The nucleoside diphosphate kinase of human neutrophils

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Nucleoside diphosphate kinase (NDP kinase) catalyses the phosphate transfer between nucleoside triphosphates and nucleoside diphosphates. As formation of guanosine triphosphate could be dependent on ATP in neutrophils, the presence of NDP kinase was tested in these phagocytic cells. Both membrane and cytosolic fractions of human neutrophils were found to contain NDP kinase activity. The specific activity measured in the cytosol appeared 10-fold higher than in the membrane and was not modified when the cells were activated with phorbol 12-myristate 13-acetate. Interestingly, stimulation with *N*-formylmethionyl-leucylphenylalanine in the presence of cytochalasin B showed an increase in membrane NDP kinase activity together with the translocation of the enzyme from the cytosol to the membrane, suggesting a possible role of NDP kinase in regulating G-proteins as previously reported. In addition, activation with opsonized zymosan induced an increase in cytosolic activity,

suggesting different regulation depending on the signal transduction pathway. The neutrophil enzyme consisted of two subunits of 21 kDa (NDPKA) and 18 kDa (NDPKB) again essentially present in the cytosol of the cell. Separation of proteins by two-dimensional PAGE demonstrated that each subunit consisted of at least four isoforms, indicating post-translational modifications. A characteristic of this family of enzymes is the stability of the phosphorylated intermediate. In neutrophils, only one acidic isoform of each NDPKA and NDPKB was labelled in the presence of EDTA. In addition, non-denatured complexes were apparent between 91 and 130 kDa, suggesting a hexameric structure as was also proposed for NDP kinases from other eukaryotic cells. These complexes were found to differ in their isoelectric points, indicating the existence of various isoenzymes probably resulting from combination between several isoforms of each subunit.

INTRODUCTION

Nucleoside diphosphate kinase (NDP kinase; EC 2.7.4.6) is a ubiquitous enzyme that catalyses phosphorylation of nucleoside 5'-diphosphate (NDP) to the corresponding triphosphate (NTP), following a Ping-Pong mechanism which includes the formation of a phosphohistidine intermediate [1]:

Although ATP is believed to be the usual phosphate donor in the cell, NDP kinase is not nucleotide specific. It accepts ribose and deoxyribose forms of both purine and pyrimidine nucleotides [1]. NDP kinase has been cloned and sequenced from several sources including the prokaryotes *Myxococcus xanthus* [2] and *Escherichia coli* [3], the eukaryotes *Dictyostelium discoideum* [4], *Drosophila* [5] and mammals [6,7]. Sequence analysis indicated that this family of enzymes is highly conserved.

Interest in NDP kinase has been revived with recent reports suggesting that this enzyme may be involved in cell differentiation and proliferation. In *Drosophila*, mutants of the gene *awd* encoding NDP kinase show strong developmental defects [5]. In human, expression of *nm23-H1*, a putative metastasis suppressor gene [5], is strongly increased in malignant tissues [8–10]. The gene *nm23-H1* [11] encodes the subunit A of NDP kinase (NDPKA), whereas its homologous allele *nm23-H2* [12] encodes the subunit B (NDPKB) [7]. Recently, it has been demonstrated *in vitro* that NDPKB is identical to the *c-myc* transcription factor PuF [13].

On the other hand, a putative role of NDP kinase in the activation of GTP-binding proteins is emerging. Indeed, NDP

kinase was found associated with different GTP-binding proteins, such as the initiation factor EIF2 [14], the α -subunit of G-protein [15], small GTP-binding proteins in rat liver [16] and microtubules [17,18]. NDP kinase activity was also found in membranes of myeloid differentiated human leukaemia (HL-60) cells [19,20].

In the present study, the presence of NDP kinase from human neutrophils was determined by measuring its activity in the membrane and in the cytosol of the cell. The enzyme and its phosphorylated intermediate were further characterized by two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Materials

Enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Rahn AG, Zürich, Switzerland); alkaline phosphatase conjugate substrate kit and nitrocellulose membranes were obtained from Bio-Rad (Glattbrugg, Switzerland); Ficoll-Paque, Percoll, Ampholine pH 3.5–9.5, Ampholine pH 4.0–6.5 and Immobiline DryStrips pH 3–10 NL 18 cm were purchased from Pharmacia Biotech (Dübendorf, Switzerland); purified NDP kinase, Nonidet P-40, 3-[(cholamidopropyl)dime-thylammonio]-1-propanesulphonate (CHAPS), leupeptin, pepstatin A, PMSF, phorbol 12-myristate 13-acetate (PMA), cytochalasin B (CB), *N*-formylmethionylleucylphenylalanine (fMLP) and zymosan A from *Saccharomyces cerevisiae* were all from Sigma (Buchs, Switzerland); other chemical products were obtained from Fluka (Buchs, Switzerland) and Merck (Dietikon, Switzerland).

Abbreviations used: NDP, nucleoside 5'-diphosphate; NTP, nucleoside 5'-triphosphate; NDP kinase, nucleoside 5'-diphosphate kinase; CB, cytochalasin B; fMLP, *N*-formylmethionylleucylphenylalanine; OZ, opsonized zymosan; PMA, phorbol 12-myristate 13-acetate; ECL, enhanced chemiluminescence; NDPKA and NDPKB, subunits A and B of NDP kinase; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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The polyclonal antibodies anti-NDPKA and anti-NDPKB were a generous gift from Dr. I. Lascu (Institut de Biochimie et Génétique Cellulaires, CNRS, Bordeaux, France).

Neutrophil activation and preparation of membrane and cytosol

Human neutrophils were isolated from 450 ml of fresh buffy coats by dextran sedimentation as already reported [21]. After removal of contaminating erythrocytes by hypo-osmotic lysis, the cell pellet was resuspended in Hanks buffer (pH 7.4) consisting of 137 mM NaCl, 5.3 mM KCl, 0.3 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 0.4 mM MgSO_4 , 0.5 mM MgCl_2 , 1.3 mM CaCl_2 , 4.1 mM NaHCO_3 and 5 mM glucose.

An aliquot (1 ml) of cell suspension containing 10^7 – 10^8 neutrophils was preincubated for 3 min at 37 °C, then activated or not (controls) with PMA ($1 \mu\text{g}/10^7$ cells) for 10 min, opsonized zymosan (OZ) (prepared as already described [21]; $4 \text{ mg}/10^7$ cells) for 7 min or fMLP ($1 \text{ nmol}/\text{ml}$) plus CB ($5 \mu\text{g}/\text{ml}$) for 5 min. The reaction was stopped by adding ice-cold Hanks buffer without calcium, magnesium and glucose.

The cells were centrifuged at 150 *g* for 5 min at 4 °C and resuspended in 0.34 M saccharose containing 0.5 mM PMSF, $1 \mu\text{M}$ pepstatin A and $1 \mu\text{M}$ leupeptin (anti-proteases). The cell suspension was sonicated at full power (350 W) for $3 \times 30 \text{ s}$, at 1 min intervals, at 0 °C in a Branson Sonifier system (model B-30) fitted with a cup horn. The membrane fraction was purified on a discontinuous sucrose gradient (30%/50%, w/v) as described previously [22] and the cytosolic fraction was carefully removed from the top layer of the sucrose gradient.

Alternatively, neutrophils were isolated from 20 ml of freshly drawn heparinized blood on a discontinuous Percoll gradient [23]. The cells, resuspended in Hanks buffer, were activated and sonicated as described above, then centrifuged at 100 000 *g* for 30 min at 4 °C. The cytosolic fraction was recovered in the supernatant and the membrane fraction was obtained by resuspension of the pellet in 0.34 M saccharose plus anti-proteases.

Protein concentration

Protein concentration of the cytosol and the membrane preparations was measured by using the Bradford reagent (Bio-Rad).

NDP kinase activity measurement

NDP kinase activity was measured by the coupled pyruvate kinase–lactate dehydrogenase system [24]. A final volume of 1 ml containing 50 mM Tris (pH 7.4), 8 mM MgCl_2 , 80 mM KCl, 0.4 mM ATP, 1 mM phosphoenol pyruvate, 4 kU/l pyruvate kinase, 2.8 kU/l lactate dehydrogenase, 0.15 mM NADH and 20 μl of membrane or cytosolic fractions was incubated for 3 min at 37 °C. Then, 0.2 mM deoxythymidine 5'-diphosphate (dTDP) was added and the rate of oxidation of NADH was followed by measuring the decrease in absorbance at 340 nm.

^{32}P -labelling of neutrophils

Human neutrophils were isolated from 20 ml of freshly drawn heparinized blood on a discontinuous Percoll gradient as described above. The cells were resuspended at a density of 1.8×10^7 cells/ml in Hanks-Pipes Ca^{2+} -, Mg^{2+} - and phosphate-free buffer (pH 7.4) consisting of 32 mM Pipes, 137 mM NaCl and 5.3 mM KCl. Sonication and preparation of membrane and cytosolic fractions were performed as described above.

A final volume of 100 μl containing 120 μg of membrane or cytosolic proteins, 5 mM EDTA and $2 \mu\text{M}$ ATP was incubated for 15 min at 4 °C with 35 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol)

[25,26]. Proteins were then precipitated by addition of 4 vols. of acetone and left for 10 min at 4 °C. Proteins were pelleted by centrifugation at 10 000 *g* for 10 min at 4 °C and resuspended in a sample buffer containing 2% SDS and 0.03 M 1,4-dithioerythritol. The samples were boiled for 5 min at 95 °C, then urea and Bromophenol Blue were added to a final concentration of 9 M and 0.0015% respectively. Proteins were separated by two-dimensional PAGE.

Electrophoresis, autoradiography and immunoblot

Two-dimensional PAGE

Proteins (60 μg) were first separated by IEF in capillary gels (75 mm $\times$ 1.5 mm) then in SDS/14%-polyacrylamide gels [27,28]. After migration, proteins were blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech) and were autoradiographed for 3 days with Kodak X-Omat AR films between intensifying screens at –80 °C. After autoradiography, the nitrocellulose sheets were incubated for 1 h at room temperature in Tris-buffered saline solution (pH 7.5) consisting of 20 mM Tris and 500 mM NaCl (TBS buffer) supplemented with 3% (w/v) gelatin. The blots were washed ($3 \times 10 \text{ min}$) in TBS buffer containing 0.05% Tween (TTBS buffer) and incubated overnight with a rabbit polyclonal antibody raised against purified NDPKA, but cross-reacting with the NDPKB (anti-NDPKA) [7] at a dilution of 1/3000. After three washes of 10 min with TTBS buffer, a goat anti-(rabbit IgG) antibody conjugated with horseradish peroxidase (dilution 1/3000) was added for 2 h and detected by the very sensitive ECL system after a 30 s exposure.

Non-denaturing two-dimensional PAGE

Cytosolic fractions were mixed with a final concentration of 2% (v/v) Ampholine pH 3.5–9.5 and 0.0015% Bromophenol Blue.

Proteins (40 μg) were first separated by IEF in non-denaturing capillary gels (75 mm $\times$ 1.5 mm) consisting of 4% (w/v) acrylamide, 1.6% (v/v) Ampholine pH 3.5–9.5 and 0.4% (v/v) Ampholine pH 4.0–6.5. The proteins were focused at 500 V for 30 min, then at 1000 V for 4 h. After IEF, the capillary gels were equilibrated for 3 min in a solution containing 70 mM Tris (pH 6.8) and 0.028% Bromophenol Blue [27] before application on to the second dimension consisting of 7–16% PAGE without SDS [28].

After migration, proteins were blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech). The blots were then incubated with anti-NDPKA at a dilution of 1/3000 and the primary antibody was revealed with a goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase (dilution 1/3000) as described previously [29].

Two-dimensional PAGE using Immobiline Drystrips

Proteins were precipitated from cytosolic fractions of resting neutrophils with ice-cold acetone as described above and 300 μg samples were firstly separated in precast gels containing a non-linear pH gradient (Immobiline Drystrips pH 3–10 NL 18 cm; Pharmacia Biotech, Dübendorf, Switzerland), then on SDS/PAGE in 14% acrylamide gels [27,28]. Proteins were blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech) and the nitrocellulose sheets were incubated for 1 h at room temperature in TBS buffer supplemented with 3% (w/v) gelatin. The blots were washed ($3 \times 10 \text{ min}$) in TTBS buffer and incubated overnight with anti-NDPKA at a dilution of 1/3000. After three washes of 10 min with TTBS buffer, a goat

anti-(rabbit IgG) antibody conjugated with horseradish peroxidase (Bio-Rad, Glattbrugg, Switzerland) was added for 2 h at a dilution of 1/3000 and detected by the very sensitive ECL system after a 30 s exposure.

SDS/PAGE

Proteins (10 µg) from membrane and cytosolic fractions of resting or activated neutrophils were separated by SDS/PAGE in 14 % acrylamide gels according to Laemmli [28] and blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech). The blots were incubated with anti-NDPKA (dilution 1/3000) or with a rabbit polyclonal antibody specific for NDPKB (anti-NDPKB) [7] and the primary antibody was revealed with a goat anti-(rabbit IgG) secondary antibody conjugated with alkaline phosphatase (dilution 1/3000) as described previously [29].

Non-denaturing PAGE

Proteins (40 µg) from cytosolic fractions of resting neutrophils were separated by 7–16 % acrylamide gradient gels without SDS and 2-mercaptoethanol [28]. Proteins were blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech). The blots were incubated with anti-NDPKA (dilution 1/3000) and the primary antibody was revealed with a goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase (dilution 1/3000) as described previously [29].

Densitometry

Immunoblots were scanned with a Molecular Dynamics densitometer (Perkin-Elmer, Le Mont-sur-Lausanne, Switzerland) and analysed using the ImageQuant program (v.3.3).

Statistical analysis

Comparisons were made by using the non-parametric Mann–Whitney test for unpaired values. The level of significance was accepted at $P < 0.05$. All experiments were done at least three times on neutrophils isolated from different donors unless otherwise stated.

RESULTS

NDP kinase activity in membrane and cytosolic fractions

The presence of NDP kinase in neutrophils was demonstrated by measuring the NDP kinase activity in membrane and cytosolic fractions with the coupled pyruvate kinase–lactate dehydrogenase system [24]. As presented in Table 1, the specific activity measured in the membrane fraction of resting cells was 0.062 µmol/min per mg of protein and that measured in the cytosolic fraction was 0.585 µmol/min per mg of protein. Thus, the NDP kinase activity appeared about 10-fold higher in the cytosol than in the membrane. To evaluate whether activation of neutrophils could have an influence on enzyme activity, the cells were activated with OZ, PMA or CB/fMLP [30] and membrane and cytosolic fractions were separated as described in the Materials and methods section. The NDP kinase activities measured in the membrane fractions of OZ-activated and PMA-stimulated cells were 0.085 and 0.048 µmol/min per mg of protein respectively and showed no significant difference compared with resting cells. In contrast, the NDP kinase activity measured in the membrane of CB/fMLP-stimulated cells was significantly higher than that measured in the membrane of

Table 1 Activity and distribution of the NDP kinase in the membrane and in the cytosol of resting and activated neutrophils

Neutrophils (1.8×10^7 cells/ml) were activated with OZ, PMA, CB/fMLP or left untreated (resting neutrophils). The cells were separated in membrane and cytosolic fractions and NDP kinase activity was measured in both fractions with the coupled pyruvate kinase–lactate dehydrogenase system as described in the Materials and methods section. In addition, membrane and cytosolic proteins were separated by SDS/14 % PAGE, blotted on to nitrocellulose sheets and revealed with anti-NDPKA, and the subunits A and B were quantified by densitometry. Values are means $\pm$ S.D. of the number of experiments indicated in parentheses.

Stimuli	Activity (µmol/min per mg of protein)	Distribution of NDP kinase (%)	
		Subunit A	Subunit B
Resting neutrophils			
Membrane	0.062 $\pm$ 0.011 (10)	8 $\pm$ 1 (3)	9 $\pm$ 2 (3)
Cytosol	0.585 $\pm$ 0.086 (10)	92 $\pm$ 1 (3)	91 $\pm$ 2 (3)
OZ-activated cells			
Membrane	0.085 $\pm$ 0.027 (5)	11 $\pm$ 1 (3)	12 $\pm$ 2 (3)
Cytosol	0.740 $\pm$ 0.060 (5) ¹	89 $\pm$ 1 (3)	88 $\pm$ 2 (3)
Translocation		3 $\pm$ 1	3 $\pm$ 1
PMA-activated cells			
Membrane	0.048 $\pm$ 0.014 (5)	6 $\pm$ 2 (3)	7 $\pm$ 3 (3)
Cytosol	0.512 $\pm$ 0.019 (5)	94 $\pm$ 2 (3)	93 $\pm$ 3 (3)
Translocation		0	0
CB/fMLP-activated cells			
Membrane	0.124 $\pm$ 0.016 (8) ^{2,3,4}	20 $\pm$ 3 (3)	19 $\pm$ 3 (3)
Cytosol	0.594 $\pm$ 0.117 (8)	80 $\pm$ 3 (3)	81 $\pm$ 3 (3)
Translocation		12 $\pm$ 2	10 $\pm$ 2

¹ $P < 0.02$ versus cytosol of resting neutrophils.

² $P < 0.00002$ versus membrane of resting neutrophils.

³ $P < 0.02$ versus membrane of OZ-activated cells.

⁴ $P < 0.005$ versus membrane of PMA-activated cells.

resting ($P < 0.00002$), OZ- ($P < 0.02$) or PMA-activated ($P < 0.005$) neutrophils (Table 1), suggesting different regulation depending on the signal transduction pathway. The activities measured in the cytosolic fractions of PMA- and CB/fMLP-activated cells were 0.512 and 0.594 µmol/min per mg of protein respectively and were not different from unstimulated cells, whereas that measured in the cytosol of OZ-activated cells was significantly increased to 0.740 µmol/min per mg of protein ($P < 0.02$).

Immunological characterization of the NDP kinase from neutrophils

The molecular mass of the subunit(s) of the NDP kinase was determined by separating the membrane and cytosolic proteins from resting, OZ-, PMA- and CB/fMLP-activated cells on SDS/PAGE. Proteins were then blotted on to nitrocellulose sheets and revealed with a polyclonal antibody recognizing the two subunits A and B of the erythrocytic NDP kinase (anti-NDPKA) or with a polyclonal antibody recognizing only the subunit B of the NDP kinase from erythrocytes (anti-NDPKB) [7]. As shown in Figure 1(A), anti-NDPKA recognized two subunits with molecular masses of 21 kDa and 18 kDa. In contrast, anti-NDPKB recognized only the 18 kDa protein (Figure 1B), thus identifying the 18 kDa molecule as the B subunit and the 21 kDa as the A subunit of NDP kinase. These two subunits were very weakly detected in the membrane, whereas they were clearly evident in the cytosol (Figures 1A and 1B), correlating with the NDP kinase activity measured in the two fractions (Table 1). Both anti-NDPKA and anti-NDPKB recognized other proteins at higher molecular masses, which probably

Figure 1 Immunoblotting profiles of NDPKA and NDPKB in the membrane and in the cytosol of resting or activated neutrophils

Proteins (10 μ g) of membrane (M) and cytosol (C) from resting neutrophils (—) or neutrophils activated with OZ (4 mg/ 10^7 cells), PMA (1 μ g/ 10^7 cells) and fMLP (1 nmol/ml) in the presence of CB (5 μ g/ml) were separated in SDS/14%-PAGE. Proteins were blotted on to nitrocellulose sheets and revealed with anti-NDPKA (A) or anti-NDPKB (B) as described in the Materials and methods section. The molecular mass standards used are given on the right of each Figure. The results are from one experiment representative of three.

Figure 2 Heterogeneity of NDPKA and NDPKB separated by two-dimensional PAGE

Proteins (300 μ g) of membrane (A) and cytosolic (B) fractions were separated by two-dimensional PAGE using Immobiline Drystrips for IEF, blotted on to nitrocellulose sheets and revealed with anti-NDPKA as described in the Materials and methods section. The letters a, b, c, and d indicate the different variants of NDPKA and NDPKB. The acid side on all two-dimensional PAGE is to the left and the basic side to the right. The results are from one experiment representative of three.

correspond to different complexes formed by the subunits A and B. Indeed, when commercially available purified NDP kinase was used, the antibodies recognized similar higher-molecular-mass proteins (results not shown). Moreover, no differences were observed between the membrane fraction of resting and OZ- or PMA-activated cells or between the cytosolic fractions of unstimulated and OZ- or PMA-stimulated neutrophils, suggesting that neutrophils contain membrane-bound and cytosolic forms of NDP kinase. In contrast, 12% of NDPKA and 10% of NDPKB were found to translocate from the cytosol to the membrane when neutrophils were activated with CB/fMLP

Figure 3 Complex formation between NDPKA and NDPKB

Cytosolic proteins (40 μ g) were separated in non-denaturing two-dimensional PAGE using capillary gels for IEF (A) or in non-denaturing 7–16% gradient PAGE (B) as described in the Materials and methods section. Proteins were revealed by anti-NDPKA after blotting on to nitrocellulose. The letters a–g (A) indicate the different complexes recognized by the antibody. The molecular mass standards for non-denaturing gel are indicated on the right of the Figure in B. The results are from one experiment representative of three.

(Table 1). This result correlated with the increase of NDP kinase activity observed in the membrane of CB/fMLP-stimulated neutrophils (see above).

Separation of proteins by two-dimensional PAGE (Figure 2) showed again that NDPKA and NDPKB were less abundant in the membrane fraction (Figure 2A) than in the cytosolic fraction (Figure 2B). Furthermore, it indicated that each subunit was composed of different isoforms. Indeed, an isoform with pI value of 5.7 (isoform b) was weakly recognized by anti-NDPKA in the membrane fraction (Figure 2A) compared with the cytosolic fraction (Figure 2B). A second variant of NDPKA was detected in the membrane at pI 6.2 (isoform c) as also were a succession of forms of NDPKB from pI 7.8 to pI 8.8 (Figure 2A). In the cytosolic fraction (Figure 2B), anti-NDPKA revealed four isoforms of NDPKA: a minor acidic form at pI 5.3 (isoform a) and three major variants with pI 5.7 (isoform b), 6.2 (isoform c) and 6.4 (isoform d). It also revealed four variants of NDPKB: a minor form at pI 7.8 (isoform a), and three other forms with pI values of 8.1 (isoform b), 8.6 (isoform c) and 8.8 (isoform d).

Association of the two subunits A and B of NDP kinase

As eukaryotic NDP kinases form hexamers [1,7], putative protein complexes were separated on non-denaturing two-dimensional PAGE and revealed with anti-NDPKA after blotting on nitrocellulose, as described in the Materials and methods section. As shown in Figure 3(A), anti-NDPKA recognized at least seven complexes at pI values of 5.5 (a), 5.6 (b), 5.9 (c), 6.4 (d), 6.5 (e), 6.6 (f) and 6.8 (g). To determine the molecular masses of these complexes, the proteins were separated on non-denaturing 7–16% acrylamide gels. Complexes at molecular masses of 91, 118, 130, 215 and 430 kDa were detected by anti-NDPKA (Figure 3B).

Isolation of the phosphorylated intermediate of NDP kinase

The phosphorylated intermediate of NDP kinase was isolated in the presence of EDTA [25,26]. After separation of labelled

Figure 4 Autoradiography (A, B) and immunoblotting (C) profiles of proteins separated by two-dimensional PAGE

Membrane (A) and cytosolic (B, C) proteins were labelled with [γ - 32 P]ATP, separated by two-dimensional PAGE using capillary gels for IEF and blotted on to nitrocellulose sheets. Autoradiographs are shown in (A) and (B). To identify the labelled proteins, the same nitrocellulose sheets were incubated with anti-NDPKA after autoradiography (C). The results are from one experiment representative of three.

proteins by two-dimensional PAGE, only one isoform of both NDPKA and NDPKB appeared to be phosphorylated in the membrane (Figure 4A) and in the cytosol (Figure 4B) of neutrophils. These variants were identified by anti-NDPKA (Figure 4C) as the isoform b (pI 5.7) of NDPKA and the isoform a (pI 7.8) of NDPKB. Under these conditions, the other forms of NDPKA and NDPKB were not labelled, although they reacted with anti-NDPKA on the blot after its use for autoradiography (Figure 4C). Interestingly, this antibody recognized a protein of 15 kDa which could be the product from proteolysis of the isoform c of NDPKA as it has the same pI (Figure 4C).

DISCUSSION

NDP kinases are very widely distributed from the prokaryotes to the eukaryotes. Moreover, the hexameric structure seems to be a common feature of these enzymes, although it is formed by the association of one or two kinds of polypeptides depending on the source. In *Saccharomyces cerevisiae* [31], *Myxococcus xanthus* [32], *Dictyostelium discoideum* [33] and pig brain [34], the monomers have a molecular mass of about 17 kDa. In human erythrocytes, the enzyme consists of two polypeptides chains of 20.5 kDa and 19 kDa [7]. Similarly, the NDP kinase from human neutrophils is composed of two subunits of 21 kDa (NDPKA) and 18 kDa (NDPKB). These polypeptides appeared very heterogeneous when separated by two-dimensional PAGE. Indeed, NDPKA, the acidic subunit, and NDPKB, the basic subunit, each showed four different isoforms, suggesting post-translational modifications, since each subunit is the product of a single gene [11,12].

Interestingly these two polypeptides, although present in the membrane, were predominant in the cytosol. This observation was confirmed when NDP kinase activity was measured in the two fractions. The activity found in the membrane fraction was about ten times less than the cytosolic activity. Moreover, activation of neutrophils with PMA did not modify NDP kinase activity and its immunodetection on blots either in the membrane or in the cytosol compared with resting cells. This indicates that neutrophils contain membrane-bound and cytosolic forms of NDP kinase which are not dependent on such stimulation.

Human erythrocyte NDP kinase consists of two subunits, A and B, which form a hexameric structure by random association (A6, A5B1...A1B5, B6) [7]. In accordance with these observa-

tions, the NDP kinase from neutrophils was found to form at least seven complexes with different isoelectric points indicating the occurrence of various isoenzymes and with molecular masses of 91–130 kDa also suggesting a polymeric structure. Complexes of 215 and 430 kDa were also detected, suggesting a non-specific association between two and four NDP kinases. However, our results show that each subunit may comprise at least four isoforms with various isoelectric points. Thus, the hexameric structure and the presence of various isoenzymes could not only be explained by the association of the two subunits, but might be the results of a combination between various isoforms of each subunit.

Different studies [1,32] have shown that the reaction of ATP with NDP kinase resulted in the reversible transfer of the γ -phosphate of ATP to a histidine residue of the enzyme. This phosphohistidine intermediate is stable and can be isolated by incubating the protein with ATP in the presence of EDTA. Under these conditions, the P_i release is extremely slow, and the phosphate group remains on the protein molecule [32]. In this work, only one acidic form of the subunit A and of the subunit B were phosphorylated. Interestingly, these proteins were weakly recognized by anti-NDPKA, indicating that they represented minor forms of NDP kinase, but they appeared to be highly phosphorylated which suggests that, despite their small amount, they should have an important role in the function of NDP kinase.

NDP kinase was found in preparations enriched in GTP-binding proteins [14–18]. It has been proposed that this association was not fortuitous, but reflected a physiologically relevant connection between GTP-binding proteins and NDP kinase [14,18,35,36], raising the possibility that GTP might act not only as the physiological activator, but also as a regulator of GTP-binding proteins. Seifert et al. [19] showed that HL-60 membranes possessed NDP kinase activity, catalysing the formation of guanosine 5'-[γ -thio]triphosphate (GTP[S]) from adenosine 5'-[γ -thio]triphosphate and thereby promoting activation of G-proteins. In addition, it was demonstrated [20] that the GTP[S] formed, efficiently bound to G-protein coupled to formyl-peptide receptors and regulated their agonist binding affinity. Since G-proteins are known to play a role in the production of superoxide anion by phagocytic cells [30], NDP kinase was proposed to modulate the activation of NADPH oxidase [20]. This enzyme is a multicomponent protein composed of at least six subunits. Two subunits are located in the plasma and specific granule membranes of resting neutrophils, i.e. the gp91-*phox* and p22-*phox* subunits of cytochrome b_{558} , the terminal electron carrier of the NADPH oxidase. The other subunits are located in the cytosol of resting cells and translocate to the membrane upon neutrophil activation. These proteins are p47-*phox*, p67-*phox*, p40-*phox* and Rac2, a small GTP-binding protein of the Ras family [37]. Rap 1a, another small GTP-binding protein, was found to be physically associated with the cytochrome b_{558} , but its role is still not known [37]. In a cell-free system from human neutrophils, Uhlinger et al. [38] showed that NTPs were necessary for NADPH oxidase activation and the apparent non-specificity for NTPs was explained by the presence of NDP kinase activity in the preparations. Moreover, these authors found that phosphorylation of p47-*phox*, which is important in the production of superoxide anion by the oxidase, showed a similar non-specificity for NTPs also probably caused by the presence of NDP kinase. A recent report using a cell-free system demonstrated that GTP was absolutely required in activation of human neutrophil NADPH oxidase, whereas ATP could indirectly support superoxide anion production by means of endogenous enzymes, and probably of NDP kinase, that catalyse the ATP-dependent

conversion of GDP into GTP [39]. Although the enzyme was not characterized, all these reports provide indirect evidence that HL-60 cells and neutrophils contain an NDP kinase at least in the membrane. In addition to these observations, we showed that the NDP kinase activity was significantly increased in the membrane of neutrophils activated with fMLP, an agonist known to interact with a G-coupled receptor, and that this increase correlated with the translocation of the enzyme from the cytosol to the membrane. This result suggests that the enzyme can modify its location, probably to assure a sufficient local concentration of GTP to promote G-protein stimulation. In contrast, the NDP kinase activity was increased in the cytosol when neutrophils were stimulated with OZ, a stimulus which did not modify the cell location of the enzyme. Such stimulation might reflect a more important need in cytosolic NTPs, perhaps by cytosolic GTP-binding proteins, e.g. Rac2, and could suggest a different regulation of the enzyme depending on the signal transduction pathway. Thus, NDP kinase may play an important role in supporting neutrophil activation. However, further characterization of NDP kinase will help to elucidate the exact role of this enzyme in neutrophil functions.

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