Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 509-516

RECONSTITUTION AND CHARACTERIZATION OF THE HUMAN NEUTROPHIL RESPIRATORY BURST OXIDASE USING RECOMBINANT p47-*phox*, p67-*phox* AND PLASMA MEMBRANE

David J. Uhlinger, K. Leigh Inge, Mary L. Kreck, Shiv Raj Tyagi, Nickolas Neckelmann, and J. David Lambeth

Department of Biochemistry
Emory University School of Medicine, Atlanta, GA 30322

Received May 15, 1992

Summary: Human neutrophil respiratory burst oxidase (NADPH-oxidase) activity can be reconstituted in a cell-free system consisting of plasma membrane, cytosol and an anionic amphiphile [*e.g.*, sodium dodecyl sulfate (SDS) or arachidonate]. Herein, we report reconstitution of oxidase activity using isolated neutrophil plasma membranes together with purified recombinant p47-*phox* and p67-*phox* which had been produced using a baculovirus expression system. Activity required an anionic amphiphile (SDS or arachidonate) and was potentiated by diacylglycerol and GTPγS. Serial washes of the plasma membrane failed to affect its ability to reconstitute activity, indicating that a dissociable membrane component was not present. The K_m for NADPH, 43 μM, was the same as that determined using cytosol in place of recombinant factors. The EC_{50} values for p47-*phox* and p67-*phox* under optimal activation conditions were 220 nM and 80 nM, respectively, indicating a relatively high affinity of these components in an activation complex. Since neither cytosolic component contains a nucleotide binding consensus sequence, these data indicate that the NADPH binding component of the oxidase resides in the plasma membrane.

The neutrophil NADPH-dependent respiratory burst is activated upon phagocytosis of bacteria, and provides an important defense against microbial infection. The burst is initiated by activation of the respiratory burst oxidase, an NADPH-dependent enzyme complex which reduces molecular oxygen to superoxide. The NADPH-oxidase can be activated by anionic amphiphiles (e.g., arachidonate, SDS) in a cell-free system consisting of isolated plasma membrane plus cytosol (1-3). Activity in this setting can be potentiated by GTPγS (4-6) and by diacylglycerols (*e.g.*, dioctanoyl glycerol or diC8) (7) by a protein kinase C/phosphorylation-independent mechanism (6).

The oxidase consists of multiple protein components present in both the cytosol and the plasma membrane. Cytochrome b_{558}, which consists of two subunits (gp91-*phox*, and p22-*phox*), is present in the plasma membrane, and is thought to function as the terminal electron donor to O_2 (8-10). Two additional cytosolic components, p47-*phox* and p67-*phox*, have been identified as oxidase components [based on their absence in variant forms of Chronic Granulomatous Disease (CGD) wherein oxidase activity is absent], cloned, and sequenced

0006-291X/92 $4.00

(11-13). Several *ras*-related guanine nucleotide regulatory proteins ($p21^{rac1}$, $p21^{rac2}$, and/or $p21^{rap1a}$) have also been proposed as oxidase-related components (14-16), and one or more of these is likely to mediate the activating effects of GTPγS. In addition, although an NADPH binding component is a theoretical necessity, such a component has not yet been unambiguously identified. Indirect evidence has yielded contradictory results, localizing this component in either the plasma membrane (17-20) or the cytosol (21,22). In addition, since the oxidase catalyzes the univalent reduction of O_2 by the two electron donor NADPH, it presumably possesses a flavin or other prosthetic group capable of both 1- and 2-electron transfers, and indirect evidence suggests the participation of FAD (23,24). Since the cytosolic components are present in low abundance and since most studies have been carried out using crude cytosol or impure chromatographic fractions, it has been difficult to identify the minimal number of cytosolic components required to reconstitute oxidase activity. In the present studies, we have used recombinant methodology to express large amounts of p47-*phox* and p67-*phox*, and have utilized these highly purified components together with isolated plasma membranes to investigate their role in oxidase activation.

MATERIALS AND METHODS

Isolation of Human Neutrophils and Subcellular Fractions- Human neutrophils were isolated and fractionated into cytosol and plasma membranes as described previously (7).

Superoxide Generation and Incubation Conditions- Superoxide generation was monitored by superoxide dismutase-inhibitable cytochrome *c* reduction using a Molecular Devices Thermomax Kinetic microplate reader as detailed previously (6).

Construction of Recombinant Baculovirus Containing cDNA Encoding p47-*phox* and p67-*phox*- pBluescript cDNA clones encoding the full length human p47-*phox* and p67-*phox* proteins as well as an N-terminal 3 kDa segment of β-galactosidase were the generous gift of Dr. Thomas Leto (NIAID). The cDNAs encoding the open reading frames (excluding the 5' β-galactosidase sequence) were excised by the restriction endonuclease *Eco* RI and ligated into the multiple cloning site of the non-fusion baculovirus expression vector 1393. cDNAs in the correct orientation were amplified and plasmid DNA was purified by standard protocols. AcMNPV DNA (1 μg) was combined with 2 μg of the recombinant 1393 plasmids and cotransfected into Sf9 insect cells using a cationic liposomes method. Viruses that had undergone homologous recombination were identified by visual screening of plaque isolates. Identity was ultimately confirmed by SDS-PAGE and subsequent immunoblotting of the recombinant proteins *versus* normal cytosol, and by reconstitution of activity using p47-*phox*-deficient cytosol from CGD neutrophils or heat-inactivated cytosol from normal neutrophils. The blots were probed with rabbit polyclonal antibodies to a C-terminal peptide of p47-*phox* (25) and those produced against the p67-*phox* B-gal fusion protein (D. Uhlinger, unpublished).

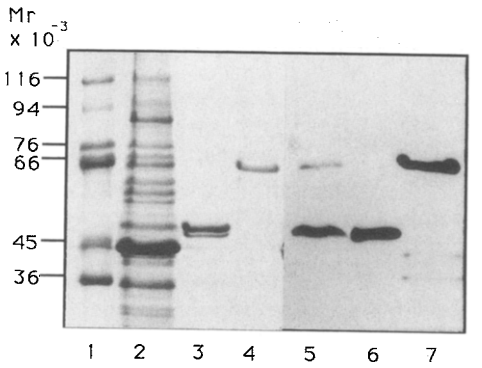

Fig. 1. SDS-polyacrylamide gel electrophoresis and corresponding Western blot of recombinant cytosolic factors. SDS-PAGE was performed as described (7). Lanes 1-4 represent the Coomassie Blue-stained SDS gel, and lanes 5-7 the Western blot. Lane 1 contains molecular weight standards, lane 2 contains 50 μg of neutrophil cytosol, lanes 3 and 4 contain 2 μg each of purified recombinant p47-*phox* and p67-*phox*, respectively. Lanes 5, 6 and 7 are the Western blot corresponding to the SDS-PAGE samples run in lanes 2, 3 and 4. The blot was probed with a combination of rabbit polyclonal antibodies to the two cytosolic oxidase components and developed with horse radish peroxidase-conjugated second antibody.

Purification of Recombinant p47-*phox* and p67-*phox*- The recombinant p47-*phox* and p67-*phox* were purified from infected Sf9 cells by ion exchange methods similar to those described previously (26). Briefly, Sf9 cells were grown to approximately 80% confluence in roller bottles at 27°C. The cells were infected with a multiplicity of infection of approximately 5 and the infection proceeded for three days at which time the cells were harvested and either processed immediately or frozen at -80°C. The cells, resuspended in 10 mM sodium phosphate, pH 7.0, and 50 mM NaCl, were disrupted by nitrogen cavitation in the presence of protease inhibitors as described (7). Unbroken cells and nuclei were removed by centrifugation at 600 x g for 10 min and the resulting supernatants were subjected to centrifugation at 100,000 x g for 1 hour. These 100K x g supernatants were then subjected to ion exchange chromatography. The p47-*phox* was purified by chromatography over CM-Sepharose (Sigma) and the p67-*phox* was purified by chromatography over a Mono-Q cartridge (BioRad) both columns were developed with a linear NaCl gradient and both proteins eluted in the range of 150-250 mM NaCl. Column fractions were assayed initially by Dot-blots and subsequently analyzed by SDS-PAGE and Western blotting to assess purity as shown in Fig. 1.

RESULTS AND DISCUSSION

To test whether the recombinant cytosolic factors were active, superoxide generation was monitored by supplementing dilute cytosol with the purified recombinant proteins in the presence of isolated plasma membrane plus activating agonists (upper panel, Fig. 2). Dilute cytosol was initially included as a source of any additional required oxidase-related factors, and was adjusted to a concentration which gave 10 % of the maximal activity. Added p47-*phox* resulted in a small (1.3-fold) increase in activity whereas p67-*phox* resulted in a 3-fold increase, indicating that p67-*phox*

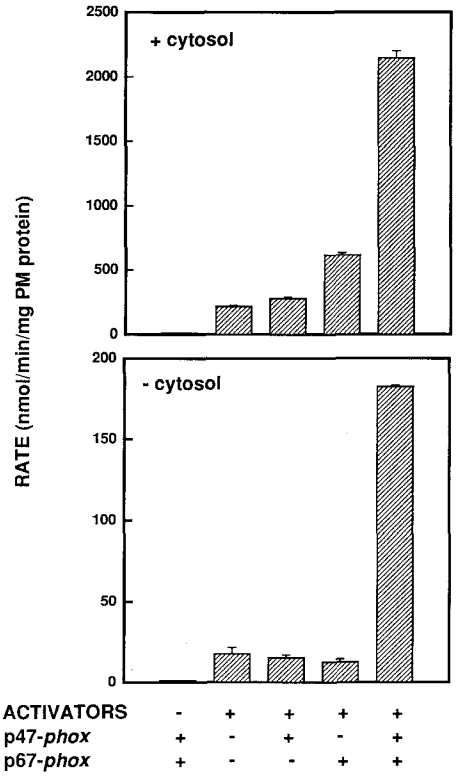

Fig. 2. Effect of recombinant cytosolic factors on superoxide generation in the cell-free systems consisting of plasma membranes and limiting normal cytosol or plasma membranes alone. All incubations contained 10 μg plasma membrane, 150 μM SDS, 200 μM dioctanoyl glycerol, 10 μM GTPγS and 5 μg of recombinant cytosolic factor(s) (corresponding to concentrations of 2.2 μM and 1.6 μM for p47-*phox* and p67-*phox*, respectively) as indicated. The cell-free system depicted in the upper panel also contained 25 μg normal cytosol in addition other components. The data presented are representative of five separate experiments carried out using three different plasma membrane preparations.

is limiting compared with p47-*phox* in normal cytosol. Addition of both proteins resulted in a marked stimulation (10-fold) which yielded an activity similar to that seen when an optimal concentration of cytosol was used (data not shown).

We also carried out the identical experiment in the absence of added cytosol. A similar experiment was reported previously (26) using recombinant p47-*phox* and p67-*phox* together with detergent-solubilized plasma membrane, but no activity was seen using this protocol. In the present studies, when intact plasma membranes were used (see Fig. 2, lower panel), significant activity was seen when both proteins were present. In contrast to results seen using dilute cytosol (upper panel), neither protein alone stimulated activity above the basal rate of superoxide generation. The maximal activity using both recombinant proteins was typically 10 - 15 % of that seen when dilute cytosol was present, indicating that cytosol contributes one or more additional stimulatory factors, but the present studies indicate that the minimal system necessary to reconstitute oxidase activity consists of p47-*phox* , p67-*phox* , and plasma membrane.

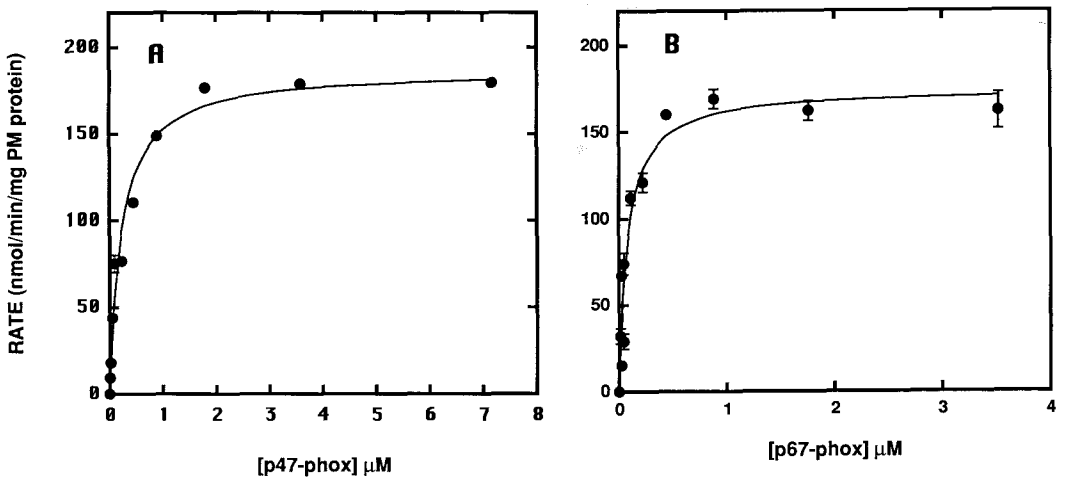

Fig. 3. Concentration dependencies for activating effects of p67-*phox* and p47-*phox*. The minimal cell-free system consisted of 10 μg PM, recombinant cytosolic factors (detailed below), 100 μM SDS, 200 μM dioctanoyl glycerol and 10 μM GTPγS. In panel A the concentration of p67-*phox* was held constant at 0.33 μM and the concentration of p47-*phox* was varied as indicated. In panel B the concentration of p47-*phox* was held constant at 0.45 μM and p67-*phox* was varied. Data presented are representative of four separate experiments.

The concentration dependencies for each recombinant protein in the presence of an optimal concentration of the other component are shown in Fig. 3. EC_{50} values were calculated to be 220 nM for p47-*phox* (panel A) and 80 nM for p67-*phox* (panel B). Recent studies indicate that p47-*phox* and p67-*phox* both translocate to the plasma membrane (25,27), and are though to be involved in a multicomponent complex with cytochrome b_{558}. The EC_{50} values determine in the present studies indicate that the affinity of p47-*phox* and p67-*phox* for such a complex is likely to be relatively high in the cell-free activation system.

To test whether plasma membrane contained a contaminating dissociable cytosolic factor, we carried out the experiment shown in Fig. 4. Plasma membranes, prepared as in Materials and Methods, were sequentially washed by centrifugation and resuspension by homogenization in fresh buffer. A parallel control was carried out by centrifugation and resuspension in the same supernatant, and the activity using washed membranes was expressed as a per cent of the control activity. As is shown, the washing had essentially no effect on the activity, indicating that plasma membrane does not contain a freely dissociable oxidase component, and that the activity which was seen was not due to contamination of plasma membranes with cytosol.

We have previously shown that diacylglycerol and GTPγS synergize with SDS to activate superoxide generation in a cell-free system consisting of cytosol plus plasma membrane (7). To determine whether the factors necessary for the guanine nucleotide and diacylglycerol synergistic responses remained present in our minimal system, the experiment in Fig. 5 was carried out. As can be seen, neither GTPγS nor diacylglycerol alone or in combination had a significant effect. SDS alone produced a modest activation, which was augmented by GTPγS. Although diacylglycerol failed to augment the SDS activation, when GTPγS was present the combination of

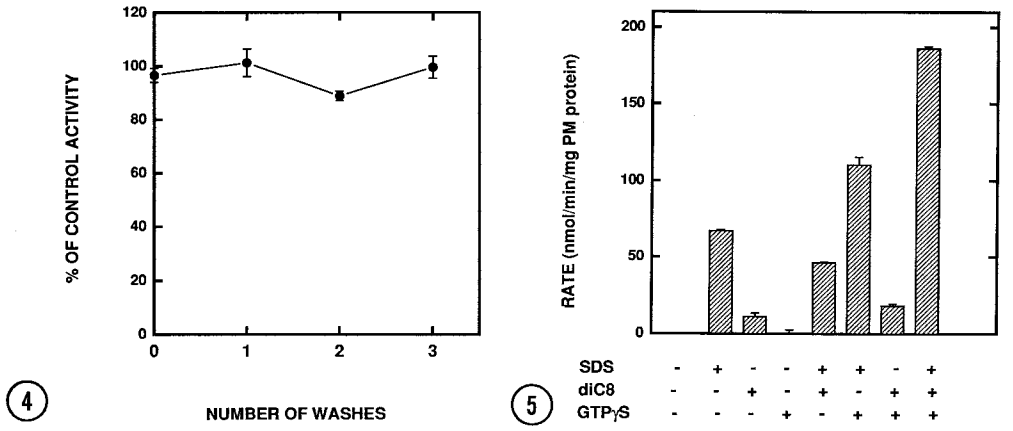

Fig. 4. Effect of repetitive plasma membrane washing on oxidase activity in the minimal cell-free system. Neutrophil plasma membranes were diluted to approximately 1 mg/ml in plasma membrane resuspension buffer (7) and stirred on ice for 30 minutes at which time the plasma membranes were recovered by centrifugation at 546,000 x g for 1 hour. The 'washed' plasma membranes were resuspended to 1 mg/ml in fresh buffer while the control plasma membranes were resuspended into the same supernatant. Oxidase activity, presented as [(wash/control) x 100%], was determined using 10 μg of plasma membrane, 2.24 μM p47-*phox*, 1.64 μM p67-*phox*, 100 μM SDS, 200 μM diC8 and 10 μM GTPγS. The data presented is from one experiment which is representative of results obtained in four separate experiments performed with different plasma membrane preparations.

Fig. 5 Agonist specificity in the minimal cell-free system. The incubations all contained 10 μg plasma membranes, 2.24 μM p47-*phox* and 1.64 μM p67-*phox*. The agonists: 150 μM SDS, 200 μM dioctanoyl glycerol (diC8) and 10 μM GTPγS were added singly or in combination as indicated. Data presented are mean ± SE of three determinations. The experiment is representative of three performed with different plasma membrane preparations.

the three agonists resulted in the highest level of activation. These results indicate that the activator specificity in the minimal system parallels that seen when cytosol is used. Thus, the target sites for diacylglycerol and GTPγS must be present to some extent in either plasma membrane or the recombinant proteins.

Because it had been reported previously that the NADPH-binding component of the oxidase is present in cytosol from resting cells, and because it seems clear that neither p47-*phox* nor p67-*phox* contains a nucleotide binding domain nor do they bind flavin (26), we determined the K_m for NADPH in the minimal oxidase system to assess whether the normal NADPH binding component was present. Fig. 6 shows a Lineweaver-Burk plot of NADPH concentration *versus* activity using the recombinant proteins plus plasma membrane. The respective K_m values obtained using normal cytosol and using recombinant proteins were 45 μM (mean of 3 experiments) and 43 μM. The respective V_{max} values were 2,042 and 360 nmol cytochrome *c* reduced/min/mg plasma membrane protein. If one accepts that neither p47-*phox* nor p67-*phox* is the NADPH binding component, these data indicate that this component is located in the plasma membrane.

In summary, we have expressed recombinant p47-*phox* nor p67-*phox* using a baculovirus expression system, and have utilized the purified recombinant proteins to define and characterize the activation characteristics of a minimal cell-free oxidase system consisting of the recombinant

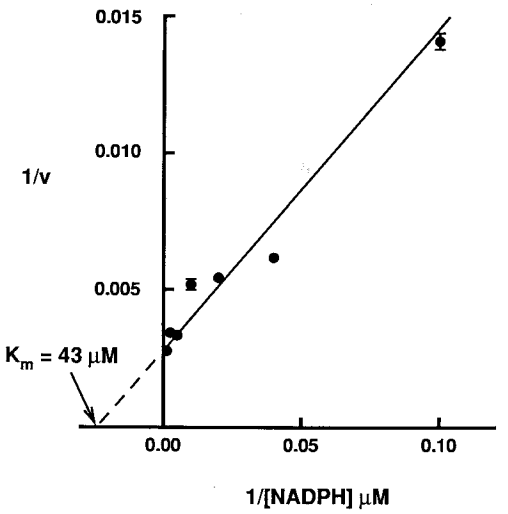

Fig. 6. NADPH concentration dependence for the minimal cell-free system. The incubations contained 10 μg plasma membranes, 2.24 μM p47-*phox* and 1.64 μM p67-*phox*., 250 μM SDS, 200 μM dioctanoyl glycerol and 10 μM GTPγS. The concentration of NADPH in the final reaction mixtures ranged from 0 to 800 μM. The velocity and substrate data are presented in a Lineweaver-Burk format. K_m and V_{max} values presented in the text were obtained from a non-linear least squares fit of the data.

proteins plus plasma membranes. We find that all components necessary for activity and response to diacylglycerol and GTPγS are present in plasma membrane plus recombinant proteins.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI22809 and CA46508. We wish to thank Dr. Thomas Leto and Dr. Harry Malech, Laboratory of Host Defenses, NIAID, NIH, Bethesda, MD 20892, for providing the p47-*phox* and p67-*phox* cDNAs in pBluescript. We are also grateful to Dr. John Curnutte for providing p47-*phox*-deficient cytosol from CGD neutrophils.

REFERENCES

1. Pick, E., Bromberg, Y., Shpungin, S. and Gadba, R. (1987) J. Biol. Chem. 262, 16476-16483

2. Curnutte, J.T. (1985) J. Clin. Invest. 75, 1740-1743

3. McPhail, L.C., Shirley, P.S., Clayton, C.C. and Snyderman, R. (1985) J. Clin. Invest. 75, 1735-1739

4. Seifert, R. and Schultz, G. (1987) Biochem. Biophys. Res. Commun. 146, 1296-1302

5. Ishida, K., Takeshige, K., Takasugi, S. and Minakami, S. (1989) FEBS Lett. 243, 169-172

6. Uhlinger, D.J., Burnham, D.N. and Lambeth, J.D. (1991) J. Biol. Chem. 266, 20990-20997

7. Burnham, D.N., Uhlinger, D.J. and Lambeth, J.D. (1990) J. Biol. Chem. 265, 17550-17559

8. Segal, A.W., Cross, A.R., Garcia, R.C., Borregaard, N., Valerius, N.H., Soothill, J.F. and Jones, O.T.G. (1983) N. England J. Med. 308, 245-251

9. Dinauer, M.C., Orkin, S.H., Brown, R., Jesaitis, A.J. and Parkos, C.A. (1987) Nature 327, 717-720

10. Parkos, C.A., Dinauer, M.C., Walker, L.E., Rodger, A.A., Jesaitis, A.J. and Orkin, S.H. (1988) Proc. Natl. Acad. Sci. USA 85, 3319-3323

11. Lomax, K.J., Leto, T.L., Nunoi, H., Gallin, J.I. and Malech, H.L. (1989) Science 245, 409-412

12. Volpp, B.D., Nauseef, W.M., Donelson, J.E., Moser, D.R. and Clark, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 7195-7199

13. Leto, T.L., Lomax, K.J., Volpp, B.D., Nunoi, H., Sechler, J.M.G., Nauseef, W.M., Clark, R.A., Gallin, J.I. and Malech, H.L. (1990) Science 248, 727-730

14. Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T. and Bokoch, G.M. (1991) Science 254, 1512-1515

15. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C.G. and Segal, A.W. (1991) Nature 353, 668-670

16. Eklund, E.A., Marshall, M., Gibbs, J.B., Crean, C.D. and Gabig, T.G. (1991) J. Biol. Chem. 266, 13964-13970

17. LaPorte, F., Doussiere, J. and Vignais, P. (1990) Biochem. Biophys. Res. Comm. 168, 78-84

18. Yea, C.M., Cross, A.R. and Jones, O.T.G. (1990) Biochem. J. 265, 95-100

19. Sakane, F., Kojima, H., Takahashi, K. and Koyama, J. (1987) Biochem. Biophys. Res. Commun. 147, 71-77

20. Markert, M., Glass, G.A. and Babior, B.M. (1985) Proc. Natl. Acad. Sci. USA 82, 3144-3148

21. Umei, T., Babior, B.M., Curnutte, J.T. and Smith, R.M. (1991) J. Biol. Chem. 266, 6019-6022

22. Smith, R.M., Curnutte, J.T., Mayo, L.A. and Babior, B.M. (1989) J. Biol. Chem. 264, 12243-12248

23. Light, D.R., Walsh, C., O'Callaghan, A.M., Goetzl, E.J. and Tauber, A.I. (1987) Biochemistry 20, 1468-1476

24. Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) J. Biol. Chem. 261, 9426-9432

25. Tyagi, S.R., Neckelmann, N., Uhlinger, D.J., Burnham, D.N. and Lambeth, J.D. (1992) Biochemistry 31, 2765-2774

26. Leto, T.L., Garrett, M.C., Fujii, H. and Nunoi, H. (1991) J. Biol. Chem. 266, 19812-19818

27. Heyworth, P.G., Curnutte, J.T., Nauseef, W.M., Volpp, B.D., Pearson, D.W., Rosen, H. and Clark, R.A. (1991) J. Clin. Invest. 87, 352-356