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COMMENTARY

TRANSPHOSPHORYLATION AND G PROTEIN ACTIVATION

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The control of a variety of essential cellular functions involves a group of proteins characterized by specific binding of guanine nucleotides and the ability to hydrolyze GTP [1]. This family of GTP-binding proteins includes tubulin, the basic unit of microtubules [2], the initiation and elongation factors of protein synthesis [3], and the G proteins that mediate transmembrane signalling [4–6]. GTP-binding proteins cycle between active (GTP bound) and inactive (GDP bound) forms, that differ markedly in terms of their interactions with other protein components of a particular system. Conversion between these forms is accomplished by sequential GDP–GTP exchange and GTP hydrolysis, and leads to transient activation of specific cellular processes [1–6].

The absolute requirement of guanine nucleotide binding proteins for GTP and the opposing effects of GDP and GTP on the activity of GTP-binding proteins imply that rephosphorylation of GDP arising from GTP hydrolysis is indispensable for their function. Extramitochondrial GTP regeneration can be accomplished through transphosphorylation reactions catalyzed by guanosine triphosphate monophosphate kinase (2GDP = GMP + GTP), guanylate kinase (GDP + ADP = GTP + AMP) and by nucleoside diphosphate kinase (GDP + NTP = GTP + NDP)† [7]. The latter enzyme is ubiquitous, and in most tissues its activity is 10- to 100-fold greater than the activity of the nucleotide monophosphate kinases [8]. Nucleoside diphosphate kinase (NDPK) is found in preparations enriched in GTP-binding proteins, such as microtubule proteins [9–11], rod outer segments [12, 13], ribosomes [14, 15] and plasma membranes (see below). It has been proposed that this association is not fortuitous, but reflects a physiologically relevant connection between GTP-binding proteins and NDPK [11, 15–20], raising the possibility that GTP may act not only as the physiological activator, but also as a regulator of GTP-binding proteins. The purpose of this review is to examine the experimental evidence that led to this proposition in the specific case of the membrane-bound G proteins that control signal transduction, and to discuss the possibility that GTP may modulate their function.

Transphosphorylation reactions in intact cells: effect on G proteins

In intact cells, effects of transphosphorylation on G protein linked systems have been observed repeatedly. For example, adult (but not embryonic [21]) atrial myocytes retain G protein dependent responses during intracellular dialysis with solutions containing ATP, but lacking GTP [22, 23]. In experiments performed with neurons, not only GTP but also combinations of ATP with GDP or GMP support G protein activation [24]. Further evidence for *in vivo* transphosphorylation reactions that affect G protein function was obtained in experiments where thiophosphate analogs of adenine or guanine nucleotides were applied intracellularly. The enzyme-catalyzed transfer of thiophosphoryl groups is usually slower than that of phosphoryl groups [25, 26] so that the products of these reactions appear gradually. Their stability towards hydrolysis, however, facilitates the detection of transphosphorylation. When atrial cells are internally perfused with either the ATP analog ATPγS or with a GTP analog such as GTPγS or GMP-PNP, muscarinic K^+ currents are activated in a receptor independent way [18, 23]. While GTP analogs elicit K^+ currents by direct interaction with G_k, the G protein that couples muscarinic receptors to K^+ channels [23], the effects of ATPγS are indirect and require its conversion into GTPγS by an endogenous phosphotransferase [18]. This enzyme was tentatively identified as an NDPK, since the GTPγS-like actions of ATPγS are inhibited by UDP, ATP and GTP, but enhanced by GDP [27]. In isolated photoreceptor cells, ATPγS also mimicks the effects of GTP analogs [28, 29], and it is likely that these effects arise by intracellular formation of GTPγS.

GDPβS, a GDP analog which usually blocks G protein function [30], can also have stimulatory effects as a result of transphosphorylation [31]. The gradual development of effects resembling those of GTP analogs has been observed when GDPβS was injected at high concentrations into neurons [32] and isolated rod photoreceptors [33]. These findings probably reflect slow intracellular synthesis of a poorly hydrolyzable GTP analog by cellular enzymes. Although the identity of the latter compound is not known, it is likely to be GTPβS, which can be formed from GDPβS and a high energy phosphate donor by a variety of kinases [30, 31, 34]. It is

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† NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; GMP-PNP, guanylyl-imidodiphosphate; R_p-cAMPS, adenosine-3′:5′-monophosphothioate, cyclic R_p isomer; and PGE_1, prostaglandin E_1.

known from *in vitro* experiments that GTPβS is a weak activator of the G protein transducin [35], and can support tubulin polymerization [36].

The realization that cells can metabolize compounds previously assumed to be relatively stable implies that the interpretation of experiments performed in intact cells involving nucleotide analogs and G proteins is not trivial. Even when the experimental observations are consistent with transphosphorylation, the precise identification of the enzyme or enzymes involved is not straightforward, since a large number of intracellular enzymes can use substrates that carry substituted phosphate groups [25, 26, 34, 37–39], and the products of such reactions can affect G protein-mediated processes at steps where the G protein is not directly involved. Thus, for example, the incorporation of modified phosphoryl groups into cellular pools might conceivably result in nucleotides like GTPγS eventually being utilized to generate R_p-cAMPS, a potent cAMP antagonist [40], or $I(PS)_3$, a phosphatase-resistant IP_3 analog [41]. Although *in vivo* synthesis of these compounds has not been demonstrated, these possibilities should be considered.

Finally, it is noteworthy that the efficiency of transphosphorylation can vary among different cells as a function of intrinsic or extrinsic factors. For example, NDPK activity is influenced by treatment of kidney cells with picolinic acid [42], and undergoes step increases during the cell cycle of yeast [43].

From the evidence discussed above, one may conclude that transphosphorylating enzymes, in general, are quite active in cells. Furthermore, it is clear that GTP (or a GTP analog) produced in such reactions is available to a variety of G proteins. Nevertheless, it would be unreasonable to assume that there is a direct link between nucleotide interconversion and G protein activation based only on these observations, since transphosphorylation effects on specific G proteins could obviously result from changes in the entire cellular GTP pool. However, as detailed below, there is evidence that a significant NDPK activity is associated with membranes enriched in G proteins, and it appears that in some circumstances these two enzymes are functionally coupled.

Characteristics of membrane-bound NDPK

Since early phases of the work on G proteins, the presence of phosphotransferases in plasma membranes was recognized, mostly as a result of their potential as a source of experimental artifacts [44, 45]. Indeed, membranes isolated from a variety of tissues can phosphorylate GDP at the expense of ATP or other high energy phosphate donors [16, 18–20, 46–50]. In many instances, these transphosphorylation processes were found to result from a prominent NDPK activity associated with plasma membranes. The enzyme is detected in purified membranes [16–18], and it is not released unless the membrane structure is disrupted by detergents [16, 51–54]. Membrane-bound NDPK (mNDPK) resembles the cytosolic form of the enzyme in many aspects, including the ability to utilize both naturally occurring nucleotides and some of their so-called "stable" analogs [18, 20, 25, 26, 31]. The NDPKs isolated from particulate fractions of brain, platelets, and Ehrlich ascites tumor cells [51–53] and from liver plasma membranes [54] have similar properties. The native enzymes are oligomers of apparent M_r 70,000–120,000 comprised of 4–6 subunits ($M_{r_{app.}} \simeq$ 17,000–21,000). They display the kinetic behavior characteristic of NDPKs, namely a Ping–Pong reaction mechanism which includes the formation of a phosphorylated intermediate [8]. These enzymes utilize purine nucleotides with affinities and V_{max} that are an order of magnitude higher than those measured for pyrimidine nucleotides. Their activity is dependent on divalent cations, the K_a for Mg^{2+} being near 0.3 mM [54].

Functional coupling between mNDPK and G-proteins

Kimura and Shimada [16, 17, 54–56] have studied in detail the intrinsic NDPK activity of plasma membranes and its relation to the hormone-sensitive adenylate cyclase. They demonstrated that GDP-supported hormonal activation of the cyclase depends on its transphosphorylation to GTP, and that the latter process is associated with an mNDPK [16]. Inhibition of NDPK was found to suppress the GDP-dependent activation of adenylate cyclase by hormone, but did not affect stimulation by hormone plus GTP (or GTP analogs). This led to the suggestion that mNDPK may have a regulatory role in the function of G_s. Although it was initially thought that the activity of NDPK might be increased by activated receptors, subsequent experiments indicated that hormonal stimulation does not affect the rate of GTP regeneration by mNDPK [16, 48, 55]. Therefore, it was proposed that in the presence of ATP, when GDP is the prevalent guanine nucleotide in the medium, addition of hormone allows the G protein G_s to bind the GTP formed at a constant rate by mNDPK rather than GDP. A similar correlation between G_s activation by PGE_1 and mNDPK has been described in platelet membranes. Incubation of human platelet membranes with ATPγS leads to persistent activation of adenylate cyclase, a result of mNDPK-mediated thiophosphate transfer to endogenous GDP and consequent formation of GTPγS [20]. The process is enhanced by PGE_1, Mg^{2+} and exogenous GDP, and does not occur in the presence of an NTP-regenerating system, which removes GDP. The inhibitory action of the regenerating system can be partially reversed by addition of PGE_1: under these conditions ATPγS recovers 45% of its ability to activate the cyclase, and small amounts of GTPγS are formed. These results are consistent with the hypothesis that activated receptors cause the mNDPK-dependent phosphorylation of a pool of GDP that is not accessible to the regenerating system. Because this particular pool of endogenous GDP is also not accessible to the mNDPK in the absence of agonist, the authors suggest that it may represent the GDP bound to the population of G proteins that is activated by PGE_1 [20]. This conjecture is supported by the remarkable agreement between the amounts of GDP made accessible to mNDPK by PGE_1 (0.3 pmol/mg protein/10 min) estimated in this work and an earlier determination of the amounts of tightly bound GMP-PNP specifically released from platelet membranes

by PGE_1 plus GTP (0.18 pmol/mg protein/3–4 min; [57]). Taken together, these data support the proposal of a preferential interaction between mNDPK and G_s in the presence of stimulatory agonists, which could result in channeling of substrates from one enzyme to the other.

The first indication of a physical connection between mNDPK and G protein came from early attempts to isolate adenylate cyclase from brain particulate material. The preparation obtained was heavily contaminated with NDPK, prompting the speculation that the kinase was loosely associated with a G_s–cyclase complex [51]. More recently, this hypothesis was tested directly by Kimura and Shimada [17]. Anti-mNDPK antibodies were used for immunoprecipitation of detergent extracts of membranes in which G_s had been labeled by incubation with [^{32}P]NAD in the presence of cholera toxin. Analysis of the immune complexes demonstrated that a fraction of G_s is specifically associated to NDPK under basal conditions. Incubation of labeled membranes with hormones and guanine nucleotides prior to solubilization affected the amounts of G_s observed in the immunoprecipitates. Glucagon had no effect on complex formation in the absence of added guanine nucleotides. From these results, the authors concluded that mNDPK and G_s interact reversibly in membranes, and that this interaction is regulated primarily by guanine nucleotides and secondarily by hormones.

The generality of a functional association between mNDPK and G proteins has yet to be demonstrated. Aside from a preliminary report of receptor-dependent coupling of G_i to mNDPK in platelets [58], there are studies demonstrating the co-purification of small GTP-binding proteins of unknown function with membrane bound forms of NDPK [19, 53]. It has been proposed that the latter type of mNDPK is able to phosphorylate directly the GDP bound to these small GTP binding proteins as well as p21-ras proteins, ribosomal EF-α_1, and a variety of purified G proteins [19]. However, the evidence presented is not sufficient to eliminate the possibility of an intervening step of aqueous diffusion of GDP. Rigorous demonstration of a direct transfer of substrate between enzymes requires detailed kinetic studies and is more convincing when corroborated by observations of protein–protein association [59].

The data on the coupling between NDPK and G proteins imply that both proteins are functional whether coupled to each other or not. Moreover, both enzymes can utilize substrate from the surrounding medium. Functional coupling seems to be favored when the G protein is activated by receptor in the presence of low GTP concentrations. A dependence on GTP regeneration appears to be inconsistent with current models of G protein function, where occupation of the guanine nucleotide binding site by GTP in the agonist · receptor · G protein complex is always favored, because the K_m for GTP is low (0.3 μM; reviewed in Ref. 5) and cellular concentrations of GTP are believed to be relatively high [4]. However, there is evidence, considered in the following section, that supports the idea that GTP may have a regulatory role in G protein dependent processes.

Can GTP be a regulator of G protein function?

Determination of cytoplasmic GTP levels yields values of 0.3 to 2 mM [60–62] which exceed the apparent K_m for GTP hydrolysis by purified G proteins [5]. Based on these findings, the proposal that GTP could regulate G protein activity would be untenable. However, it should be noted that while chemical determinations provide accurate values for total nucleotide content, they do not make a distinction between bound and free nucleotides. Recent measurements of GTP activity in intact cells, using the patch clamp technique, indicate that, at least in cardiac myocytes, the levels of free GTP are significantly lower than previously thought. In isolated frog atrial cells the concentration of GTP in the pool accessible to G_k, the G protein that couples muscarinic receptors to K^+ channels, was found to be in the range of 25 to 50 μM [23]. These values are much lower than cytoplasmic GTP concentrations measured by chemical methods. While the *in vivo* values could represent a lower limit for $[GTP]_i$, since they were measured in cells under internal dialysis, the GTP concentration in the vicinity of G_k has also been estimated in non-dialyzed cells. In these experiments, the activity of ACh-activated K^+ channels in excised patches of atrial membrane was measured in the presence of increasing concentrations of GTP, and compared with the activity observed in the same patch while still attached to the cell. This approach yields an estimate of $[GTP]_i = 106 \pm 23$ μM [63]. Therefore, in isolated atrial cells, the free cytoplasmic GTP concentration must be in the range of 50 to 150 μM.

Another important finding of the latter study [63] is that in inside–out patches of atrial membranes the half-saturation concentration of GTP for agonist-dependent activation of the G protein G_k is 24 ± 8 μM. Consequently, under physiological conditions, agonist-activated G_k is not fully saturated with GTP. This conclusion supports the proposal that GTP is not only the essential activator, but also a plausible regulator of the function of G_k.

Note that, as previously mentioned, the K_m values for GTP hydrolysis by G proteins determined *in vitro* are usually much lower than the $K_{0.5}$ reported for G_k in patches of membrane from atrial cells. Also, deactivation of G_k in cardiac myocytes, which presumably results from GTP hydrolysis, proceeds at a rate (100–200/min [23]) that is two orders of magnitude larger than the rate measured for the GTPase activity of purified G proteins [4, 5]. These discrepancies do not indicate necessarily that G_k differs markedly from other G proteins, but probably arise from differences between isolated and native G proteins. The function of G_k can be studied *in situ*, by electrophysiological methods that provide much higher time resolution than biochemical methods can afford. At present, the only other G protein whose activity can be measured at near physiological conditions with comparable time resolution is transducin (G_t), and there is increasing evidence that the behavior of G_t parallels that of G_k. Measurements of the turnover of G_t in concentrated suspensions of rod outer segments [64] or in intact photoreceptor

disks [65] yield a GTPase rate of 60–160/min. Biochemical studies [66] indicate that G_t has two GTPase activites; in illuminated preparations the K_m values for GTP are <1 and 10–25 μM, with corresponding V_{max} values of 4–5 and 20–30 GTP hydrolyzed/min/G_t. The presence of this second, low affinity binding site for GTP in G_t was also detected in binding studies [67]. Since both GTP hydrolysis and inactivation of G_t are faster at higher than lower concentrations of GTP [66], it appears that the binding of GTP with low affinity is physiologically relevant. This signifies that, like G_k, G_t may also have its activity regulated by GTP.

Biphasic velocity curves have also been noted in experiments with purified G_s [68], G_i [69, 70] and G_o [70]. In the case of G_s this phenomenon was subsequently ascribed to impurities [71]. However, the curvature in reciprocal plots obtained with highly purified brain G_o and G_i subunits is elicited not by contaminants, but by the specific interaction between α and $\beta\gamma$ subunits [70]. Therefore, the multiphasic GTP dependence of the GTPase rate appears to be a property of a number of heterotrimeric G proteins. Two explanations have been proposed to account for this type of kinetic behavior [66]: (i) the G protein has only one nucleotide binding site, but it can assume two distinct conformations that bind GTP with different affinities; and (ii) the G protein has two nucleotide binding sites that bind GTP with different affinities. Regardless of which alternative is eventually proven to be correct, a regulatory role for GTP is implicit in both, giving credibility to the hypothesis that the association of mNDPK with G proteins is an important factor in the function of transmembrane signalling pathways.

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