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DIRECT ACTIVATION OF GUANINE NUCLEOTIDE BINDING PROTEINS THROUGH A HIGH-ENERGY PHOSPHATE-TRANSFER BY NUCLEOSIDE DIPHOSPHATE-KINASE

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Summary: An in vitro study of phosphate-transfer, from the high-energy phosphates on the phosphoenzyme (enzyme-bound high-energy phosphate intermediate) of NDP-kinase to GDP on various guanine nucleotide binding proteins (G_1, elongation factor α_1, recombinant v-ras^H p21 protein, transducin, Gi and Go), revealed that the GDP acts as a phosphate-acceptor, in the presence of divalent cations (Mg^{2+} and Ca^{2+}). This finding suggests that via phosphate-transfer, NDP-kinase may be responsible for the direct activation of various guanine nucleotide binding proteins through phosphate-transfer by the enzyme.

Recently, we reported that (i) nucleoside diphosphate (NDP)-kinase [EC 2.7.4.6], which catalyzes a phosphate-transfer in a wide variety of nucleoside 5'-di- and triphosphates (1-3), rapidly forms a phosphoenzyme (an enzyme-bound high-energy phosphate intermediate) when incubated with one of the nucleoside 5'-triphosphates (NTPs), in the presence of divalent cations (Mg^{2+} and Ca^{2+}) (4,5); (ii) incubation of the phosphoenzyme with one of the nucleoside 5'-diphosphates (NDPs) in the presence of these divalent cations results in the formation of NTPs, without strict base-specificity (6); (iii) guanine

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Abbreviations: NDP-kinase, nucleoside-diphosphate kinase; p21 protein, ras p21 protooncogene product; r-ras p21, recombinant v-ras^H p21 protooncogene product; Gt, transducin; G-proteins, guanine nucleotide binding proteins (Gi, Gs and Go); EF-α_1, α-subunit of elongation factor; NDP, nucleoside 5'-diphosphate; NTP, nucleoside 5'-triphosphate.

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nucleotide binding proteins, which have biochemical characteristics similar to r-ras p21, are associated with these enzymes (5,6); and (iv) GDP on the guanine nucleotide binding proteins (G_1 and G_2) from NDP-kinases, as well as GDP on r-ras p21, acts as a phosphate-acceptor for the high-energy phosphates on the phosphoenzyme in the presence of divalent cations (7). The present study was undertaken to determine any common characters, of the phosphate-transfer between the phosphoenzyme of NDP-kinase and GDP-bound guanine nucleotide binding proteins, such as the EF-α_1, r-ras p21, Gt, Gi and Go. The results show that, in the presence of 0.2 mM Ca^{2+}, GDP acts as a phosphate acceptor for the high-energy phosphates on the phosphoenzyme, producing GTP, which is an essential activator for these guanine nucleotide binding proteins. This finding suggests that, via phosphate-transfer, NDP-kinase may be responsible for the direct activation of various guanine nucleotide binding proteins.

MATERIALS AND METHODS

Chemicals: [γ-^{32}P]ATP (20.0 Ci/mmol), [α-^{32}P]GTP (200.0 Ci/mmol) and [^{35}S]GTP-γ-S (1262 Ci/mmol) were obtained from Amersham International plc (England), nucleoside 5'-diphosphates (ADP, GDP, CDP and UDP), and dithiothreitol from Sigma Chemical Co., Ltd. (USA), nitrocellulose membrane filters (type TM-1, 0.65 μm) from Toyo Roshi Co., Ltd. (Tokyo) and PEI-cellulose F thin-layer plate from Merck (USA).

Guanine nucleotide binding proteins: Six different highly purified guanine nucleotide binding proteins (G_1, r-ras p21, EF-α_1, Gt, Gi and Go) were used in this study. NDP-kinase-associated guanine nucleotide binding protein (G_1) was isolated from the purified NDP-kinase F-1 fraction of HeLa S3 cells, as reported previously (5). Gt (bovine eye), Gi and Go from porcine cerebrum (8) were kindly supplied by Dr. T. Haga (Hamamatsu Univ. Sch. Med., Hamamatsu, Japan). EF-α_1 from wheat germ (9) and r-ras p21 were generously supplied by respectively, Dr. S. Ejiri (Iwate Univ., Morioka, Japan) and Dr. S. Hattori (Univ. of Tokyo, Tokyo).

Assay for GTP binding activity: The reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 0.1 mM EDTA, 5 mM Mg^{2+}, 10 μM [^{35}S]GTP-γ-S (1262 Ci/mmol) and the indicated amount of various guanine nucleotide-binding proteins (G_1, r-ras p21, EF-α_1, Gt, Gi and Go). The mixtures were incubated for 10 min in an ice bath and [^{35}S]GTP-γ-S incorporation into the proteins was arrested by the addition of 2.0 ml of 0.1 M sodium pyrophosphate/10 mM EDTA. The solution was passed through a nitrocellulose membrane filter (type TM-1) and washed with the same buffer. After drying, the [^{35}S]radioactivity on the membrane filter was measured using a liquid scintillation spectrometer (5,6).

Assay for phosphoenzyme formation of NDP-kinase: As reported previously (2,3,5), NDP-kinase rapidly forms a phosphoenzyme (an enzyme-bound, high-energy phosphate intermediate) when incubated with [γ-^{32}P]ATP, in the presence of divalent cations such as 1 mM Ca^{2+} and 1 mM Mg^{2+}. The [^{32}P]phosphoenzyme

formed was determined quantitatively by the nitrocellulose membrane method (2).

Purification of NDP-kinases: NDP-kinase (F-I) was extracted from HeLa S3 cells (in cell pellets of approximately 5 ml) and highly purified by Sephacryl S200 gel filtration and DEAE-cellulose column chromatography (3,5). Guanine nucleotide binding proteins ([^{35}S]GTP-γ-S binding proteins) can be separated from NDP-kinases by Sephacryl S200 gel filtration with 20 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol and 1 mM EDTA in the presence and absence of 7 M urea, since only NDP-kinase subunits [α-subunit (Mr 21,000) and β-subunit (Mr 18,000)] can be reassociated in the absence of 7 M urea (4,5).

Preparation of [^{32}P]labeled phosphoenzymes: For the preparation of [^{32}P]labeled phosphoenzyme, 10 μg of purified (free of guanine nucleotide binding protein) NDP-kinase F-I from HeLa S3 cells was incubated, for 10 min in an ice bath, with 10 μM [γ-^{32}P]ATP (20.0 Ci/mmol) in the presence of 0.5 mM Ca^{2+}. To remove free [γ-^{32}P]ATP, the reaction mixture was immediately passed through a Sephadex G-25 column (0.7 x 16 cm), previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.5 M KCl, 1 mM dithiothreitol and 1 mM EDTA. [^{32}P]Labeled NDP-kinase F-I (approximately 1 x 10^5 cpm/μg) was obtained.

RESULTS AND DISCUSSION

The kinetics of the phosphate-transfer between the [^{32}P]high-energy phosphates on the [^{32}P]phosphoenzyme of NDP-kinase from HeLa S3 cells (5) and various nucleoside 5'-diphosphates (ADP, GDP, UDP and CDP) were investigated. The findings confirm those of earlier studies on the NDP-kinases from Ehrlich ascites tumor cells (6,7). (i) The [^{32}P]phosphates on the [^{32}P]phosphoenzyme of NDP-kinase can directly transfer to one of the NDPs, in the presence of divalent cations (Mg^{2+} and Ca^{2+}), resulting in the formation of [^{32}P]NTPs without strict base-specificity. (ii) This [^{32}P]NTP was formed in a time-dependent manner within about 40 sec, even at low temperatures (below 4°C). (iii) [^{32}P]ATP was preferentially formed when the [^{32}P]phosphoenzyme as incubated with nucleoside 5'-diphosphate mixtures (ADP, GDP, CDP and UDP, each 5 μM) in the presence of 0.2 mM Ca^{2+}, since the enzyme has a higher affinity for ADP than the other nucleoside 5'-diphosphates (7,10). However, (iv) selective [^{32}P]GTP formation occurred when the [^{32}P]phosphoenzyme was incubated with GDP-bound guanine nucleotide binding protein (G_1) from NDP-kinase F-I in the presence of any NDPs except GDP. Under these conditions, [^{32}P]ATP was undetectable. Similar [^{32}P]GTP formation was observed on addition of GDP-bound r-*ras* p21 instead of GDP-bound G_1, even if nucleoside 5'-diphosphates (ADP and UDP, 5 μM each) were present in the reaction mixtures.

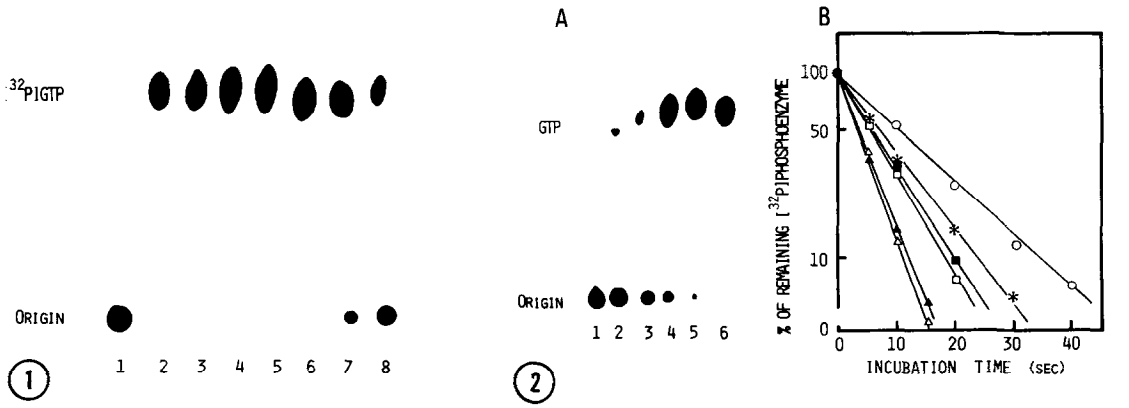

Fig. 1. [^{32}P]GTP formation from the [^{32}P]phosphoenzyme and GDP-bound guanine nucleotide binding proteins. The reaction mixtures (50 µl) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 0.1 mM EDTA, 1 mM Mg^{2+}, 5 µM ADP, various GDP-bound guanine nucleotide binding proteins (protein, 0.5 µg; GDP, approximately 1 mol/molecule protein) and [^{32}P]phosphoenzyme (0.1 µg of protein, approximately 10^4 cpm), were incubated separately for 3 min in an ice bath. After addition of 0.15 M EDTA (final concentration 25 mM), the [^{32}P]GTP formed was detected by PEI thin-layer chromatography, followed by autoradiography, as described in previous our reports (6,7). Lane 1, no incubation of the [^{32}P]phosphoenzyme; lane 2, incubation of the [^{32}P]phosphoenzyme with GDP-bound Gt; lane 3, with GDP-bound Gi; lane 4, with GDP-bound Go; lane 5, with GDP-bound EF-α_1; lane 6, with GDP-bound r-*ras* p21; lane 7, with GDP-bound r-*ras* p21 and Y13-259 (2 µg); and lane 8, with GDP-bound r-*ras* p21 and Y13-259 (10 µg).

Fig. 2. Kinetics of [^{32}P]GTP formation from the [^{32}P]phosphoenzyme of NDP-kinase and GDP or GDP-bound guanine nucleotide binding proteins. The [^{32}P]phosphoenzyme (0.1 µg of protein, approximately 10^4 cpm) was added to the reaction mixtures (50 µl), which contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM Ca^{2+}, 0.1 M KCl and 10 µM GDP. After the mixtures were incubated for the indicated periods in an ice bath, the enzyme reaction was arrested by the addition of 0.15 M EDTA (final concentration 25 mM). Aliquots of 5 µl were spotted separately on a PEI-cellulose F thin-layer plate. The [^{32}P]GTP formed was detected by autoradiography after development in 0.75 M potassium phosphate at room temperature. [A] No incubation of [^{32}P]phosphoenzyme (time 0, lane 1); incubation of the [^{32}P]phosphoenzyme (0.1 µg) with 10 µM GDP in an ice bath for 10 sec (lane 2), 20 sec (lane 3), 30 sec (lane 4), 40 sec (lane 5) and 50 sec (lane 6), respectively. Figure B (right) represents the percentage of the [^{32}P]phosphoenzyme remaining after incubation with GDP (o) or GDP-bound various guanine nucleotide binding proteins [guanine nucleotide binding protein from NDP-kinase F-I (G_1, △), r-*ras* p21 (▲), EF-α_1 (x), Gt (□) and Go (■)] for the indicated incubation times. 100% radioactivity in 50 µl of the control reaction mixture (time 0, ●, total 50 µl) was approximately 1 x 10^4 cpm.

To determine the phosphate-transfer from the high-energy phosphates on the phosphoenzyme of NDP-kinase to GDP on various guanine nucleotide binding proteins [EF-α_1, r-*ras* p21, Gt, Gi and Go], analytical experiments were carried out. The results are shown in Fig. 1, (i) [^{32}P]GTP was formed within 40 sec at 0°C after addition of the [^{32}P]phosphoenzyme of NDP-kinase from HeLa S3 cells to the reaction mixtures, which contained a different guanine

nucleotide binding proteins saturated previously with cold GDP (free GDP being removed by Sephadex G-25 gel filtration). (ii) [^{32}P]GTP formation occurred in all tested GDP-bound guanine nucleotide binding proteins, although these proteins were heterogeneous materials purified from different cell sources. (iii) The monoclonal antibody Y13-259 (12,13), which reacts with all known mammalian and yeast ras-encoded proteins, can prevent the phosphate-transfer between GDP-bound r-ras p21 and the phosphoenzyme. Recently, Hattori et al. reported that the monoclonal antibody Y13-259 severely hampers the nucleotide exchange reaction between ras p21-bound and exogenous guanine nucleotides but does not interfere with the association of GDP to ras p21 (14). This report is basically consistent with our observation since the antibody impairs the activation of ras p21. Moreover, in the presence of 0.2 mM Ca^{2+}, the kinetics of the phosphate-transfer between the [^{32}P]phosphoenzyme and five different GDP-bound guanine nucleotide binding proteins were determined (Fig. 2). The most rapid rates of phosphate-transfer were between the phosphoenzyme and GDP-bound G_1, and GDP-bound r-ras p21. Under the same condition, similar kinetics for Gt and Go were obtained. The reaction rate of this phosphate-transfer (GTP formation) seems to be due to the binding affinity of these proteins for the enzyme. This is supported by the evidence that G_1 and r-ras p21 can be reassociated tightly with the enzyme (5), whereas the others (Gt, Gi and Go) show less affinity for the enzyme. In addition, our preliminary experiments showed that (i) G_1 and r-ras p21 were modified by ADP ribosyltransferase purified from hen liver nuclei (11), but not much with bacterial toxins (pertussis or cholera toxins) (15,16); and (ii) the GTPase activity of the modified G_1 or r-ras p21 was highly reduced, but no affect of their modification on the GTP forming and GTP binding activities was observed. Finally, we concluded that the protein-bound GDP acts as a phosphate acceptor for the high-energy phosphates on the phosphoenzyme in the presence of 0.2 mM Ca^{2+}.

Recent studies have revealed that G-proteins (Gi, Gs, Go and Gt) are a family of guanine nucleotide binding proteins involved in a variety of

receptor-mediated signal transduction systems (17). Gs and Gi are involved in the hormonal stimulation and inhibition, respectively, of adenylate cyclase activity (15), whereas Gt, which is present predominantly in the retinal rod outer segment, regulates cGMP phosphodiesterase activity (16,18). The α-subunits of EF-1 (9) and eIF-2 (19,20) have characterized guanine nucleotide binding proteins. [eIF-2 and EF-1 are initiation and elongation factors which govern, respectively, initiation and elongation of polypeptide chains eukaryotes (21,22)]. Although these proteins and factors have different physiological functions, their α-subunits all show (i) guanine nucleotide (GDP and GTP) specific binding activities; (ii) low GTP hydrolytic activities; (iii) modification by bacterial toxins (15,16); and (iv) highly homologous DNA sequences at their GTP binding and GTPase domains (23,24). Moreover, it is important to note that these proteins are physiologically active only when complexed to GTP, and become inactive when bound GTP is hydrolyzed to GDP. It is assumed that an inactive form (GDP-protein complex) of these proteins can be converted to an active form (GTP-protein complex) by a GDP-GTP exchange reaction. Recently, Hall and Self have reported that this exchange is mediated by an activated receptor in the presence of low levels of Mg^{2+} (5 μM), which may lead to a conformational change of r-$\underline{ras}^{N}$ p21 (25). GDP-GTP exchange under similar conditions was reported for Gt (26,27) and yeast $\underline{ras}$ gene product (28). However, in such a low Mg^{2+} (5 μM) conditions, no phosphate-transfer could be detected between the high-energy phosphates on the phosphoenzyme and GDP-bound r-$\underline{ras}$ p21. Data presented here, and indirect evidence in early reports (17,24-27), suggest that there are two possible mechanisms involved in the activation of guanine nucleotide binding proteins: (i) a GDP-GTP exchange reaction, which requires a relatively higher concentration of GDP and GTP, and specific mediating factors; and (ii) direct GTP formation from GDP-bound guanine nucleotide binding proteins (inactive forms) and the phosphoenzyme of NDP-kinase. The latter seems apply during the initiation of signal transduction, which is a type of instantaneous reaction, since direct activation of the guanine nucleotide binding proteins by NDP-

kinase is an ATP dependent reaction. In contrast to this, the GDP-GTP exchange reaction may be involved in the recycling of guanine nucleotide binding proteins (α-subunits) in the regulation of adenylate cyclase activity during signal transduction and in the recycling of eIF-2 and EFs during protein synthesis. Further analytical studies are needed to explain the physiological discrepancies between these two activation mechanisms.

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