Biochimica et Biophysica Acta 1793 (2009) 469–476

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Direct binding of cytosolic NDP kinases to membrane lipids is regulated by nucleotides

Kimberly A.P. Mitchell, Gabor Szabo *, Angela de S. Otero

Department of Molecular Physiology and Biological Physics, University of Virginia Medical School, P.O. Box 800736, Charlottesville, VA 22908-0736, USA

ARTICLE INFO

Article history:
Received 28 June 2008
Received in revised form 30 November 2008
Accepted 3 December 2008
Available online 25 December 2008

Keywords:
Nucleoside-diphosphate kinase
nm23
Cytosol
Membrane
Phospholipid
Nucleotide

ABSTRACT

In spite of their complete lack of any structural features that characterize membrane proteins, cytosolic nucleoside-diphosphate kinases (NDPKs) have been found repeatedly to associate with membranes. In some instances the recruitment of cytosolic NDPKs to membranes was attributed to interactions with peripheral or integral membrane proteins, but in many cases the mechanism underlying the association of NDPKs with membranes remained unknown. We show here that cytosolic NDPKs bind directly to membrane lipids in a dynamic process that is controlled by its substrates, nucleoside tri- and diphosphates, and can be fully reconstituted with chemically defined, protein-free phospholipids and recombinant NDPK, or with purified NDPK. Our results uncover a novel mechanism for the reversible targeting of soluble NDPKs to membranes, where they may act as a reservoir of high energy phosphate, supporting the operation of membrane-based processes that utilize nucleotides other than ATP, such as intracellular traffic and phospholipid biosynthesis.

1. Introduction

Nucleoside-diphosphate kinases (NDPKs) play a central role in a broad range of cellular functions, including nucleic acid synthesis, lipid, and carbohydrate metabolism, through their ability to phosphorylate nucleoside diphosphates utilizing the terminal phosphate of ATP [1,2]. These enzymes show broad base specificity, but display a preference for adenine and guanine nucleotides. The phosphate transfer requires Mg^{2+} and follows ping-pong kinetics [3], taking place via a phospho-histidine intermediate. The reaction occurs in two steps,

$$E + N_1TP \rightleftharpoons E \cdot N_1TP \rightleftharpoons E\sim P + N_1DP \quad (a)$$

$$E\sim P + N_2DP \rightleftharpoons E\sim P \cdot N_2DP \rightleftharpoons E + N_2TP \quad (b)$$

which when combined give the overall reaction sequence:

$$N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP. \quad (1)$$

The most ubiquitous members of this family of proteins, NDPK A and B (the products of the nm23-1 and -2 genes), are hexamers of two highly (88%) homologous polypeptides of ~20 kDa [4], with similar kinetic parameters [5]. NDPK A and B are abundant proteins: their estimated cytosolic concentration ranges from 1 to 10 μM. While human NDPK A and B differ markedly in isoelectric point (theoretical pI values are 5.8 and 8.5 for A and B, respectively), their murine equivalents have pIs around 7. The three-dimensional structures for human NDPK A and B are very similar [6,7], and the monomers of the two proteins can associate, forming hetero-hexamers [4].

NDPK A and B have all the characteristics of cytosolic proteins, with no exposed hydrophobic segments or membrane-binding motifs. Yet, purification and characterization of NDPK from rat liver plasma membranes reveal that it is identical to the soluble protein [8]. Indeed, in many instances cytosolic NDPKs associate with membranes of a wide variety of intracellular compartments. At the plasma membrane NDPKs influence the function of ion channels [9], receptor activation and desensitization by agonists [10], activation of phagocyte NADPH oxidase [11], dynamin-mediated endocytosis [12–14] and integrin-mediated adhesion [15]. Soluble NDPKs also localize to endosomes [14], membranes from the endoplasmic reticulum [16–19], the Golgi and vesicles budding from the trans-Golgi [16], as well as phagosomes [20]. In some cases the recruitment of cytosolic NDPKs to membranes was ascribed to interactions with peripheral or integral membrane proteins such as G proteins [21–23], integrin cytoplasmic domain associated protein 1-α [15], the SET complex [17] and the potassium channel KCa3.1 [9]. Overexpression of cytosolic NDPKs revealed novel associations with the GTP-bound form of ARF6 [13], the thromboxane A2 β receptor [10] and the von-Hippel Lindau protein [14], and these binding partners were proposed to anchor NDPK to membranes. Recently, NDPKs A and B were found to associate directly with purified phospholipids [19,24].

Intracellular transport of membranes is largely mediated by the microtubular network, and treatment of cellular extracts with taxol, followed by pelleting of the resulting microtubules and associated structures, allows the rapid isolation and characterization of a

* Corresponding author. Fax: +1 434 982 1616.
E-mail address: gs3z@virginia.edu (G. Szabo).

0167-4889/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2008.12.009

fraction enriched in trafficking vesicles. This fraction (MT/Ves) has been well characterized, and contains microtubules to which are tethered endosomes, exocytic and transcytotic vesicles, as well as putative intra-Golgi transport vesicles [25–28]. We have previously shown that NDPK is present in MT/Ves and that NDPK binds to the membranes in this fraction, but not to the microtubules [28]. Furthermore, the binding of NDPK to intracellular membranes is disrupted by high concentrations of nucleotides, with an order of selectivity typical of NDPKs. The present work seeks to further characterize the association of NDPK with the intracellular vesicles isolated by microtubule stabilization. The results indicate that NDPK is present in most of the membrane vesicles obtained in this manner. Additionally, it is eluted from these membranes by nucleotides not as a complex, but as a single protein, and this is a reversible process: as nucleotide concentrations are reduced to micromolar levels, NDPK re-binds to membranes. Thus, the levels of nucleotides in the cell have a profound effect on the reversible interaction of soluble NDPKs with membranes. Furthermore, the nucleotide sensitivity of this process is a consequence of the intrinsic properties of NDPK and can be replicated in a simple system consisting of purified soluble NDPKs and chemically defined phospholipids. Taken together, our observations reveal a simple and novel mechanism for the reversible targeting of soluble NDPKs to the membranes of intracellular transport vesicles, and support the hypothesis that NDPK may play a role in localized regeneration of nucleotides such as CTP and GTP, that are essential for the biosynthesis of phospholipids as well as intracellular traffic.

2. Materials and methods

2.1. Materials

Nucleotides were from Roche (GTP, GTPγS, GMP-PNP) and Sigma-Aldrich (ATP, ADP, GDPβS). Taxol was purchased from Tocris, Sigma-Aldrich or LC Laboratories. SDS-PAGE gels (NuPage, 10 or 12%, run in MOPS/SDS buffer) were from Invitrogen. PIP strips™ were from Echelon Biosciences. Anti-nm23 H1/2/3, anti-Arf6, and anti-von Hippel–Lindau protein were from Santa Cruz Biotechnology, anti-NDPK (Ab-1) and anti-α-tubulin (Ab-2) from LabVision, anti-dynamin from Calbiochem, antibodies to Rac1 were from Santa Cruz and Upstate Biotechnology (clone 23A8). Polyclonal antibodies to NDPK C (Anti-C), affinity-purified antibodies to NDPK A depleted of antibodies cross-reacting with NDPK B (Anti-A), and affinity-purified polyclonal antibodies to NDPK B were a generous gift of Dr. Ioan Lascu (Université de Bordeaux, France). For specific detection of NDPK B, IgGs cross-reacting with NDPK A were removed by chromatography on columns of immobilized NDPK A [29]; the fraction obtained is designated here as Anti-B. Other antibodies were from sources previously described [28]. Secondary antibodies labeled with infrared dyes (IRDye 800® anti-rabbit and anti-goat, IRDye700® anti-mouse) were from Rockland Immunochemicals. Phospholipids (dioleolylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS), 1,2-dioleoyl-*sn*-glycerol-3-phosphate (DOPA), and carboxyfluorescein-labeled dioleoylphosphatidylethanolamine) were from Avanti Polar Lipids.

2.2. Methods

2.2.1. Cell culture

NIH-3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/l penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. For ATP depletion experiments, cultures were transferred to low glucose medium, and were either left untreated in controls, or exposed to 50 mM 2-deoxyglucose and 10 mM sodium azide for 1 h.

2.2.2. Isolation of microtubule-associated vesicles and cytosol

Microtubule-associated vesicles (MT/Ves) were isolated as described previously [28]. Briefly, NIH-3T3 cells were homogenized in MEPS (35 mM PIPES pH 7.1 with NaOH, 5 mM $MgSO_4$, 5 mM EGTA, 200 mM sucrose, 1 mM DTT; the calculated free Mg^{2+} is 4.3 mM) containing protease inhibitors (2 mM PMSF, 1 mM benzamidine, 2 μg/ml leupeptin). A post-nuclear supernatant (S1) was centrifuged at 40,000 ×*g* for 20 min. The supernatant (S2; small vesicles and cytosol) was incubated at 37 °C for 30 min with 20 μM taxol (and nucleotides when indicated) to polymerize tubulin, and pelleted at 16,000 ×*g* for 30 min at 4 °C. The pellets were brought to the original volume with MEPS, taxol was added to 20 μM and the samples were centrifuged at 16,000 ×*g*. After a second wash, the pellets were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting. In some instances the pellets were incubated with nucleotides after two washes, and then processed for analysis. Samples were immunoblotted in parallel with antibodies to α-tubulin (and Rab4 or LAMP1, when appropriate) to make sure that treatments did not affect tubulin polymerization or co-pelleting of membrane vesicles. Proteins were detected by chemiluminescence and quantitated with Maxim DL 2.12 (Diffraction Limited) software, or with the LI-COR® Odyssey infrared imaging system. Results shown are representative of 3–10 separate experiments. Cytosol from NIH-3T3 cells was prepared by centrifugation of the S2 supernatant in a TLA 100.3 rotor at 335,000 ×*g* for 1 h at 4 °C.

2.2.3. Immunofluorescence and confocal microscopy

A 10 μl aliquot of MT/Ves pellet suspended in MEPS was spread onto polylysine-coated coverslips and allowed to adhere for 3 min. The specimen was fixed with 4% paraformaldehyde for 10 min, washed three times with PBS, incubated with $NaBH_4$ to quench free aldehyde groups and processed for indirect immunofluorescence as in [28], using an antibody to NDPK (Ab-1; 1:50), followed by Texas Red anti-rabbit (1:100). Confocal and differential interference contrast (DIC) images were acquired with an Olympus FluoView 300 confocal laser scanning microscope (Olympus, Center Valley, PA).

2.2.4. Purification of NDPK from human erythrocytes

Soluble NDPK was purified from human erythrocytes using a modification of an earlier protocol [30]. Briefly, red blood cells were lysed in buffer A (25 mM MOPS, 1 mM EDTA, 1 mM PMSF, pH 7.5) on ice, and centrifuged at 17,600 ×*g* for 1 h. The supernatant was removed and fractionated with 45%–85% ammonium sulfate. The 85% precipitate was suspended in buffer B (20 mM Tris, 1 mM EDTA, pH 8.0), dialyzed to the same buffer, concentrated on an Amicon XM50 membrane, centrifuged for 15 min at 48,000 ×*g*, and applied to a Reactive Yellow 3 column. Wash and elution with ATP were as described [30], and yielded a mix of NDPKs A and B estimated to be 88% pure by SDS-PAGE, with a specific activity of 1200 U/mg when tested by the coupled assay with 1 mM ATP and 0.2 mM TDP as substrates [30]. The purified preparation (eNDPK) was stored at −20 °C in PBS/50% glycerol.

2.2.5. Bacterial expression and purification of human NDPK A

Wild-type NDPK A was expressed in *E. coli* from a pET 21b vector generously provided by Dr. Marie-Lise Lacombe (INSERM U 402, Paris, France), as described [31]. Purification was by sequential chromatography in Q-Sepharose and Blue Sepharose as described by Lascu et al. [32]. After concentration and desalting the preparation was stored in liquid nitrogen. The purified protein (rNDPK A) was electrophoretically homogeneous and had a specific activity of 1600 U/mg.

2.2.6. Size exclusion chromatography

MT/Ves pellets were isolated, and incubated as described above in MEPS buffer with 20 μM taxol, with or without 1 mM GTP. After centrifugation at 16,000 ×*g* the supernatant was removed and applied

to a Superose 12 HR 10/30 (Amersham Biosciences) column developed with MEPS buffer at a flow rate of 0.4 ml/min and fractions of 1.0 ml were collected. The protein elution profile was monitored at 280 nm, and aliquots were assayed for NDPK activity. The samples were precipitated with trichloroacetic acid, dissolved in sample buffer and analyzed by SDS-PAGE followed by silver staining and/or immunoblotting. The column was calibrated with standards (IgG, BSA, β-lactoglobulin, cytochrome C and vitamin B12) according to the manufacturer's instructions. In earlier experiments we utilized a Sepharose 6 HR 10/30 column with similar results.

2.2.7. Lipid overlay assay

Nitrocellulose membranes pre-spotted with phospholipids (PIP strips™) were blocked for 1 h at 25 °C with 3% fatty-acid free bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBST). Blots were overlaid with 4 ml of 3.75 μg/ml purified rNDPK A in blocking solution, with or without 1 mM GTP, for 1 h at 4 °C. The membranes were washed 6 times for 5 min with TBST, and then incubated with primary antibody against NDPK for 1 h. After 4 washes, the blot was incubated with secondary antibody, washed 4 more times, and developed using enhanced chemiluminescence. The same procedure was followed with cytosol from NIH-3T3 cells.

2.2.8. Liposome binding assays

The lipid combinations used were (in molar ratios): DOPC:DOPS, DOPC:DOPE and DOPC:DOPA (80:20), and DOPC:DOPE:DOPS, DOPC:DOPE:DOPA (60:20:20). Small unilamellar vesicles were prepared by mixing the appropriate combination of lipid stock solutions in chloroform in a glass tube. The solvent was evaporated under a stream of nitrogen and the samples were further dried in a SpeedVac for 1 h. Lipids (1.8 μmol) were incubated for 1 h at room temperature in 90 μl of MEPS buffer with 400 mM sucrose, resuspended in a vortex mixer and sonicated on ice in a bath sonicator (Model G112SPIT, Laboratory Supply Company, Hicksville, NY) for 8–12 min. Prior to experiments, cytosol or purified proteins were diluted in MEPS with 100 mM sucrose and centrifuged in a Beckman Airfuge at 167,000 ×g for 10 min to remove any aggregates. Cytosol (1.5–1.8 mg/ml) or purified NDPK (0.8–5 μM) were incubated with liposomes (0.4 mg/ml) at 30 °C for 30 min. After centrifugation at 200,000 ×g for 30 min at 25 °C in a TLA 120.1 rotor, the supernatants were removed and the pellets were resuspended in MEPS buffer with or without 1 mM GTP. The samples were incubated for 30 min at 30 °C and pelleted as above. The supernatants and pellets were resolved by SDS-PAGE and immunoblotted. Lipid recovery was uniform, as established using carboxyfluorescein-labeled phosphatidylethanolamine (0.2 mol%) as a marker. All results shown are representative of 3–6 separate experiments.

3. Results

We have previously shown that nucleoside diphosphate kinase (NDPK) co-purifies with intracellular vesicles associated with taxol-stabilized microtubules (MT/Ves), but dissociates from this fraction upon incubation with 1 mM GTP or ATP [28]. Guanine nucleotide analogs also elute NDPK from membranes: at 1 mM, GTPγS and GDPβS are as effective as GTP, whereas GMP-PNP only induces a partial release. This order of efficacy is not the one expected from the binding site of GTP-binding proteins, where GTPγS and GMP-PNP act alike, and have opposite effects to those of GDPβS. Rather, it parallels the rank order of affinity of these compounds for NDPK, leading to the suggestion that the nucleotide binding site involved in the dissociation process is that of NDPK [28]. The simplest interpretation of this behavior is that binding of nucleotides to the active site triggers the dissociation of NDPK from its binding site in the vesicles. On the other hand, nucleotides often disrupt interactions between microtubules, motors and their membrane cargo [33–35]. We found that dynein and kinesin, as well as Rab4 and Lamp1, remained in the pellet after treatment with 1 mM GTP or ATP [28], suggesting that the association of motors plus early and late endosomal membranes with microtubules was intact. However, the elution of NDPK by nucleotides could be linked to the release of yet another component of the fraction.

3.1. NDPK is the main protein released from membranes by GTP

To determine whether 1 mM GTP dissociated other components concomitantly with NDPK, we washed MT/Ves pellets in MEPS buffer with or without 1 mM GTP, centrifuged the samples and examined the protein composition of the supernatants by SDS-PAGE followed by silver staining and immunoblotting. A prominent band migrating at the position expected for NDPK (~18 kDa) is present in the GTP extract, but not in the control (Fig. 1A, arrow). Aside from

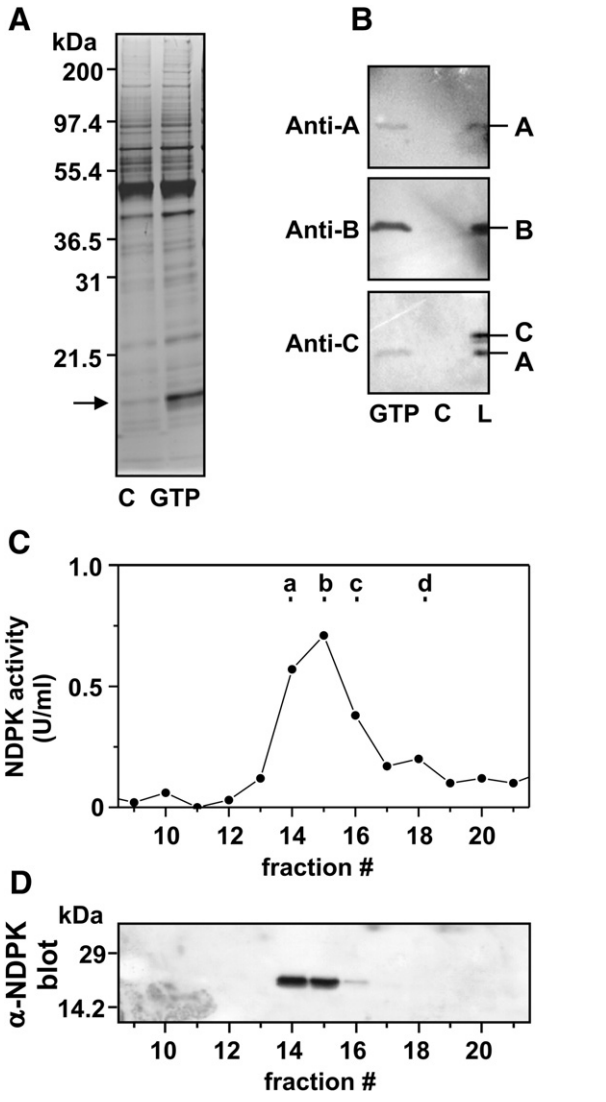

Fig. 1. NDPK-B is the major protein dissociated from membranes by GTP, and behaves as a hexamer, without associated proteins. (A) MT/Ves pellets were washed twice with MEPS (containing protease inhibitors and 20 μM taxol), and then incubated in the same buffer in the absence (Ctr) or presence of 1 mM GTP. After centrifugation for 30 min at 4 °C the supernatants were processed for SDS-PAGE, resolved in a 12% NuPage gel and stained with silver. (B) Immunoblot analysis of the control (C) and GTP extracts obtained as above, as well as whole cell lysates (L) from NIH-3T3 cells, with antibodies specific to NDPK-A (Anti-A), NDPK-B (Anti-B) and polyclonal antibodies to NDPK-C (Anti-C). (C) GTP extracts were obtained as above and fractionated on Superose 12 as described in Materials and methods. Fractions were assayed for NDPK activity. The elution positions of standards are indicated by (in kDa): a, 158; b, 67; c, 35, and d, 12.4. (D) Immunoblot of column fractions from C with antibodies to NDPK B (Anti-B). Results shown are representative of 5 independent experiments.

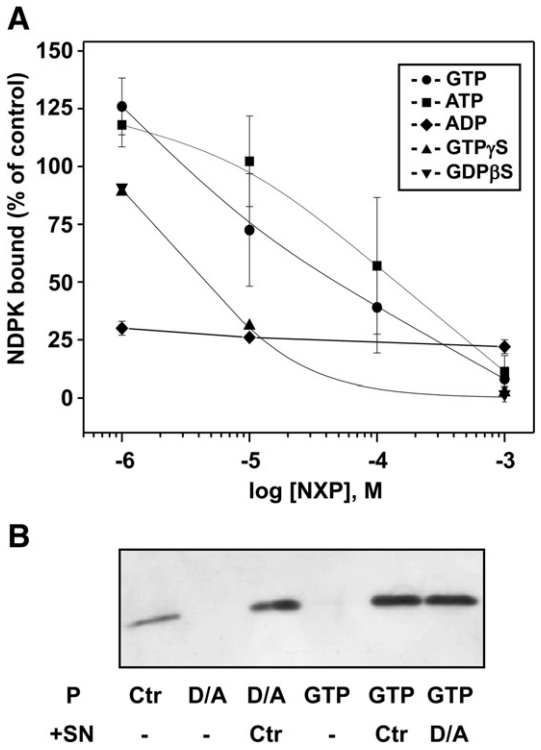

Fig. 2. The interaction of NDPK with membranes is controlled by its substrates, and is abolished by ATP depletion. (A) Nucleotides were present during taxol-induced polymerization of microtubules at the concentrations indicated. The amounts of NDPK in the resulting MT/Ves pellets were assessed as in Materials and methods. Values shown are ratios of the amounts of NDPK in nucleotide-treated samples to those measured in controls run in parallel with no added nucleotides, expressed as percent of control. n=2–3 for GTPγS and GDPβS, and 3–9 for ATP, GTP and ADP. Error bars indicate S.E. of means. (B) Cells were kept in low glucose medium (4 samples) or treated with deoxyglucose and azide (D/A; 2 samples) as in Materials and methods. Following polymerization with taxol and centrifugation, supernatants (Ctr SN, D/A SN) were removed and pellets were washed twice. Three Ctr samples were extracted with 1 mM GTP and pelleted (GTP pellet). Pellets were then resuspended as follows: one each of Ctr, D/A, and GTP pellets in MEPS buffer (−), one each of D/A and GTP pellets in Ctr SN, and one GTP pellet in D/A SN. After 30 min on ice, samples were centrifuged and the pellets were analyzed by SDS-PAGE followed by immunoblotting with antibodies to NDPK B (Anti-B). Results are representative of 3 experiments.

this band, the protein profiles of control and GTP extracts are essentially the same. Immunoblotting with antibodies to NDPKs A, B and C indicated that this band consists mostly of NDPK B (Fig. 1B), which is expressed at higher levels in murine tissues than other isoforms [16], as well as trace amounts of NDPK A. NDPK C is detectable only in the cell lysate.

3.2. NDPK is released as an active hexamer, without associated proteins

To confirm the identity of this protein as NDPK, the supernatants obtained in the presence of GTP were further analyzed by size-exclusion chromatography, and the NDPK content of individual fractions was measured using enzyme activity assays, SDS-PAGE and immunoblotting. As seen in Fig. 1C, the GTP extract contains NDPK activity that elutes in one peak centered at approximately 100 kDa, the size expected for a hexamer of 18 kDa subunits. The enzyme activity overlaps with a band identifiable as NDPK by immunoblotting (Fig. 1D). A similar analysis of control extracts shows no NDPK activity above basal levels and no reactivity with antibodies to NDPK in immunoblots (not shown). These experiments verify that the 18 kDa band seen in Fig. 1A is NDPK, and show that GTP releases enzymatically active NDPK, in its native, hexameric state. Moreover, the apparent molecular mass of 100 kDa demonstrates that NDPK is released alone, and not in a complex with other proteins.

We also performed immunoblot analyses of the MT/Ves pellet with antibodies against known NDPK binding partners, such as Arf6, dynamin, Rac1, and the von Hippel–Lindau protein. Only Rac1 and Arf6 were detected in the MT/Ves pellet, and neither was released upon nucleotide treatment (not shown). In addition, although [α-^{32}P]-GTP overlay assays of the MT/Ves fraction revealed the presence of several small GTP binding proteins, none were released by GTP treatment (not shown).

3.3. The binding of NDPK to intracellular vesicles is controlled by its catalytic cycle

GTP, ATP and the thiophosphate analogs GTPγS and GDPβS, which bind tightly to NDPKs [5] release similar amounts of NDPK from the MT/Ves fraction, whereas GMP-PNP, which binds with low affinity to NDPK, is far less effective [28]. This suggests that the nucleotide effects on the association of NDPK to membranes are intimately linked to its catalytic properties. To address this issue in depth, we determined the dose–response relationships for release of NDPK from the MT/Ves fraction by GTP, ATP, ADP, GTPγS and GDPβS.

As the concentration of GTP or ATP increases from 1 μM to 1 mM, the amounts of NDPK bound to the MT/Ves pellet in the presence of GTP or ATP decreases markedly (Fig. 2A). GTPγS and GDPβS inhibit binding in a dose-dependent manner and with significantly higher potency than ATP or GTP. In contrast, ADP decreases the amount of NDPK bound by 70–80% at all concentrations tested.

While the results obtained at high concentrations of nucleotides confirm our previous data on the dissociation of NDPK from membranes, examination of the effects of low concentrations of these compounds offers a new insight into the association process. Unexpectedly, the lowest concentration of GTP and ATP, 1 μM, consistently raised the amount of NDPK associated with MT/Ves to 120–125% of the control value. This increase is not observed with the GTP analog GTPγS which binds to NDPK with relatively high affinity, but is a poor substrate for phosphate transfer [5], and suggests that only nucleotides that transfer phosphate to NDPK can promote the association of NDPK with membranes. If so, reducing the concentration of E~P should promote dissociation. Indeed, ADP, which is an acceptor for the phosphate of E~P and thus dephosphorylates the enzyme, induces dissociation even at 1 μM. In contrast, at 1 μM, GDPβS, which is a poor phosphate acceptor, has little or no effect on the association of NDPK with membranes. Collectively, these data indicate that the association of NDPK with intracellular vesicles is a reversible process whose dynamics are controlled by the relative amounts of the phosphorylated intermediate, E~P, and the free enzyme, E.

If this reasoning is correct, a reduction in the [NTP]/[NDP] ratio in cells, which necessarily decreases the [E~P]/[E] ratio, should decrease significantly the amounts of NDPK bound to MT/Ves. To test this hypothesis, we assessed the levels of NDPK in MT/Ves fractions isolated from cells pre-incubated with the glycolysis inhibitor 2-deoxyglucose and the mitochondrial poison sodium azide (D/A), a treatment that markedly depletes cytosolic ATP (and consequently other NTPs), and reduces markedly the [ATP]/[ADP] ratio, as well as the levels of free ATP and GTP [36]. Fig. 2B shows that MT/Ves pellets prepared from cells pre-treated with D/A contain only traces of NDPK (7±5% of control, n=4), as would be expected if the association of NDPK to MT/Ves depended on the levels of ATP. There is, however, the possibility that the decrease in binding is due to a secondary effect of the D/A treatment, which could decrease the number of NDPK binding sites on the MT/Ves fraction or induce a decline in the actual amounts of cytosolic NDPK. To address this point, we performed several control experiments: in one, we incubated MT/Ves pellets obtained from D/A-treated cells with cytosol from the control cells, centrifuged the preparation and measured NDPK in the pellet. This same procedure was repeated using cytosol from D/A-treated cells, and from control

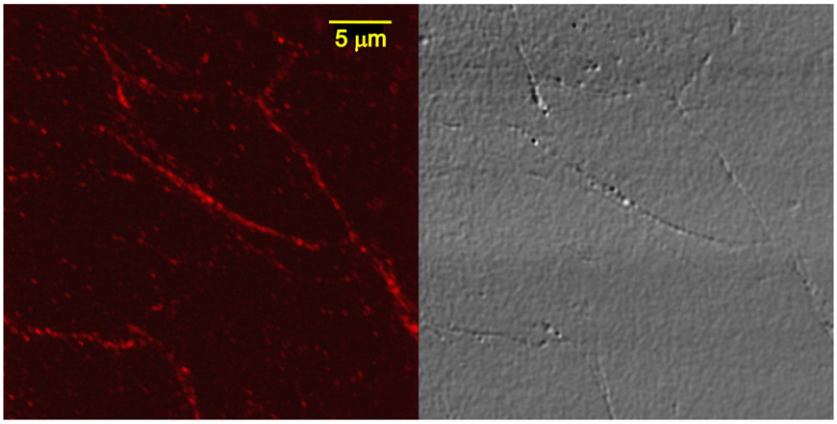

Fig. 3. NDPK localizes to most vesicular structures associated with microtubules. Samples were prepared as in Materials and methods; the antibody to NDPK was Ab-1. Left panel: Confocal immunofluorescence of MT/Ves pellets showing localization of NDPK (red) to many vesicular structures of varying sizes and shapes that are often distributed along linear tracks. Right panel: the corresponding DIC image demonstrates that most of these vesicles are associated with the underlying microtubular network. Comparison of the two panels reveals that nearly all the vesicles seen in the DIC image contain NDPK.

cells, which were incubated with MT/Ves pellets that had been stripped of NDPK with 1 mM GTP and therefore contain significant amounts of residual GTP. The results in Fig. 2B show that in all cases NDPK re-bound to the MT/Ves fraction. This set of experiments indicates that both MT/Ves membranes and cytosolic NDPK from D/A-treated cells retain their ability to associate, as long as the NTP concentration in the incubation mixture is at a minimum level. Therefore, the lower binding of NDPK to MT/Ves isolated from cells treated with D/A is likely to be the consequence of a lowered [ATP]/[ADP] ratio.

3.4. NDPK localizes to the majority of vesicular structures associated with MT

Our previous biochemical data indicate that in the MT/Ves fraction NDPK is not associated directly with microtubules [28], given that when the polymerization of tubulin with taxol was performed after membranes have been removed by centrifugation at 230,000 ×g, NDPK was absent from the microtubular pellet [28]. This lack of direct interaction between NDPK and microtubules is in agreement with observations by others [37,38]. Analysis of the taxol-stabilized MT/Ves fraction by confocal immunofluorescence and DIC confirms this idea. MT/Ves pellets isolated from NIH-3T3 cells were placed on polylysine-coated coverslips, and processed for indirect immunofluorescence using an antibody to NDPK. As shown in Fig. 3, NDPK localizes to numerous structures of varied size and shape, most of them organized in linear arrays along the microtubules that are clearly visible in the corresponding DIC image. Similar DIC images have been obtained with microtubules polymerized with taxol from purified tubulin [39,40]. The heterogeneity of the vesicles and their irregular distribution along the microtubules is typical of similar fractions obtained from hepatocytes [25,26] and 3T3-L1 adipocytes [27] by the same protocol utilized here. Thus, transmission electron microscopy of this fraction has revealed microtubules with many vesicles of variable size and appearance attached along their lengths [25,26]. Inspection of Fig. 3 also demonstrates that NDPK localizes to nearly all of the vesicular structures visible in the DIC image. The widespread distribution of NDPK seen in experiments like the one in Fig. 3 suggests that it binds to a universal component of intracellular membranes, namely lipids. This would agree with recent results showing that NDPKs A and B bind to acidic phospholipids [19, 24]. Thus, our next step was to determine whether nucleotides affect the direct association of NDPK with lipids.

3.5. NDPK binds to phospholipids in a nucleotide sensitive manner

In preliminary tests we used a protein lipid overlay assay. Membrane strips pre-spotted with different phospholipids were incubated with cytosol from NIH-3T3 cells, as well as purified NDPKs, and then probed with antibodies against NDPK. Fig. 4 illustrates the results obtained with rNDPK A, in the presence and absence of GTP. The enzyme displays a preference for PtdIns(5)P, PtdIns(3)P, PtdIns(4)P, PtdIns and PS. When GTP is added, the binding of rNDPK A to all these phospholipids is decreased, but the extent of the GTP-induced reduction varies from 66% (PS) to 35% (PtdIns(3)P) of the control value. The enzyme from cytosol also binds to PS and phosphoinositides, albeit with a slightly distinct selectivity (not shown). There was no detectable binding of murine or human NDPK to immobilized PC, PE, sphingosine phosphate or lysophospholipids. Although this type of assay does not necessarily reflect binding affinity or specificity in solution, and sometimes fails to detect all the phospholipids to which a protein actually binds [41], it is clear from

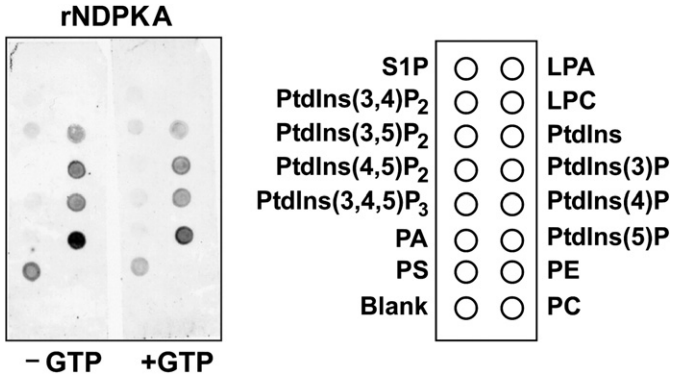

Fig. 4. rNDPK A binds to immobilized phospholipids in a GTP-sensitive manner. PIP strips™ were incubated with purified rNDPK A as described in Materials and methods, in the presence and absence of 1 mM GTP, and the bound protein was detected with antibodies to NDPK A (Anti-A). The location of each lipid is shown in the diagram on the right. Abbreviations are: S1P, sphingosine-1-phosphate; LPA, lysophosphatidic acid; PtdIns(3,4)P_2, phosphatidylinositol (3,4) bisphosphate; LPC, lysophosphocholine; PtdIns(3,5)P_2, phosphatidylinositol (3,5) bisphosphate; PtdIns, phosphatidylinositol; PtdIns(4,5)P_2, phosphatidylinositol (4,5) bisphosphate; PtdIns(3)P, phosphatidylinositol (3) phosphate; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5) trisphosphate; PtdIns(4)P, phosphatidylinositol (4) phosphate; PA, phosphatidic acid; PtdIns(5)P, phosphatidylinositol (5) phosphate; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

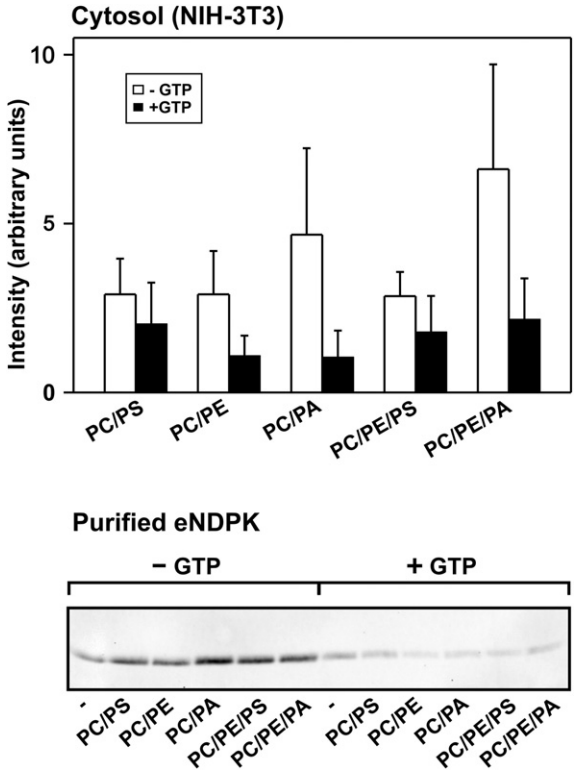

Fig. 5. Interaction of NDPK from cytosol and purified eNDPK with liposomes of defined composition. Liposomes were incubated with cytosol from NIH-3T3 cells (upper panel) or with purified eNDPK (lower panel) as described in Materials and methods, centrifuged, and washed with buffer alone or buffer containing 1 mM GTP. The bound protein was detected by SDS-PAGE and immunoblotting with Anti-nm23 H1/2/3. Abbreviations are: PC: dioleylphosphatidylcholine, PE: dioleylphosphatidylethanolamine, PS: dioleylphosphatidylserine and PA: 1,2-dioleoyl-*sn*-glycero-3-phosphate. The bar graph shows the average band intensity measured in 4 experiments, normalized to protein. Error bars are S. E. of means.

Fig. 4 that purified recombinant NDPK A can bind to pure phospholipids, as reported by Tokarska-Schlattner et al. [24], and does so in a GTP-sensitive manner.

We then examined how the nucleotide-sensitive binding of NDPK to pure lipids translated into a more physiological experimental system, that is, liposomes, using a sedimentation method [42]. Briefly, NDPK from cytosol was incubated with sucrose-loaded small unilamellar vesicles of defined phospholipid composition. The samples were pelleted by ultracentrifugation and the level of NDPK bound to the liposomes was measured as in Materials and methods. Fig. 5 shows that cytosolic NDPK from NIH-3T3 bound to liposomes of different compositions, showing a preference for PA-containing mixes. There was negligible binding to liposomes made of pure PC (not shown). This result is in agreement with the finding that purified NDPK A and B prefer anionic to neutral phospholipids [19,24]. More importantly, in the presence of 1 mM GTP (or ATP), the levels of cytosolic NDPK associated with liposomes were noticeably reduced. Similar results were obtained with purified eNDPK (Fig. 5, lower panel) and rNDPK A (not shown): the purified proteins bound to several combinations of phospholipids, and the binding was decreased by 1 mM GTP or ATP. Note that the last step of the purification procedures for both rNDPK A and eNDPK involves elution of the enzyme from a dye column with ATP; as a result, both preparations are likely to be partially phosphorylated [43] and therefore able to associate with liposomes in the absence of added NTP.

Taken together, these findings imply that the mechanism of binding to the liposomes and to the MT/Ves isolated from cells is essentially the same. Furthermore, the results shown in Figs. 4 and 5 (lower panel) demonstrate conclusively that the nucleotide-sensitive binding of NDPK to intracellular membranes can be reconstituted with a minimal set of components, namely purified enzyme and lipids.

4. Discussion

Our results confirm recent work [19,24] showing that soluble NDPKs can associate with membranes by binding directly to lipids. The interaction does not require additional proteins, although it is conceivable that it might be strengthened by additional interactions with other membrane components. Also, the data suggest that the form with highest affinity for lipids is the phosphoenzyme. Therefore, the equilibrium between membrane-bound and soluble NDPK will be determined by the availability of the phosphorylated enzyme.

The equilibrium constant of reaction (a),

$$[E{\sim}P][NDP] \rightleftharpoons [E][NTP]$$

lies in the range of 0.15 to 0.5 for NDPKs of various species (reviewed in [44]). The concentration of ATP in vivo exceeds those of other NTPs, so [NTP]/[NDP] is approximately equal to the [ATP]/[ADP] ratio, which in most cells, including NIH-3T3 fibroblasts [45], is around 10. Under these conditions, the ratio [E~P]/[E] is 1.5–5, allowing the binding of NDPK to membranes.

Because of the ping-pong mechanism of NDPK (Fig. 6), NDPs not only dephosphorylate E~P, but also bind to the free enzyme, E, acting as dead end inhibitors [3]. In either case, if E~P is the form that associates with membranes, NDPs, in this case ADP, should inhibit binding at all concentrations, either by removing free enzyme available for phosphorylation, or by dephosphorylating E~P, and this is what is seen in Fig. 2.

NDPK's K_m for ATP is 0.3 mM, one order of magnitude lower than the K_i for dead end inhibition, 3 mM [3]. Thus, the binding of NTPs to E~P forming abortive complexes (E~P·NTP) (Fig. 6) is only observed at

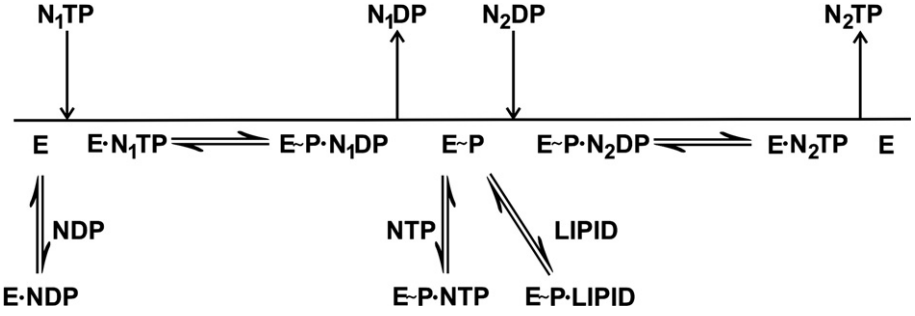

Fig. 6. Scheme of NDPK-catalyzed phosphate transfer in the presence of phospholipids (Cleland notation). E: free enzyme; E·N_1TP: enzyme–substrate (nucleoside triphosphate$_1$) complex; E~P·N_1DP: phosphorylated enzyme–product (nucleoside diphosphate$_1$) complex; E~P: phosphorylated enzyme intermediate; E~P·N_2DP: phosphorylated enzyme–substrate (nucleoside diphosphate$_2$) complex; E·N_2TP: enzyme–substrate (nucleoside triphosphate$_2$) complex; E·NDP, E~P·NTP: dead-end complexes formed at high concentrations of NDP or NTP; E~P·Lipid: proposed phosphorylated enzyme–phospholipid complex.

very high concentrations of NTP, and in the absence of significant amounts of NDPs. This explains the progressive reduction in the amounts of NDPK associated with membranes as the concentration of ATP (and GTP) is raised. The marked difference in the behavior of phosphorylating (ATP, GTP) and non-phosphorylating (GTPγS) nucleotides is a result of the balance between phosphorylation and inhibition, which is significant for the natural nucleotides, but is heavily biased towards inhibition in the case of the thio-modified nucleotide.

Two critical questions remain: where does the lipid bind, and how is the catalytic cycle of NDPK coupled to the association and dissociation from membranes? The 3D structures of hexameric NDPK show a symmetrical arrangement that can be described as two identical trimers superimposed horizontally, forming a disk with a diameter of 70Å and a thickness of 50Å (reviewed in [46]). There is a single nucleotide-binding site per subunit, and the top and bottom surfaces of the disk contain three active sites each. The binding site lies in a crevice lined with basic residues, between a loop and a hairpin formed by helices αA and α2. Preliminary studies using in silico docking indicate that this site might accomodate a phospholipid such as PA. In this aspect NDPK is reminiscent of the phosphatidylethanolamine-binding protein (PEBP), a 23 kDa basic protein that binds phospholipids as well as GTP [47]. Similarly to NDPK, in the crystal structure of PEBP the ligand binding site lies in a narrow crevice near the surface, proximal to a strip of basic residues. Like NDPK, the bovine PEBP structure is not related to other nucleotide binding proteins, or to known lipid-binding proteins. Notably, the loop connecting helices αA and α2 contains the conserved basic residues K56 and R58, newly identified as essential for the binding of NDPK B to acidic phospholipids [19]. A previous, groundbreaking report by Epand et al. [48] demonstrated novel properties of the mitochondrial NDPK isoform, NDPK D (or NM23-H4), namely the ability to cross-link bilayers and transfer lipids between them, provided that cardiolipin is present. These observations were recently expanded by Tokarska-Schlattner et al. [24], who demonstrated that NDPK-D binds strongly to other acidic phospholipids as well, and that residue R90 (D57 in NDPKs A and B) from the same loop is essential for the binding of NDPK-D to artificial and native membranes. Additionally, results from Orlov et al. [21] are consistent with an involvement of the αA-α2 hairpin in the association process. Briefly, a synthetic peptide with a sequence that encompasses helix αA dissociates NDPK B from rod outer segment membranes, whereas peptides homologous to other portions of the protein have no effect.

There are no detectable differences between the 3D structures of the phosphorylated NDPK and the free enzyme [49], but binding of nucleotides to the active site results in a small conformational change, where the αA-α2 hairpin moves 2Å and locks the nucleotide in place. This suggests a mechanism by which the conformational change associated with nucleotide binding could trigger dissociation of NDPK from bilayers: in E~P, the nucleotide binding site is in the "open" conformation, allowing its binding to phospholipids, while occupation of the site, followed or not by phosphate transfer to NDP, closes the cleft and releases the enzyme from the lipid. Soluble NDPK is eventually rephosphorylated by NTP, and is again capable of associating with membrane lipids.

In conclusion, our present studies suggest that in vivo the high [ATP]/[ADP] ratio promotes phosphorylation of NDPK and its association with membranes, where it could transfer phosphate to locally generated NDPs. Schneider et al. [50] suggested that because of its high concentration in the cytosol and the expected prevalence of its phosphorylated state at normal [ATP]/[ADP] ratios, soluble NDPKs could serve as a cellular reservoir of high energy phosphate. Targeting of such a reservoir to membranes may be relevant to the operation of the pathways of phospholipid synthesis, which require CTP (reviewed in [51]), as well as the multiple GTPases that control intracellular membrane transport.

Acknowledgements

We thank Dr. Ioan Lascu and Dr. Marie-Lise Lacombe for generously sharing constructs and antibodies. We are grateful to Dr. Avril Somlyo and Dr. Ruoya Ho for supplying their expertise and equipment for confocal immunofluorescence and DIC imaging. We are indebted to Dr. Peter Horanyi for the in silico docking work. This work was supported by DHHS grants GM57833 to A.O. and GM62762 to G.S.

References

[1] R.E. Parks, R.P. Agarwal, Nucleoside diphosphokinases, in: P.D. Boyer (Ed.), The Enzymes, Vol. VIII, Academic Press, N.Y., 1973, pp. 307–333.
[2] M.L. Lacombe, L. Milon, A. Munier, J.G. Mehus, D.O. Lambeth, The human Nm23/nucleoside diphosphate kinases, J. Bioenerg. Biomembr. 32 (2000) 247–258.
[3] E. Garces, W.W. Cleland, Kinetic studies of yeast nucleoside diphosphate kinase, Biochemistry 7 (1969) 633–640.
[4] A.M. Gilles, E. Presecan, A. Vonica, I. Lascu, Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme, J. Biol. Chem. 266 (1991) 8784–8789.
[5] S. Schaertl, M. Konrad, M.A. Geeves, Substrate specificity of human nucleoside-diphosphate kinase revealed by transient kinetic analysis, J. Biol. Chem. 273 (1998) 5662–5669.
[6] P.A. Webb, O. Perisic, C.E. Mendola, J.M. Backer, R.L. Williams, The crystal structure of a human nucleoside diphosphate kinase, NM23-H2, J. Mol. Biol. 251 (1995) 574–587.
[7] Y. Chen, S. Gallois-Montbrun, B. Schneider, M. Véron, S. Moréra, D. Deville-Bonne, J. Janin, Nucleotide binding to nucleoside diphosphate kinases: X-ray structure of human NDPK-A in complex with ADP and comparison to protein kinases, J. Mol. Biol. 332 (2003) 915–926.
[8] N. Kimura, N. Shimada, Membrane-associated nucleoside diphosphate kinase from rat liver. Purification, characterization, and comparison with cytosolic enzyme, J. Biol. Chem. 263 (1988) 4647–4653.
[9] S. Srivastava, Z. Li, K. Ko, P. Choudhury, M. Albaqumi, A.K. Johnson, Y. Yan, J.M. Backer, D. Unutmaz, W.A. Coetzee, E.Y. Skolnik, Histidine phosphorylation of the potassium channel KCa3.1 by nucleoside diphosphate kinase B is required for activation of KCa3.1 and CD4 T cells, Mol. Cell 24 (2006) 665–675.
[10] M.D. Rochdi, G. Laroche, E. Dupre, P. Giguere, A. Lebel, V. Watier, E. Hamelin, M.C. Lepine, G. Dupuis, J.-L. Parent, Nm23-H2 Interacts with a G protein-coupled receptor to regulate its endocytosis through a Rac1-dependent mechanism, J. Biol. Chem. 279 (2004) 18981–18989.
[11] A. Mizrahi, S. Molshanski-Mor, C. Weinbaum, Y. Zheng, M. Hirshberg, E. Pick, Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase, J. Biol. Chem. 280 (2005) 3802–3811.
[12] K.S. Krishnan, R. Rikhy, S. Rao, M. Shivalkar, M. Mosko, R. Narayanan, P. Etter, P.S. Estes, M. Ramaswami, Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling, Neuron 30 (2001) 197–210.
[13] J.K. Palacios, J.K. Schweitzer, R.L. Boshans, C. Souza-Schorey, ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly, Nat. Cell Biol. 4 (2002) 929–936.
[14] T. Hsu, Y. Adereth, N. Kose, V. Dammai, Endocytic function of von Hippel–Lindau tumor suppressor protein regulates surface localization of fibroblast growth factor receptor 1 and cell motility, J. Biol. Chem. 281 (2006) 12069–12080.
[15] H.N. Fournier, S. Dupe-Manet, D. Bouvard, M.L. Lacombe, C. Marie, M.R. Block, C. Albiges-Rizo, ICAP-1alpha interacts directly with the metastasis suppressor nm23-H2 and both proteins are targeted to newly formed cell adhesion sites upon integrin engagement, J. Biol. Chem. 277 (2002) 20895–20902.
[16] P. Barraud, L. Amrein, E. Dobremez, S. Dabernat, K. Masse, M. Larou, J.Y. Daniel, M. Landry, Differential expression of nm23 genes in adult mouse dorsal root ganglia, J. Comp. Neurol. 444 (2002) 306–323.
[17] Z. Fan, P.J. Beresford, D.Y. Oh, D. Zhang, J. Lieberman, Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor, Cell 112 (2003) 659–672.
[18] L. Kapetanovich, C. Baughman, T.H. Lee, Nm23H2 facilitates coat protein complex II assembly and endoplasmic reticulum export in mammalian cells, Mol. Biol. Cell 16 (2005) 835–848.
[19] C. Baughman, J. Morin-Leisk, T. Lee, Nucleoside diphosphate kinase B (NDKB) scaffolds endoplasmic reticulum membranes in vitro, Exp. Cell Res. 314 (2008) 2702–2714.
[20] J. Garin, R. Diez, S. Kieffer, J.F. Dermine, S. Duclos, E. Gagnon, R. Sadoul, C. Rondeau, M. Desjardins, The phagosome proteome: insight into phagosome functions, J. Cell Biol. 152 (2001) 165–180.
[21] N.Y. Orlov, T.G. Orlova, K. Nomura, N. Hanai, N. Kimura, Transducin-mediated, isoform-specific interaction of recombinant rat nucleoside diphosphate kinases with bleached bovine retinal rod outer segment membranes, FEBS Lett. 389 (1996) 186–190.
[22] N.Y. Orlov, N. Kimura, Interaction of nucleoside diphosphate kinase with membranes of bleached bovine retinal rod outer segments. Effects of pH, salts, and guanine nucleotides, Biochemistry (Mosc.) 63 (1998) 171–179.

[23] T. Wieland, Interaction of nucleoside diphosphate kinase B with heterotrimeric G protein betagamma dimers: consequences on G protein activation and stability, Naunyn-Schmiedebergs Arch. Pharmacol. 374 (2007) 373–383.
[24] M. Tokarska-Schlattner, M. Boissan, A. Munier, C. Borot, C. Mailleau, O. Speer, U. Schlattner, M.L. Lacombe, The nucleoside diphosphate kinase D (NM23-H4) binds the inner mitochondrial membrane with high affinity to cardiolipin and couples nucleotide transfer with respiration, J. Biol. Chem. 283 (2008) 26198–26207.
[25] J.S. Goltz, A.W. Wolkoff, P.M. Novikoff, R.J. Stockert, P. Satir, A role for microtubules in sorting endocytic vesicles in rat hepatocytes, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7026–7030.
[26] C.J. Soroka, M.K. Pate, J.L. Boyer, Canalicular export pumps traffic with polymeric immunoglobulin A receptor on the same microtubule-associated vesicle in rat liver, J. Biol. Chem. 274 (1999) 26416–26424.
[27] A.L. Olson, A.R. Trumbly, G.V. Gibson, Insulin-mediated GLUT4 translocation is dependent on the microtubule network, J. Biol. Chem. 276 (2001) 10706–10714.
[28] B.C. Gallagher, K.A. Parrott, G. Szabo, A.S. Otero, Receptor activation regulates cortical, but not vesicular localization of NDP kinase, J. Cell Sci. 116 (2003) 3239–3250.
[29] V.P. Pinon, A. Munier, I. Lascu, A.S. Lajarte-Thirouard, S. Prévot, J. Capeau, M.L. Lacombe, Preparation of specific polyclonal antibodies against protein isoforms: application to nucleoside diphosphate kinase A, Sci. Tools Amersham Pharmacia Biotech. 3 (1998) 10–11.
[30] X.B. Yi, N.S. Sweitzer, A.S. Otero, Neutralizing antibodies to nucleoside diphosphate kinase inhibit the enzyme in vitro and in vivo. Evidence for two distinct mechanisms of activation of atrial currents by ATPγS, Biochim. Biophys. Acta 1310 (1996) 334–342.
[31] L. Milon, P. Meyer, M. Chiadmi, A. Munier, M. Johansson, A. Karlsson, I. Lascu, J. Capeau, J. Janin, M.L. Lacombe, The human nm23-H4 gene product is a mitochondrial nucleoside diphosphate kinase, J. Biol. Chem. 275 (2000) 14264–14272.
[32] I. Lascu, S. Schaertl, C. Wang, C. Sarger, A. Giartosio, G. Briand, M.L. Lacombe, M. Konrad, A point mutation of human nucleoside diphosphate kinase A found in aggressive neuroblastoma affects protein folding, J. Biol. Chem. 272 (1997) 15599–15602.
[33] J. Scheel, T.E. Kreis, Motor protein-independent binding of endocytic carrier vesicles to microtubules in vitro, J. Biol. Chem. 266 (1991) 18141–18148.
[34] H. Oda, R.J. Stockert, C. Collins, H. Wang, P.M. Novikoff, P. Satir, A.W. Wolkoff, Interaction of the microtubule cytoskeleton with endocytic vesicles and cytoplasmic dynein in cultured rat hepatocytes, J. Biol. Chem. 270 (1995) 15242–15249.
[35] F. Rajas, V. Gire, B. Rousseau, Involvement of a membrane-bound form of glutamate dehydrogenase in the association of lysosomes to microtubules, J. Biol. Chem. 271 (1996) 29882–29890.
[36] E.D. Schwoebel, T.H. Ho, M.S. Moore, The mechanism of inhibition of Ran-dependent nuclear transport by cellular ATP depletion, J. Cell Biol. 157 (2002) 963–974.
[37] K. Islam, R.G. Burns, Microtubules and nucleoside diphosphate kinase. Nucleoside diphosphate kinase binds to co-purifying contaminants rather than to microtubule proteins, Biochem. J. 232 (1985) 651–656.
[38] R. Melki, I. Lascu, M.F. Carlier, M. Véron, Nucleoside diphosphate kinase does not directly interact with tubulin nor microtubules, Biochem. Biophys. Res. Commun. 187 (1992) 65–72.
[39] T.A. Schroer, B.J. Schnapp, T.S. Reese, M.P. Sheetz, The role of kinesin and other soluble factors in organelle movement along microtubules, J. Cell Biol. 107 (1988) 1785–1792.
[40] A.T. Fullerton, M.Y. Bau, P.A. Conrad, G.S. Bloom, In vitro reconstitution of microtubule plus end-directed, GTPgammaS-sensitive motility of Golgi membranes, Mol. Biol. Cell 9 (1998) 2699–2714.
[41] E. Jo, J. McLaurin, C.M. Yip, P. St George-Hyslop, P.E. Fraser, alpha-Synuclein membrane interactions and lipid specificity, J. Biol Chem. 275 (2000) 34328–34334.
[42] K. Robbe, B. Antonny, Liposomes in the study of GDP/GTP cycle of Arf and related small G proteins, Methods Enzymol. 372 (2003) 151–166.
[43] J.B. Robinson, D.N. Brems, E. Stellwagen, A monoisozymic nucleoside diphosphate kinase capable of complete phosphorylation, J. Biol. Chem. 256 (1981) 10769–10773.
[44] I. Lascu, P. Gonin, The catalytic mechanism of nucleoside diphosphate kinases, J. Bioenerg. Biomembr. 32 (2000) 237–246.
[45] T. Takahashi, K. Matsushita, M.J. Welsh, J.B. Stokes, Effect of cAMP on intracellular and extracellular ATP content of C1-secreting epithelia and 3T3 fibroblasts, J. Biol. Chem. 269 (1994) 17853–17857.
[46] J. Janin, C. Dumas, Y. Xu, P. Meyer, M. Chiadmi, J. Cherfils, Three-dimensional structure of nucleoside diphosphate kinase, J. Bioenerg. Biomembr. 32 (2000) 215–225.
[47] L. Serre, B. Vallée, N. Bureaud, F. Schoentgen, C. Zelwer, Crystal structure of the phosphatidylethanolamine-binding protein from bovine brain: a novel structural class of phospholipid-binding proteins, Structure 6 (1998) 1255–1265.
[48] R.F. Epand, U. Schlattner, T. Wallimann, M.L. Lacombe, R.M. Epand, Novel lipid transfer property of two mitochondrial proteins that bridge the inner and outer membranes, Biophys. J. 92 (2007) 126–137.
[49] S. Moréra, M. Chiadmi, G. LeBras, I. Lascu, J. Janin, Mechanism of phosphate transfer by nucleoside diphosphate kinase: X-ray structures of a phosphohistidine form of the enzymes from *Drosophila* and *Dictyostelium*, Biochemistry 34 (1995) 11062–11070.
[50] B. Schneider, A. Norda, A. Karlsson, M. Véron, D. Deville-Bonne, Nucleotide affinity for a stable phosphorylated intermediate of nucleoside diphosphate kinase, Protein Sci. 11 (2002) 1648–1656.
[51] D.R. Voelker, Interorganelle transport of aminoglycerophospholipids, Biochim. Biophys. Acta 1486 (2000) 97–107.