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Specific Biochemical Inactivation of Oncogenic Ras Proteins by Nucleoside Diphosphate Kinase[1]

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ABSTRACT

Activating mutations of *Ras* have been implicated in ~30% of human cancers. In every case, the biochemical consequence of such mutations is to disrupt the GTPase activity of Ras and to render Ras resistant to the actions of GTPase activating proteins. Consequently, oncogenic Ras mutants are "locked" in a GTP-bound active state. We detected a potent activity in *Escherichia coli* extract that can efficiently convert mutationally activated GTP-bound Ras to the inactive GDP-bound form. Purification of the protein responsible for this activity led to the identification of the enzyme nucleoside diphosphate kinase (Ndk). The human orthologue of Ndk is the NM23 metastasis suppressor, which we found to exhibit a similar activity. Purified Ndk effectively inactivates several of the oncogenic forms of Ras that are seen frequently in human cancers, including RasD12, the most commonly detected Ras mutation. Significantly, Ndk does not detectably affect wild-type Ras or an activated form of the Ras-related Rho GTPase. These results demonstrate that it is possible, through biochemical means, to specifically inactivate oncogenic Ras as a potential therapeutic approach to tumors that harbor Ras mutations. Moreover, the results suggest that the loss of NM23 expression that is commonly observed during tumor progression could lead to increased potency of oncogenic Ras proteins.

INTRODUCTION

Oncogenic mutations in the human *Ras* genes (*H-*, *N-*, and *K-Ras*) are observed in ~30% of human cancers (1). Tumors that are most often associated with *Ras* mutations include those of the pancreas, colon, and lungs. Most mutations have been detected in the *K-Ras* gene, and they typically involve missense substitutions of the encoded GTPase in one of three amino acid positions (12, 13, or 61) that occupy the catalytic site of GTP hydrolysis (2). Such mutational substitutions invariably lead to diminished intrinsic GTPase activity, as well as resistance to GTPase stimulation by Ras-specific GAPs.[3] Thus, these mutated forms of Ras remain GTP-bound, and transduce constitutive signals for cell proliferation (3–5).

The biochemical basis for the GTPase defect in oncogenic Ras has been partially elucidated. Glutamine-61 of Ras appears to play a role in activating a water molecule for nucleophilic attack, and its substitution by any other amino acid abolishes both intrinsic and GAP-stimulated GTPase activity. The crystal structure of the Ras-RasGAP complex revealed that any mutation of glycine-12 or glycine-13 would position a side-chain that both displaces glutamine-61 and sterically occludes the catalytic "arginine finger" of GAP (R789), resulting in a loss of intrinsic and GAP-stimulated GTPase activity (6). One exception is the Ras proline-12 mutant, which exhibits a 100-fold increased intrinsic GTPase activity compared with other codon 12 mutants. Interestingly, despite its resistance to GAP, Ras proline-12 cannot transform cultured fibroblasts, suggesting that partial restoration of the GTPase activity of oncogenic Ras mutants might be sufficient to prevent their ability to promote oncogenesis (7).

Because of the prominence of *Ras* mutations in human cancers, Ras has been considered an important target for the development of drug-based cancer therapies. Currently, the majority of small molecule-based therapies designed to target Ras are based on inhibition of the enzyme FTase. FTase catalyzes the COOH-terminal farnesylation of Ras, a post-translational modification that is essential for Ras function. Although FTase inhibitors can block the proliferation of Ras-transformed cells *in vitro*, clinical trials with these molecules have been disappointing thus far, and it is possible that their antiproliferative effects on transformed cells are because of inhibition of any one of numerous other cellular FTase substrates (8, 9). Moreover, this strategy does not selectively target the oncogenic forms of Ras, and, therefore, is likely to disrupt the functions of wild-type Ras that are required in normal cells.

Another potential therapeutic strategy to target oncogenic Ras is the development of GTP analogues that can be hydrolyzed by GTPase-deficient oncogenic Ras proteins. On binding these analogues, mutant Ras catalyzes its own conversion to the GDP-bound state (10). However, GTP analogues are unlikely to have therapeutic utility considering the many cellular GTP-binding proteins of which the function would be perturbed by such molecules.

Whereas characterizing the biochemical properties of several K-Ras (hereafter "Ras") mutants commonly found in human cancers, we made the surprising observation that a crude *E. coli* cell extract contains an activity that efficiently converts GTP-bound mutant forms of Ras to an inactive GDP-bound form *in vitro*. This activity was characterized, purified, and identified as the enzyme Ndk. In its purified form, Ndk was demonstrated to biochemically inactivate these Ras mutants, but not wild-type Ras or a mutationally activated Rho GTPase. Thus, these results establish the "proof-of-principle" that it is possible to promote the selective inactivation of oncogenic Ras proteins.

MATERIALS AND METHODS

Generation of Recombinant DNA Plasmids and Site-directed Mutagenesis. Human *K-Ras4B* and *NM23* were amplified by PCR from full-length cDNA and cloned into the pGEX-KG vector (11). Site-directed mutagenesis was performed using a standard primer-directed PCR-based strategy to generate five plasmids encoding mutant *K-Ras4B* genes: G12D, G13D, G13R, Q61L, and Q61R. The GST-*RhoV14* plasmid was kindly provided by Dr. Alan Hall, University College London, London, UK.

Expression and Purification of Recombinant Proteins. GST fusion proteins corresponding to K-Ras4B, RhoV14, and GST-NM23 were purified from *E. coli* extracts after induction of expression by isopropyl-1-thio-β-D-galactopyranoside, using procedures recommended by Stratagene. K-Ras4B and RhoV14 were liberated from GST by thrombin cleavage, and the thrombin was subsequently removed using *p*-aminobenzamadine agarose beads. GST-NM23 was eluted from glutathione agarose beads with free glutathione. Proteins were dialyzed against a solution containing 50 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT, and 20% glycerol, and quantified by SDS-PAGE and Coomassie Blue staining, using BSA as a standard.

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[3] The abbreviations used are: GAP, GTPase-activating protein; FTase, farnesyltransferase; Ndk, nucleoside diphosphate kinase; GST, glutathione *S*-transferase; PEI, polyethylenimine; FPLC, fast protein liquid chromatography.

Purification, Sequencing, and Identification of the Ras-inactivating Protein. *E. coli* BL21 cells were grown overnight in 8 liters of Luria-Bertani medium. Cells were harvested, freeze-thawed, and resuspended in 144 ml of a solution containing 25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 5 mg/ml chicken egg white lysozyme (Sigma). The lysate was sonicated, cleared by centrifugation, and then 1.44 ml of a 50% (w/v) solution of PEI (Sigma) was added. The lysate was cleared of the resultant precipitate by centrifugation and loaded onto a Q Sepharose 26/10 FPLC column (Amersham Pharmacia Biotech) equilibrated previously in Buffer A [25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT]. A linear gradient of NaCl (100–600 mM) was run, and eluted fractions were assayed for activity in the 96-well GTP hydrolysis assay described below. The fractions representing the peak of activity were pooled, and 35% $(NH_4)_2SO_4$ was added. The solution was cleared by centrifugation and loaded onto a phenyl Sepharose 26/10 FPLC column equilibrated previously in Buffer A containing 20% $(NH_4)_2SO_4$. A linear gradient of $(NH_4)_2SO_4$ was run (20% to 0%), and pooled fractions representing the peak of activity were increased to 60% $(NH_4)_2SO_4$. The resultant precipitate was pelleted by centrifugation, resuspended in 2 ml Buffer A, and dialyzed extensively against Buffer A. The dialysate was loaded onto a MonoQ 5/5 FPLC column equilibrated in Buffer A. A linear NaCl gradient (250–600 mM) was run, and pooled fractions representing the peak of activity were increased to 35% $(NH_4)_2SO_4$. The solution was cleared by centrifugation and loaded onto a phenyl Superose 5/5 FPLC column equilibrated in Buffer A containing 20% $(NH_4)_2SO_4$. A linear gradient of $(NH_4)_2SO_4$ was run (20% to 0%), pooled fractions representing the peak of activity were increased to 150 mM NaCl, and the solution was loaded on a Sephacryl S-100 16/60 FPLC column equilibrated previously in a solution containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT. Fractions representing the peak of activity were pooled, analyzed by SDS-PAGE, and found to contain five distinct bands. Each band was excised and sequenced by tandem mass spectrometry (Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, MA). Database searches of the resultant sequences revealed three unique proteins, including Ndk.

GTP Hydrolysis Assays. For all of the assays, the nucleotide exchange reaction was performed by incubating 3 μg Ras (143 pmol) with 25 μCi of either [γ-^{32}P]GTP (NEN, 6000 Ci/mmol) or [α-^{32}P]GTP (NEN, 800 Ci/mmol) in 100 μl of a solution containing 50 mM HEPES (pH 7.4), 50 mM NaCl, 0.1 mM DTT, 0.1 mM EGTA, 5 mM EDTA, and 1 mg/ml BSA (Sigma) for 10 min at 37°C. After incubation, $MgCl_2$ was added to 10 mM, and the reaction was incubated on ice for 10 min.

For all of the 96-well assays, the Ras-[α/γ-^{32}P]GTP was diluted into 2–6 ml Buffer A containing 1 mg/ml BSA, and a 100 μl aliquot was used for each reaction. During characterization and purification of the lysate activity, assays were performed in the presence of 0.1 mM GTP and 0.1 mM ATP. Assays to determine the GTP-dissociation rate from Ras proteins were performed in the presence of 0.1 mM GTP. Candidate Ras-inactivating protein(s) were added (detailed below), and reactions were incubated at 37°C for 1 h (characterization/purification), 30°C for 10 min (p120 RasGAP activity), or 25°C for 30 min (GTP-dissociation rate). After incubation, the reactions were filtered through a nitrocellulose membrane (Schleicher & Schuell BA85) on a vacuum manifold and washed twice with cold Buffer A. Filters were recovered, and the quantity of bound nucleotide was determined either by autoradiography or liquid scintillation.

The standard GTP hydrolysis assay was modified to 96-well format to enable rapid parallel evaluation of samples. A plate-sized vacuum manifold was fitted with a bottomless 96-well plate, and a seal was formed by a rectangular sheet of nitrocellulose membrane (Schleicher & Schuell BA85). Filter-bound radioactivity was visualized by autoradiography.

Ras-inactivating molecules were added before incubation as follows. To determine the activity of p120 RasGAP against Ras proteins, 20 ng p120 RasGAP (12) was added. To determine the effects of boiling and dialysis on the lysate activity, crude *E. coli* lysate was either boiled for 5 min or dialyzed against Buffer A (SpectraPor, 10,000 molecular weight cut-off), and 8 μg lysate was added. To determine the effects of phosphatase inhibitors on lysate activity, either phenylphosphate or sodium fluoride was added to the lysates at 5 mM, and 8 μg lysate was added. To determine the activity of crude *E. coli* lysates both lacking and overexpressing Ndk, three *E. coli* strains were obtained as a gift from Dr. Linda Wheeler (Oregon State University, Corvallis, OR): JC7623 (parental strain), QL7623 (Ndk$^-$), and pKT8P3 (Ndk overexpressing). Lysates were prepared from each strain as described above, and 8 μg lysate was added.

To determine the activity of purified Ndk toward each mutant K-Ras protein, the assay was performed with the following modifications. Products of the nucleotide exchange reaction were desalted over a PD10 gel filtration column (Amersham Pharmacia Biotech), eluted in 2 ml of Buffer A, and 1 mg/ml BSA was added to prevent nucleotide dissociation. Fifty μl aliquots were incubated with either 0.39 μg (26 pmol) of bovine Ndk (Sigma) or 1 μg purified GST-NM23 at 25°C for 30 min. "0 min" time points were incubated on ice for the duration of the reaction. Reactions were stopped by the addition of 10–15 mM EDTA and 0.1–1.0% SDS, and bound nucleotides were liberated by incubation for 10 min at 65°C. Ten μl of each reaction was spotted on a PEI-cellulose TLC plate (EM Science), and the labeled nucleotides were separated with a mobile phase of 0.75 M KH_2PO_4 (pH 3.4). Nucleotides were visualized by autoradiography, and then corresponding spots were excised from the TLC plates and radioactivity was quantified by liquid scintillation.

RESULTS AND DISCUSSION

Detection of a Ras-inactivating Activity in *E. coli* Extracts. We routinely use a conventional filter-binding assay to measure Ras GTP-hydrolyzing activity. Specifically, purified recombinant Ras is "loaded" with [γ-^{32}P]GTP, incubated with candidate Ras-inactivating molecules, and then passed through a protein-binding filter that traps radiolabeled phosphate only when it is bound to Ras. After filter washing, the degree of GTP hydrolysis can be quantified by measuring filter-trapped (Ras-associated) radioactivity. A 96-well version of this assay was used for some experiments to enable rapid parallel evaluation of multiple samples (Fig. 1*a*). Using this assay, we made the surprising observation that an *E. coli* cell extract contains an activity that promotes the complete loss of radioactivity associated with mutant K-Ras within 1 h of incubation (Fig. 1*b*). We next determined that this activity is nondialyzable and largely inactivated by boiling, suggesting that it is associated with a protein (Fig. 1*c*).

The observed loss of filter-bound radioactivity in such an assay could potentially reflect only one of several biochemical activities: (*a*) a protease that degrades Ras; (*b*) a protein that promotes nucleotide dissociation from Ras; (*c*) a phosphatase that removes phosphates from Ras-bound GTP; and (*d*) a protein that specifically promotes release of the γ-phosphate from Ras-GTP, yielding Ras-GDP.

To address the first two possibilities, we performed the same analysis using Ras that was loaded with [α-^{32}P]GTP (Fig. 1*d*). We found that, unlike with the γ-phosphate-labeled GTP, Ras-bound α-phosphate-labeled nucleotide was retained on the filter after incubation with *E. coli* extract. This result indicates that the observed activity is because of the selective loss of the γ-phosphate from mutant Ras, and is not because of degradation of Ras protein or nucleotide release. To address the third possibility, we performed the assay in the presence of the general phosphatase inhibitors phenylphosphate and sodium fluoride (Fig. 1*e*). In both cases, no inhibition of activity is seen, suggesting that a phosphatase is not involved. Together, these results suggest that the protein activity in bacterial extracts is GAP-like in that it promotes the conversion of Ras-GTP to Ras-GDP.

Purification and Identification of Ras-inactivating Activity as Ndk. To identify the Ras-inactivating protein in *E. coli*, we performed a series of conventional protein purification steps. Using the oncogenic Ras mutant RasD13 and the 96-well GTP hydrolysis assay (Fig. 2*a*), we followed a single major Ras-inactivating activity through several column chromatography steps. After the strategy outlined in Fig. 2*b,* we obtained a protein fraction that contained substantial activity (Fig. 2*c*) and was associated with three detectable protein bands by SDS-PAGE and Coomassie Blue staining. The proteins were sequenced by tandem mass spectrometry, and a 15 kDa

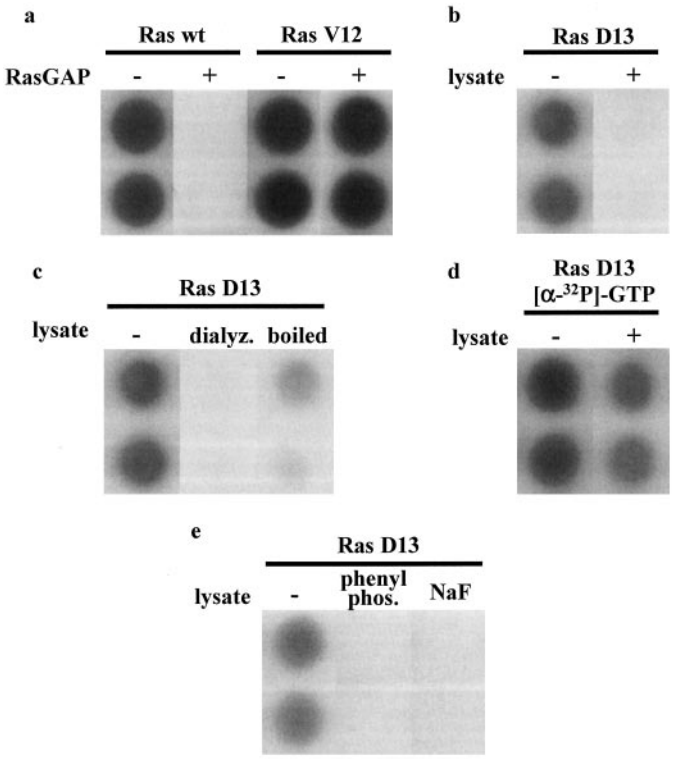

Fig. 1. A protein in *E. coli* lysate inactivates oncogenic Ras. *a,* wild-type Ras and RasV12 were loaded with [γ-^{32}P]GTP, and incubated in the presence and absence of p120 RasGAP at 30°C for 10 min. After incubation, the reactions were vacuum-filtered through a nitrocellulose membrane, and bound radioactivity was visualized by autoradiography. Results of duplicate assays are shown for each experiment. *b,* RasD13 was loaded with [γ-^{32}P]GTP, and then incubated in the presence and absence of crude *E. coli* extract at 37°C for 1 h. After incubation, radioactivity was analyzed as in *a. c,* RasD13 was loaded with [γ-^{32}P]GTP, and then incubated at 37°C for 1 h in the presence and absence of crude *E. coli* lysate that had either been boiled for 5 min or dialyzed through a 10,000 MWCO membrane. After incubation, radioactivity was analyzed as in *a. d,* RasD13 was loaded with [α-^{32}P]GTP, and then incubated in the presence and absence of crude *E. coli* lysate at 37°C for 1 h. After incubation, radioactivity was analyzed as in *a. e,* RasD13 was loaded with [γ-^{32}P]GTP, and then incubated at 37°C for 1 h in the presence and absence of crude *E. coli* lysate that had been pretreated with either phenylphosphate or sodium fluoride (*NaF*). After incubation, radioactivity was analyzed as in *a*.

protein was identified as Ndk. Ndk was a particularly attractive candidate because its mammalian orthologue had been shown previously to bind and/or regulate the nucleotide state of several other GTPases, including Rad (13), Arf (14), Pra (15), menin (16), β-tubulin (17), dynamin (18), and Gα (19). Ndk is a highly evolutionarily conserved enzyme that catalyzes the interconversion of nucleoside di- and triphosphates through the generation of a phosphoenzyme intermediate (20). Several previous reports have demonstrated the ability of Ndk to interact with a variety of cellular proteins, suggesting that its nucleotide-converting activity is only one of its functions (21, 22).

To confirm that Ndk accounts for the observed activity on RasD13, we examined the activity in extracts from an *E. coli* strain that is genetically deficient in *Ndk*, and a strain that has been engineered to overexpress Ndk (Fig. 3, *a* and *b*). Lysate prepared from the Ndk$^-$ strain exhibits an ~5-fold reduction in the Ras-regulatory activity relative to that seen in wild-type lysate, whereas lysate prepared from the overexpressing strain exhibits an ~10-fold increase in the activity relative to wild-type lysate. These results strongly implicate Ndk as the relevant Ras-regulating protein in *E. coli* extract. Notably, the strain lacking Ndk has been reported to exhibit ~15% of the classical Ndk nucleotide-converting activity seen in wild-type *E. coli*, suggesting that a second *E. coli* protein can also perform this activity (23). This probably accounts for the fact that we did not observe a complete loss of the Ras-regulatory activity in extract from the mutant strain, and importantly, also suggests that the previously defined enzymatic activity of Ndk is responsible for the observed effects on mutant Ras.

Mammalian Orthologues of Ndk Inactivate Oncogenic Ras. To determine whether mammalian forms of Ndk have a similar Ras-inactivating activity, we tested the ability of purified bovine Ndk to regulate RasD13. Using TLC to visualize the conversion of mutant Ras from a GTP-bound to a GDP-bound state, we found that bovine Ndk exhibits a similar time- and dose-dependent Ras-inactivating activity (Fig. 3*c*; data not shown). After a 45-min incubation, mutant Ras could be nearly completely inactivated by bovine Ndk. However, because Ndk has been reported to associate with a variety of cellular proteins, we cannot exclude the possibility that an additional co-purifying protein contributes to the Ras-inactivating activity of "purified" Ndk.

Interestingly, the human orthologue of Ndk is the NM23 metastasis suppressor (24). Reduced expression of NM23 has been correlated with the progression of several tumor types in a many clinical studies. However, the mechanism by which NM23 regulates tumorigenesis is unclear, and could potentially involve any of several reported cellular functions, including the regulation of nucleotide pools, transcriptional activity, DNA cleavage activity, and a signal transduction role (22, 25). We determined that the human NM23 protein, expressed in bacteria as a GST-fusion, also exhibits the Ras-inactivating activity (Fig. 3*d*). It has been suggested that NM23 influences oncogenesis by regulating the activity of members of the small GTPase family (22). Our results support this hypothesis and suggest that selective loss of

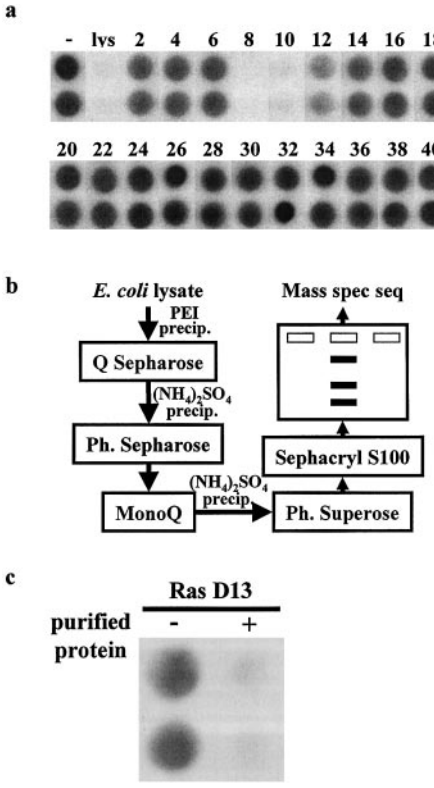

Fig. 2. Purification of the protein responsible for the activity from *E. coli* lysate. *a,* RasD13 was loaded with [γ-^{32}P]GTP and incubated with anion-exchange (*MonoQ*)-fractionated *E. coli* lysate. After incubation at 37°C for 1 h, reactions were vacuum-filtered through a nitrocellulose membrane, and bound radioactivity was visualized by autoradiography. Fraction numbers are indicated, and fractions 8–10 correspond to the peak of activity. *b,* schematic description of the purification as described in "Materials and Methods." *c,* RasD13 was loaded with [γ-^{32}P]GTP and incubated with the enriched protein fraction corresponding to the peak of activity from the Sephacryl S-100 column. After incubation at 37°C for 1 h, the reactions were vacuum-filtered through a nitrocellulose membrane, and bound radioactivity was visualized by autoradiography.

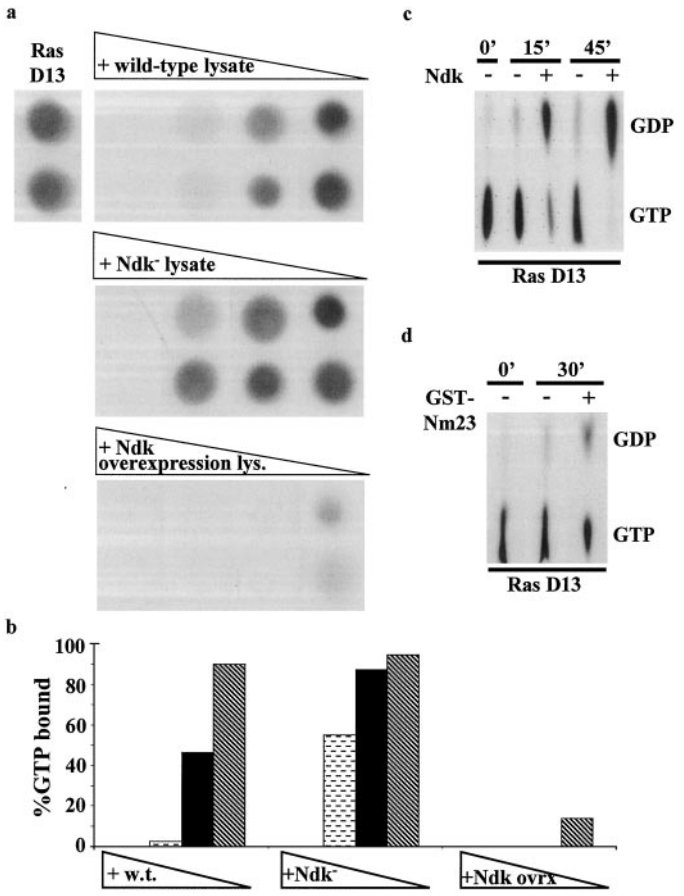

Fig. 3. Ndk is the protein responsible for the Ras-inactivating activity in *E. coli* lysate. *a,* RasD13 was loaded with [γ-^{32}P]GTP and incubated at 37°C for 1 h with lysates prepared from *E. coli* strains deficient in Ndk, overexpressing Ndk, or a wild-type strain. After incubation, reactions were vacuum-filtered through a nitrocellulose membrane, and bound radioactivity was visualized by autoradiography. *b,* radioactivity was quantified by densitometry using the public domain NIH Image program.[4] *c,* RasD13 was loaded with [α-^{32}P]GTP. Purified bovine Ndk (Sigma) was added, and the reaction was incubated at 25°C for 15 or 45 min. After incubation, guanine nucleotides were separated by TLC and visualized by autoradiography. *d,* RasD13 was loaded with [α-^{32}P]GTP. Purified GST-NM23 was added, and the reaction was incubated at 25°C for 30 min. After incubation, guanine nucleotides were separated by TLC and visualized by autoradiography.

NM23 in human tumors harboring a Ras mutation could increase the potency of the encoded oncogenic Ras proteins. In addition, because activation of Ras and loss of NM23 have both been implicated in tumor invasion, it is possible that NM23 loss facilitates Ras-mediated tumor invasion (24, 26).

Ndk Can Specifically Inactivate Several of the Commonly Detected Oncogenic Ras Proteins. Having determined that Ndk can efficiently promote the inactivation of the oncogenic Ras mutant RasD13, we next examined the effect of Ndk on several of the oncogenic forms of Ras that are most commonly observed in human cancers. These include RasD12, RasR13, RasL61, and RasR61, each of which is resistant to the activity of a Ras GTPase activating protein (p120 RasGAP; Fig. 4*a*). As shown, Ndk can promote the inactivation of each of these mutants to various degrees (Fig. 4*b*). The effect on RasD12 and RasD13 is the strongest, the effect on RasR13 and RasL61 is slightly weaker, and the effect on RasR61 is very weak. Significantly, RasD12 is the most commonly detected Ras mutation in human cancers (2). Interestingly, we determined that Ndk exhibits no detectable activity on wild-type Ras (Fig. 5*a*), consistent with previous findings (13). Moreover, in an additional test of specificity, we found that Ndk does not detectably influence the nucleotide state of an activated Rho mutant, RhoV14 (Ref. 27; Fig. 5*b*). Thus, Ndk promotes the selective inactivation of oncogenic Ras proteins.

Ndk Acts on Ras-bound GTP. Although Ndk has been implicated in a variety of biochemical and cellular processes, its precise biological function remains somewhat unclear. Previous studies have revealed that Ndk can influence the nucleotide state of several GTPases, although there has been some controversy as to whether those results, in some cases, might reflect the action of Ndk on nucleotide that has become dissociated from the GTPase during incubation (21, 28). Therefore, we have performed two experiments to rule out this possibility in the case of Ndk activity toward mutant Ras. First, as described above, we determined that when the assay is performed with Ras loaded with [α-^{32}P]GTP, there is no detectable loss of associated nucleotide (Fig. 1*d*). This is true despite the presence of 1000-fold excess of "cold" GTP present in the reaction, which would effectively replace any labeled nucleotide released during incubation.

In addition, we used an assay to quantify nucleotide dissociation from each of the Ras mutants under the conditions used for the demonstration of Ndk activity (Fig. 6). As shown, essentially all of the labeled nucleotide that is bound to Ras at the start of the incubation remains associated with Ras 30 min later. Together, these results indicate that the possibility of nucleotide dissociation from Ras, followed by nucleotide conversion by Ndk, cannot account for the observed effects. Therefore, Ndk must be interacting directly with the

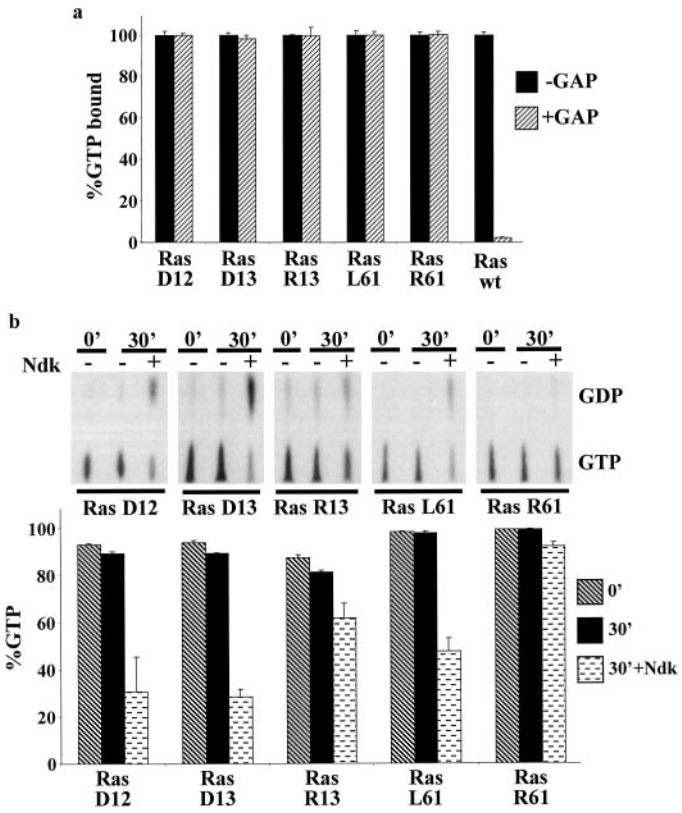

Fig. 4. Ndk promotes the inactivation of several oncogenic Ras proteins. *a,* RasD12, RasD13, RasR13, RasL61, RasR61, and wild-type Ras were loaded with [γ-^{32}P]GTP, and incubated in the presence and absence of p120 RasGAP at 30°C for 10 min. After incubation, the reactions were vacuum-filtered through a nitrocellulose membrane, and bound radioactivity was quantified by liquid scintillation. The graph shows the average of four experiments. *b,* RasD12, RasD13, RasR13, RasL61, and RasR61 were loaded with [α-^{32}P]GTP. Purified bovine Ndk (Sigma) was added and reactions were incubated at 25°C for 30 min. After incubation, guanine nucleotides were separated by TLC and visualized by autoradiography. Corresponding spots were excised from the TLC plate, and radioactivity was quantified by liquid scintillation. A representative TLC is shown, and the graph indicates the average of at least four experiments; *bars,* ± SD.

[4] Internet address: http://rsb.info.nih.gov/nih-image/.

Ras-bound GTP molecule. Considering that Ndk can directly accept the γ-phosphate from a nucleoside triphosphate, we imagine the most likely scenario is that Ndk is able to interact with Ras-GTP in such a way that results in the transfer of the γ-phosphate directly to Ndk, yielding Ras-GDP and an Ndk phosphoenzyme intermediate. This interaction would potentially be sensitive to structural differences in the Ras nucleotide-binding pocket that influence the "accessibility" of the γ-phosphate to an interacting protein. Interestingly, the crystal structures of several oncogenic Ras proteins, including RasD12 and RasL61, reveal structural differences between the wild-type and mutant enzymes in the nucleotide-binding pocket that affect the local environment of the γ-phosphate (7, 29), and would, therefore, be predicted to alter its accessibility to an interacting protein. This is consistent with our finding that Ndk exhibits a range of activities toward the various oncogenic Ras proteins and the wild-type enzyme.

Significance of the Ndk-Ras Interaction. In addition to raising the possibility that loss of NM23 expression during tumor progression increases the potency of oncogenic Ras proteins, our findings also suggest an alternative therapeutic strategy for Ras inactivation. However, we believe that the therapeutic potential of the Ndk/NM23 protein itself is probably quite limited, because NM23 interacts with myriad cellular proteins and has been reported to affect a wide variety of cellular processes. Thus, it seems likely that therapeutic delivery of high levels of NM23 would be toxic to normal cells, and we do not imagine that such an approach would provide an effective Ras-specific therapy. Moreover, because NM23 can interact with numerous cellular proteins (several small GTPases among them), it would be difficult to prove conclusively in a cell-based assay that the interaction between NM23 and Ras is specifically responsible for blocking Ras transformation. Indeed, previous studies have demonstrated the ability of overexpressed NM23 to inhibit the tumorigenic properties of human cancer cell lines that do not harbor oncogenic Ras mutations (25).

Nevertheless, our results establish the proof-of-principle that it is possible to achieve the specific biochemical inactivation of oncogenic forms of Ras *in vitro*. Future experiments to establish the precise mechanism of Ndk activity toward oncogenic Ras could form the basis of a novel therapeutic strategy to recapitulate this effect with a small molecule. Similarly, it is possible that a small molecule could be identified that promotes the selective interaction of Ndk with oncogenic Ras proteins *in vivo*. Furthermore, if Ndk and Ras form a stable complex, its X-ray crystal structure would yield valuable information regarding the structural requirements for the inactivation of mutant Ras. Thus, it will be interesting to explore the mechanistic and structural implications of the Ndk-Ras interaction toward the goal of developing novel treatments for Ras-associated cancers.

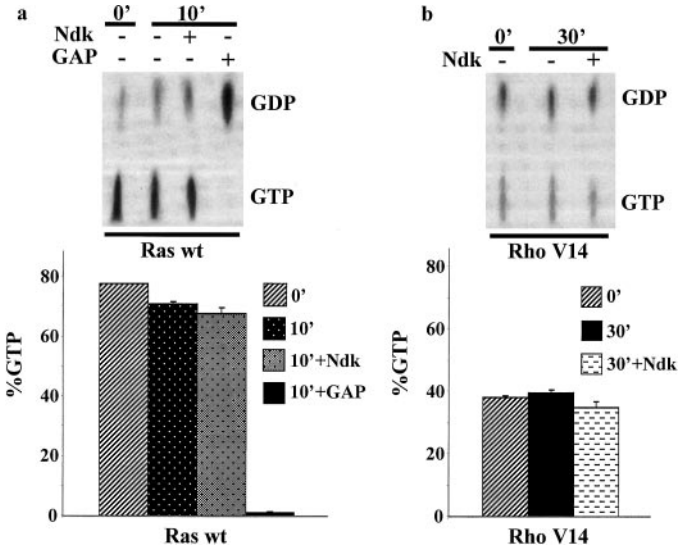

Fig. 5. Ndk activity is specific to oncogenic Ras. *a*, wild-type Ras and (*b*) RhoV14 were each loaded with [α-^{32}P]GTP. Purified bovine Ndk (Sigma) or p120 RasGAP was added and reactions were incubated at (*a*) 30°C for 10 min or (*b*) 25°C for 30 min. After incubation, guanine nucleotides were separated by TLC and visualized by autoradiography. Corresponding spots were excised from the TLC plate, and radioactivity was quantified by liquid scintillation. A representative TLC is shown, and the graph indicates the average of four experiments; *bars,* ±SD.

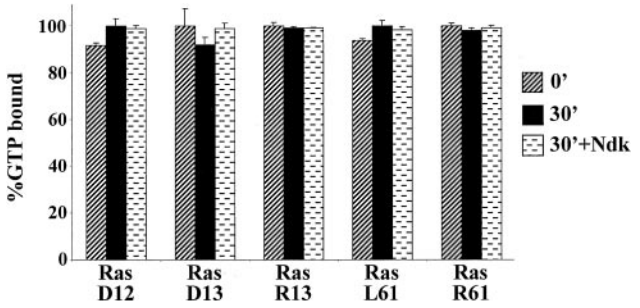

Fig. 6. GTP does not dissociate from the oncogenic Ras proteins over the time course of the experiment. RasD12, RasD13, RasR13, RasL61, and RasR61 were each loaded with [α-^{32}P]GTP and incubated in the presence of a 1000-fold excess of cold GTP at 25°C for 30 min. Purified bovine Ndk was added where indicated. After incubation, reactions were vacuum filtered through a nitrocellulose membrane, and bound radioactivity was quantified by liquid scintillation. The graph indicates the average of four experiments; *bars,* ±SD.

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REFERENCES

1. Bos, J. L. ras oncogenes in human cancer: a review. Cancer Res., *49:* 4682–4689, 1989.
2. Andreyev, H. J., Norman, A. R., Cunningham, D., Oates, J. R., and Clarke, P. A. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J. Natl. Cancer Inst., *90:* 675–684, 1998.
3. Bourne, H. R., Sanders, D. A., and McCormick, F. The GTPase superfamily: a conserved switch for diverse cell functions. Nature (Lond.), *348:* 125–132, 1990.
4. Bourne, H. R., Sanders, D. A., and McCormick, F. The GTPase superfamily: conserved structure and molecular mechanism. Nature (Lond.), *349:* 117–127, 1991.
5. Lowy, D. R., and Willumsen, B. M. Function and regulation of ras. Annu. Rev. Biochem., *62:* 851–891, 1993.
6. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science (Wash. DC), *277:* 333–338, 1997.
7. Franken, S. M., Scheidig, A. J., Krengel, U., Rensland, H., Lautwein, A., Geyer, M., Scheffzek, K., Goody, R. S., Kalbitzer, H. R., Pai, E. F., and *et al.* Three-dimensional structures and properties of a transforming and a nontransforming glycine-12 mutant of p21H-ras. Biochemistry, *32:* 8411–8420, 1993.
8. Downard, J. Targeting Ras signalling pathways in cancer therapy. Nat. Rev., *3:* 11–22, 2003.
9. Kloog, Y., Cox, A. D., and Sinensky, M. Concepts in Ras-directed therapy. Exp. Opin. Investig. Drugs, *8:* 2121–2140, 1999.
10. Ahmadian, M. R., Zor, T., Vogt, D., Kabsch, W., Selinger, Z., Wittinghofer, A., and Scheffzek, K. Guanosine triphosphatase stimulation of oncogenic Ras mutants. Proc. Natl. Acad. Sci. USA, *96:* 7065–7070, 1999.
11. Guan, K. L., and Dixon, J. E. Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem., *192:* 262–267, 1991.
12. Hu, K. Q., and Settleman, J. Tandem SH2 binding sites mediate the RasGAP-RhoGAP interaction: a conformational mechanism for SH3 domain regulation. EMBO J., *16:* 473–483, 1997.
13. Zhu, J., Tseng, Y. H., Kantor, J. D., Rhodes, C. J., Zetter, B. R., Moyers, J. S., and Kahn, C. R. Interaction of the Ras-related protein associated with diabetes rad and the putative tumor metastasis suppressor NM23 provides a novel mechanism of GTPase regulation. Proc. Natl. Acad. Sci. USA, *96:* 14911–14918, 1999.

14. Palacios, F., Schweitzer, J. K., Boshans, R. L., and D'Souza-Schorey. C. ARF6-GTP recruits Nm23–H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat. Cell Biol., *4:* 929–936, 2002.
15. Chopade, B. A., Shankar, S., Sundin, G. W., Mukhopadhyay, S., and Chakrabarty, A. M. Characterization of membrane-associated Pseudomonas aeruginosa Ras-like protein Pra, a GTP-binding protein that forms complexes with truncated nucleoside diphosphate kinase and pyruvate kinase to modulate GTP synthesis. J. Bacteriol., *179:* 2181–2188, 1997.
16. Yaguchi, H., Ohkura, N., Tsukada, T., and Yamaguchi, K. Menin, the multiple endocrine neoplasia type 1 gene product, exhibits GTP-hydrolyzing activity in the presence of the tumor metastasis suppressor nm23. J. Biol. Chem., *277:* 38197–38204, 2002.
17. Lombardi, D., Sacchi, A., D'Agostino, G., and Tibursi, G. The association of the Nm23–M1 protein and β-tubulin correlates with cell differentiation. Exp. Cell Res., *217:* 267–271, 1995.
18. Krishnan, K. S., Rikhy, R., Rao, S., Shivalkar, M., Mosko, M., Narayanan, R., Etter, P., Estes, P. S., and Ramaswami, M. Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling. Neuron, *30:* 197–210, 2001.
19. Cuello, F., Schulze, R. A., Heemeyer, F., Meyer, H. E., Lutz, S., Jakobs, K. H., Niroomand, F., and Wieland, T. Activation of heterotrimeric G proteins by a high-energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits I. Complex formation of NDPK B with G$\beta\gamma$ dimers and phosphorylation of His266 in Gβ. J. Biol. Chem., *278:* 7220–7226, 2003.
20. Lascu, I., and Gonin, P. The catalytic mechanism of nucleoside diphosphate kinases. J. Bioenerg. Biomembr., *32:* 237–246, 2000.
21. Otero, A. S. NM23/nucleoside diphosphate kinase and signal transduction. J. Bioenerg. Biomembr., *32:* 269–275, 2000.
22. Roymans, D., Willems, R., Van Blockstaele, D. R., and Slegers, H. Nucleoside diphosphate kinase (NDPK/NM23) and the waltz with multiple partners: possible consequences in tumor metastasis. Clin. Exp. Metastasis, *19:* 465–476, 2002.
23. Lu, Q., and Inouye, M. Adenylate kinase complements nucleoside diphosphate kinase deficiency in nucleotide metabolism. Proc. Natl. Acad. Sci. USA, *93:* 5720–5725, 1996.
24. Salerno, M., Ouatas, T., Palmieri, D., and Steeg, P. S. Inhibition of signal transduction by the nm23 metastasis suppressor: possible mechanisms. Clin. Exp. Metastasis, *20:* 3–10, 2003.
25. Hartsough, M. T., and Steeg, P. S. Nm23/nucleoside diphosphate kinase in human cancers. J. Bioenerg. Biomembr., *32:* 301–308, 2000.
26. Mareel, M., and Leroy, A. Clinical, cellular, and molecular aspects of cancer invasion. Physiol. Rev., *83:* 337–376, 2003.
27. Ihara, K., Muraguchi, S., Kato, M., Shimizu, T., Shirakawa, M., Kuroda, S., Kaibuchi, K., and Hakoshima, T. Crystal structure of human RhoA in a dominantly active form complexed with a GTP analogue. J. Biol. Chem., *273:* 9656–9666, 1998.
28. Randazzo, P. A., Northup, J. K., and Kahn, R. A. Regulatory GTP-binding proteins (ADP-ribosylation factor. Gt, and RAS) are not activated directly by nucleoside diphosphate kinase. J. Biol. Chem., *267:* 18182–18189, 1992.
29. Krengel, U., Schlichting, L., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E. F., and Wittinghofer, A. Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. Cell, *62:* 539–548, 1990.