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DIRECT INTERACTION BETWEEN MEMBRANE-ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE AND GTP-BINDING PROTEIN(Gs), AND ITS REGULATION BY HORMONES AND GUANINE NUCLEOTIDES

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SUMMARY: In previous studies we have proposed that the membrane-associated nucleoside diphosphate kinase(m-NDP kinase) may play a role as a GTP channeling machinery for adenylate cyclase regulation by hormones. In this study, whether the m-NDP kinase has a direct interaction with the component(GTP-binding protein(Gs)) of the glucagon- and β-adrenergic agonist-sensitive adenylate cyclase systems in rat liver membranes was examined by extraction with octylglucoside, followed by immunoprecipitation by affinity-purified monospecific anti-NDP kinase antibodies. The results demonstrated that the m-NDP kinase and the Gs were extractable as a complexed form and that the complex formation was reversibly regulated, through cell surface receptors, by hormones which had an ability to cause activation of the rat liver adenylate cyclase. Also, it was suggested that guanine nucleotides rather than hormones were primary regulators of the m-NDP kinase-Gs interaction. These results were discussed in relation to the regulatory cycle of the Gs of adenylate cyclase system.

Nucleoside diphosphate kinase(NDP kinase, EC 2.7.4.6.) plays a major role in the enzymatic synthesis of nucleoside triphosphates other than ATP and is mainly located in the cytosol of cells(s-NDP kinase)(1). Since the activity of NDP kinases in many tissues is relatively high compared with that of other enzymes involved in the synthesis of triphosphate nucleotides-(NTPs), it has been believed that the NDP kinase is unlikely to play a regulatory role in the pathways of triphosphate nucleotide syntheses(1). Recently, however, reports suggesting that the NDP kinase may play a role in supplying NTPs into NTP-requiring reactions without complete equilibration with the bulk cellular fluid (channeling(2)) have been accumulating(3-7). Possible involvement of the NDP kinase in channeling of high energy phosphate was first proposed by Lehninger(8). Since then, transfer of NTPs

Abbreviations used are: NDP kinase, nucleoside diphosphate kinase; m-, membrane-associated; s-, soluble(cytosolic); Gs and Gi, the stimulatory and inhibitory GTP binding proteins of adenylate cyclase system, respectively; octylglucoside, n-octyl-β-D-glucoside; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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by the enzyme into various biological reaction systems through such channeling mechanism has been reported (e.g. DNA(3), RNA(4,5) and protein(6) syntheses, and microtubule assembly(7)).

Recently, we found that the NDP kinase activity was also present in plasma membranes(m-NDP kinase) from several sources(9-11), and suggested that the m-NDP kinase may play a role as a GTP supply machinery for hormone sensitive adenylate cyalase systems(9,10,12-14). The interaction between the m-NDP kinase and the adenylate cyclase system seems to be specific in that the introduction of GTP occurs hormone-dependently (9,13) and takes place into a GTP-binding protein(Gs) of adenylate cyclase, but not into an another GTP binding protein, ADP ribosylation factor(14). These observations make it likely that this GTP supply via the m-NDP kinase occurs by the channeling mechanism and suggest that direct interaction of the m-NDP kinase with a component(s) of the adenylate cyclase system could occur by the action of hormones.

To date, at least three components(hormone receptor, GTP-binding proteins(Gs, Gi) and catalyst) have been known to be involved in hormone sensitive adenylate cyclase system(15). Among these, the Gs in stimulatory systems has a hetero-trimeric structure and requires GTP to convert into Gsα-GTP which interacts with and activates adenylate cyclase catalyst(15). Since the Gs bears GTPase activity on the GTP binding site(16,17), rapid and effective replenishment of GTP is thought to be an important process in keeping its active form during stimulation. The Gs, therefore, is a most plausible and suitable partner with which the m-NDP kinase interacts. In this communication, the methods to detect such m-NDP kinase-Gs complexes sensitively were devised by means of solubilization with octylglucoside, followed by immunoprecipitation by the recently developed monospecific anti-NDP kinase antibodies (18). Here we provide evidence that the m-NDP kinase and the Gs can be extracted as a complexed form from the rat liver plasma membranes. Moreover, this interaction can be regulated, in combination with guanine nucleotides, by glucagon and, in a certain condition, a β-adrenergic agonist.

MATERIALS AND METHODS

Materials [^{32}P]NAD was purchased from New England Nuclear. A Yamasa cyclic AMP assay kit was a generous gift from Yamasa Shoyu Co(Choshi, Japan). Glucagon, insulin, isoproterenol, propranolol, PMSF, GTP, GDP, and NAD were obtained from Sigma. Affi-Gel 15, and Affi-Gel Protein A were purchased from Bio-Rad. ATP(GTP-free) and Gpp(NH)p were from Boehringer Mannheim. Pansorbin and forskolin were from Calbiochem-Behring. Cholera toxin was obtained from Seikagaku Kogyo Co.(Japan). Octylglucoside was from Wako Pure Chemical Co. Vasopressin and angiotensin II were from Peptide Institute Inc.(Osaka, Japan). Prostaglandin E_1 was from Funakoshi Pharmaceutical Co. Epidermal growth factor was purchased from Toyobo Co(Osaka, Japan).

Preparation of liver plasma membranes Liver plasma membranes from rats 10 weeks after birth were prepared as described previously(19). The membranes from rats 2 weeks after birth were prepared similarly except that sucrose density gradient centrifugation was performed once in an SW 27 rotor.

[^{32}P]ADP ribosylation of Gs in membranes The Gs in the purified rat liver plasma membranes was [^{32}P]ADP ribosylated in incubation mixture containing, in a total volume of 2ml, 200mM potassium phosphate, pH 7.1, 5μM [^{32}P]NAD(1mCi), 1mM GTP, 1mM ATP, 10mM thymidine, 100μg/ml of preactivated cholera toxin and 2.5mg of membranes. Incubation was carried out for 5min at 37°C and then terminated by adding 100μl of 40mM NAD. The labeled membranes were washed twice by centrifugation at 20,000xg for 15min in a buffer of 50mM Tris/HCl, pH 7.4 containing 0.2% bovine serum albumin and 0.25mM phenylmethylsulfonyl fluoride(PMSF).

Analyses on m-NDP kinase-Gs complexes The labeled membranes(~ 0.3 mg) were routinely treated without, or with 1μM glucagon and/or 0.1mM Gpp(NH)p or other reagents for 5min at 30°C in 50mM Tris/HCl, pH 7.4 containing 1mM $MgCl_2$, 0.2% bovine serum albumin and 0.25mM PMSF in a total volume of 60 μl, subsequently cooled on ice and then solubilized for 30min at 0°C by adding equal volume of 2% octylglucoside prepared in the above buffer. The samples were ultracentrifuged at 105,000xg for 30min and 100-μl aliquots of the supernatants were treated overnight at 0°C with the anti-NDP kinase antibodies or the nonimmune rabbit IgG. The immune complexes formed were adsorbed to formaldehyde-fixed Staphylococcus aureus cells(Pansorbin), detached in SDS sample buffer with boiling and then subjected to SDS-PAGE with a 10% gel, followed by autoradiography.

Preparation and purification of antibodies The affinity-purified anti-NDP kinase antibodies and the affinity purified nonimmune rabbit IgG were prepared as described previously(18).

Adenylate cyclase assay Adenylate cyclase activity was determined by measuring cyclic AMP with radioimmunoassay after succinylation as described previously(13).

Miscellaneous Protein concentration was determined as described by Lowry et al.(20) using bovine serum albumin as a standard.

Densitometry was done using Shimazu CS-910 TLC scanner by monitoring absorption at 600nm.

Autoradiography was performed using Kodak XAR film with intensifying screens.

RESULTS

The radioactivity corresponding to the Rf value of the Gs(Gsα) was clearly detected by treatment with the anti-NDP kinase antibodies, while only a small amount of radioactivity was precipitated by the nonimmune rabbit IgG(Fig.1). The precipitation of the Gs by the anti-NDP kinase antibodies can be ascribed to an indirect action of the antibodies as a result of the complex formation between the m-NDP kinase and the Gs. The following observations favor this view. 1)The isolated radioactive Gs(Gsα) on SDS-PAGE was not immunoprecipitated by the antibodies. 2)Precipitation of the Gs depended on detergents used; detergents including Lubrol PX and digitonin did not cause such a result despite their ability to solubilize the labeled Gs. 3)Even with octylglucoside(1%), only a small portion(up to 5%) of the solubilized Gs was precipitable by the antibodies.

The interaction between m-NDP kinase and Gs was essentially unaffected by glucagon alone, enhanced to a small extent by Gpp(NH)p, and

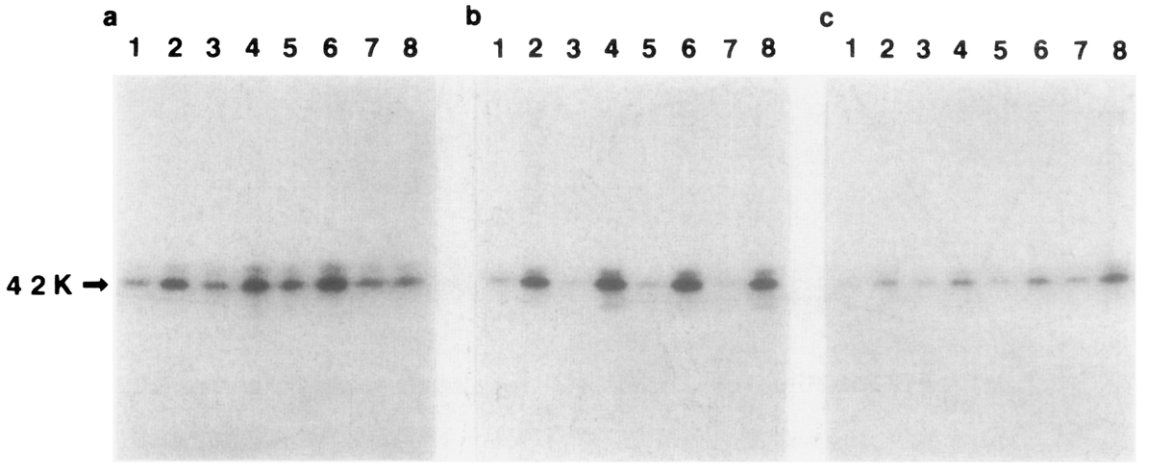
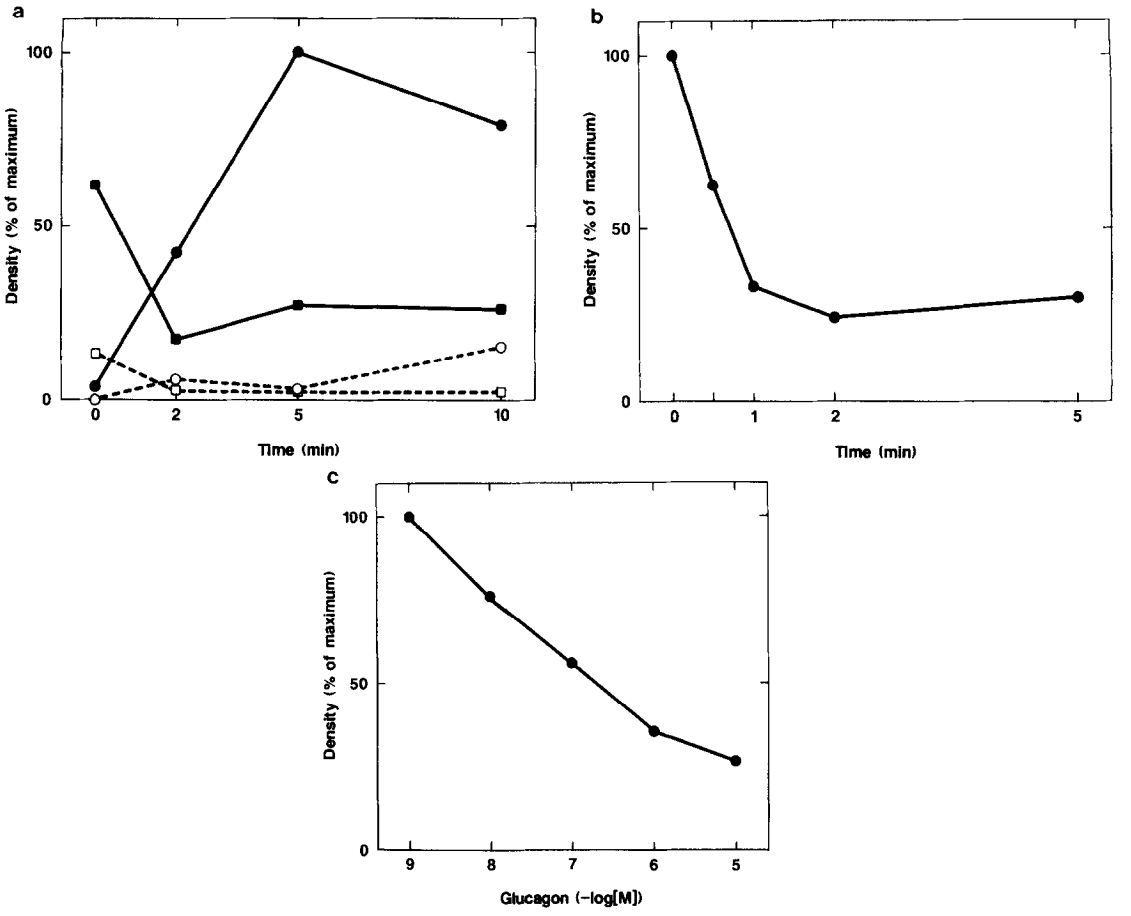
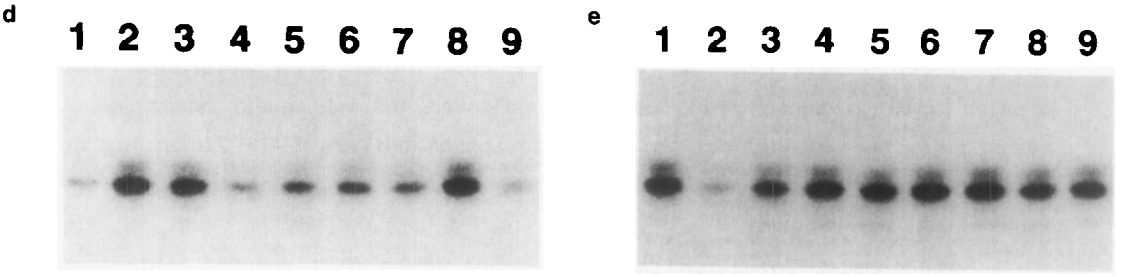


Fig. 1. Precipitation of octylglucoside-solubilized m-NDP kinase-Gs complex by affinity-purified anti-NDP kinase antibodies.
a) The reagents were added before solubilization and processed as described under "MATERIALS AND METHODS". Additions were: 1 and 2, none; 3 and 4, 1μM glucagon; 5 and 6, 0.1mM Gpp(NH)p; 7 and 8, glucagon plus Gpp(NH)p. 1,3,5, and 7, the purified nonimmune IgG; 2,4,6, and 8, the purified anti-NDP kinase antibodies. b) The labeled membranes were treated with glucagon and/or Gpp(NH)p for 5min at 30°C, subsequently washed by centrifugation at 20,000xg for 15min to remove these compounds and then solubilized with octylglucoside. Other methods were as in a. c) The labeled membranes were solubilized, and the solubilized samples were treated with glucagon and/or Gpp(NH)p for 30min at 0°C and then processed as in a.

almost completely disrupted by glucagon plus Gpp(NH)p when these compounds were added before solubilization(Fig.1,a). By contrast, glucagon plus Gpp(NH)p rather enhanced the complex formation when they were added after solubilization(Fig.1,c). It should be noted that when membranes were treated with glucagon plus Gpp(NH)p, followed by subsequent washings to remove them and solubilization, the interaction between m-NDP kinase and Gs recovered to the original level(Fig.1,b), demonstrating that this interaction occurs reversibly.

The complex formation in the presence of Gpp(NH)p and the inhibitory effect of glucagon on it were time- and dose-dependent(Fig.2, a-c). The former reaction reached maximum at 5 min of incubation(Fig.2,a), while the latter reaction to disaggregate the preformed m-NDP kinase-Gs complex was almost completed within 1 min of incubation(Fig.2,b). The concentration of glucagon to cause half maximal effect was approximately 50nM(Fig.2,c), which was somewhat higher than that for adenylate cyclase activation(12,19). On the other hand, GDP or GTP alone was inhibitory for the m-NDP kinase-Gs complex formation, and glucagon rather enhanced the complex formation with GDP(Fig.2,d), which are in contrast with the results obtained with Gpp(NH)p. NaF and forskolin, receptor-independent strong activators of adenylate cyclase, were virtually ineffective upon the interaction between m-NDP





kinase and Gs(Fig.2,e). Isoproterenol and prostaglandin E_1(PGE_1), weak activators for adenylate cyclase from rat liver(9), showed essentially no effect for the interaction(Fig.2,e; Fig.3,b(PGE_1)). Other reagents tested seemed to be ineffective(Fig.2,e).

In order to confirm that the m-NDP kinase-Gs interaction is hormone receptor dependent phenomenon, we tried another hormone, a β-adrenergic agonist, whose action occurs through adenylate cyclase activation. The adenylate cyclase in liver membranes prepared from rats 10 weeks after birth, however, has poor response to the β-agonist (9). Since it has been observed that young rat liver loses response to β-adrenergic agonist during

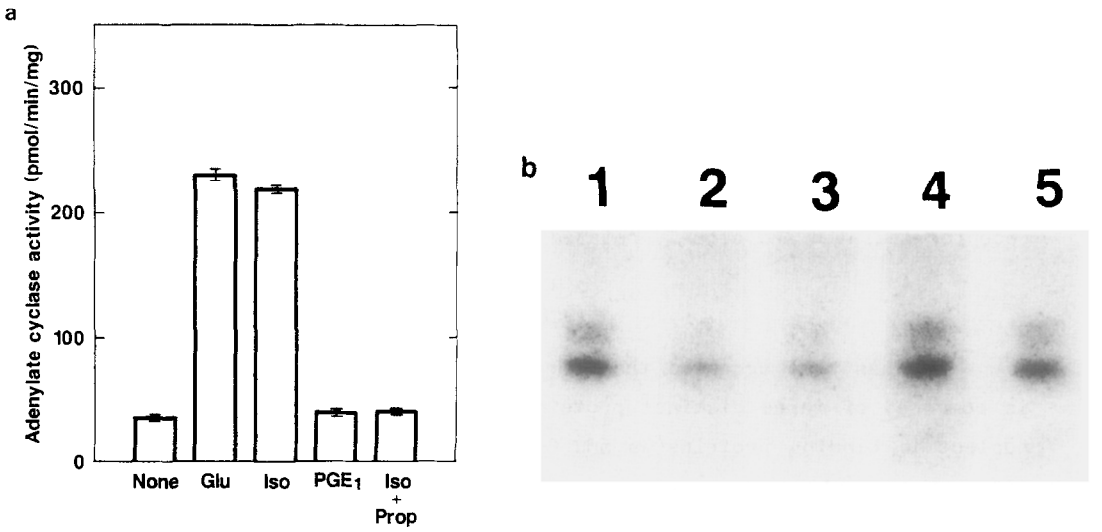


Fig. 3. Effects of isoproterenol on adenylate cyclase activity(a) and complex formation between m-NDP kinase and Gs(b) in liver plasma membranes from rats 2 weeks after birth.
a) Adenylate cyclase assay was done as described under "MATERIALS AND METHODS". Additions, all together with 10μM GTP, were: glucagon(Glu), 1μM; isoproterenol(Iso), 0.1mM; prostaglandin E_1(PGE_1), 10μM; ioproterenol(Iso) plus propranolol(Prop), both 0.1mM. b) The complex formation was examined with 0.1mM Gpp(NH)p and the followings: 1, none; 2, 1μM glucagon; 3, 0.1mM isoproterenol; 4, 10μM prostaglandin E_1; 5, 0.1mM isoproterenol plus 0.1mM propranolol.

development due to the decrease of β-adrenergic receptor(21), we examined the effect of β-adrenergic agonist with the membranes from younger rats. As shown in Fig.3a, the liver adenylate cyclase from the rats 2 weeks after birth displayed a good response to isoproterenol as well as glucagon. In

Fig. 2. Characterization of the interaction between m-NDP kinase and Gs.
a) Time course of the m-NDP kinase-Gs complex formation. The labeled membranes were treated with 0.1mM Gpp(NH)p for indicated periods in the absence(●,○) and presence(■,□) of 1μM glucagon. Closed symbols(●,■), anti-NDP kinase antibodies; open symbols(○,□), nonimmune IgG. b) Time course of the effect of glucagon. The labeled membranes were pretreated for 5min with 0.1mM Gpp(NH)p alone, and subsequently 1μM glucagon was added and incubation was further continued for the indicated periods. c) Effect of the concentration of glucagon. Gpp(NH)p(0.1mM) and glucagon, at indicated concentrations, were added for 5min at 30°C. d) Effects of various guanine nucleotides. Guanine nucleotides were added at 0.1mM without(lanes 1,2,4,6,8) or with(lanes 3,5,7,9) 1μM glucagon for 5min at 30°C. Additions were: 1-3, none; 4 and 5, GDP; 6 and 7, GTP; 8 and 9, Gpp(NH)p. Lane 1 was with the nonimmune IgG, and lanes 2-9 were with the anti-NDP kinase antibodies. e) Effects of various reagents. The labeled membranes were treated for 5min at 30°C with reagents alone(NaF and forskolin) or reagents plus 0.1mM Gpp(NH)p before solubilization. Additions were: 1, none; 2, 1μM glucagon; 3, 10mM NaF; 4, 10μM forskolin; 5, 1μM insulin; 6, 0.1mM isoproterenol; 7, 1μM vasopressin; 8, 1μM angiotensin II; 9, 1μg/ml epidermal growth factor. In these experiments(a-e), unless otherwise mentioned, the solubilized m-NDP kinase-Gs complexes were precipitated with the affinity purified anti-NDP kinase antibodies. Other conditions were as in Fig.1a. Densitometry(a-c) was performed using Shimazu CS-910 TLC scanner.

the membranes, isoproterenol was found to be as effective as glucagon in disrupting the interaction between m-NDP kinase and Gs(Fig.3b). Moreover, both actions of isoproterenol were blocked by the further addition of β-adrenergic antagonist, propranolol. This result, together with the aforementioned ones, strongly suggests that the hormone action on the m-NDP kinase-Gs interaction and that on adenylate cyclase regulation are both receptor-mediated and closely related phenomena.

DISCUSSION

It has been accepted that hormone sensitive adenylate cyclase system is composed of three distinct protein components, hormone receptor, guanine nucleotide binding proteins(Gs and Gi), and catalyst(15). The reconstitution experiments with these purified proteins in lipid vesicles have comfirmed that these three components are required to reconstitute hormone sensitive adenylate cyclase(22). Nevertheless, there have been observations which suggest possible involvement of some other factors in regulation of the adenylate cyclase system(22-29). The m-NDP kinase is one of such protein components, whose role is presumed to supply GTP, an essential cofactor of adenylate cyclase regulation, into the Gs by channeling mechanism(9,10,12-14). Although the present observations are preliminary, they are, to our knowledge, the first which have shown direct interaction between the m-NDP kinase and the Gs under the influence of hormones and guanine nucleotides.

The m-NDP kinase-Gs complex was very sensitive to detergent; Lubrol PX and digitonin failed to solubilize these components as the complexed form. Octylglucoside and, to a lesser extent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate(CHAPS) were found to be satisfactory. Even though octylglucoside was used, only a small portion (up to 5%) of the solubilized Gs was recovered as the complex. However, this does not necessarily mean that the Gs is present in excess of the m-NDP kinase in membranes since both amounts are estimated to be approximately 1 pmol/mg of membrane protein (30,18). Lability of the m-NDP kinase-Gs complex is not surprizing since the observation of such lability of the interaction between membrane adenylate cyclase components in detergent solution is not unprecedented (31). Therefore, despite the lability of the m-NDP kinase-Gs complex, the potential importance of the hormone and guanine nucleotide dependent m-NDP kinase-Gs interaction is suggested in terms of adenylate cyclase regulation. Molecular interaction between the m-NDP kinase and the Gs (in conjunction with hormone receptor) with the purified preparations remains to be studied. The purified m-NDP kinase has been recently purified(18) and is now available for such studies.

The following conclusions can be drawn from this study. 1)The m-NDP kinase and the Gs interact physically in membranes. 2)This interaction can be regulated reversibly by hormones through cell surface receptors only coupled to adenylate cyclase activation. 3)Guanine nucleotide seems to be a primary regulator of this interaction probably by acting through the Gs because the guanine nucleotides alone affected the complex formation, whereas hormones required guanine nucleotides to do so. 4)GDP inhibits, whereas Gpp(NH)p somewhat stimulates the complex formation(the effect of GTP is considered to be the mixed ones of itself and its hydrolytic product, GDP.). 5) Glucagon enhances the complex formation with GDP, while the hormone disrupts it with Gpp(NH)p in a similar dose-response relationship to that for adenylate cyclase activation. These results suggest that the m-NDP kinase-Gs complex formation and adenylate cyclase regulation are closely related; probably both regulations proceed by sharing the regulatory cycle of the Gs(15,32). The fact that isoproterenol became active in disaggregating the complex in parallel with its action on the liver adenylate cyclase strengthens this view.

Whether the hormone dependent GTP channeling mechanism, that is, the transfer of GTP by the m-NDP kinase without complete equilibration with the bulk cellular fluid plays a physiological role and whether the m-NDP kinase-Gs interaction is the underlying mechanism of GTP channeling remain to be fully elucidated. Previous studies have demonstrated that GDP-GTP exchange reaction is the central mechanism in regulation of GTP binding proteins including the Gs(15,32,33). However, in view of our previous studies, it seems likely that the GTP channeling mechanism may operate in concert with the GDP-GTP exchange mechanism in response to external signals, at least, in hormone-stimulable adenylate cyclase systems. In a cultured cell system(10,34), it has been shown that increased hormonal responsiveness which was linked to adenylate cyclase activation was accompanied by an enhancement of the m-NDP kinase activity with no appreciable alteration in s-NDP kinase activity. Recently, a factor capable of regulating GDP-GTP exchange reaction has been reported in yeast(35). It can be envisioned that the m-NDP kinase could be a forth component of the adenylate cyclase system, which endows cells with a rapid response to external signals by channeling GTP into the Gs.

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