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Receptor activation regulates cortical, but not vesicular localization of NDP kinase

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Summary

We used immunofluorescence techniques to determine the localization of nucleoside diphosphate (NDP) kinase in NIH-3T3 fibroblasts. We found that cytoplasmic NDP kinase can be separated into two populations according to subcellular localization and response to extracellular stimuli. Specifically, within minutes of stimulation of resting fibroblasts with serum, growth factors or bombesin, a portion of NDP kinase becomes associated with membrane ruffles and lamellipodia. Another pool of NDP kinase accumulates independently of stimulation around intracellular vesicles. Transfection of cells with activated Rac mimics, whereas expression of dominant negative Rac inhibits, the effects of extracellular stimulation on the translocation of NDP kinase to the cell cortex. Neither Rac mutant affects the vesicle-associated pool. Association of NDP kinase with vesicles depends on microtubule integrity

and is disrupted by nocodazole. In cell-free assays NDP kinase binds tightly to membrane vesicles associated with taxol-stabilized microtubules. Binding of NDP kinase to this fraction is reduced by ATP and abolished by GTP, as well as guanine nucleotides that are NDP kinase substrates. Thus, the localization of the two NDP kinase pools identified here is regulated independently by distinct cellular components: the appearance of cortical NDP kinase is a consequence of Rac activation, whereas vesicular NDP kinase is responsive to microtubule dynamics and nucleotides, in particular GTP. These results suggest that in fibroblasts NDP kinase participates in Rac-related cortical events and in GTP-dependent processes linked to intracellular vesicle trafficking.

Key words: NDP kinase, NM23, Microtubules, Rac

Introduction

The NM23/NDP kinase gene family encodes a group of eight homologous proteins with conserved structure. In higher organisms NDP kinases A and B are the most abundant members of the group and they are the best characterized to date (Lacombe et al., 2000). The bulk of mammalian NDP kinase A and B are cytosolic and form stable heterohexamers that can only be resolved into homogeneous preparations of NDP kinase A and B polypeptide chains after denaturation (Gilles et al., 1991). Using ATP as a phosphate donor for the synthesis of other nucleotides such as GTP, UTP and CTP, NDP kinases play an important role in the synthesis of nucleic acids and proteins, as well as in the metabolism of sugars and lipids (Lacombe et al., 2000). In recent years, it has become apparent that changes in the expression levels or modifications in the structure of NDP kinases alter functions as diverse as development, cell migration and differentiation in unexpected ways, leading to the suggestion that NDP kinases are multifunctional proteins (Otero, 2000; Kimura et al., 2000; Postel et al., 2002). For instance, members of this family were reported to inhibit growth-factor-induced cell motility of breast cancer cells (Kantor et al., 1993), attenuate the desensitization of muscarinic-activated atrial K⁺ channels (Xu et al., 1996; Otero et al., 1999) and regulate synaptic vesicle internalization in *Drosophila* (Krishnan et al., 2001).

At the molecular level, the finding that NM23-H1 (human NDP kinase A) interacts with Tiam1, a Rac guanine nucleotide

exchange factor, and downregulates Tiam1-Rac1 signaling, implied that it could affect remodeling of the actin cytoskeleton (Otsuki et al., 2001). More recently, Palacios et al. demonstrated that constitutively activated ARF6 binds NM23-H1 and recruits NM23-H1 to cell junctions (Palacios et al., 2002). The presence of NM23-H1 at these sites facilitates dynamin-dependent endocytosis and downregulates Rac1 activity. Additionally, NM23-H2 (human NDP kinase B) was found to be linked to β -integrins through integrin cytoplasmic domain associated protein 1- α (ICAP1- α) (Fournier et al., 2002), which inhibits activation of Rac1 and Cdc42 GTPases during integrin-mediated cell adhesion (Degani et al., 2002). Taken together, these reports hint at the multiple ways by which NDP kinases A and B could affect cell adhesion, signaling and motility. One important question is whether activation of surface receptors that trigger signal transduction pathways can modulate the subcellular distribution of the essentially cytosolic NDP kinases A and B in a manner that is spatially and temporally consistent with a role in signaling. In the present study, we examined the ability of NDP kinases A and B to respond to activation of receptor tyrosine kinases and G-protein-coupled receptors (GPCRs) by monitoring their spatial distribution in NIH-3T3 fibroblasts. These experiments allowed us to identify two distinct pools of NDP kinase in these cells: one population is rapidly translocated to the cell periphery when receptors are activated, whereas a second pool binds constitutively to microtubule-associated vesicles.

Vesicular NDP kinase is released by nucleotides, with GTP being more efficient than ATP; this suggests that NDP kinase might associate with intracellular vesicles when GTP levels are low, in order to provide the substrate used by the many GTPases that control intracellular trafficking.

Materials and Methods

Materials

Epidermal growth factor (EGF) and nucleotides were from Roche Molecular Biochemicals. Rhodamine-labeled phalloidin, Taxol, nocodazole, bombesin, 2,3-butanedione monoxime (BDM) and platelet-derived growth factor (PDGF) were from Sigma-Aldrich. Texas Red dextran (lysine-fixable, 70,000 M_r) was from Molecular Probes. The expression plasmid for N-terminal 3 \times -hemagglutinin (HA)-tagged human Rac1 (G12V and T17N mutants) was from the Guthrie cDNA Resource Center (Sayre, PA); the green fluorescent protein (GFP) vector pGreen Lantern was from Life Technologies. Gradient SDS-PAGE gels were from BioRad.

The monoclonal antibody specific for NDP kinase A (NM301) was from Santa Cruz Biotechnology; this antibody does not recognize NDP kinase B. Immunofluorescence staining of NDP kinase was also performed with a polyclonal antibody, Ab-1 (Labvision, Fremont, CA), raised to a homologous inner region of human NDP kinases A and B (amino acids 86-102). This antibody reacts with two proteins with the relative mobility of NDP kinase A and B in immunoblots of lysates from NIH-3T3 cells; no cross-reactivity with other proteins was detected (not shown). Although Ab-1 yielded similar results to NM301, it often stained cell nuclei, in agreement with the finding that NDP kinase B can localize to the nucleus (Kraeft et al., 1996; Pinon et al., 1999; Barraud et al., 2002). Like other rodent cells (Kimura et al., 2000; Barraud et al., 2002), NIH-3T3 fibroblasts express much higher levels of NDP kinase B than NDP kinase A (not shown). Therefore, the signal obtained with Ab-1 is presumably dominated by the B isoform. NDP kinase was detected in immunoblots with a rabbit polyclonal antibody that recognizes mammalian NDP kinases A and B (a generous gift from I. Lascu, Université de Bordeaux). Other antibodies used were: polyclonal anti-Rab4 (StressGen), polyclonal anti-Tiam1 (Santa Cruz), monoclonal anti-Rac (clone 23A8, Upstate Biotechnology) monoclonal anti-HA epitope tag (12CA5, Exalpha), monoclonals anti-LAMP-1, anti-kinesin heavy chain and Na⁺,K⁺-ATPase (1D4B, SUK4 and α 5, respectively, from the Developmental Studies Hybridoma Bank, Iowa) and monoclonal anti- α -tubulin (clone DM1A, Sigma-Aldrich). Texas Red-transferrin and all secondary antibodies (Texas Red or Cy2 conjugates, multiple labeling grade) were from Jackson Immunoresearch Laboratories. Specificity of immunofluorescence labeling was established by pre-incubation with excess antigen, which effectively blocked cell staining. The secondary antibodies did not stain cells in the absence of primary antibodies.

Cell culture and transfection

NIH-3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U l⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were subcultured at 50-70% confluence; assays were performed on cultures derived from the same stock, between passages 2 and 8. Transfections were performed using LipofectAMINE™ 2000 as recommended by the manufacturer.

Immunofluorescence

Cells were grown on No. 1 thickness acid-washed glass coverslips. For experiments, cells 30-40% confluent were placed in serum-free medium for 16-24 hours and then treated with 10 ng ml⁻¹ EGF, 10

nM bombesin or 10% serum for the times specified in the figure legends. Treatment with 30 mM BDM was performed in serum-free medium supplemented with 0.5% bovine serum albumin (BSA) and 10 mM glucose; cells were then stimulated with EGF for 2 minutes.

Cells were fixed for 10 minutes at room temperature in 4% paraformaldehyde/0.1% glutaraldehyde. Free aldehyde groups were quenched with NaBH₄, and cells were then stained as described (Pinon et al., 1999). Briefly, after a wash with PBS, cells were transferred to a blocking solution composed of 0.1% saponin and 3% BSA in PBS. This same buffer was used to dilute primary and secondary antibodies. Coverslips were incubated overnight with primary antibodies at 4°C, washed extensively with PBS and incubated for 45 minutes at room temperature with secondary antibodies. After washes with PBS, coverslips were mounted in Mowiol (Calbiochem) containing 2% *n*-propylgallate. To stain F-actin, rhodamine-conjugated phalloidin (0.05 ng ml⁻¹) was added to the secondary antibody solution. The coverslips were examined on a Nikon Diaphot microscope equipped with a 40 \times oil immersion objective. Digital images were obtained with a Nikon CoolPix 990 camera. The phase-contrast images of the immunolabeled cells were routinely collected to confirm the identification of specific cell features. Figures were assembled using Adobe Photoshop software. Results shown are representative of 4-25 independent experiments.

For fluorescence ratio imaging, NIH-3T3 cells transfected with GFP were fixed and immunostained with NM301 followed by Texas-Red-conjugated anti-mouse IgG. Paired images of GFP and Texas Red fluorescence were acquired under conditions designed to avoid pixel saturation, inspected to verify alignment and saved as TIFF (8-bit) files. Ratio images (NDP kinase A/GFP) were obtained with Scion Image (Scion). Line profiles obtained with Maxim DL 2.12 (Diffraction Limited) were used for quantitative analysis of fluorescence values in digitized images.

Measurements of the diameters of NDP-kinase-labeled vesicles were performed in images of cells immunolabeled with Ab-1 or NM301. Histograms were constructed using a bin size of 0.5 μ m.

Subcellular fractionation

Membrane preparation

Isolation of the particulate fraction from quiescent and serum stimulated cells was performed by the method of Del Pozo et al. (Del Pozo et al., 2002) with slight modifications. Briefly, serum-deprived cells in 10 cm dishes were treated with medium containing 0% or 10% serum for 10 minutes, rinsed with PBS and incubated in ice-cold lysis buffer (10 mM Tris, pH 7.4 with HCl, 1.5 mM MgCl₂, 5 mM KCl, 1 mM DTT, 0.2 mM sodium vanadate, 1 mM PMSF, 1 μ g ml⁻¹ each aprotinin and leupeptin) for 5 minutes. Cells were scraped, homogenized by 15 passes in a Dounce homogenizer and the lysates were centrifuged at 700 *g* for 3 minutes. The supernatants were spun for 15 minutes at 167,000 *g* in a Beckman Airfuge; the cytosolic fraction was removed, the membrane pellet was washed once with lysis buffer. Samples containing equal amounts of protein were solubilized in SDS-PAGE sample buffer with 20 mM DTT, alkylated with 60 mM iodoacetamide and resolved in 15% or 4-20% minigels. Proteins were transferred to nitrocellulose and immunoblotted with antibodies to Rac and NM23 as described previously (Otero, 1997). Immunoblotting with the plasma membrane marker Na⁺,K⁺-ATPase was used to verify equal loading of control and serum-treated samples. Results are expressed as the proportion of the protein detected in control membrane fractions. Proteins were detected by chemiluminescence and quantified using Scion Image.

Isolation of microtubule-associated endocytic vesicles and proteins

Endocytic vesicles associated with endogenous microtubules were

isolated essentially as described by Wolkoff and colleagues (Goltz et al., 1992; Oda et al., 1995). Briefly, 80% confluent cells in 15 cm plates were washed with PBS, scraped into 0.6 ml MEPS buffer (35 mM PIPES pH 7.1 with NaOH, 5 mM MgSO₄, 5 mM EGTA, 200 mM sucrose, 1 mM DTT) containing protease inhibitors (2 mM PMSF, 1 mM benzamide, 2 µg ml⁻¹ leupeptin) and homogenized by 10-14 passes through a 27G needle. Homogenates were centrifuged at 1000 *g* for 10 minutes to sediment nuclei and large debris. The post-nuclear supernatant was centrifuged at 40,000 *g* for 20 minutes. The supernatant (small vesicles and cytosol) was incubated at 37°C for 30 minutes with 20 µM Taxol (and nucleotides when indicated) to polymerize tubulin. The resulting microtubules and associated vesicles were pelleted at 16,000 *g* for 30 minutes at 4°C. The pellets were brought to the original volume with MEPS, taxol was added and the samples were centrifuged at 16,000 *g*. After a second wash, pellets were solubilized in SDS-PAGE sample buffer and analysed as described above. Results shown are representative of 3-12 separate experiments.

To determine whether NDP kinase is initially associated with vesicles or soluble tubulin, the supernatant of the 40,000 *g* step was centrifuged at 230,000 *g* for 1 hour in a TLA 100.3 rotor (Beckman Coulter) to sediment membrane vesicles. The supernatant was incubated with taxol as described above to polymerize soluble tubulin, and centrifuged at 16,000 *g*. Both pellets were washed twice with MEPS and analyzed by immunoblotting.

Results

NDP kinase is associated dynamically with membrane ruffles and constitutively with intracellular vesicles

In quiescent, serum-starved NIH-3T3 fibroblasts, staining of NDP kinase with an antibody that recognizes NDP kinases A and B (Ab-1) reveals a cytosolic distribution pattern, more intense in the thicker central area (Fig. 1a). In many cells, NDP kinase forms ring-like structures, and phase-contrast microscopy (Fig. 1b) shows that most of these rings correspond to phase-bright vesicles of various sizes that are scattered through the cytoplasm, particularly around the nucleus. NDP kinase is associated with the outer rim of these structures, and is most conspicuous around large perinuclear vesicles (Fig. 1a).

In cells treated with EGF, NDP kinases are present not only in the cytosol and in vesicular structures but also at well-defined areas of the cell cortex. Fig. 1c shows several features of NDP kinase localization in cells exposed to EGF for 2 minutes; similar results were obtained with 5 nM PDGF and with 10% fetal bovine serum (not shown). In stimulated cells, NDP kinase staining is apparent in the submembranous space of cell protrusions such as ruffling lamellipodia; the large, phase-bright vesicles labeled by NDP kinase in quiescent cells are still prominent after stimulation with EGF (Fig. 1c,d). Translocation of NDP kinase to ruffles is a rapid and transient event, being evident within 1 minute of exposure to growth factors (Fig. 2) and becoming less pronounced after 30 minutes of continued stimulation (data not shown).

Areas heavily stained for NDP kinase were not always

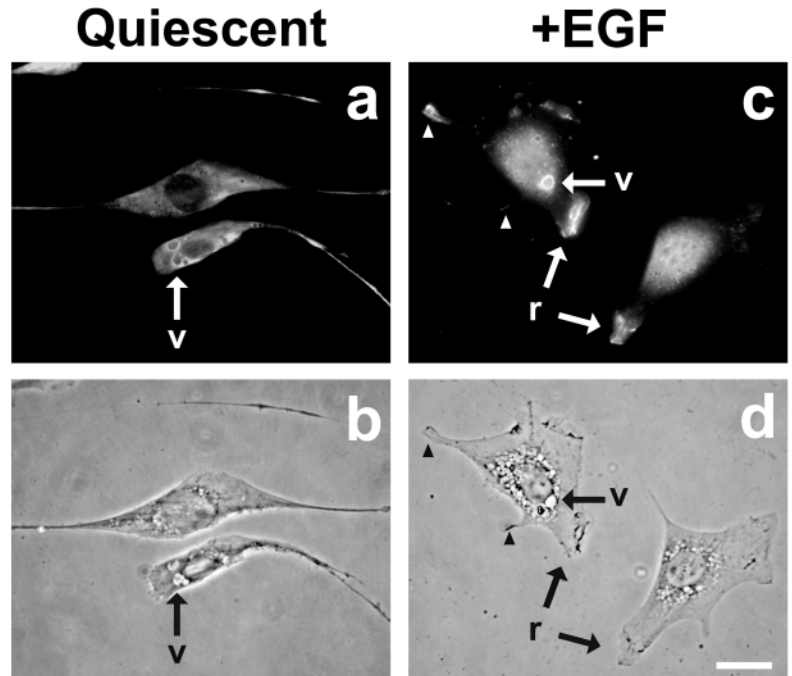


Fig. 1. Localization of NDP kinase in quiescent and EGF-treated NIH-3T3 cells. (a) In resting cells, NDP kinase accumulates in ring-like structures (v) that correspond to phase-bright cytoplasmic vesicles (b). (c) In cells treated with 10 ng ml⁻¹ EGF for 2 minutes, NDP kinase is present in lamellipodia and ruffles (r) as well as around cytoplasmic vesicles (v); (d) the corresponding phase-contrast image. Arrowheads indicate areas where the intensity of the fluorescence signal does not coincide with phase-dark areas. NDP kinase was visualized with polyclonal Ab-1. Scale bar, 25 µm.

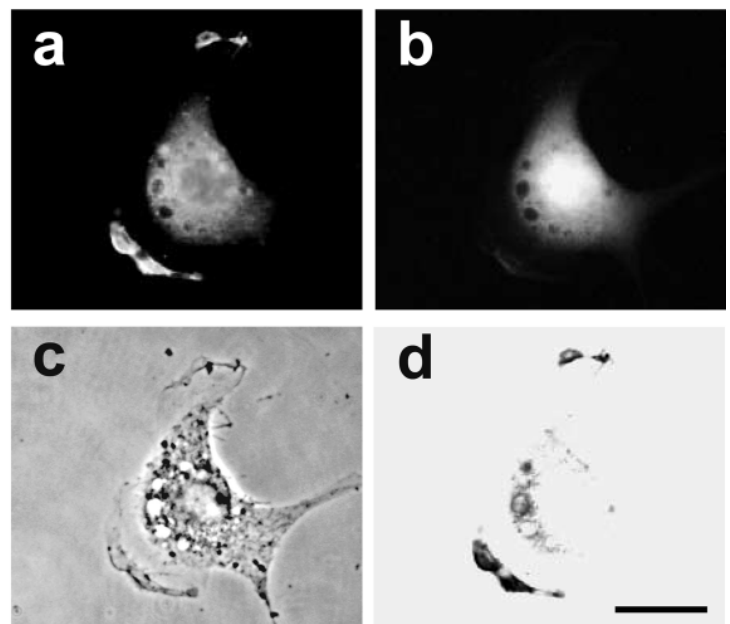


Fig. 2. The strong signal for NDP kinase around vesicles and in ruffling membranes is not a volume effect. Fluorescence ratio imaging of cells transfected with GFP and stained with anti-NDP kinase-A after treatment with 10 ng ml⁻¹ EGF for 1 minute. (a) NDP kinase A, (b) GFP and (c) corresponding phase-contrast image. (d) Ratio image showing high NDP kinase A/GFP ratios (arbitrarily set as dark areas) at edge ruffles and around large vesicles in the perinuclear area. Scale bar, 25 µm.

phase dark; by the same token, NDP kinase did not accumulate in all phase-dense regions (Fig. 1c,d, arrowheads), suggesting that volume effects are not the main reason for its enrichment in specific areas. Nevertheless, given that NDP kinases A and B are abundant proteins, we tested the possibility that the increased fluorescence signal observed in ruffles and around vesicles reflected the increased thickness in these locations and not actual accumulation of the antigen. To measure the enrichment of NDP kinase around vesicles and in ruffles, we examined its distribution in cells transfected with GFP, a soluble protein that behaves as a volume marker for the cytosol (Kaksonen et al., 2000) and analysed the data by fluorescence ratio imaging. Fig. 2 shows a GFP-expressing cell stained with the antibody specific for NDP kinase A after a short (1 minute) exposure to EGF. Although the signal for both GFP and NDP kinase A is strong in the cell body, the two patterns are mostly distinct. NDP kinase A (Fig. 2a) is concentrated at ruffling lamellipodia and forms a ring around one of several perinuclear vesicles seen in the phase-contrast image (Fig. 2c). By contrast, GFP is not present in ruffles and is excluded from, but does not accumulate around, vesicles (Fig. 2b). The ratio image (Fig. 2d) illustrates the high ratios of NDP kinase A to GFP in the ruffling edge (15:1 – 20:1) and at the rim of the vesicle seen in Fig. 2a (2:1 – 3:1), and reveals a feature that is not obvious from the fluorescent image (Fig. 2a), namely that other vesicles are faintly positive for NDP kinase A. This analysis is consistent with a genuine enrichment of NDP kinase around vesicles as well as in ruffling membranes.

Comparison of Figs 1 and 2 shows that the monoclonal antibody specific to NDP kinase A and the polyclonal antibody that recognizes both NDP kinase A and B yield similar results, suggesting that these two isoforms coexist within the same areas of a cell. Therefore, in the following sections we refer to NDP kinase A and B jointly as NDP kinase.

NDP kinase is translocated to ruffles in response to bombesin, a GPCR agonist

To determine whether the intracellular distribution of NDP kinase could be changed by stimulation of membrane receptors other than receptor tyrosine kinases (RTKs) such as the EGF and PDGF receptors, we also examined the localization of NDP kinase in cells stimulated by bombesin, which acts through a GPCR (Kjöller and Hall, 1999). Exposure of quiescent cells for 5–10 minutes to bombesin induced membrane ruffles that stained brightly for NDP kinase (Fig. 3a). Thus, stimulation of GPCRs, as well as RTKs, triggers the translocation of NDP kinase to the cell cortex.

Membrane ruffling results from profound changes in the organization of the actin cytoskeleton, with increased accumulation of F-actin at the cortex (Ridley et al., 1992). To determine whether the translocation of NDP kinase to ruffles is related to the redistribution of F-actin or actin binding proteins, we labeled stimulated cells with phalloidin and anti-NDP kinase. Fig. 3b shows the distribution of F-actin in a cell incubated with bombesin; similar results were obtained in cells treated with EGF. There is some overlap between NDP kinase staining and the intense F-actin signal at the edge of cells and at the tip of ruffles but the NDP kinase signal in ruffles typically forms broad patches that extend towards the center of the cell (Fig. 3a, arrowheads), well beyond the areas rich in F-actin. There is little or no co-localization of the two proteins elsewhere, with NDP kinase being absent from the stress fibers detected by phalloidin and the focal adhesions outlined by an antibody to vinculin (not shown). Notably, the vesicles labeled by NDP kinase antibodies (Fig. 3a, arrows) do not stain with phalloidin. *In vitro* studies with the purified proteins show no interaction between actin and NDP kinase (not shown). Thus, it appears that localization of NM23 to the cell periphery does not depend on a direct interaction with F-actin. To verify this hypothesis, we used BDM, which reduces ruffling (Rottner et

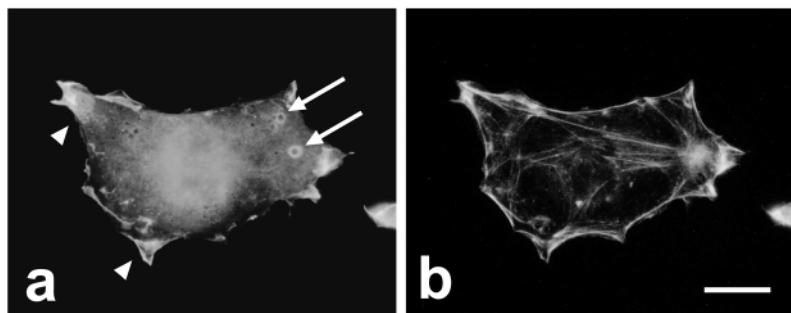


Fig. 3. Stimulation of cells with bombesin, a GPCR agonist, also induces translocation of NDP kinase to the actin-rich cell cortex. Serum-starved cells were treated with 10 nM bombesin for 10 minutes, then stained with Ab-1 antibody (a) and rhodamine-phalloidin (b). Arrows show accumulation of NDP kinase around vesicles. The arrowheads indicate patches where NDP kinase staining extends beyond the actin-rich ruffling edge. Scale bar, 25 μ m.

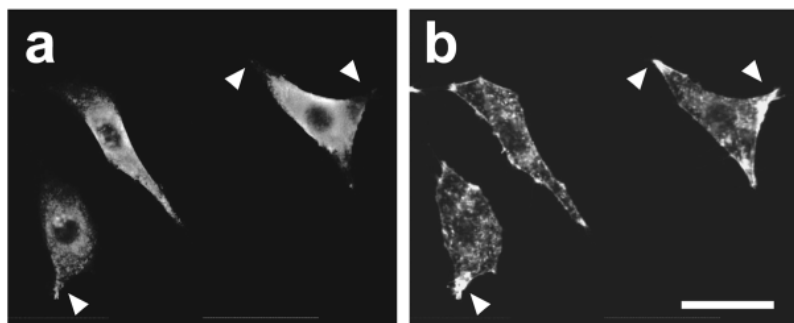


Fig. 4. BDM suppresses NDP kinase translocation to the cell cortex. Cells were pre-incubated with 30 mM BDM for 30 minutes as described in Materials and Methods. The incubation was continued in the presence of 10 ng ml⁻¹ EGF for 2 minutes, followed by fixation and labeling with anti-NDP kinase A (a) and rhodamine-phalloidin (b). Notice the absence of NDP kinase staining in cell protrusions that remain enriched in F-actin (b, arrowheads). Scale bar, 50 μ m.

al., 1999) but does not interfere with actin polymerization (Cramer and Mitchison, 1995). When cells were treated with BDM prior to stimulation with EGF, NDP kinase remained in the cell body (Fig. 4a), whereas significant amounts of F-actin were observable at the cortex (Fig. 4b). BDM has no effect on the enzymatic activity of purified erythrocyte NDP kinase (B. K.A.P. and A.d.S.O., unpublished). Taken together, these data imply that NDP kinase does not interact directly with F-actin and is translocated to the cell periphery through a mechanism distinct from binding to microfilaments. Given that BDM is a low-affinity myosin ATPase inhibitor, it is conceivable that a member of the large myosin family is involved on NDP kinase translocation. We are currently exploring this possibility.

Rac controls the translocation of NDP kinase to ruffles in response to activation of membrane receptors

Activation of Rac, a member of the Rho family of small GTPases that regulates the formation of lamellipodia and membrane ruffles, is a shared step in the response of fibroblasts to stimulation of different types of receptors (Kj  ller and Hall, 1999). Our observations in cells responding to growth factors and bombesin suggest that localization of NDP kinase to the cell periphery is linked to a common event, possibly Rac activation. To test this hypothesis, we examined the distribution of NDP kinase in cells expressing a constitutively activated form of Rac1, RacG12V. Expression of RacG12V (Fig. 5a) induced extension of lamellipodia in unstimulated, serum-starved cells as expected (Ridley et al., 1992) and also promoted the appearance of NDP kinase at the periphery (Fig. 5b). By contrast, NIH-3T3 cells expressing the dominant negative Rac mutant RacT17N did not extend lamellipodia in response to EGF (Fig. 5d) and showed the perinuclear staining for NDP kinase typical of quiescent cells (Fig. 5e). The

numbers and appearance of NDP kinase-labeled intracellular vesicles were similar in cells expressing Rac mutants and in neighboring untransfected cells (Fig. 5b,e), indicating that Rac controls only the recruitment of NDP kinase to the cortex.

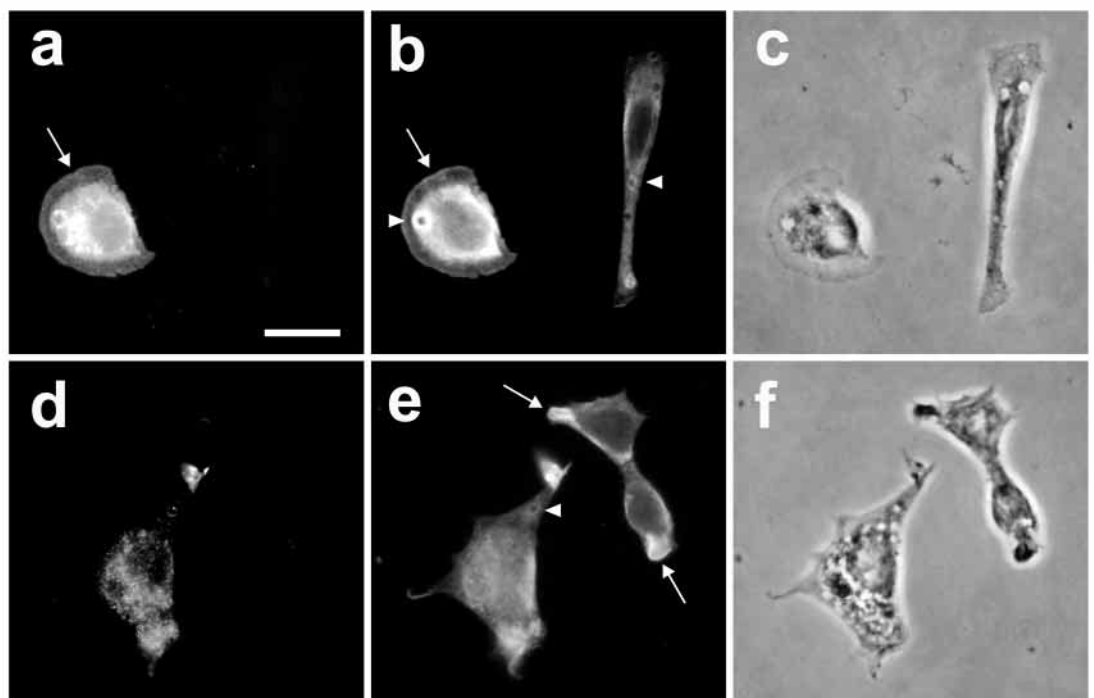
Tiam1 is not involved in NDP kinase translocation

Although NDP kinase and activated Rac co-localize in lamellipodia (Fig. 5a,b, arrow), NDP kinase does not bind directly to Rac (Otsuki et al., 2001) (K.A.P. and A.d.S.O., unpublished). Therefore, its connection to activated Rac is likely to be mediated by another cell component. NDP kinase A associates with a Rac1-specific nucleotide exchange factor, Tiam1 (Otsuki et al., 2001; Palacios et al., 2002). However, the idea of a Tiam1-mediated redistribution of NDP kinase to the cortex as a response to growth factors and bombesin is not supported by our results. Fig. 6 shows that there is limited overlap of the signals for NDP kinase A and Tiam1 at ruffles following exposure to EGF for 2 minutes. In particular, NDP kinase was visible throughout ruffling areas in cells, whereas Tiam1 staining was confined to small areas of the cell periphery (Fig. 6). This result is consistent with previous work showing that Tiam1's ability to translocate to membranes and to act as a GEF for Rac is markedly reduced in cells cultured in low or no serum media (Michiels et al., 1997; Bourguignon, 2000). Thus, under our experimental conditions, Tiam1 is not likely to be responsible for the translocation of NDP kinase to the cell cortex.

Cell stimulation promotes association of NDP kinase with membranes

The cortical staining of stimulated cells with NDP kinase antibodies might reflect an actual translocation from the

Fig. 5. Rac induces recruitment of NDP kinase to ruffles. Cells transfected with HA-tagged RacG12V (a,b,c) or RacT17N (d,e,f) were serum starved. Cells expressing RacG12V were fixed directly, whereas those expressing T17N Rac were first treated with EGF for 2 minutes. Samples were double labeled for the HA tag (a,d) and NDP kinase (b,e) with Ab-1. (c,f) Phase contrast images. In the cell that expresses activated Rac (a), NDP kinase is visible at the edge of the lamellipodium (b, arrow) and around vesicles (b, arrowheads). Expression of T17N Rac (d) inhibits accumulation of NDP kinase in ruffles in response to EGF (e; compare the transfected cell on the left with those on top right) but does not affect vesicular NDP kinase (e, arrowhead). Scale bar, 20 μ m.



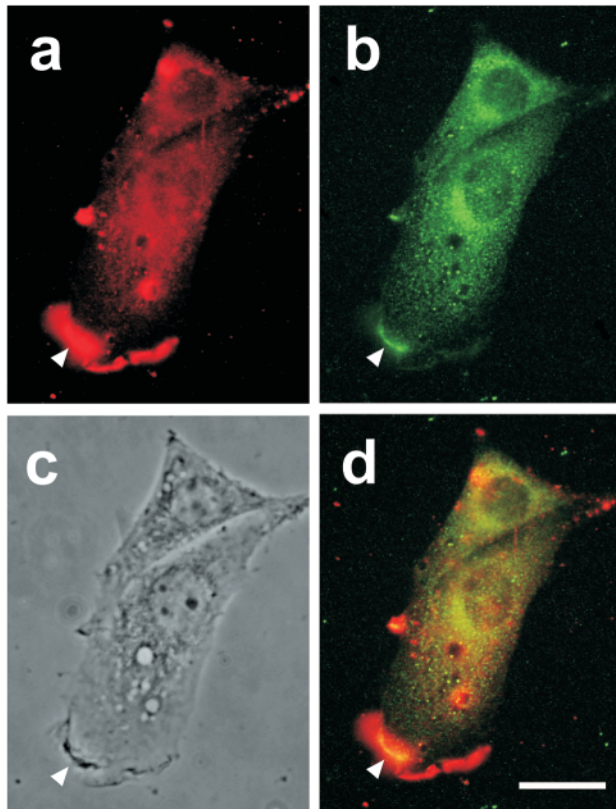


Fig. 6. Limited overlap of Tiam1 and NDP kinase in ruffles. Serum-starved cells were treated with 10 ng ml^{-1} EGF for 2 minutes. Cells were stained with anti-NDP kinase A (a) and anti-Tiam1 (b) antibodies; (c) phase contrast image. Notice that NDP kinase is present throughout the ruffle, whereas Tiam1 is confined to a small area (arrow). The merged image (d) highlights the limited overlap between the signals for Tiam1 and for NDP kinase. (c) Scale bar, $25 \mu\text{m}$.

cytosol to the plasma membrane, as observed with activated Rac. To test this hypothesis, we compared the distribution of Rac and NDP kinase by two independent approaches. First, we performed a line scan analysis of images of cells treated with EGF and fluorescently stained for endogenous Rac and NDP kinase. Fig. 7A shows that the fluorescence intensities for both NDP kinase and Rac are correlated only in peripheral ruffles. The two proteins do not co-localize elsewhere in the lamellipodium and, as seen also in Fig. 3b, the signal for NDP kinase, but not Rac, increases across a large patch immediately behind the ruffling region. Neither protein localizes to less active areas of the plasma membrane, indicating that the appearance of cortical NDP kinase is associated with ruffling activity. To confirm this result, we also compared the NDP kinase content of membrane fractions isolated from quiescent and serum-stimulated cells. Immunoblotting with antibodies to Rac and NDP kinase shows that the amounts of both proteins in the particulate fraction are similarly increased (3.5 times) following serum stimulation, whereas the plasma membrane marker $\text{Na}^+\text{K}^+\text{-ATPase}$ remains unchanged, as expected (Fig. 7B).

Overall, these results demonstrate that, in NIH-3T3 cells, stimulation of surface receptors that lead to Rac activation

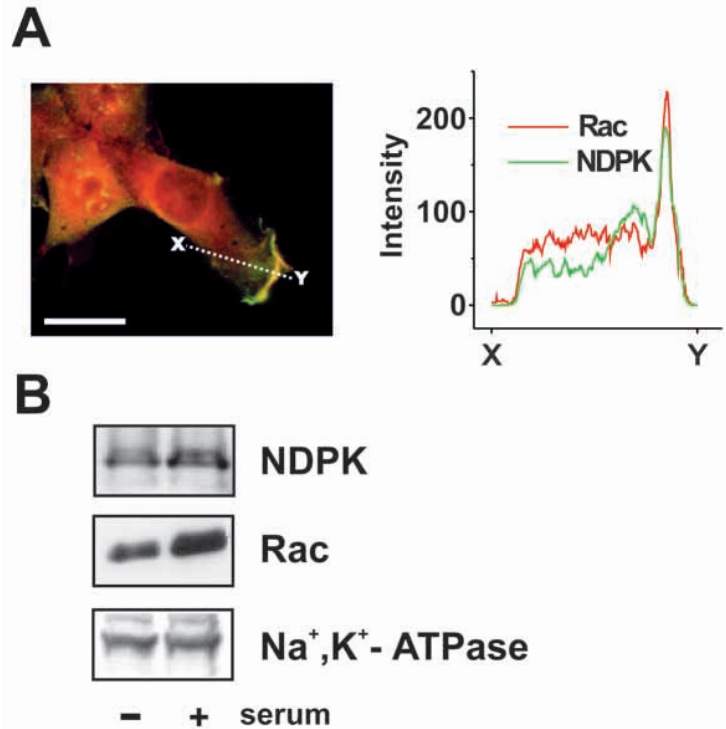


Fig. 7. Cell stimulation increases the association of NDP kinase and Rac with cell membranes. (A) Merged image of serum-starved cells treated with EGF for 2 minutes and stained for Rac (red) and NDP kinase (Ab-1; green); areas of overlap appear in yellow. The line scan analysis shows the fluorescence intensities of Rac and NDP kinase along the white line in the image. Scale bar, $25 \mu\text{m}$. (B) Subcellular fractionation of quiescent and serum-treated cells was as in Methods. The particulate fraction was immunoblotted with antibodies to NDP kinase (NDPK), Rac and $\text{Na}^+\text{K}^+\text{-ATPase}$, a plasma membrane marker.

triggers the translocation of a pool of NDP kinase to the cell periphery without affecting vesicular NDP kinase. The shift of NDP kinase towards the cell cortex is rapid, being evident within 1 minute of treatment with EGF, and results in extensive co-localization of NDP kinase and activated Rac at lamellipodia but does not involve a direct interaction with Rac, Tiam1 or F-actin.

Characterization of the vesicles labeled by NDP kinase

The results above show that the Rac-related events that lead to the dynamic targeting of NDP kinase to ruffles differ considerably from the biochemical processes responsible for its constitutive association with vesicles. To define more clearly the mechanism by which NDP kinase assembles around vesicles, we first characterized their morphology and protein composition. Fig. 8A shows the size distribution of NDP-kinase-labeled vesicles, which covers a range of $0.7\text{-}6.9 \mu\text{m}$ with a median diameter of $2.3 \mu\text{m}$ ($n=209$). There is no statistically significant difference ($P=0.19$) between the size distribution of NDP-kinase-labeled vesicles in resting and stimulated cells. The large size and variable diameter of these vesicles, as well as lack of overlap of NDP kinase staining with internalized transferrin (not shown), indicate that they do not represent small, uniform sized clathrin-coated pits. Although

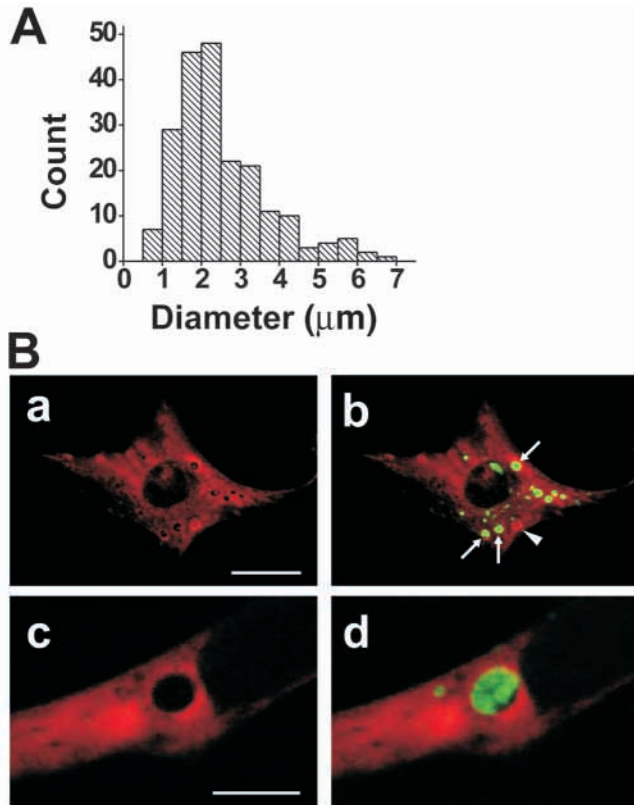


Fig. 8. Characterization of the vesicles labeled by NDP kinase. (A). Size distribution of NDP kinase-labeled vesicles. The histogram was built as described in Materials and Methods. (B) NDP kinase is associated with vesicles labeled by LAMP-1. This image shows the immunolocalization of NDP kinase with Ab-1 (red) and LAMP-1 (green) in quiescent cells. NDP kinase labels the periphery (a) of vesicles, whereas LAMP-1 is visible in the lumen (b, arrows); a circular ruffle (arrowhead) is stained only by NDP kinase. Scale bar, 25 μm . A large vacuole positive for both NDP kinase (c) and LAMP-1 (d) has a multivesicular appearance. Scale bar, 5 μm .

NDP-kinase-labeled vesicles have many characteristics of the macropinosomes formed by the closing of ruffles (broad size distribution, appearance in phase-contrast optics) (Swanson and Watts, 1995), they lack basic features of these structures. For instance, if NDP kinase were associated with macropinosomes, one would expect to see an increase in the number of labeled vesicles following Rac activation, which stimulates macropinocytosis in murine fibroblasts (Ridley et al., 1992), yet we observed no significant effects of Rac activation on vesicle-associated NDP kinase. More importantly, the structures coated by NDP kinase are not accessible to a fluorescent endocytic tracer (Texas Red dextran) even after an 18 hour chase (not shown), and therefore do not appear to be involved in fluid phase uptake.

The vesicles labeled by NDP kinase are also distinct from structures containing Rab4 (data not shown), a marker of early endosomes (Van der Sluijs et al., 1991). However, double labeling of quiescent NIH-3T3 fibroblasts for NDP kinase and the lysosome-associated membrane protein 1 (LAMP-1) sometimes shows a significant overlap of the two signals in phase-bright vesicles. NDP kinase accumulates around the

cytoplasmic surface of the vacuoles, whereas LAMP-1 labels the lumen (Fig. 8Ba,b). Because LAMP-1 is a membrane protein, its apparent localization in the lumen of late endosomes is likely to be a consequence of smaller vesicles being present in the lumen of the larger vacuoles. Indeed, occasionally, the LAMP-1-positive vacuoles labeled by NDP kinase are very large and contain clusters of smaller, round vesicles (Fig. 8Bc,d). The morphological features and strong labeling with anti-LAMP-1 suggests that these structures are multivesicular bodies (MVBs), which are part of the late endosomal compartment and participate in the sorting of endocytosed proteins and lipids (Felder et al., 1990). However, late endosomes and MVBs should accumulate internalized fluorescent dextran, whereas the vesicles labeled by NDP kinase do not. Thus, the NDP-kinase-positive vesicles, including those with multivesicular appearance, belong to an atypical intracellular compartment that is not active in the classical endocytic pathway.

NDP-kinase-labeled vesicles co-localize with microtubules

MVB-like organelles that are not involved in fluid phase uptake have been identified in neurons through their association with a member of the kinesin superfamily of microtubule motors, KIFC2 (Saito et al., 1997). Given that NDP kinase co-localizes partially with the microtubular network in epithelial cells (Pinon et al., 1999) and the centrosome of C6 glioma cells (Roymans et al., 2001), we investigated the relationship between microtubules and the vesicles coated with NDP kinase by double labeling with antibodies to α -tubulin and NDP kinase (Fig. 9). Although treatment with EGF induces pronounced accumulation of NDP kinase in ruffles, microtubules are largely absent from ruffling areas (not shown). However, the signals for the two proteins overlap distinctly around the vesicles labeled by NDP kinase, indicating that the vesicles interact simultaneously with microtubules and NDP kinase. The co-localization of tubulin and NDP kinase at the periphery of vesicles is not a result of serum deprivation because it is also observed in cells cultured in complete medium (Fig. 9). Depolymerization of microtubules with nocodazole eliminates association of tubulin and NDP kinase with large intracellular vesicles (Fig. 9). Thus, clustering of NDP kinase at the periphery of vesicles depends on an intact microtubular network.

NDP kinase is associated with membranes that bind to microtubules in vitro

Our observations are suggestive of an interaction between NDP kinase and microtubule-bound vesicles. To determine whether the overlap in staining reflects a physical association between NDP kinase and microtubule-associated vesicles, we took advantage of a cell-free assay that reproduces the *in vivo* association between endosomes and microtubules (Goltz et al., 1992; Oda et al., 1995), thus putting the morphological results to a biochemical test. A fraction containing light membranes and cytosol was obtained from NIH-3T3 cells. Endogenous tubulin was polymerized with taxol, and pelleted at *g* forces sufficient to sediment microtubules and associated structures, but not isolated membranes. The pellet (MT/Ves) was washed

extensively and subjected to immunoblot analysis. The MT/Ves fraction contains NDP kinase, tubulin, Rab4 and LAMP-1 (Fig. 10), indicating that both early and late endosomal membranes associate with microtubules under our conditions. Stimulation of cells with EGF does not affect the amount of NDP kinase, tubulin or LAMP-1 in the MT/Ves fraction but eliminates Rab4 from the pellet (Fig. 10). Translocation of Rab4 from endosomes to the cytosol in response to stimulation was previously reported in adipocytes treated with insulin (Cormont et al., 1993) and our data show that EGF has the same effect in fibroblasts. The lack of effect of EGF on the levels of NDP kinase bound to the MT/Ves fraction is consistent with our observations that the association of NDP kinase with vesicles is not altered by extracellular stimulation (Fig. 9) or by expression of activated or dominant-negative Rac (Fig. 5).

NDP kinase co-immunoprecipitates from cell lysates with tubulin (Lombardi et al., 1995; Roymans et al., 2001) and co-

localizes partially with microtubules in intact cells (Pinon et al., 1999), although purified NDP kinase does not bind directly to microtubules (Melki et al., 1992). To determine whether the NDP kinase pool bound to the MT/Ves pellet was associated with tubulin prior to the formation of microtubules, the postnuclear supernatant was centrifuged at 230,000 *g* to sediment membranes. The supernatant (cytosol) was removed, incubated with taxol to polymerize soluble tubulin and centrifuged at 16,000 *g*. When both pellets were washed and examined for their NDP kinase content by immunoblotting, we found that NDP kinase is present in the membrane pellet but not in the microtubule pellet (not shown). Thus, the NDP kinase in the MT/Ves pellet derives from membranes, not the tubulin component. This result demonstrates that the association of NDP kinase with the MT/Ves fraction is specific and not due to trapping of this abundant cytosolic protein in the microtubule pellet.

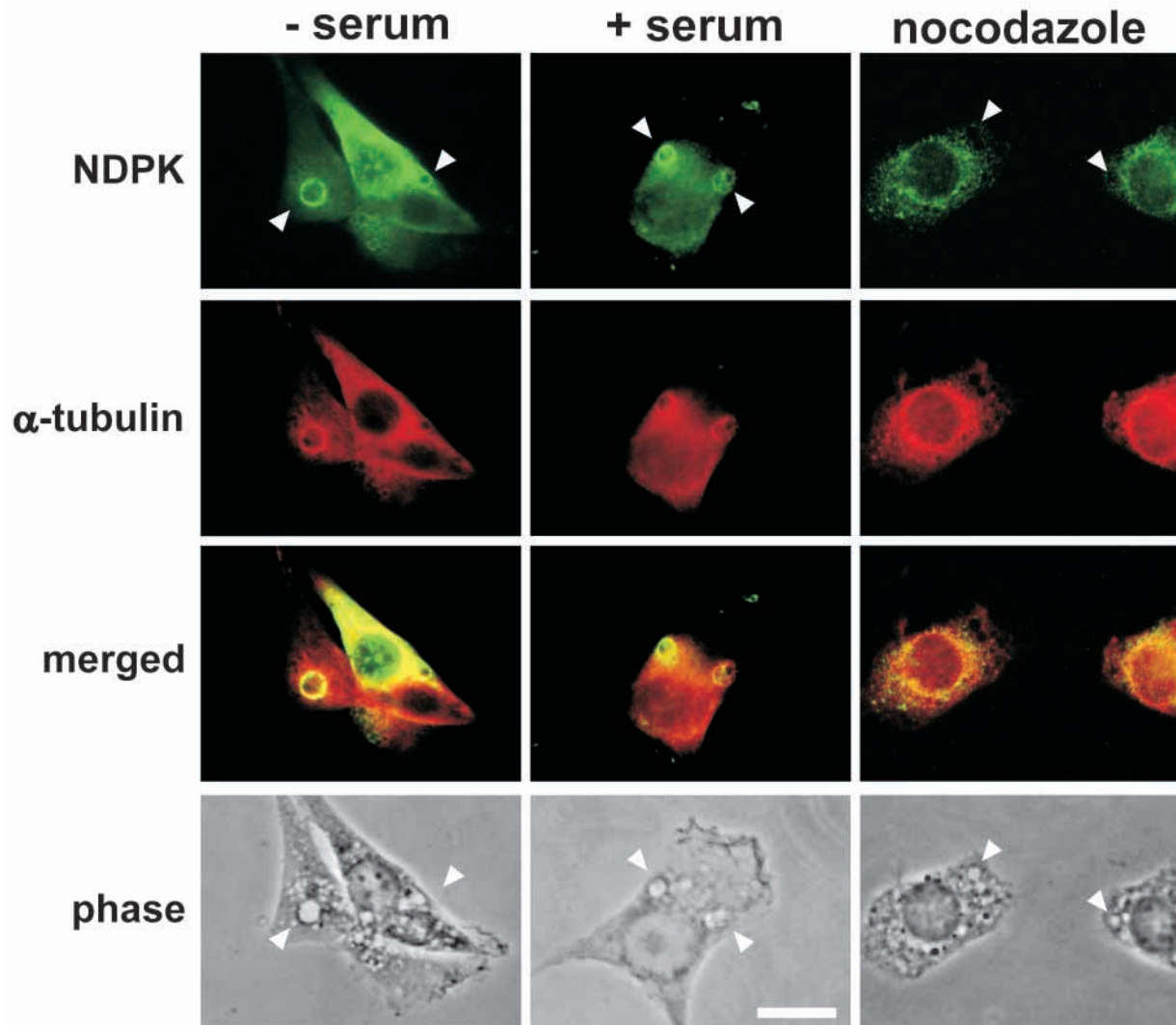
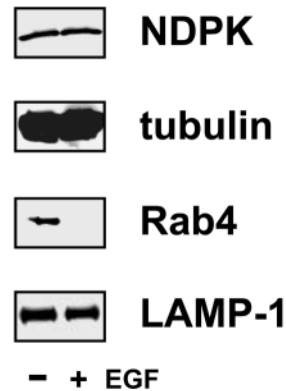


Fig. 9. Microtubular association of NDP kinase-labeled vesicles. Cells were fixed and double stained with antibodies to NDP kinase (Ab-1) and α -tubulin after being serum starved for 18 hours (-serum), kept in complete medium (+serum) or treated with 33 μ M nocodazole for 1 hour (nocodazole). Arrowheads show vesicular structures visible in phase contrast, which are labeled by antibodies to α -tubulin and NDP kinase in serum-starved and serum-treated cells (left and middle, respectively). Labeling of vesicles is lost in cells treated with nocodazole (right). Scale bar, 20 μ m.

Fig. 10. NDP kinase pellets with microtubules and endosomal vesicles. Prior to homogenization, cells were incubated in the absence and presence of 10 ng ml^{-1} EGF for 15 minutes. Taxol-stabilized microtubules and associated membranes were then isolated as described in Materials and Methods, in the absence of nucleotides. Fractions were analysed by immunoblotting with antibodies to NDP kinase, tubulin and the endosomal markers Rab4 and LAMP-1.



Nucleotides release NDP kinase from microtubule-bound endosomes

Formation of taxol-stabilized microtubules is usually performed in the presence of GTP, even though (at appropriate concentrations) taxol alone is sufficient to induce complete polymerization (Oda et al., 1995). Indeed, inclusion of 1 mM GTP in the incubation with Taxol does not affect the amount of tubulin in the MT/Ves pellet; neither does it affect the association of early and late endosomal membranes with the microtubule pellet, as indicated by the unchanged levels of Rab4 and LAMP-1 (Fig. 11A). By contrast, GTP induces a striking release of NDP kinase from the pellet; ATP also reduces the association of NDP kinase with MT/Ves, but is noticeably and consistently ($n=6$) less effective than GTP (Fig. 11A). Thus, NDP kinase is released from MT/Ves through a nucleotide-sensitive site that shows some specificity for the base.

The nucleotide-induced release of NDP kinase from the MT/Ves fraction qualitatively resembles the behavior of the molecular motors dynein and kinesin, whose binding to microtubules and associated endosomal vesicles is also responsive to nucleotides (Oda et al., 1995). However, we find that the amounts of dynein and kinesin associated with the MT/Ves fraction are not altered by 1 mM GTP or ATP (not shown). Presumably the interaction of NDP kinase with MT/Ves is more sensitive to nucleotides than that of molecular motors, so the nucleotide concentration used in this work is too low to cause elution of significant amounts of dynein and kinesin. For instance, 10 mM ATP releases only 50% of bound dynein and kinesin from an analogous fraction obtained from rat liver (Oda et al., 1995), with GTP being slightly less effective. Thus, the dissociation of NDP kinase from the MT/Ves pellet by nucleotides is not linked to the release of motor proteins and their cargo.

NDP kinases bind nucleotides with affinities in the range of 10–200 μM , and guanine nucleotides are somewhat preferred over other substrates (Schaertl et al., 1998; Cervoni et al., 2001; Schneider et al., 2002). Therefore, the simplest explanation for the preferential release of NDP kinase from the MT/Ves fraction by GTP is that it involves binding of the nucleotide directly to its catalytic site. However, it is also possible that the release of NDP kinase from the MT/Ves fraction is secondary to the interaction of GTP with a highly selective binding site, perhaps a GTP-binding protein, and that the effect of ATP is indirect, requiring its conversion into GTP. To distinguish

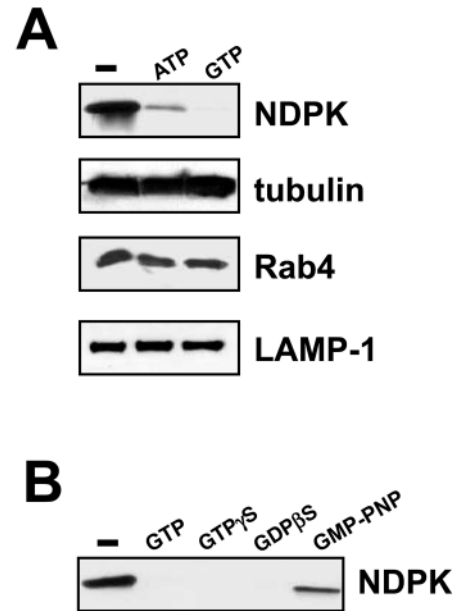


Fig. 11. Nucleotides release NDP kinase from taxol-stabilized microtubules and associated vesicles. (A) An extract from resting cells was divided into equal portions that were incubated with Taxol in buffer supplemented or not with ATP or GTP (both at 1 mM); after washes, pellets were tested for NDP kinase, tubulin, Rab4 and LAMP-1. (B) As above; binding of NDP kinase to the MT/Ves pellets was assessed in samples where GTP or guanine nucleotide analogs were included in the incubation with taxol.

between these possibilities, we examined the effects of the guanine nucleotide analogs guanosine-5'-*O*-(3-thio)triphosphate (GTPγS), guanylyl-imidodiphosphate (GMP-PNP) and guanosine-5'-*O*-(2-thio)diphosphate (GDPβS) on the association of NDP kinase with the MT/Ves fraction. If release of NDP kinase by GTP is related to a GTP-binding protein, the activating GTP analogs GTPγS and GMP-PNP are expected not only to act alike but also to have opposite effects to those of GDPβS, which locks GTP-binding proteins in the inactive form. However, if the release is mediated by the catalytic site of NDP kinase, the thiophosphate analogs GTPγS and GDPβS, which are substrates for NDP kinases (Schaertl et al., 1998), should behave similarly to GTP. Imidodiphosphate analogs bind to NDP kinases with low affinity and are not substrates (Cervoni et al., 2001), so GMP-PNP should have modest effects, if any, on the retention of NDP kinase by the MT/Ves fraction. As seen in Fig. 11B, 1 mM GTPγS and GDPβS, but not GMP-PNP, are as effective as GTP in eluting NDP kinase, suggesting that the nucleotide binding site involved is that of NDP kinase.

Discussion

To investigate further the role of NDP kinase in transmembrane signaling, we sought to identify subcellular compartments in which it is present in resting cells and to determine whether activation of surface receptors can cause rapid changes in the pattern of NDP kinase localization. We found that, in NIH-3T3 cells, there are two discernible cytoplasmic pools of NDP kinase whose location is regulated independently by distinct

cellular signals: a proportion of the cellular NDP kinase is rapidly translocated to lamellipodia and ruffles in response to activation of RTKs and GPCRs, whereas another NDP kinase pool is associated with cytoplasmic vesicles in both quiescent and stimulated cells. Comparison with the distribution of the cytosolic protein GFP shows that the intense staining seen around vesicles and in ruffles is not a volume effect but reflects real accumulation of NDP kinase. The clear differences between cortical and vesicular NDP kinase pools in terms of localization and response to extracellular stimulation suggest strongly that they use different targeting devices.

Although we used two different antibodies as tools, a monoclonal specific to NDP kinase A and a polyclonal that recognizes both NDP kinase A and B both highlight the same structures. This is not surprising, given that the bulk of mammalian NDP kinases A and B are present in cells as stable mixed hexamers (Gilles et al., 1991). This finding is also consistent with a report that isoforms A and B are similarly distributed in the cytoplasmic space under normal culture conditions (Pinon et al., 1999); nevertheless, the possibility that the ratio of one isoform to another might change substantially as a function of the compartment examined (Barraud et al., 2002) cannot be overlooked. Another caveat is that the antibodies used here were tested against NDP kinase A and B, which are by far the most abundant isoforms in mammals (Lacombe et al., 2000). Given that some NDP kinase proteins are yet to be isolated, it is possible (although unlikely) that our results reflect the localization of cross-reacting NDP kinase isoforms that are expressed at low levels and undetectable by immunoblotting but might be concentrated in specific cell locations; a definite answer will have to await the purification of all NDP kinase isoforms and development of high-affinity isoform-specific tools.

Translocation of molecules into ruffles is often achieved by binding to proteins enriched in these structures, such as F-actin or activated Rac1. However, NDP kinase does not bind to F-actin or Rac in vitro. NDP kinase also does not carry consensus sequences found in Rac effectors, Rac GEFs or in Rac GTPase activating proteins. Therefore, its connection to Rac activation is probably indirect. Otsuki et al. reported that NDP kinase A associates with the Rac1 nucleotide exchange factor Tiam1 and that overexpression of NDP kinase A inhibits the activating effect of Tiam1 on Rac (Otsuki et al., 2001). However, our experiments show that, in serum-starved NIH-3T3 cells stimulated briefly with EGF, ruffles that are strongly stained for NDP kinase contain little Tiam1, suggesting that association with Tiam1 does not mediate translocation of NDP kinase A upon Rac activation. Recently, Palacios et al. demonstrated that, in MDCK epithelial cells, the small GTPase ARF6 mediates the recruitment of heterologously expressed human NDP kinase A to areas of cell-cell contact (Palacios et al., 2002). Once translocated to these sites, NDP kinase A would facilitate dynamin-dependent endocytosis and downregulate Rac activation by binding Tiam1. We are currently investigating whether an analogous interaction with ARF6 can mediate the rapid translocation of endogenous NDP kinase to the cortex of stimulated NIH-3T3 fibroblasts.

NDP kinase B was also found to be directed to ruffles, through interaction with ICAP1- α (Fournier et al., 2002). However, we use adherent cells and ICAP1- α reportedly affects cell morphology and behavior only during cell adhesion

(Degani et al., 2002), so a similar mechanism is not likely to be responsible for the rapid translocation of NDP kinase in response to EGF and bombesin reported here.

The marked effect of BDM on NDP kinase localization (Fig. 4) suggests yet another alternative, namely that a member of the myosin family might be involved. However, because BDM is also known to affect calcium homeostasis and microtubule function (Kiehart, 1999), the precise mechanism of its action requires careful investigation. Despite the uncertainty as to the underlying mechanism, the demonstration that a proportion of cellular NDP kinases is quickly redistributed following cell stimulation and Rac activation strongly supports previous data indicating that these proteins are involved in signaling (reviewed in Otero, 2000).

The finding that, in NIH-3T3 cells, NDP kinase localizes constitutively to the surface of intracellular vesicles was surprising. Based on their inaccessibility to a fluid phase uptake marker, we conclude that these structures are not macropinosomes. Some of the structures ringed by NDP kinase in quiescent cells contain LAMP-1, a marker of late endosomes/lysosomes; occasionally, the NDP kinase/LAMP-1-positive vesicles resemble multivesicular bodies, which are part of the late endosomal compartment. However, double labeling with markers for endocytic compartments indicates that the vesicles labeled by NDP kinase antibodies are distinct from early and recycling endosomes, and lack fundamental features of late endosomes and MVBs. Thus, most of the NDP-kinase-positive vesicles are not conventional endocytic organelles and might be part of the secretory pathway. Although the analysis of the protein composition of the vesicles coated by NDP kinase is currently being performed, the possibility of a link between NDP kinase and secretory vesicles is backed by recent work showing that, in neurons, NDP kinases localize to membranes from the Golgi and endoplasmic reticulum, and to vesicles budding from the trans-Golgi (Barraud et al., 2002).

The morphological evidence presented here points to an interaction between NDP kinase and discrete vesicles that associate with microtubules. The co-localization of NDP kinase and tubulin seen by immunofluorescence is not coincidental: a separate experimental approach using a cell-free assay demonstrates that the overlap reflects a physical association between NDP kinase, stabilized microtubules and membrane vesicles. The tight binding of NDP kinase to this fraction and the full release induced by GTP and other substrates suggest that the interaction is specific. We hypothesize that the dynamic association of NDP kinase with microtubule-bound vesicles in fibroblasts is relevant to the operation of the multiple GTPases that control intracellular membrane transport. Namely, in vivo, NDP kinase might associate with microtubule-bound vesicles when GTP levels are low, using nucleoside triphosphates such as ATP to phosphorylate GDP, whereas a rise in GTP levels leads to its release. Our data agree with studies by other groups implicating NDP kinase in intracellular vesicle trafficking processes. Thus, NDP kinase B is a component of isolated phagosomes (Garin et al., 2001) and the *Drosophila* homolog of NDP kinase, Awd, regulates dynamin-dependent synaptic vesicle recycling through a mechanism that requires its intrinsic NDP kinase activity, presumably GTP regeneration (Krishnan et al., 2001). Facilitation of dynamin-based

endocytosis by NM23-H1 was also reported for mammalian cells (Palacios et al., 2002). Recently, Baillat et al. (Baillat et al., 2002) demonstrated a direct interaction between NDP kinase and dynamin I, as well NDP kinase and phocein, which is homologous to the σ subunits of clathrin adaptor subunits, supporting the idea that NDP kinase plays a role in vesicular traffic.

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