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Guanine Nucleotide Exchange Factors Operate by a Simple Allosteric Competitive Mechanism†

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ABSTRACT: Guanine nucleotide exchange factors are essential components of the mode of action of GTPases involved in signal transduction. Their fundamental mechanism is generally accepted to derive from stabilization of the nucleotide-free form of GTPases, which is reflected in an increase in the rate of GDP dissociation when such an exchange factor is bound to a GTPase. The known kinetic properties of exchange factors can be explained on the basis of this simple allosteric competitive mechanism. Here, we describe experiments designed to distinguish this mechanism from a newer model, which invokes an active role for the incoming (i.e., displacing) nucleotide, implying the transient formation of a quaternary complex consisting of an exchange factor, a GTPase, and two nucleotides, one which is being displaced while the other stimulates this displacement. We show that for a well-known system (the small GTPase Ras and its exchange factor Cdc25) there is no evidence for an effect of the concentration or the nature (i.e., GDP or GTP) of the displacing nucleotide on the rate constant of GDP release from the Cdc25·Ras·GDP complex, consistent with the simple allosteric competitive model, and in disagreement with the newer suggestion. In addition, we present arguments, which demonstrate how the erroneous conclusions leading to the alternative model were derived.

Guanine exchange factors (GEFs)[1] are essential partners of GTP/GDP binding proteins (or GTPases) involved in intracellular signal transduction and regulatory mechanisms. They are known for most classes of such GTPases, in particular for ribosomal translation factors (*1*), heteromeric G-proteins (*2*), and small GTPases of the Ras superfamily (*3*−*6*). They operate on those GTPases that have a low intrinsic rate of GDP release and catalyze the generation of the active GTP-bound state from the inactive GDP-bound form. This process is often a result of the GEFs themselves being activated or recruited to the vicinity of the corresponding GTPase in response to extracellular signaling events.

GEFs have been characterized extensively both from a structural and mechanistic point of view (*7*). At the structural level, binding of a GEF to a GTPase results in disruption of the GTP/GDP binding site, leading in particular to an opening of the two important regions of GTPases referred to as switch I and switch II (*8*). These regions are involved in essential

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[1] Abbreviations: GDP, guanosine 5′-diphosphate; mGDP or mant-GDP, 2′(3′)-*O*-(*N*-methylanthraniloyl)-GDP; mdGDP, mant-2’-deoxy-GDP; GEF, guanine nucleotide exchange factor.

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functions of GTPases including the recognition of partner molecules, including effectors, and the hydrolysis of GTP (*9*).

It was shown approximately 10 years ago that a simple model could explain the kinetics of nucleotide exchange catalyzed by GEFs (*10*). According to this model, which can be classified as an allosteric competitive mechanism, GTPases can form stable complexes with nucleotides or with exchange factors or can form less stable complexes with both GEF and nucleotides simultaneously. In the ternary complex, both ligands (GDP and GEF) are bound orders of magnitude less strongly than in the binary complexes. In general, this reduction of affinity occurs mainly as a result of an increase of the rate constants for release of GDP or GEF, respectively, so that the observed and physiologically meaningful phenomenon of acceleration of GDP release can be adequately explained by these observations. The kinetics and thermodynamics of these interactions have been reviewed and discussed in terms of this model (*11*).

Despite the satisfactory and detailed explanation of the kinetics of GEF-catalyzed nucleotide exchange, it has recently been suggested that the mechanism is actually more complex and that dissociation of a bound nucleotide under the influence of an exchange factor is dependent on the displacing nucleotide in an associative mechanism (*12*, *13*). This implies a quaternary complex between a GEF, a GTPase, and two nucleotides, one dissociating (GDP) and the other associating (GTP). We show here that this mechanism does not pertain for the Ras/Cdc25 system and that the suggestion of the more complex mechanism for a very closely related pair of proteins (Ras/GRF1) (*12*) arose as a result of incorrect interpretation of the experimental data.

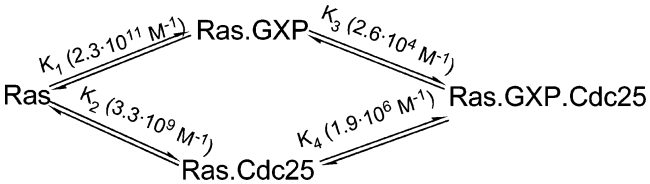
MATERIALS AND METHODS

Proteins and Nucleotides. Recombinant H-Ras and $Cdc25^{Mm}$ were expressed and purified as previously described (*14*). The recombinant GST (glutathione *S*-transferase) fusion proteins were isolated by affinity chromatography on a glutathione−sepharose column (Pharmacia, Uppsala) in 30 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 3 mM dithiothreitol, and 2.5% glycerol. After thrombin cleavage, the proteins were purified by the same affinity chromatography.

The nucleotide-free form of Ras was prepared as described (*15*). Fluorescent methylanthraniloyl-GDP (mantGDP or mGDP) was synthesized according to protocols described (*15*). The corresponding derivative of 2'deoxy GDP, referred to here as mdGDP, was obtained from JenaBioscience (Jena, Germany). Ras·mGDP or RasmdGDP were obtained by mixing the nucleotide-free form form of Ras and mGDP or mdGDP in a molar ratio of 1:1.2 and removing the unbound nucleotides using prepacked gel filtration columns (NAP-5, Pharmacia). The concentration of Ras·mGDP was determined by HPLC as described (*16*). All proteins were snap-frozen in liquid nitrogen and stored at −80 °C.

Equilibrium Measurements. All fluorescence measurements were performed at 25 °C in 30 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM dithiothreitol. Titration of Ras·mGDP (1 μM) with $Cdc25^{Mm}$ was performed as previously described (*10*) using a FluoroMax spectrofluorimeter (Spex Instruments, NJ) and excitation and emission wavelengths of 366 and 450 nm, respectively. The data obtained were fitted using the programs Grafit and Scientist.

Scheme 1: Equilibrium Constant for Interactions in the Ras, Guanine Nucleotide, and Cdc25 System, Where GXP Symbolizes GDP or GTP[a]



[a] The values given for the constants are for mdGDP and are derived from the experiment of Figure 2.

Kinetic Measurements. The interaction of Ras·mGDP or RasmdGDP (concentration as indicated) with $Cdc25^{Mm}$ in the absence and presence of excess free GDP (20 μM) or at variable concentrations of GDP or GTP was monitored in a time-dependent manner using an Applied Photophysics SX16MV stopped-flow instrument. mGDP was excited at 366 nm, and detection was through a cutoff filter at 408 nm. The data obtained were evaluated with the program Grafit.

The association kinetics of nucleotide-free Ras with mGDP or mGDP were measured under pseudo-first-order conditions as described (*14*). The intrinsic mGDP dissociation rate constant from the GTPase was measured by displacement of excess free GDP (20 μM).

RESULTS AND DISCUSSION

One of the main considerations leading to the newer suggestion of the importance of the incoming (i.e., displacing) nucleotide in GEF-catalyzed reactions is the purported observation that GDP dissociation from GTPases in the presence of GEFs only occurs, with one or two exceptions (Ran/RCCI and Ras/smgGDS are quoted), if a "displacing" nucleotide is also present (*12*). In general, this is not true. Whether a given exchange factor will dissociate GDP from a cognate GTPase in the absence of a displacing nucleotide is dependent on all equilibrium constants in the scheme (Scheme 1) showing the formation of binary and ternary complexes, and on the concentrations of all species involved. In a fairly recent study of Rab exchange factors, it was shown that, while one (DSS4) could displace GDP or its analogues very efficiently under certain concentration conditions in the absence of a displacing nucleotide, others (Vps9, Rabex) could only do this much less efficiently (*17*). These results are easily explainable in terms of the affinity relationships of Scheme 1. Indeed, the displacement of GDP (usually its fluorescent derivative mGDP) by a GEF can be used to derive the remaining equilibrium constants in Scheme 1, as long as the affinity of the GTPase for the nucleotide is known, as explained in detail by Klebe et al. (*10*).

The experiments of Zhang et al. were performed using mGDP as a fluorescent GDP analogue. Although not explicitly stated, this must be the mixture of 2'- and 3'-*O*-methylanthraniloyl isomers of the derivative, which equilibrate with each other to form a mixture containing approximately 60% of the 3'-isomer and 40% of the 2'-isomer (*18*). These do not behave identically with Ras, which is the reason quantitative kinetic and thermodynamic experiments have often used the corresponding 2'-deoxy derivative, in which the fluorescent label is attached unambiguously to the 3'-

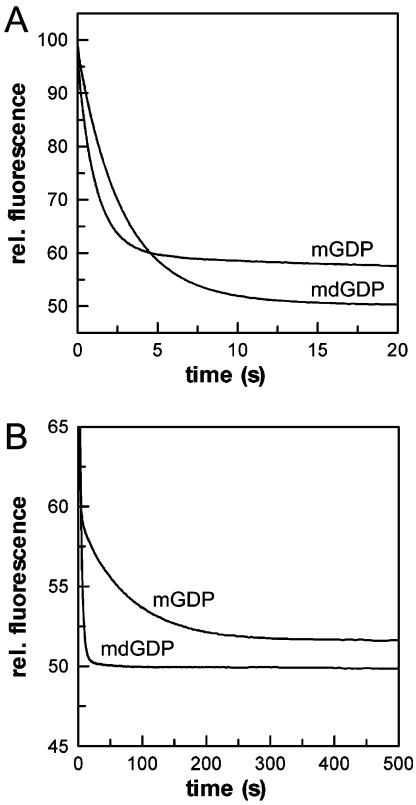


FIGURE 1: Mono- and biphasic signals on dissociation of mdGDP and mGDP from Ras under the influence of Cdc25. Displacement of mGDP and mdGDP from their complexes with Ras (0.1 μM) was measured in the presence of 20 μM Cdc25 and 20 μM free GDP under the conditions described in Materials and Methods. While the fast first phase (A) is comparable for both mant-nucleotides, the slow second phase (B) is absent for the mdGDP dissociation. The data of panel B are from the same experiment as for panel A, which means that both curves actually start at a relative fluorescence of 100 although the *y*-axis scale has been changed to show the slow phase for mGDP in more detail. The rate constants for the faster and slower phases in the case of mGDP are 0.90 and 0.015 s^{-1}, respectively; that for mdGDP is 0.36 s^{-1}.

postion. Before addressing the data of the Zhang et al. publication, we checked whether the already reported difference in the rate constant for release of the isomers from Ras (*18*) in the absence of exchange factors also applies in their presence. As shown in Figure 1, there is indeed a biphasic time course when mGDP is displaced from its complex with Ras in the presence of 20 μM Cdc25, but a simple monoexponential decrease in the fluorescent signal in the case of mdGDP. It is clear that the presence of two phases in the dissociation of mGDP is related to the presence of two isomers in mGDP, but there are two possible explanations of the biphasic signal in the displacement kinetics. In the case of uncatalyzed release of mGDP from Ras, two phases were seen which differed by a factor of approximately 10 (*18*). The difference of rates in the experiment of Figure 2 is considerably larger (factor of 60), and this could mean that another explanation applies. In accordance with this possibility, the slow second phase is due to equilibration of the two isomers of mGDP after faster release of both from Ras. As shown previously, the isomers have quite different fluorescence intensities in solution, and equilibration takes place with a half-life of about 500 s at 37 °C and pH 7.0 (*18*). The second phase in Figure 2 is approximately a factor of 10 faster than this, despite the lower

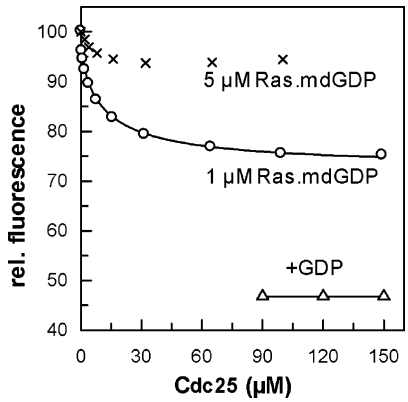


FIGURE 2: Titration of Ras·mdGDP complexes with Cdc25. Increasing amounts of Cdc25 were added to 1 μM Ras·mdGDP under conditions described in Materials and Methods. The decrease of fluorescence was followed until the signal remained stable. Unlabeled GDP (20 μM) was added to determine the theoretical end point for total displacement of mdGDP. Fitting the data using the program Scientist provided K_3, K_4, and K_1/K_2, defined in Scheme 1.

temperature (25 °C), but the higher pH value would lead to a higher rate of equilibration, so that this possibility has to be considered. Interestingly, in experiments with Ran and RCC1, we observed a similar behavior, and the rate of the second phase was identical to that seen here. Moreover, repeating the experiment of Figure 2 in the presence of 5 or 10 μM Cdc25 instead of 20 μM did not affect the rate constant of the slow phase, although it reduced the rate constant of the first phase significantly (see Figure 3b). This is the expected result if the second phase is due to isomerization of mGDP released at a much faster rate in the first phase. As shown previously, the ratio of isomers in the bound state tends to differ from that in the free state, depending on the exact protocol used for preparing the Ras·mGDP complex (*18*), and this is the reason for re-equilibration after release from Ras. In principle, the large difference in the rate constants for the two phases means that it is possible to analyze the kinetic properties for the rapid phase without disturbance by the slow phase, at least at high exchange factor concentrations, but analyses in which the amplitudes of the signals are also of importance require a detailed and complex approach.

To avoid these complications, we performed an equilibrium titration to establish the equilibrium constants for each of the four processes shown in Scheme 1 unequivocally using mdGDP. This experiment is equivalent to that described by Klebe et al. (*10*), which is based on a much earlier study of the influence of actin on the affinity of myosin for nucleotides (*19*). As shown in Figure 2, starting with 1 μM Ras·mdGDP as a 1:1 complex, addition of Cdc25 led to progressive displacement of mdGDP, which tended toward saturation at high concentrations of the exchange factor. However, this did not correspond to complete displacement of mdGDP, as shown by the fact that addition of excess GDP or GTP led to a further reduction in fluorescence intensity. These data were evaluated using the program scientist and Scheme 1 as a model.

The fluorescence intensity corresponding to complete displacement was taken as the value in the presence of excess GDP and was held constant during the fitting procedure, as was the affinity of Ras for mdGDP, which was determined

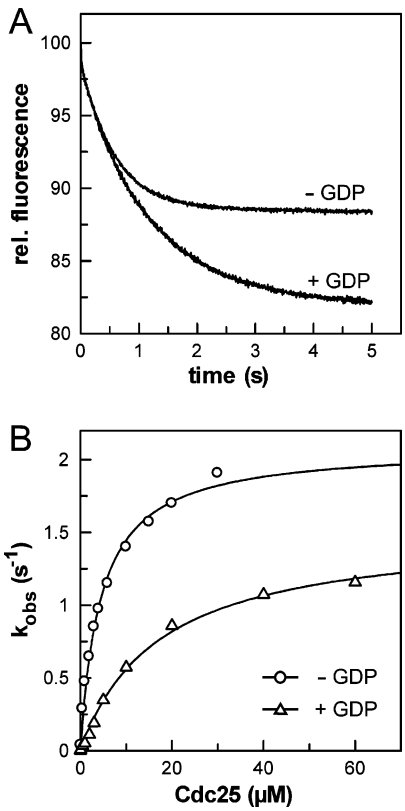


FIGURE 3: Comparison of the Cdc25-induced kinetics of dissociation of mGDP from the Ras·mGDP complex in the absence and in the presence of GDP. (A) Cdc25 (20 μM) was mixed with 0.1 μM Ras·mGDP in the stopped-flow apparatus in the absence and in the presence of 20 μM GDP under conditions described in Materials and Methods. (B) Fitted observed rate constants for the initial rapid phase corresponding to dissociation are plotted against the Cdc25 concentration.

in a separate experiment as described by John et al. (*15*). Note that the extraction of the remaining equilibrium constants of Scheme 1 is dependent on the fact that, under the concentration conditions used, saturating concentrations of Cdc25 do not lead to complete dissociation of mdGDP. Essentially, this defines K_4 in Scheme 1. If mdGDP displacement at saturating GEF concentration is complete, which is the case with Ras/Cdc25 at lower starting Ras·mdGDP concentrations or with exchange factors which have a more drastic effect on the nucleotide affinity, only the ratio K_1/K_2 can be calculated, which corresponds to the value of K_3/K_4 because of thermodynamic consistency. Of course, K_2 calculation is then possible if K_1 is known.

Evaluation of the data of Figure 2 led to the values given in Scheme 1. In approximate agreement with the results of Lenzen et al. (*14*), this means that Cdc25 is bound ca. 70-fold more weakly than mdGDP to Ras. Nevertheless, saturating concentrations of Cdc25 lead to significant displacement of mdGDP from Ras·mdGDP when the latter is at micromolar or lower concentrations, for the simple reason that the K_d of Ras·Cdc25 for mdGDP is ca. 0.5 μM ($1/K_4$).

To interpret the results of Figure 2, we made the assumption that there was no substantial fluorescence change upon binding of Cdc25 to Ras·mdGDP. If this were not true, the fluorescence change seen in the titration of Figure 2 could arise from a lower fluorescence of the ternary complex than the binary complex, and the evaluation would be invalid. A test for this possibility is shown in the upper titration curve of Figure 2. Increasing the starting concentration of Ras·mdGDP led to a dramatic reduction in the amplitude of the fluorescence change at saturating Cdc25 concentrations. This is the expected result based on our original premise that the fluorescence change is due to mdGDP dissociation and not simply to binding of Cdc25. The lower amplitude of the signal is due to the fact that a concentration of free mdGDP which is high in comparison with the value of $1/K_4$ is reached with a much lower fractional degree of displacement of mdGDP than when starting at lower Ras·mdGDP concentrations. Increasing the Ras·mdGDP concentration to 10 μM and higher led to complete loss of signal change upon addition of Cdc25 (data not shown). If the alternative explanation applied (i.e., fluorescence quenching on formation of the ternary complex without dissociation of mdGDP), there should still be a signal change when starting with very high concentrations of Ras·mdGDP. Even for the mixed situation in which both binding of Cdc25 and dissociation of mdGDP lead to signal changes, there should still be a detectable signal starting with high Ras·mdGDP concentrations. In support of these observations, transient kinetic data of the type shown in Figure 1 did not show any sign of double-exponential behavior using Ras·mdGDP at any concentration of Cdc25, as would be expected if there was a significant change in intensity on ternary complex formation. In the case of RCC1 and Ran·mdGDP, there is actually an increase in intensity upon formation of the ternary complex, and this was easily seen in transient kinetic experiments (*10*).

Having established that Cdc25 can displace mdGDP from Ras·mdGDP in the absence of free nucleotide (qualitatively similar results were obtained with mGDP), we can now perform an experiment that is a direct test of the model of Zhang et al. (*12*). This is a comparison of the kinetics of dissociation of mGDP from the Ras·mGDP complex in the absence and in the presence of a displacing nucleotide. These experiments were performed with mGDP because of its easier availability than mdGDP, and only the more rapid phase of the dissociation kinetics (see Figure 1) was used for the evaluation. This approach is valid since the first phase in the curves corresponds to mGDP dissociation, was easily separated from the second (isomerization phase), and could be treated independently. The comparison of dissociation with and without GDP as displacing nucleotide is shown for a single Cdc25 concentration in Figure 3A.

The most striking difference in the two traces is the difference in amplitudes. In light of the experiment of Figure 2, this is not surprising, since dissociation of mGDP is not complete in the absence of a displacing nucleotide. Of more significance with respect to the suggested model of Zhang et al. are the kinetics of mGDP dissociation. According to this model, there should be a dependence of the rate of mGDP displacement on the presence and concentration of displacing nucleotide, in the sense that the rate should increase with increasing concentration. Fitting the curves of Figure 3A with single-exponential functions led to rate constants of 1.70 s^{-1} in the absence of 20 μM GDP and of 0.86 s^{-1} in its presence. Thus, superficially, the effect appears to be in the opposite direction to that predicted by the Zhang et al. model. However, it is exactly what is expected from the simple model of exchange factor action established in

other earlier works (*10*, *14*) and arises because, in the first situation (i.e., without GDP), there is a contribution from the reverse reaction in the rate of approach to equilibrium. This is a familiar classical situation in kinetics, that is, the somewhat counterintuitive fact that the rate of establishment of the endpoint in a reversible reaction is faster than that of an irreversible one, even if the rate constant in the forward direction is the same in both cases. When the system is examined more rigorously, we find that the time course in the absence of displacing nucleotide should actually be more complex than single exponential, since the reverse process, that is, reassociation of mGDP with the Ras·Cdc25 complex, is a second-order reaction, and there is not an excess of the product mGDP, so its concentration will increase during the reaction and lead to more complex behavior. This problem is resolved using a numerical simulation approach described below. However, the main point emerging from this experiment is clear, that is, that there is no indication of a "helping" effect of the incoming nucleotide in the kinetics of the displacement of mGDP, and the further analysis presented below confirms that the simple model involving allosteric competition is sufficient to explain all available data on exchange kinetics.

Figure 3B shows the dependence of the observed rate constant for mGDP displacement from its complex with Ras on the Cdc25 concentration in the absence and in the presence of excess GDP as displacing nucleotide. In both cases, there is a hyperbolic approach to a maximal rate constant of 2.1 s^{-1} in the absence of GDP and 1.5 s^{-1} in its presence. Again, the higher apparent rate constant in the absence of GDP is due to incomplete dissociation of mGDP (i.e., due to the fact that an equilibrium is being established and both the forward and reverse rate constants contribute to the time dependence). Hyperbolic fits to the data led to values of 4.5×10^{-6} M and 1.7×10^{-5} M for the apparent dissociation constant of Cdc25 from the Ras·mGDP complex. In the experiment in the absence of GDP, the complex kinetic situation arising from only partial dissociation of mGDP means that the significance of this value is not clear, but in the presence of GDP, the value should, based on the Scheme 1, correspond to $1/K_3$, the dissociation constant of Cdc25 from the ternary complex. In fact, the value obtained from the kinetic experiment is ca. 2-fold lower than the value obtained from the equilibrium titration of Figure 2. In view of the fact that the approach is very different in the two experiments and also of the fact that we are comparing values for mdGDP and mGDP, the agreement can be regarded as satisfactory.

For a complete evaluation of the comparison between the nucleotide dissociation kinetics in the absence and presence of unlabeled GDP, mdGDP was used, since the amplitude of the resulting signal contains essential information. The results are shown in Figure 4. At the concentration of Ras·mdGDP used, the difference in the amplitude in the two curves is large, and as in the experiment of Figure 3, the rate constant in the absence of unlabeled GDP is considerably faster than in its presence (0.4 cf. 1.7 s^{-1}, respectively). For quantitative evaluation using numerical simulation, there is not enough information in the individual curves to fit all constants, so we made the reasonable assumption, based on earlier work, that the kinetics of GDP and mdGDP were similar. The main point of this exercise was to show that

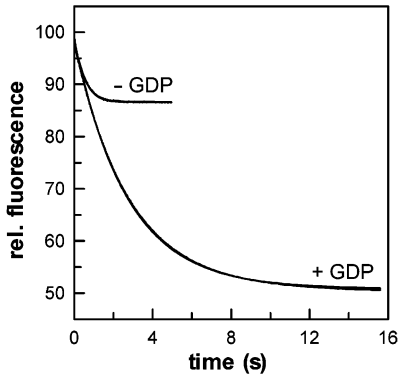


FIGURE 4: Comparison of the Cdc25-induced kinetics of dissociation of mdGDP from the Ras·mdGDP complex in the absence and in the presence of GDP. Experiment as in Figure 3A but using 1 μM Ras·mdGDP. The time courses were evaluated using a numerical integration approach to simulate the time course according to the differential equations describing the system. There was no information from this type of experiment on the values for the rate constants for formation of the binary Ras·mdGDP and Ras·Cdc25 complexes, so the assumption was made that, due to the high affinities, free Ras (and therefore free mdGDP) was not presnt. The remaining eight equations (four for mdGDP, four for GDP) were implemented using the program Scientist. The fitted values for the rate constants in the absence of GDP were (nomenclature from Scheme 1) $k_{+3} = 7.2 \times 10^5$ M^{-1} s^{-1}; $k_{-3} = 49$ s^{-1}; $k_{+4} = 3.8 \times 10^6$ M^{-1} s^{-1}; $k_{-4} = 1.78$ s^{-1}. For the evaluation of the data in the presence of GDP, only the value of k_{-4} was allowed to vary, since the effective irreversibility of the exchange reaction means that there is no information on the other constants, which in any case should be the same in both experiments. The fitted value of k_{-4} was 1.72 s^{-1}. The fitted curves cannot be distinguished from the experimental data in this plot.

the same set of constants can be used to fit the curves both in the presence and absence of GDP and, in particular, to show that the faster observed rate constant seen in the absence of GDP is a natural consequence of the mechanism and the values of the rate and equilibrium constants. The fitted value for the rate constant of mdGDP dissociation from the ternary complex (k_{-4} in Scheme 1) are the same within error for the experiment with GDP (1.72 s^{-1}) and without GDP (1.78 s^{-1}). From the experiment in the absence of GDP, the values for k_{+4} and k_{-4} can be used to calculate a value of 2.1×10^6 M^{-1} for K_4, in good agreement with the value of 1.9×10^6 M^{-1} obtained from the equilibrium titration of Figure 2, confirming the consistence of the results.

To exclude the possibility that there is indeed an effect of the "displacing" nucleotide on the rate constant of the released nucleotide at higher concentrations of the displacer, we examined the effect of increasing GTP concentration on the rate constant of mGDP release in the presence of 10 μM Cdc25. Between 10 μM and 1 mM GTP, there was no change in rate constant, as long as care was taken to keep the concentration of free Mg^{2+} constant (data not shown). If this precaution was not taken, there was an increase in the rate of mGDP release as the GTP concentration approached that of the Mg^{2+} concentration. This is due to the well-characterized dependence of the rate of nucleotide release on the metal ion concentration (*20*) and the fact that GTP chelates Mg^{2+} and thus reduces its free concentration.

In the publication by Zhang et al. (*12*), the suggestion is made that the nature of the displacing nucleotide is of importance for the displacement reaction. As discussed later, this is based on an incorrect interpretation of the data

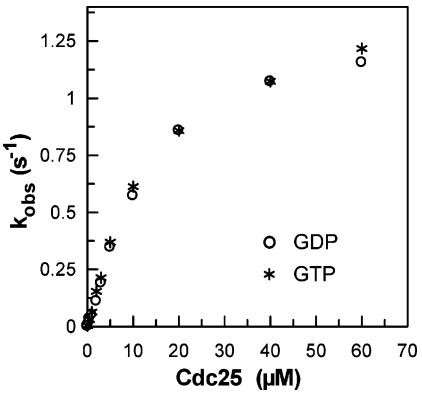


FIGURE 5: Comparison of the kinetics of dissociation of mGDP from the Ras·mGDP complex in the presence of GDP and GTP. The rate constant of the first phase in the fluorescent transient seen after rapidly mixing Ras·mGDP (0.1 μM) with different concentrations of Cdc25 in the presence of 20 μM GDP or 20 μM GTP is plotted against the Cdc25 concentration.

presented, but we performed the experiments shown in Figure 5 to show that there is no difference in the displacement kinetics with GTP or GDP, in agreement with the original model. This again argues against an active role for incoming nucleotide in the displacement reaction.

CONCLUSIONS

The experiments described here have been performed to distinguish between two alternative models for the mechanism of action of GTP/GDP exchange factors. The experiments show conclusively that the displacing nucleotide does not play an active role in their mechanism of action. Instead, as originally proposed (*10*), the complex between a GTPase and its displacement factor, which is generated directly by release of nucleotide on binding the GEF, is available to interact with guanine nucleotides that are present. If the displacing nucleotide is present in excess over the displaced nucleotide, which is normally the case under cellular conditions with GTP being in excess over GDP, this will occur predominantly with the displacing nucleotide, which in turn leads to dissociation of the GEF. In general, GEFs do not exert their effects in a specific fashion with respect to the nucleotide (GTP or GDP) bound to the GTPase, and it has been shown theoretically that this would in fact have disadvantageous consequences in a system in which nucleotide exchange itself is the main purpose of the interaction (*11*).

It is noteworthy that the C-terminal domain of rat RasGRF1 (used by Zhang et al. (*12*)) contains in part an additional Ras·GTP binding site upstream of the Cdc25 domain, a site which is important for the feedback mechanism during the process of nucleotide exchange on Ras (*21*). The fact that our mouse RasGRF1 ($Cdc25^{Mm}$) construct lacks this region is of no consequence for studies on the basic mechanism of nucleotide exchange, which is very likely to be conserved irrespective of details concerning additional regulation mechanisms.

Since the results presented here, as well as in other detailed kinetic studies of exchange factors (*10*, *14*, *17*, *22*), can all be explained by the original model involving allosteric competition between nucleotide and GEF binding to a GTPase, the question of how a different conclusion was reached from recent studies (*12*) arises. The answer is that a nonrigorous approach was taken to interpreting the kinetic data produced. Thus, curves that are described as "faster" are actually those with larger amplitude (see Figures 2B and 3B in Zhang et al. (*12*)), and the rate constant in several cases is actually higher in those curves described as "slower". Thus, the GTP titration of the kinetics of mGDP release from Ras (Figure 2b of Zhang et al.) under the influence of an exchange factor is actually a titration of the amplitudes, that is, the *extent* of mGDP release, which is simply explained on the basis of competition between mGDP and relatively low concentrations of GTP (or GTP analogues in Figure 3b). No fits are shown in the publication, but this would have quickly revealed the fallacy of the arguments, and the described fitting procedure (fitting to the same end-point in curves obtained at different GTP concentrations or with different displacing nucleotides as in Figure 3b of Zhang et al.) is blatantly incorrect, since the end-point varies obviously and dramatically, and the nature of the errors made is such that those curves which reach their end point more quickly (e.g., the curve for GPPCP in Figure 3b) are actually calculated to have a lower rate constant, because the curve would actually never reach the end point enforced upon it (clearly ca. 95% and not 65% of the starting signal as assumed by the authors). As correctly pointed out by the authors, in the experiments to investigate the effect of nucleotide structure on their displacing ability, the nature of the curves changes according to the known affinities of the analogues (to Ras). However, it is not the *rate constant* which changes, but the *amplitude* (i.e., the degree of exchange), in accordance with the known affinities and expressing their ability to compete thermodynamically with mGDP, *not* their ability to influence its kinetics of dissociation.

In summary, all evidence so far obtained on the kinetics of guanine nucleotide exchange by GEFs is commensurate with the original model of allosteric competition proposed by Klebe et al. (*10*) and much earlier for an analogous situation with ATP and actomyosin by Hofmann and Goody (*19*). The experiments reported in the present contribution have been designed to determine whether the recently proposed addition to this mechanism, which involves transient generation of an intermediate in which the incoming and outgoing nucleotides are bound simultaneously, applies to Ras exchange factors, in this case Cdc25. We have obtained similar results to those shown here for the Ran/RCCI system (Guo et al., unpublished results), for the Rho/p115RhoGEF system (Hemsath et al., unpublished results) and for Rab/RabGEF systems (*17*), and it seems likely that the principles are general. The results described by Zhang et al. (*12*) are for a different RasGEF (GRFI), but this encompasses a Cdc25-related domain with high sequence identity to that used in the present study, suggesting similar structural and biochemical properties to the GEF used in this study.

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