predicted disease could have features of congenital thrombocytopenia and a thrombotic tendency due to spontaneous platelet aggregation. Because some myeloproliferative syndromes are due to clonal proliferation of stem cells that affects platelets (*25*), somatic mutations that truncate α_{IIb} could result in an acquired thrombotic tendency. Somatic mutations that affect other integrin α subunit–cytoplasmic domains in malignant cells may also influence their invasive and metastatic properties.

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10. The $\beta_3\Delta 728$ construct was made by Eco RI digestion of BS3a (*26*), isolation of the coding fragment, filling in overhangs with T4 polymerase (*27*), and ligation to an Xba I linker with a termination codon in all three reading frames. This fragment was cut with Xba I, its ends polished with mung bean nuclease, ligated to a Bst XI linker, digested with Bst XI, and ligated to Bst XI–digested CDM8 (*26*). The $\alpha_{IIb}\Delta 991$ construct was made by generating a Sal I site in BS2b (*26*) at bases 3061 to 3066 (*28*). After digestion with Sal I and isolation of the fragment, the ends were partially filled in with T4 polymerase and polished with mung bean nuclease (*27*). This fragment was ligated to an Xba I linker containing a termination codon in all three reading frames, digested with Xba I, and ligated to Xba I–cut and dephosphorylated CDM8. The $\alpha_{IIb}\alpha_5$ chimera was generated in two steps. A 600-bp Hind III–Xba I fragment that contained the cytoplasmic sequence of α_5 was isolated from α_5 cDNA and ligated to Hind III + Xba I–digested CDM8 generating $CD\alpha_5 3'$. Then, a Hind III site was introduced into α_{IIb} at bases 3058 to 3063. After Hind III digestion, the coding fragment was isolated and ligated to Hind III–digested and dephosphorylated $CD\alpha_5 3'$.
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Mechanism for Regulating Cell Surface Distribution of Na^+, K^+-ATPase in Polarized Epithelial Cells

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Restriction of sodium, potassium adenosine triphosphatase (Na^+, K^+-ATPase) to either the apical or basal-lateral membrane domain of polarized epithelial cells is fundamental to vectorial ion and solute transport in many tissues and organs. A restricted membrane distribution of Na^+, K^+-ATPase in Madin-Darby canine kidney (MDCK) epithelial cells was found experimentally to be generated by preferential retention of active enzyme in the basal-lateral membrane domain and selective inactivation and loss from the apical membrane domain, rather than by vectorial targeting of newly synthesized protein from the Golgi complex to the basal-lateral membrane domain. These results show how different distributions of the same subunits of Na^+, K^+-ATPase may be generated in normal polarized epithelia and in disease states.

SODIUM, POTASSIUM ADENOSINE TRIphosphatase (Na^+, K^+-ATPase) is a plasma membrane protein in animal cells that functions in the maintenance of homeostasis. In many cell types, Na^+, K^+-ATPase is uniformly distributed over the cell surface. However, in polarized epithelial cells the cell surface distribution of the same subunits of Na^+, K^+-ATPase may be restricted to either the apical [for example, choroid plexus (*1*), retinal pigmented epithelium (*2*)] or basal-lateral [for example, intestine, kidney (*3*)] membrane domain. Localization of Na^+, K^+-ATPase to either domain establishes a transepithelial Na^+ gradient that drives vectorial transport of ions and solutes between the two compartments separated by the epithelium. In some epithelial diseases, such as polycystic kidney disease (*4*) and ischemia (*5*), the cell surface polarity of Na^+, K^+-ATPase appears to be partially or completely reversed.

Several mechanisms have been proposed to explain how cell surface distributions of membrane proteins are regulated in polarized epithelial cells. These include sorting of newly synthesized proteins in the Golgi complex and vectorial delivery to the appropriate membrane domain, rerouting of protein from one membrane to the other, and interaction of sorted proteins with the membrane-cytoskeleton (*6, 7*). To investigate mechanisms that regulate the cell surface distribution of Na^+, K^+-ATPase, we analyzed stages during differentiation of renal epithelial cells when unpolarized precursor cells are converted to polarized epithelial cells (*6*). During this time, the initially uniform distribution of Na^+, K^+-ATPase becomes restricted to the basal-lateral membrane. We have used MDCK cells as an in vitro model of polarized renal epithelia (*6, 7*), in which induction of Ca^{2+}-dependent cell-cell contacts through E-cadherin results in the development of structural and functional polarity in stages similar to those that occur during renal epithelial development in vivo (*6, 8*).

Ca^{2+}-dependent cell-cell contacts were induced in confluent monolayers of MDCK cells (*9*), and the distribution of Na^+, K^+-ATPase was visualized by indirect immunofluoresence with laser-scanning confocal microscopy (Fig. 1A). For as long as 48 hours after cell-cell contact, we detected staining of the α subunit of Na^+, K^+-ATPase at both the apical membrane and at

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sites of cell-cell contacts (lateral membranes). Later, the staining of lateral membranes was very prominent when compared with that of cells labeled with an antibody specific for the apical marker protein, gp135-170 (*10*) (Fig. 1A).

The cell surface distribution of Na^+, K^+-ATPase was quantified by labeling cell surface proteins with a biotinylating reagent, sulfosuccinimidobiotin (sulfo-NHS-biotin), at various times after cell-cell contact (*11*). Biotinylated Na^+, K^+-ATPase was recovered initially (24 to 48 hours) from both the forming apical and basal-lateral membrane domains (Fig. 1, B and C). Between 48 and 72 hours after cell-cell contact the amount of Na^+, K^+-ATPase on the basal-lateral membrane increased four- to fivefold (Fig. 1, B and C), which may reflect the increase in the surface area of the lateral membranes after cell-cell contact (*12*). Between 36 and 72 hours, the amount of biotinylated Na^+, K^+-ATPase detected on the apical membrane decreased by >65% (Fig. 1, B and C). After 96 hours of cell-cell contact, >85% of the total cell surface Na^+, K^+-ATPase was localized to the basal-lateral membrane domain.

To determine whether direct targeting of newly synthesized Na^+, K^+-ATPase to the basal-lateral membrane contributed to the establishment of the restricted distribution of this protein, we quantitatively analyzed the delivery of newly synthesized Na^+, K^+-ATPase to each cell surface domain. Approximately equal amounts of newly synthesized α subunit of Na^+, K^+-ATPase were delivered to the apical and basal-lateral membrane domains at all times tested (Fig. 2, A and B). These results indicate that there is little or no sorting of Na^+, K^+-ATPase in the Golgi complex at these times, and that the development of cell surface polarity of Na^+, K^+-ATPase in these cells appears not to result from vectorial targeting to only one membrane domain.

The apparent lack of polarized delivery of Na^+, K^+-ATPase to the cell surface does not reflect a general inability of these cells to vectorially deliver proteins from the Golgi complex to the basal-lateral membrane (*13*). For example, the epithelial cell adhesion protein desmoglein 1 (*14*) was rapidly restricted to the basal-lateral membrane after cell-cell contact (Fig. 2C). Furthermore, newly synthesized desmoglein 1 was vectorially delivered to the basal-lateral membrane (Fig. 2C). Differences between the sorting pathways of newly synthesized Na^+, K^+-ATPase and desmoglein 1 may reflect differences in the affinities of each protein for sorting machinery in the trans Golgi network.

These results contrast with those of Caplan *et al.* who concluded that Na^+, K^+-ATPase was delivered to only the basal-lateral membrane in polarized MDCK cells (*15*). In that study, ouabain, which binds specifically to an extracellular domain of the α subunit of active Na^+, K^+-ATPase (*16*), was used to detect the appearance of Na^+, K^+-ATPase at the cell surface. To resolve this difference, we analyzed the distribution of ouabain binding sites at different times after cell-cell contact. Initially, we detected ouabain binding to both the forming apical and basal-lateral membranes (Fig. 3A). However, within 12 hours after cell-cell contact, binding of ouabain to the apical membrane decreased, as reported (*17*), and by 24 hours, little or no ouabain binding was detected on the apical membrane, although binding sites remained on the basal-

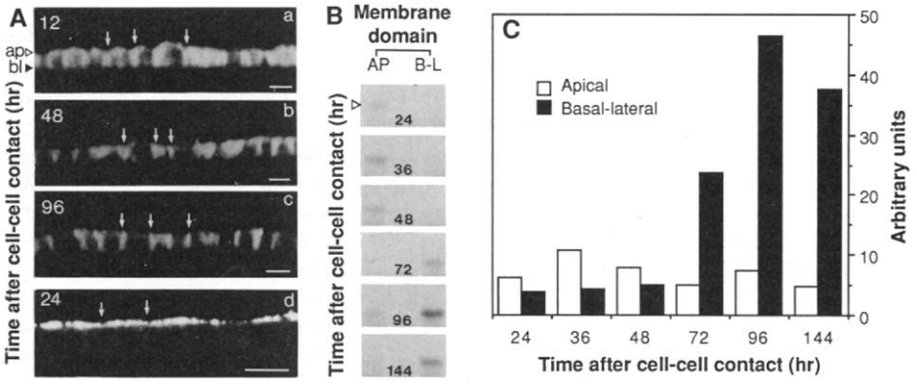


Fig. 1. Development of cell surface polarity of Na^+, K^+-ATPase after cell-cell contact. (**A**) Indirect immunofluoresence and confocal microscopy of the distribution of Na^+, K^+-ATPase. At different times after cell-cell contact, cells were fixed and permeabilized in 100% methanol at −20°C for 10 min, and then processed for indirect immunofluorescence microscopy with antibodies specific for α-subunit Na^+, K^+-ATPase (*22*) (a–c) or antibodies to the apical marker protein, gp135 (*10*) (d) and the appropriate secondary antibody conjugated to rhodamine isothiocyanate (RITC) (Boehringer Mannheim). Filters were viewed in a modified Bio-Rad MRC laser scanning confocal system coupled to a Zeiss IM35 microscope. Sections (x–z) were constructed by averaging five sections over a line at each z position and moving the computer-controlled motorized focus of the microscope in 0.3 μm steps. Bleaching of the sample under these illumination conditions was negligible. Arrows indicate the lateral membrane; bars, 10 μm. (**B**) Autoradiograms showing the distribution of biotinylated α subunit of Na^+, K^+-ATPase (M_r 97000) at different times after cell-cell contact. Either the apical (AP) or basal-lateral (B-L) membrane domain of cells on pairs of filters was treated with NHS-SS-biotin (*11*) at different times after cell-cell contact. Na^+, K^+-ATPase was isolated with specific antibody (*22*), separated by SDS-polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to nitrocellulose (*25*). The biotinylated proteins were detected by probing the nitrocellulose with ^{125}I-labeled streptavidin. After two hours of cell-cell contact, >99.5% of the diffusion of [^3H]inulin across the monolayer was inhibited, indicating that tight junctions had formed. (**C**) The amount of protein in bands from the autoradiograms was quantitated with a scanning densitometer (Hoeffer Scientific) linked to a Macintosh IIcx equipped for automatic integrations and expressed as arbitrary units. Results are from an experiment representative of seven experiments.

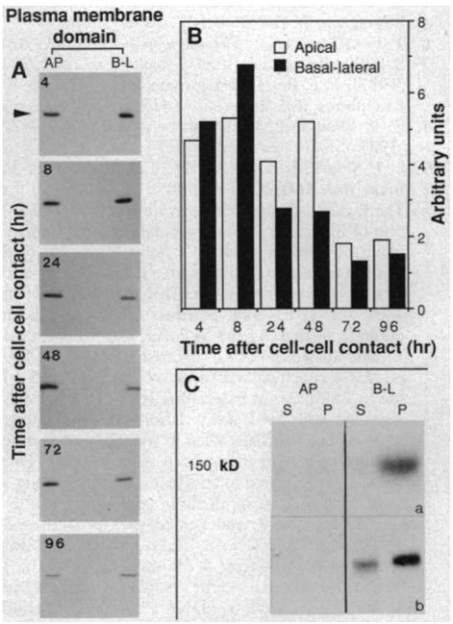


Fig. 2. Delivery of newly synthesized Na^+, K^+-ATPase and desmoglein 1 to the cell surface after cell-cell contact. The results of a representative experiment of six trials are presented. (**A**) At different times after cell-cell contact, cells on pairs of filters were labeled for 1 hour with [^{35}S]methionine-cysteine, and then biotinylated with NHS-S S-biotin on the apical (AP) or basal-lateral (B-L) membrane (*11*) to capture the first wave of newly synthesized protein to arrive at the cell surface. Cells were extracted, and Na^+, K^+-ATPase was sequentially precipitated with specific antibody (*22*) and avidin-agarose (*11*). Precipitates were processed for SDS-PAGE and fluorography (*9*). (**B**) Autoradiograms were quantitated by scanning densitometry and the values obtained expressed as arbitrary units. (**C**) Total (a) (Fig. 1) and newly synthesized (b) (Fig. 2) desmoglein 1 detected by cell surface biotinylation (*8*) on either on the apical (AP) or basal-lateral membrane (B-L) domain 8 hours (a) and 72 hours (b) after cell-cell contact; cell extracts were separated by centrifugation into Triton X-100 soluble (S) and insoluble (P) fractions prior to immunoprecipitation (*14*).

lateral membrane (Fig. 3A). Because substantial amounts of Na^+, K^+-ATPase protein are present in the apical membrane at these times (Fig. 1) we suggest that this population of enzyme is inactive and does not bind ouabain. Thus, functional polarity of Na^+, K^+-ATPase is rapidly established in these cells. These results may explain why delivery of newly synthesized Na^+, K^+-ATPase to the apical membrane was not detected with ouabain in the previous study.

Loss of ouabain binding to Na^+, K^+-ATPase in the apical membrane did not appear to be due to delivery of an abnormal Na^+, K^+-ATPase subunit complex. Analysis of newly synthesized or total biotinylated Na^+, K^+-ATPase on the apical membrane at these times revealed the presence of a biotinylated αβ-subunit complex (Fig. 3B) similar to that found in the basal-lateral membrane (*18*). However, we cannot rule out the possibility that the apical Na^+, K^+-ATPase complex is abnormally folded. A more likely possibility is that loss of ouabain binding results from selective inactivation of Na^+, K^+-ATPase. The apical membrane of MDCK cells has a relatively high concentration of glycosphingolipids and decreased membrane fluidity compared to the basal-lateral membrane (*19*), and these properties may influence Na^+, K^+-ATPase activity (*20*).

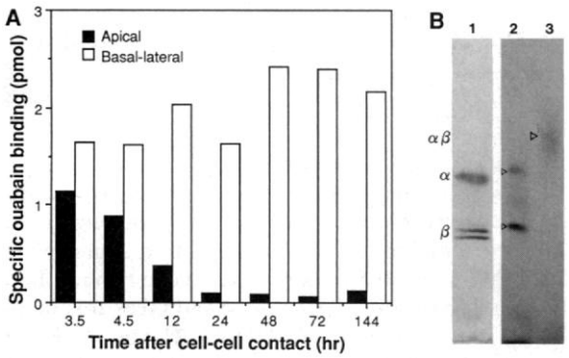
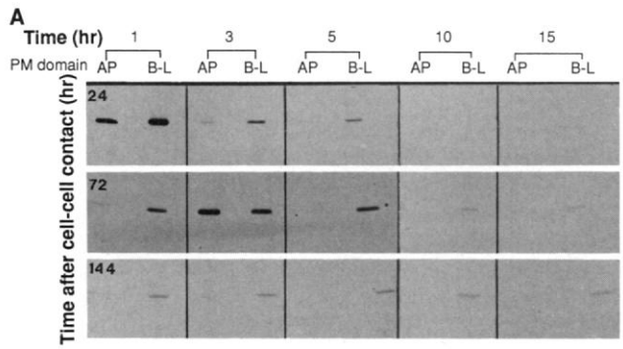


Fig. 3. Plasma membrane distribution of ouabain binding sites at different times after cell-cell contact. The results are representative of four experiments. At different times after cell-cell contact, the apical or basal-lateral membranes of cells on pairs of filters were incubated in Ringer's buffer with [^{3}H]ouabain (Dupont/NEN; 15.4 Ci/mmol, final concentration, 10^{-6} M) for 10 min at 22°C. Cells were washed 3 times in 0.1M $MgCl_2$ in Ringer's buffer for 20 min (*17*), and radioactivity was counted directly in 5 ml of scintillation fluid (Beckman) in a Beckman LS 5000CE spectrometer. Binding of ouabain to the apical and basal-lateral surfaces is expressed as specific binding per filter (~2.8×10^6 cells). More than 98% [^{3}H]ouabain binding was inhibited by the addition of a 100-fold excess of unlabeled ouabain. (**B**) Detection of an αβ-subunit complex of Na^+, K^+-ATPase in the apical membrane 24 hours after cell-cell contact. Na^+, K^+-ATPase biotinylated on the apical membrane was immunoprecipitated with antibody to the α subunit; analysis of the immunoprecipitates with ^{125}I labeled streptavidin revealed that biotinylated β-subunit co-immunoprecipitated with the α subunit (lane 1). The α and β subunits of Na^+, K^+-ATPase were labeled with [^{35}S]methionine and cysteine, biotinylated on the apical surface, and isolated as a complex by precipitation with antibodies specific to the α subunit and avidin-agarose (lane 2). When immunoprecipitates isolated as in lane 1 were boiled prior to SDS-PAGE, the subunits formed an αβ-subunit complex and migrated as a single protein species (M_r ~140,000) (lane 3).



B

Time after induction of cell-cell contact (hr)	Residence time: $t_{0.5}$ (hr)			
	Apical membrane		Basal-lateral membrane	
	Total	Na^+,K^+-ATPase	Total	Na^+,K^+-ATPase
4	9	3	12	3
24	14	1-2	12	6
72	14	1-2	13	12
144	9	1-2	10	>36

Fig. 4. Residence time of newly synthesized Na^+, K^+-ATPase on the cell surface after cell-cell contact. The results are representative of three experiments (**A**). At intervals after cell-cell contact (4, 24, 72, 144 hours), cells on pairs of filters were labeled for 30 min with [^{35}S]methionine and cysteine and then incubated to stop the labeling in medium containing unlabeled methionine and cysteine. Na^+, K^+-ATPase was biotinylated with sulfosuccinimidyl-2-(biotinamide)ethyl-1-1, 3′-dithiopropionate (NHS-SS-biotin) on the apical (AP) or basal-lateral (B-L) membrane at intervals after the labeling had been stopped and then isolated with antibodies to the α subunit and avidin-agarose (*11*). Precipitates were processed for SDS-PAGE and fluorography. During the 48 to 72 hours after cell-cell contact, we detected the delayed appearance of an equal amount of Na^+, K^+-ATPase from an intracellular pool to both cell surfaces. Regardless of the lag in delivery, Na^+, K^+-ATPase exhibited differences in residence times in the two domains. (**B**) Residence times of Na^+, K^+-ATPase and total protein on the apical and basal-lateral membranes calculated by regression analysis of the rate at which proteins were lost from each cell surface domain during the period after labeling was terminated; decay followed first-order kinetics. Total cell surface proteins were isolated from the cell extracts with avidin-agarose beads and directly counted in a liquid scintillation spectrometer.

We tested whether preferential stabilization of the Na^+, K^+-ATPase in the basal-lateral rather than the apical membrane domain could account for the restricted distribution of the protein. At various times after cell-cell contact, cells were briefly labeled with [^{35}S]methionine and cysteine. At various times after labeling was stopped, newly synthesized Na^+, K^+-ATPase was biotinylated on either the apical or basal-lateral membrane to determine the residence times of the protein in each membrane domain. Four hours after cell-cell contact, Na^+, K^+-ATPase had a very short residence time (~3 hours) on the forming apical membrane (Fig. 4A). At all subsequent times analyzed, Na^+, K^+-ATPase that was delivered to the apical membrane continued to have a short residence time (<2 hours). The fate of Na^+, K^+-ATPase removed from the apical cell surface is not known; however, we did not detect an increase in the amount of Na^+, K^+-ATPase on the basal-lateral membrane after the labeling had been terminated, which would correspond to insertion of Na^+, K^+-ATPase derived from the apical membrane (see Fig. 4A).

The residence time of Na^+, K^+-ATPase delivered to the basal-lateral membrane gradually increased after cell-cell contact; by 144 hours after cell-cell contact the residence time was >36 hours (Fig. 4A) or approximately 40 times longer than the residence times of Na^+, K^+-ATPase in the apical membrane domain (Fig. 4B). These results indicate that the difference between retention times of Na^+, K^+-ATPase in the apical and basal-lateral membranes can account for the development of cell surface polarity of Na^+, K^+-ATPase in the absence of vectorial delivery of protein from the Golgi complex to the basal-lateral membrane.

We propose that increased retention time on the membrane is the result of selective exclusion of Na^+, K^+-ATPase from endocytic vesicles due to its assembly into the membrane-cytoskeleton. Na^+, K^+-ATPase binds with high affinity to a complex of membrane-cytoskeletal proteins containing ankyrin and fodrin (*21, 22*), and in MDCK cells Na^+, K^+-ATPase accumulates at sites

of cell-cell contact where membrane-cytoskeletal complexes are assembled, possibly by linkage to E-cadherin (*23*) (Fig. 1). In membranes that lack membrane-cytoskeletal complexes (such as the apical membrane of MDCK cells) Na^+, K^+-ATPase is rapidly internalized at a rate (Fig. 4) similar to that of constitutive endocytosis of cell surface markers (~2 hours) (*23*). Assembly of the membrane-cytoskeletal complexes in selective domains may provide a flexible mechanism for generating different distributions of Na^+, K^+-ATPase in other polarized epithelial cells in which the same subunits are localized to the apical membrane (*1, 2*) or to both (*4*) the apical and lateral membranes.

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Activation of a Small GTP-Binding Protein by Nucleoside Diphosphate Kinase

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Genes that encode nucleoside diphosphate kinases (NDKs) have been implicated as regulators of mammalian tumor metastasis and development in *Drosophila melanogaster*. However, the cellular pathways through which NDKs function are not known. One potential mechanism of regulation is phosphorylation of guanosine diphosphate (GDP) bound to regulatory guanosine triphosphate (GTP) binding proteins. NDK-catalyzed phosphorylation of bound GDP was investigated for the adenosine diphosphate ribosylation factor (ARF), a 21-kilodalton GTP-binding protein that functions in the protein secretion pathway. Bovine liver NDK, recombinant human NDK, and the protein product of the mouse gene nm23-1, which suppresses the metastatic potential of certain tumor cells, used ARF·GDP as a substrate, thereby allowing rapid and efficient production of activated ARF (ARF·GTP) in the absence of nucleotide exchange. These data are consistent with the proposed function of NDK as an activator of a small GTP-binding protein and provide a mechanism of activation for a regulatory GTP-binding protein that is independent of nucleotide exchange.

NUCLEOSIDE DIPHOSPHATE KINASE (NDK) is a commonly occurring enzyme that catalyzes the phosphorylation of nucleoside diphosphates by nucleoside triphosphates. The NDKs have been proposed to function in the maintenance of nucleoside triphosphate pools (*1, 2*). Cloning of two genes encoding homologs of NDK, nm23, (expression of which reduces the metastatic potential of certain tumor cells) and awd (a developmental gene) (*3*), has renewed previous speculation (*2*) that NDKs may serve regulatory functions. One possible site of NDK action is as an activator of regulatory guanine nucleotide-binding proteins. If NDK serves as an activator of a regulatory GTP-binding protein the enzyme must utilize GDP as a substrate while GDP is bound to the regulatory protein, and the NDK-catalyzed production of GTP must result in activation of the regulatory protein. Several regulatory GTP-binding proteins, including members of both the heterotrimeric G protein family and the monomeric 20- to 25-kD family, have been examined as potential NDK substrates (*4, 5*). However, it has not been shown that NDK can use bound GDP as a substrate or that a GTP-binding protein can be activated by such a mechanism. ADP ribosylation factor (ARF), a 21-kD GTP-binding protein implicated in Golgi function, has two characteristics that helped us to examine these issues. First, the activity of ARF can be assessed with a simple in vitro assay, that is, ARF is a required cofactor for the cholera toxin–catalyzed ADP ribosylation of G_s, a heterotrimeric G protein (*6, 7*). This activity is found only for GTP-bound ARF and not the GDP-bound protein. Second, nucleotide exchange kinetics of the purified protein have been well characterized, and in the absence of phospholipid and detergent the nucleotide dissociation rate is negligible (*7*). Even under optimal conditions for nucleotide exchange the release of GDP is slow and the stoichiometry of GTP binding is less than 0.10.

Addition of NDK to purified ARF that was first bound with [α-^{32}P]GDP caused conversion to [α-^{32}P]GTP at a rate proportional to the quantity of added NDK (Fig. 1A) (*8*). At the highest concentration of NDK examined (140 nM), 80% of the bound GDP was converted to GTP within 3 min (*9*). We obtained similar results using commercially available bovine liver NDK or purified recombinant nm23-H1, nm23-H2, or nm23-1 proteins (*10, 11*). Our results

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