, or phosphorylation of the intracellular components of the NADPH oxidase complex (Fig. 3E). Similarly, ROS production was not observed upon transduction with Hph-1-EGFP protein in addition to fMLP in control neutrophils. The similar results were obtained when neutrophils were treated with Hph-1-Gal4 (data not shown).

2.2. Hph-1-p47^{phox} and Hph-1-p67^{phox} compensate for defective NADPH oxidase activity in neutrophils of autosomal recessive CGD patients

We hypothesized that restoring intracellular p47 or p67 by protein delivery would correct defective NADPH activity, if the transduced protein were functional. To test this, we designed constructs

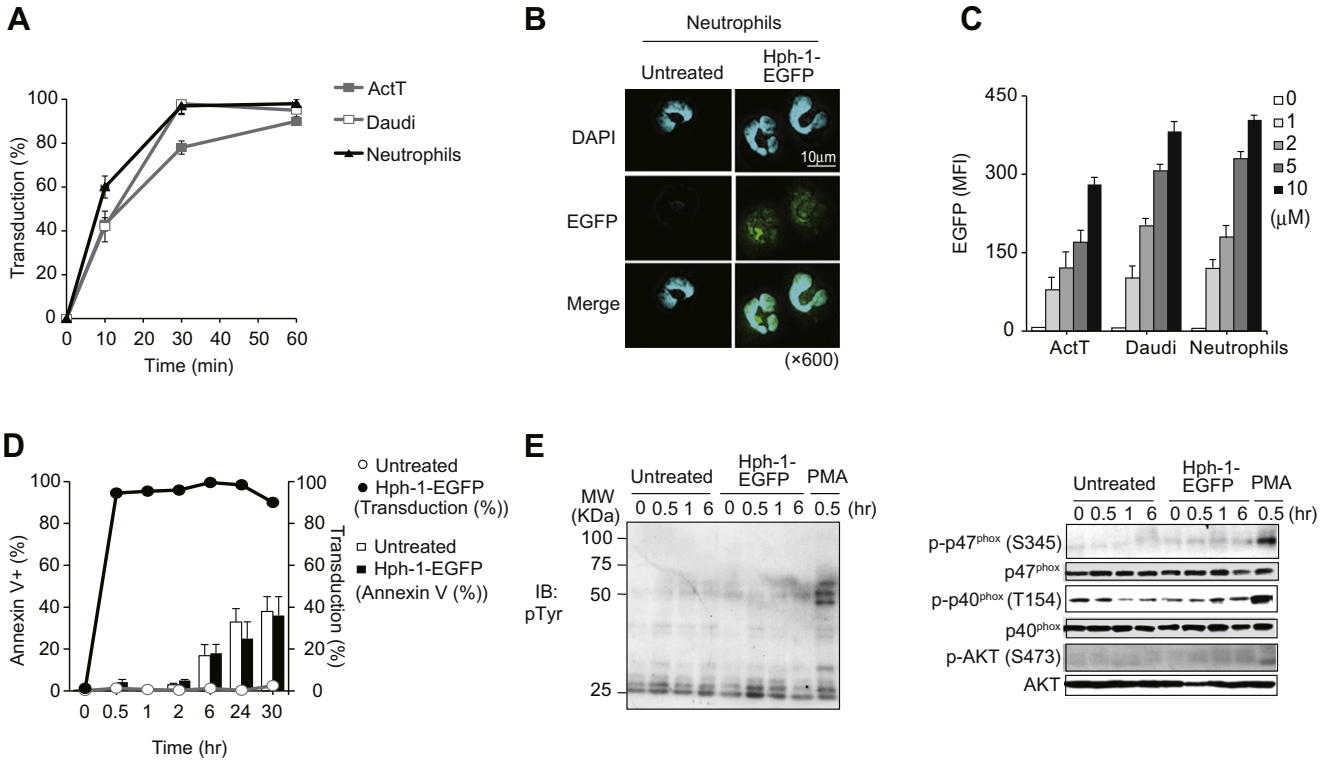


Fig. 3. Time-dependent and concentration-dependent transduction of Hph-1-EGFP in human neutrophils, and the effect of transduction on cellular activation and apoptosis. (A) Time dependent transduction kinetics of Hph-1-EGFP in human neutrophils, activated T-cells (ActT), and Daudi cells. (B) CFM analysis of transduced protein. (C) Concentration dependent intracellular delivery of EGFP in human neutrophils, activated T-cells (ActT), and Daudi cells ($n = 3$). (D) Percentage of neutrophil apoptosis and EGFP-positivity after EGFP protein delivery. Summary of three independent experiments is shown. (E) Tyrosine phosphorylation of neutrophil proteins, activation of AKT, or phosphorylation of cytosolic factors of NADPH oxidase complex after transduction of Hph-1-EGFP. One representative Western blot out of three independent experiments is shown. Mean + SD is indicated in (A, C, and D).

for Hph-1-p47^{phox} and Hph-1-p67^{phox} (Fig. 2A) and investigated their capacity to confer the ability to produce ROS in CGD neutrophils either lacking in p47 or p67. Hph-1-p47^{phox} and Hph-1-p67^{phox} were expressed abundantly and recovered insoluble fraction in BL21DE3 *Escherichia coli* strain at 37 °C incubation (Fig. 4A).

To determine the efficacy of transduction, the p47 protein was incubated with 10^6 purified neutrophils from p47-deficiency at various concentrations, and their expression was checked by Western blotting (Fig. 4B). The expression level of p47 was equivalent to that in control neutrophils when incubated at 1–5 μM for 30 min.

To confirm that Hph-1 induced p47 into the cells, we carried out CFM and Western blotting. CFM visualized the presence of p47 in the cytoplasm prior to stimulation, and the colocalization of p47 and gp91 at the membrane after PMA treatment in >95% of the cells (Fig. 4C). We also prepared cytoplasmic and membrane fractions from Hph-1-p47^{phox} transduced neutrophils and carried out anti-p47 Western blotting. The analysis further confirmed that the incorporated p47 protein, and endogenous p67, were located in the cytoplasm, but not in the membrane (Fig. 4C).

We then asked whether the transduced Hph-1-p47^{phox} was functional by measuring PMA-induced ROS production in the neutrophils from Pt-01. The Hph-1-p47^{phox} delivery restored the capacity to generate ROS in p47-deficient CGD neutrophils, while the additional expression did not result in augmented ROS release in control neutrophils (Fig. 4D). Transduction of the p47 recombinant did not enhance apoptosis as examined by Annexin V staining until 24 h post protein delivery (Fig. 4E). The delivery did not induce cellular activation in neutrophils detected by anti-phosphotyrosine blot (Fig. 4F). In addition, the transduced p47 was still detectable at 24 h as observed in Hph-1-EGFP transduction.

Five-times more expression of p67 was observed in p67-deficient neutrophils compared to control neutrophils when Hph-1-p67^{phox} was incubated at 5 μM for 30 min (Fig. 4G). The p67 expression was observed in >90% of the transduced cells by enumeration under CFM. DHR123 assay and luminol assay demonstrated that the intracellular delivery of p67 protein via Hph-1 restored the capacity to generate ROS in response to PMA in p67-deficient CGD neutrophils (Fig. 4H and I). ROS production in the transduced cells was slightly reduced compared to normal neutrophils, when the expression level of the recombinant was adjusted to the level of endogenous p67 (by incubation at 1 μM), but the difference was not statistically significant (Fig. 4H and I). Neither apoptosis nor ROS production was observed in control neutrophils transduced with Hph-1-p67^{phox}.

3. Discussion

In this paper, we have demonstrated that Hph-1-based protein delivery restores neutrophil ROS production in p47^{phox}-deficient and p67^{phox}-deficient CGD patients. The Hph-1-p47^{phox} and Hph-1-p67^{phox} was recovered in soluble fraction in large quantity in bacteria, and the transduction efficacy to neutrophils was at least more than 80%. The transported protein was localized in the cytosol of neutrophils and was translocated, upon stimulation, to the membrane associating with flavocytochrome b558 to cause the activation of NADPH oxidase. The cellular concentration of the recombinant was observed in a concentration-dependent manner, and thus was adjustable to the target level of choice. The PTD-mediated protein delivery *per se* did not trigger neutrophil activation or affect neutrophil cell survival.

To date, many groups in wide arenas of clinical and basic biology are working toward PTD-based delivery of therapeutic molecules. The diseases being treated *in vitro* or *in vivo* animal systems range from cancer, ischemia, neurodegenerative disease, and enzyme deficiency [8,11,14]. Despite the notable successes and high expectations, the use of PTD to deliver proteins has yet to become common place in cell biology, especially in fields using primary cells. This can be ascribed to inefficient protein expression, insolubility of protein, and biodegradation in transduced cells. In particular, research on primary neutrophils using protein delivery system has been limited; and most of the existing research employed peptide fragments for functional modulation of neutrophils [21,22].

Correction of defective molecule in neutrophils has not yet been attempted with PTD-based approach, much less for the replacement of cytoplasmic protein defective in CGD phagocytes. A pioneering work by Polack et al. has shown that the *Pseudomonas aeruginosa* strain harboring a plasmid encoding ExoS-N-terminal p67 fusion protein, CHA-pBP31, can infect an EBV-transformed cell line from p67-deficient CGD [23]. CHA-pBP31 was able to reconstitute the NADPH oxidase activity, to approximately 40% of normal at MOIs of 5 or 10. The system, however, is labor-intensive, has limits in deliverable molecular size, and is toxic at higher MOI. However, the intracellular delivery of p67 protein can now be achieved more easily, safely, effectively, and in more controlled manner with our PTD-based system.

The only curative therapies available for CGD are hematopoietic cell transplantation and gene therapy, both of which are associated with therapy-related toxicity and adverse effects [1,4]. In addition, infection control is critically important for the success of these curative therapies [1,4]. The true potential of the Hph-1-p47^{phox} and Hph-1-p67^{phox} as a therapeutic measure to correct deficient ROS production is yet to be tested in animal models. This protein delivery can be used, however, in local control of infection of the CGD patients, for example, by using as an ointment, or by applying the protein directly to the site of infection.

PTD-based enzyme replacement therapy has been proven effective in only limited cases or primary immunodeficiency. Purine nucleotide phosphorylase (PNP) is an intracellular enzyme critical for purine degradation, and PNP defects result in severe T-cell immunodeficiency. One study has reported that PNP fused to TAT rapidly enters PNP-deficient lymphocytes and increases intracellular enzyme activity for 96 h [12]. The same group has demonstrated in PNP$^{-/-}$ mice that TAT induced rapid and efficient delivery of active PNP into many tissues, including the brain, leading to correction of metabolic diseases and immune defects [13].

The protein transduction system thus can be applied for the correction of other immuno deficiencies lacking the intracellular enzyme or cytoplasmic signal molecule, either in part or entirely. The examples include severe congenital neutropenia due to mutation in *HAX1*, *ELA2*, or *MPO* and severe combined immunodeficiency caused by mutation in *JAK3*, *LCK*, *DCLRE1C*, *NHEJ1*, and *LIGIV*. Although the efficacy and safety should be examined in detail with an *in vivo* animal model system, clinical applications of these approaches would become a useful therapeutic option, until the time the patients receive curative therapy (e.g., hematopoietic cell transplantation or gene therapy). The unique ability of PTD will facilitate the design of therapeutic proteins that are defective in primary immunodeficiency.

4. Materials and methods

4.1. Case presentation

Pt-01 is 31-year-old female with recurrent skin infection, otitis, and genital candidiasis. She developed CGD colitis at the age of 19. PMA-driven ROS production of neutrophils as assessed by DHR123 staining is minimal (Fig. 1A). Sequencing analysis of the four NADPH oxidase genes (*CYBA*, *CYBB*, *NCF1*, and *NCF2*) revealed the homozygous c.73_74delGT mutation in *NCF1* (Fig. 1B).

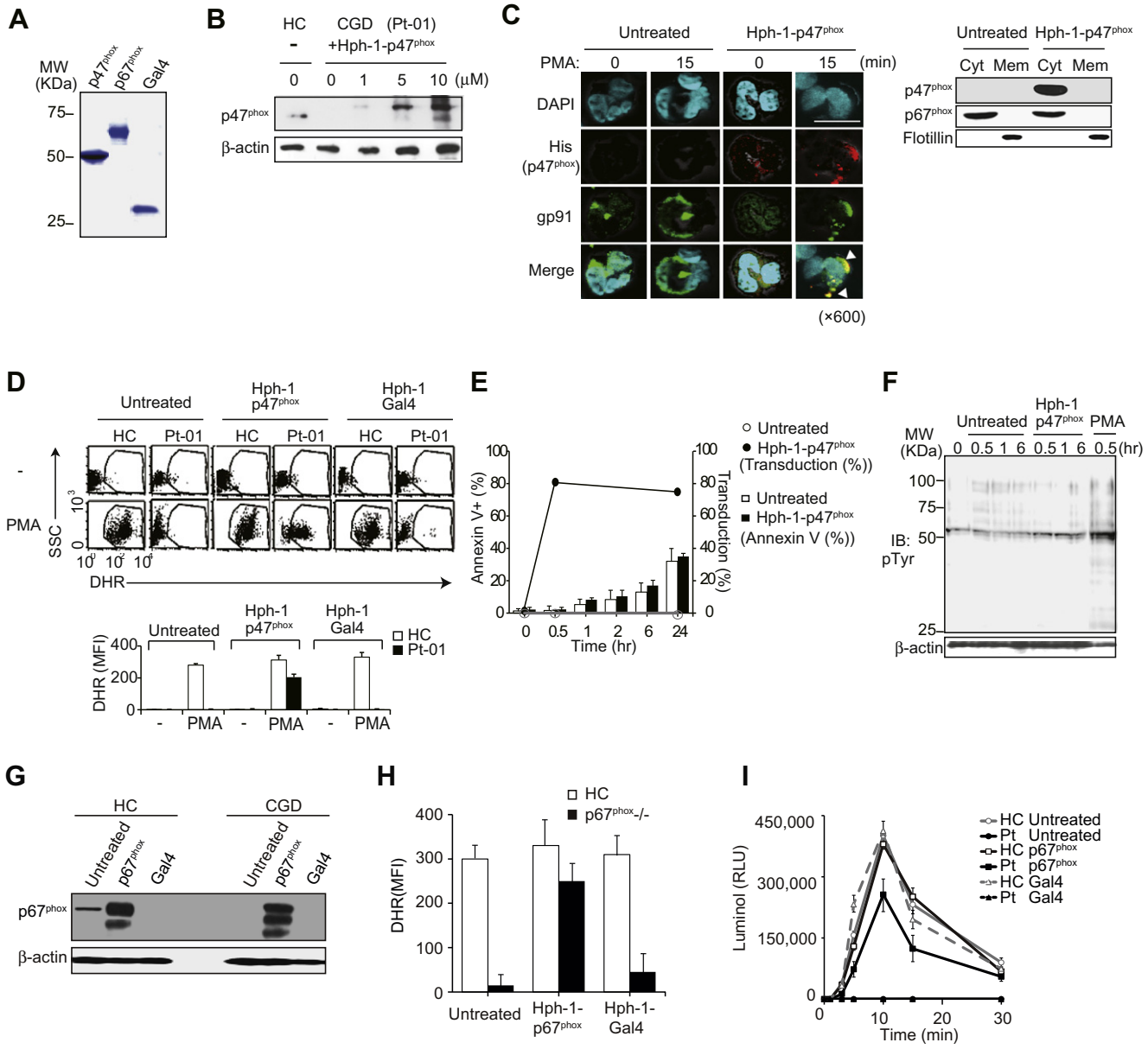


Fig. 4. The delivery of a cytosolic factor of NADPH oxidase complex via Hph-1 PTD results in the expression in the cytoplasm, co-localization with gp91 in the membrane upon stimulation, and restored ROS production in neutrophils from p47- and p67-deficiency. (A) Coomassie Brilliant Blue staining of the purified Hph-1-p47^{phox}, -p67^{phox}, and -Gal4 protein. (B) Expression of p47 in control neutrophils and in p47 deficiency transduced with indicated concentration of Hph-1-p47^{phox}. A representative data out of three independent experiments is shown. Actin is shown as a loading control. (C). Intracellular localization of transduced p47 assessed by CFM (left panel) and by WB (right panel). Flotillin was used as a membrane marker. (D) ROS production assessed by DHR123 staining in control neutrophils and in p47-deficient neutrophils that were transduced with Hph-1-p47^{phox} or with Hph-1-Gal4, and were stimulated as indicated. DMSO was used as a control reagent. Summary of three independent experiments is shown in the lower panel. (E) Percentage of apoptotic cells and p47-expressing cells in p47-negative CGD neutrophils incubated with or without Hph-1-p47^{phox}. Apoptosis was assessed by Annexin V staining, and percentage of p47-positive cells was enumerated by counting the cells with cytoplasmic p47 expression under CFM. (F) Anti-phosphotyrosine blot following transduction of Hph-1-p47^{phox} in p47-deficient neutrophils. (G) Expression of p67 in neutrophils from HC and from p67-deficiency with or without transduction of indicated recombinant protein. One representative data out of three independent experiments for Pt-02 and Pt-03 is shown. (H) Mean DHR123 fluorescence in control neutrophils and in p67-deficient neutrophils, transduced with or without the indicated recombinant. Combined results from three independent experiments for Pt-02 and Pt-03 are shown. (I) Time course of PMA-driven H_2O_2 production, measured by a luminol assay, in control neutrophils and p67-deficiency neutrophils with or without Hph-1-p67^{phox} transduction. One representative data is shown. The experiment was repeated three times in three HCs and p67 deficient patient (Pt-02). HC: healthy control. Mean + SD is shown in D, E, H, and I.

Pt-02 is 8-year-old boy who developed perianal abscess and cervical lymphoadenopathy at 1-year old. Bacterial culture of the abscess revealed the presence of *Serratia marcescens* and Group A *Streptococci*. The DHR123 assay revealed positive but significantly attenuated ROS production (Fig. 1C). Sequencing of the four NADPH oxidase genes revealed a homozygous c1233delA mutation in *NCF2* (Fig. 1D).

Pt-03 is currently 2-year-old girl and is a younger sister of Pt-02. The patient harbored the same mutation in *NCF2* detected in Pt-02. Pt-03 was well until 2-months old when she developed diarrhea of unknown origin lasting for >8 weeks. Pt-02 and Pt-03 have been on Sulfamethoxazole-Trimethoprim and Itraconazole after diagnosis of CGD and have been well without major infection. However, perianal abscess frequently recurs in Pt-02.

Written informed consent was obtained from all subjects (or their parents). The study protocol was approved by the ethics committee of the Faculty of Medicine, Tokyo Medical and Dental University.

4.2. Reagents

Anti-phosphotyrosine mAb (4G10) and rabbit polyclonal antibody to gp91^{phox} were from Upstate. Mouse mAb to flotillin-1, p67^{phox}, and isotype-matched FITC-mouse IgG were from BD Pharmingen. Mouse mAbs to phospho-AKT, to p47^{phox} were from Rockland Immunochemicals, and from Santa-Cruz, respectively. MAbs to 6x His and to phospho-p40^{phox} were obtained from Cell signaling technology. Antibody directed against phosphor-Ser345 was generated in rabbits by injection with ovalbumin conjugated to the phosphopeptide sequence of p47^{phox} (QARPGPQS [phospho]PGSPLEEE). PMA, dihydrorhodamine 123 (DHR123), DAPI, and luminol were from Sigma–Aldrich.

4.3. Sequencing of NCF1 and NCF2

Sequencing was performed for all exons and exon–intron boundaries of *NCF1* and *NCF2* as previously described [24] using ABI310 automated genetic analyzer using *NCF1* and *NCF2* specific primers.

4.4. Isolation of peripheral blood neutrophils

Neutrophils were purified using a standard technique from heparinized peripheral blood using MonoPoly mixture (Flow Laboratories, McLean, VA). The neutrophil-enriched fraction was further purified to >97% by immunomagnetic negative selection (StemCell Technologies). All procedures were carried out under sterile and endotoxin-free conditions.

Subcellular fractionation of neutrophils was carried out according to standard technique previously described. Flotillin was used as a membrane marker.

4.5. Preparation of activated T-cells

Activated T-cells were prepared by incubating peripheral blood mononuclear cells in an OKT3-coated flask in the presence of 350 U/ml IL-2 as previously described [24].

4.6. Measurement of ROS production

Purified neutrophils were loaded with DHR123 at 5 μg/mL for 5 min at 37 °C. Cells were washed, stimulated with PMA (100 ng/ml for 30 min at 37 °C), and ROS production was quantified via flow cytometry (FACSCalibur, Becton Dickinson) by measuring intracellular rhodamine. Alternatively, ROS production was quantified using a standard chemiluminescence method.

4.7. Generation and purification of Hph-1-fusion protein and protein transduction

Hph-1-protein constructs were generated by using the primers shown in Fig. 2B. EGFP, p47^{phox}, and p67^{phox} were amplified from pEGFP-N1 plasmid, NCF-1 cDNA clone (FCC117E05 obtained from TOYOBO), and cDNA from control peripheral lymphocytes, respectively. The amplified fragment and Hph-1 was combined and cloned into pET28b (+) plasmid (Novagen) as previously described. Gal4 construct was described elsewhere.

Protein induction was carried out as previously described [16]. Prepared protein was treated with Detoxi-Gel™ Endotoxin Removing gel (Takara Bio) to eliminate endotoxin.

The cells were incubated with Hph-1 recombinants in PBS at indicated concentrations for indicated time, washed and then were subjected to further analysis.

4.8. Western blotting

Neutrophil lysates were prepared using a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.25 M sucrose, 5 mM EGTA, 5 mM EDTA, 15 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aporotinin, 2.5 mM PMSF, 1.0% NP-40, 0.25% sodium deoxycholate, 10 mM sodium pyrophosphate, 25 mM NaF, 5 mM Na_3VO_4, 25 mM β-glycerophosphate). Western blotting was carried out as described previously [25].

4.9. Immunofluorescence staining

Cytospin preparations of neutrophils were air-dried and fixed for 10 min with paraformaldehyde in PBS, and then permeabilized using acetone at −20 °C for 20 min, washed, and incubated with the indicated antibodies. Nuclei were counterstained with DAPI. The slides were analyzed with a fluorescence microscope (FV10i, Olympus) equipped with Fluoview viewer and review station.

4.10. Statistical analysis

Student's *t*-test was used for statistical analysis.

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