Identification of a splice variant mRNA of p40phox, an NADPH oxidase component of phagocytes[1]

Takeshi Hasebe, Akimasa Someya, Isao Nagaoka*

Department of Biochemistry, Juntendo University, School of Medicine, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

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Abstract Northern blot analysis using p40phox cDNA probe revealed that two sizes of p40phox mRNAs were expressed in human promyelocytic HL-60 and bone marrow cells. To characterize these mRNAs, we performed reverse transcription using total RNA from HL-60 cells, and amplified the coding region of p40phox by polymerase chain reaction with oligonucleotide primers. Two cDNA fragments with different sizes were isolated. One was identical to a known p40phox cDNA (1054 bp) which encoded a protein of 339 residues (39 031 Da) with a calculated p*I* of 6.5. The other cDNA (1299 bp) contained an additional 245 bp intron 8 sequence in the open reading frame and encoded a protein of 348 residues (39 000 Da) with a calculated p*I* of 9.3. N-terminal 253 residues were identical between p40phox and the variant protein, whereas C-terminal 254–348 residues of the variant protein shared low homology with p40phox. Interestingly, the variant protein lacked PC (Phox and Cdc24p) motif of p40phox, which is assumed to be important for the interaction with p67phox. In addition, Western blot analysis revealed that the variant protein was not detected in HL-60 cells and neutrophils. Together, these observations suggest that alternatively spliced variant mRNA of p40phox is expressed, but its protein is hardly present in myeloid cells.

Key words: NADPH oxidase; p40phox; Cytosolic factor; Alternative splicing; Neutrophil

1. Introduction

Superoxide-producing NADPH oxidase is a multicomponent system which consists of the membrane-bound cytochrome *b*558, cytosolic factors (p47phox, p67phox, p40phox and small GTP-binding proteins Rac1/2) [1]. In resting cells the enzyme is inactive, and its components are distributed between the cytosol and membrane. During activation of NADPH oxidase, cytosolic proteins translocate to the membrane and form the catalytically active complex.

p40phox was originally identified as the fourth cytosolic component in guinea pig and human neutrophils by us and other investigators [2–4]. p40phox is complexed with p67phox and p47phox in resting cells [2–4], and translocated to the membrane during activation of NADPH oxidase [3,5,6]. However, in the cell-free system, NADPH oxidase activation is fully restored with cytochrome *b*558, p47phox, p67phox and rac proteins [7,8], suggesting that p40phox is not an essential oxidase component. Interestingly, we have suggested that p40phox may be involved in the activation of NADPH oxidase, because synthetic peptide corresponding to the amino acid sequence of p40phox inhibits the translocation of cytosolic factors and NADPH oxidase activation [5]. Moreover, Sathyamoorthy et al. have recently revealed using cell-free reconstitution and whole cell co-transfection techniques that p40phox is involved in the down-regulation of NADPH oxidase activity [9]. These observations indicate that p40phox has a role in modulating the activity of NADPH oxidase.

The human p40phox gene is a single copy in chromosomal location 22q13.1 and spans approximately 18 kb with 10 exons [10]. A 1.2-kb p40phox mRNA is transcribed from the gene. p40phox mRNA is expressed in myeloid cells such as neutrophils, eosinophils, basophils, monocytes, and megakaryocytes [3,10]. In a preliminary experiment, we have found by Northern blot analysis that two sizes of p40phox mRNA transcripts (1.2 and 1.5 kb) are expressed in bone marrow and promyelocytic leukemia HL-60 cells. Interestingly, for other NADPH oxidase components rac1 and p47phox, two mRNA species with different lengths have been detected by Northern blot and RNase protection analysis [11–13]. However, these mRNAs have not been characterized. In this study, we have identified the two p40phox mRNAs by the reverse transcription-polymerase chain reaction (RT-PCR) technique and studied the expression of p40phox in neutrophils and HL-60 cells.

2. Materials and methods

2.1. Cell culture and preparation of cells

Human promyelocytic leukemia HL-60 cells were obtained from the American Type Culture Collection (ATCC CCL-240; Rockville, MD, USA) [14]. Human monoblastic leukemia U-937 cells were obtained from the Health Science Research Resource Bank (JCRB9021; Osaka, Japan) [15]. The cells were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For the induction of granulocytic differentiation, HL-60 cells were treated with 1.3% dimethyl sulfoxide (DMSO) [16]. For the induction of monocytic differentiation, U-937 cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) [15]. To estimate maturation, the morphological changes were assessed by staining with May/Grünwald/Giemsa. The cell growth was assessed by cell-counting. Bone marrow cells and mature neutrophils were prepared as described previously [17].

*Corresponding author. Fax: +81 (3) 3813-3157.
E-mail: nagaokai@med.juntendo.ac.jp

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Abbreviations: phox, phagocyte oxidase; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide; SH3, src homology 3; PC, Phox and Cdc24p; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate, 13-acetate; p*I*, isoelectric point; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2.2. Isolation of RNA and Northern blot analysis

Total cellular RNA was isolated from HL-60 and bone marrow cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method [18]. Total RNA (2.5 μg) was separated by electrophoresis on 1.2% agarose-formaldehyde gel and transferred by capillary blotting onto nylon membranes (Hybond N^+, Amersham-Pharmacia Biotech., Bukinghamshire, UK). RNA was cross-linked with Funa-UV Linker (Funakoshi Co. Ltd., Tokyo, Japan), and the blots were hybridized with cDNA probes labeled with DIG high prime DNA labeling kit (Roche Diagnostics, Mannheim, Germany). Probes used for Northern blot analysis were the 539-bp p40phox cDNA, 245-bp p40phox splice variant cDNA (see below), and 2.1-kb (g)-actin cDNA (pHF(g)A; graciously provided by P. Gunning and L. Kedes, Stanford University) [19].

2.3. cDNA synthesis of p40phox and splice variant

To characterize p40phox mRNAs, we performed RT-PCR using Titan One Tube RT-PCR kit (Roche Diagnostics). Approximately 1 μg total RNA was added to a reaction mixture containing 1×RT-PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 mM DTT, 5 units RNasin, AMV reverse transcriptase, expand PCR enzyme mixture, and 0.5 μM of each primer. The first strand cDNA synthesis was carried out by reverse transcription at 50°C for 30 min. The RNA-cDNA hybrid was denatured at 94°C for 2 min. Then, cDNA was amplified for 10 cycles at 94°C for 30 s; 55°C for 30 s; 68°C for 60 s. In the next steps, additional cDNA amplification was performed for 30 cycles at 94°C for 30 s; 55°C for 30 s; 68°C for 60 s with 5 s extension after each cycle. Finally, the elongation step was added at 68°C for 7 min. Oligonucleotide primers used are as follows: for amplification of the coding region of p40phox and splice variant cDNA, 5′-primer (5′-cggaattcag-ACTCTCCACCTGCTCCCT-3′) corresponding to nt 1–18 of p40phox and splice variant, and 3′-primer (5′-gctctagagc-TCATGGCATCGTGTTGTA-3′) corresponding to nt 1037–1054 and nt 1282–1299 of p40phox and splice variant, respectively; for amplification of 539-bp cDNA probe which recognizes both p40phox and splice variant, 5′-primer (5′-cggaattcag-AACA-TTGCTGACATCGAGGA-3′) corresponding to nt 107–126, and 3′-primer (5′-gctctagagc-TGTTGATCCGACTGAGGAGG-3′) corresponding to nt 628–645 of p40phox and splice variant; for amplification of p40phox splice variant-specific probe, 5′-primer (5′-cggaattcag-GTCTGTGGCCTGGGAGGGAG-3′) corresponding to nt 793–812, and 3′-primer (5′-gctctagagc-CTAAGGGGGTGACAGGCT-GG-3′) corresponding to nt 1018–1037 of the splice variant. The lowercase letters represent *Eco*RI or *Xba*I recognition sequence included to facilitate the subcloning of PCR products.

The amplified cDNA fragments (approximately 1.1 or 1.3 kb) were purified with Gene Mate PCRpure SPIN Purification Kit (Intermountain Scientific Co. Ltd., UT, USA), digested with *Eco*RI and *Xba*I, and subcloned into a plasmid pBluescript SK(−) (Stratagene, CA, USA). The sequences of cDNAs were determined by the dideoxy chain termination method using the Thermosequenase II dye terminator cycle sequencing kit (Amersham-Pharmacia Biotech.) and the model 373A DNA Sequencer (Applied Biosystems). Both strands of multiple clones were sequenced using T3 and T7 primers (Promega, WI, USA), and synthetic oligonucleotide primers based on the cDNA sequences. Analyses of nucleotide and amino acid sequences were performed with GENETYX-MAC software (Software Development Co. Ltd., Tokyo, Japan), and homology search was carried out on the BLAST program [20].

2.4. In vitro translation of p40phox and splice variant RNA

To perform in vitro translation, pBluescript SK(−) plasmids carrying p40phox and splice variant cDNA were digested with *Xba*I, and 5 μg of linear templates were transcribed in vitro with T7 RNA polymerase (Stratagene) at 37°C for 90 min. After transcription, DNA templates were digested with RQ1 RNase-Free DNase (2 units, Promega) at 37°C for 15 min. Synthesized RNAs were purified with Biospin chromatography column 30 (Bio-Rad Laboratory, CA, USA) followed by ethanol precipitation. Each RNA (500 ng) was translated with rabbit reticulocyte lysate (Novagen Inc., WI, USA) at 30°C for 1 h, and then RNA templates were digested with 10 μg RNase A at room temperature for 5 min. Aliquots (5 μl) of reaction mixtures were added with 20 μl sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol, 2% SDS, 6.25

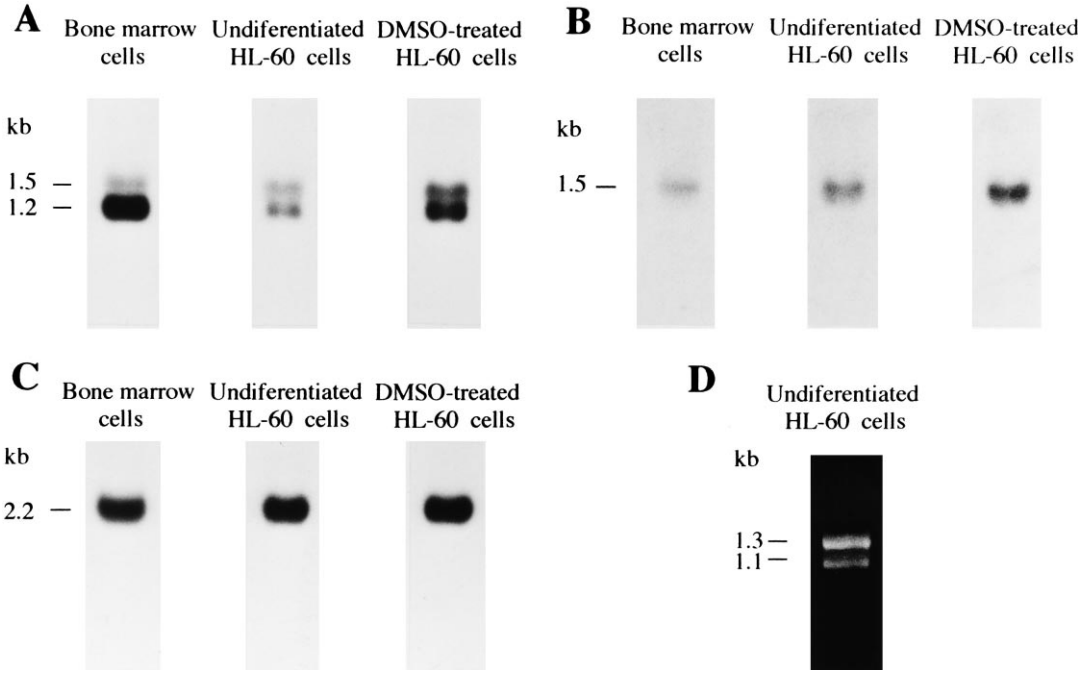

Fig. 1. Analysis of the expression of p40phox and splice variant mRNA in human promyelocytic HL-60 and bone marrow cells. Total RNAs (2.5 μg) from bone marrow, undifferentiated HL-60 and DMSO-treated HL-60 cells were electrophoresed on an agarose-formaldehyde gel and blotted onto a nylon membrane. The blots were hybridized to digoxigenin-labeled 0.5-kb p40phox cDNA probe corresponding to 107–645 of p40pox cDNA (A), p40phox splice variant-specific cDNA probe containing 245-bp intron 8 sequence (B) and (g)-actin cDNA probe (C). The 1.2-kb p40phox mRNA (A), the 1.5-kb p40phox splice variant mRNA (A or B) and the 2.2-kb γ-actin mRNA (C) are indicated. D: p40phox cDNA (nt 1–1054) and splice variant cDNA (nt 1–1299) were amplified by RT-PCR using undifferentiated HL-60 cell RNA. PCR products were electrophoresed on an agarose gel. The 1.1- and 1.3-kb cDNA fragments are indicated.

mM Tris-HCl, pH 6.8, 0.02% bromophenol blue, 5% 2-mercapto-ethanol), and 15 μl of aliquots were subjected to SDS-PAGE.

2.5. Western blot analysis

HL-60 cells and neutrophils were treated with 5 mM diisopropyl fluorophosphate (DFP) and disrupted in ice by sonication (Tomy Ultrasonic Disruptor UD-201, Tominaga Works Ltd). The sonicates were subjected to SDS-PAGE on 12% polyacrylamide gel and transferred nitrocellulose membrane (Schleicher and Shuell, Dassel, Germany) [21]. The membrane was blocked in Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) and probed with a 1:1000 dilution of rabbit anti-p40phox C-terminal peptide-specific antiserum [5,22], anti-p40phox N-terminal peptide antiserum or anti-p40phox splice variant C-terminal peptide-specific antiserum (see below). The membrane was further probed with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika Co., USA), and p40phox proteins were finally detected with ECL Western blotting detection reagents (Amersharm-Pharmacia Biotech.).

Antisera against p40phox N-terminal and p40phox splice variant C-terminal peptides were raised in rabbits by injection of keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to M^{1}AVAQQLRAESDFEQ^{15} and T^{336}SRWRKISAA^{345}, respectively [5,22].

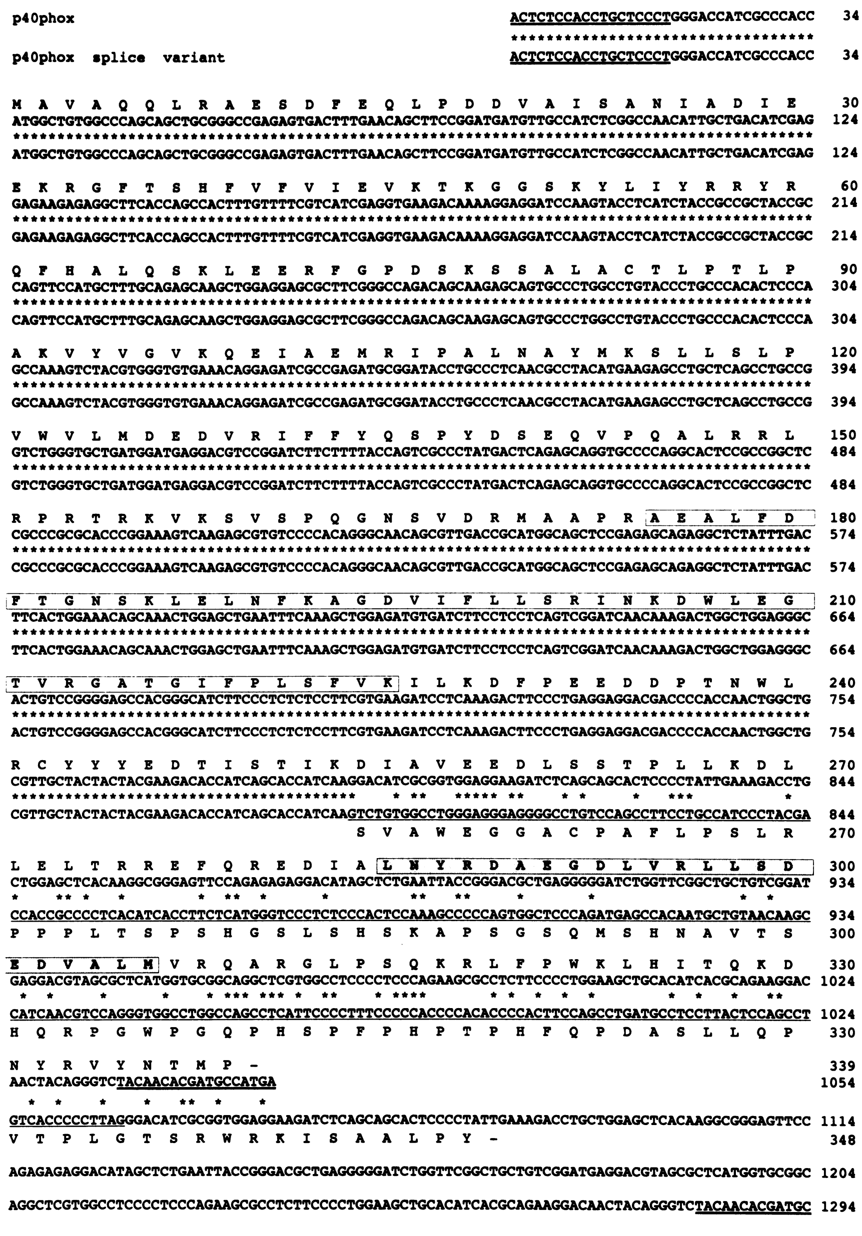

Fig. 2. Nucleotide and predicted amino acid sequences of p40phox and splice variant. The nucleotide sequences of p40phox and splice variant cDNAs are numbered from 5′-ends. The start codons of p40phox and splice variant are located at nt 35. Stop codons (TGA) of p40phox and splice variant indicated by hyphen (-) are located at nt 1052 and 1118, respectively. The nucleotide sequences of intron 8 inserted into p40phox mRNA are underlined. The deduced amino acid sequences of p40phox are shown above the nucleotide sequences, and numbered from the N-terminal methionine. The deduced amino acid sequences of splice variant cDNA are identical to p40phox between Met^{1} and Lys^{253}, but different from Ser^{254} to Tyr^{348}, which are shown under the nucleotide sequence of p40phox splice variant. Identical nucleotides are indicated by asterisks. Nucleotide sequences used for 5′- and 3′-PCR primers are indicated by bold underline. SH3 domain (Ala^{175}-Lys^{226}) and PC motif (Leu^{282}-Met^{309}) are indicated by open and shaded boxes, respectively. The sequence data in this paper have been submitted to the DDBJ/EMBL/GenBank DNA database under accession numbers AB025219 and AB025220.

3. Results and discussion

3.1. Analysis of p40phox mRNA expression

To examine the expression of p40phox mRNA, we performed Northern blot analysis using 0.5-kb p40phox cDNA probe corresponding to nt 107–645 of p40phox cDNA. Two sizes (1.2 and 1.5 kb) of p40phox mRNAs were observed in the bone marrow, undifferentiated HL-60 and DMSO-treated HL-60 cells (Fig. 1A). One was a strong signal (1.2-kb lower band) with high mobility in the gel, whereas the other was a slightly weak signal (1.5-kb higher band) with low mobility. As a control, the expression of (g)-actin mRNA was assessed. Two sizes of p40phox mRNA transcripts were also detected in undifferentiated and PMA-treated U-937 cells by Northern blot analysis (data not shown).

3.2. cDNA cloning of p40phox and spliced variant

To characterize these mRNAs, we amplified p40phox cDNA fragments using HL-60 cell RNA by RT-PCR technique. Using 5′- and 3′-end primers, two cDNA fragments with different sizes (1054 bp and 1299 bp) were obtained (Fig. 1D). The products were subcloned into a plasmid pBluescript SK(−) and sequenced. The 1054-bp fragment was identical to a known p40phox cDNA (EMBL/GenBank/DDBJ accession number X77094) which encoded a protein of 339 residues (39 031 Da) with a calculated isoelectric point (p*I*) of 6.5 (Fig. 2) [3]. The cDNA had two nucleotide substitutions at nt 472–473 from CA to AC, predicting an amino acid change from Ala^{146}(GC<u>C</u>)-Ile^{147}(<u>A</u>TC) to Ala^{146}(GC<u>A</u>)-Leu^{147}(<u>C</u>TC). The other 1299-bp fragment contained an additional 245-bp sequence in the open reading frame of p40phox and encoded a protein of 48 residues (39 000 Da) with a calculated p*I* of 9.3. As compared with the p40phox genomic DNA sequence (EMBL/GenBank/DDBJ accession numbers AL008637, U50727 and U50728), the 245-bp sequence was identical to the intron 8 sequence [10]. The 1054- and 1299-bp fragments were also obtained from bone marrow cell RNA by RT-PCR, and the amplified cDNA contained the same substitutions (CA to AC). Northern blot analysis using 245-bp p40phox splice variant-specific cDNA probe corresponding to 793–1037 nt (intron 8 sequence) revealed that low mobility bands represented p40phox splice variant mRNA containing intron 8 sequence (Fig. 1B). These results suggest that p40phox mRNA is alternatively spliced in myeloid cells. Densitometric analysis of mRNA bands in Fig. 1A using a scanning densitometer (MasterScan System; Scanalytics, USA) showed the splice variant mRNA levels in bone marrow, undifferentiated HL-60 and DMSO-treated HL-60 cells were 23%, 88% and 80% of p40phox mRNA levels, respectively. These observations indicate that the bone marrow cells express the splice variant mRNA at a relatively low level compared with p40phox mRNA, whereas HL-60 cells express p40phox and splice variant mRNAs at almost the same level.

p40phox protein has two motif sequences which are assumed to be important for protein-protein interactions. One is an src homology 3 (SH3) domain, in which p40phox interacts with the proline-rich region of p47phox [23–25]. The other is PC (Phox and Cdc24p) motif, in which p40phox interacts with p67phox [26]. N-terminal 253 residues were identical between p40phox and the variant protein, and the SH3 domain was conserved in the variant protein. In contrast, C-terminal 254–348 residues of the variant protein shared low homology with p40phox, and lacked PC motif.

3.3. Evaluation of the expression of splice variant protein in myeloid cells

To evaluate the potential translation products of alternatively spliced p40phox mRNA, we first translated p40phox and splice variant RNAs in vitro and subjected to Western blotting (Fig. 3). Both p40phox and splice variant proteins (approximately 40 kDa) were detected by Western blot analysis using anti-p40phox N-terminal peptide antibody. In contrast, p40phox protein could be detected only by anti-p40phox C-terminal peptide antibody, but not by p40phox splice variant C-terminal peptide-specific antibody. Moreover, p40phox splice variant protein could be detected by anti-p40phox splice variant C-terminal peptide-specific antibody, but not by anti-p40phox C-terminal peptide antibody. Interestingly, p40phox was detected in neutrophils by anti-p40phox N-terminal and C-terminal peptide-specific antibodies, but not detected by anti-p40phox splice variant-specific antibody. Furthermore, we evaluated the expression of variant protein in undifferentiated and DMSO-treated HL-60 cells by Western blot analysis. The variant protein was not detected in these cells. Taken together, these observations suggest that alternatively spliced variant mRNA of p40phox is expressed, but its protein is hardly present in myeloid cells. It is not clear at this stage why p40phox splice variant protein can not be detected in neutrophils and HL-60 cells. It might be possible that translation efficiency of p40phox splice variant mRNA is low and/

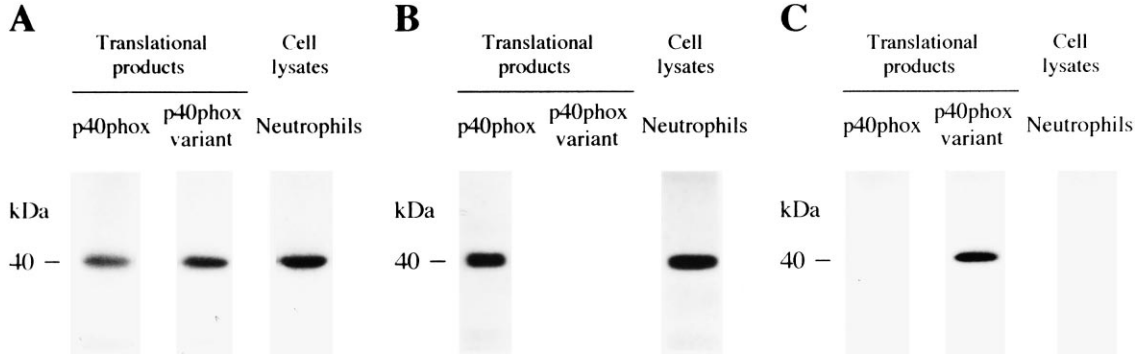

Fig. 3. Western blot analysis of p40phox and splice variant protein. Peripheral blood neutrophils (1×10^5 cell equivalents), in vitro translated p40phox and spliced variant proteins were subjected to 12% SDS-PAGE. Western blotting was performed using anti-p40phox N-terminal peptide antibody (A), anti-p40phox C-terminal peptide-specific antibody (B) and anti-p40phox splice variant C-terminal peptide-specific antibody (C).

or cationic p40phox splice variant protein with p*I* 9.3 is unstable in myeloid cells. These possibilities remain to be clarified in future.

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