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In vitro activation of the *Saccharomyces cerevisiae* Ras/adenylate cyclase system by glucose and some of its analogues

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Using crude membrane preparations of *Saccharomyces cerevisiae*, we have demonstrated that glucose and glucose analogues which are not efficiently phosphorylated activate the guanine nucleotide-dependent adenylate cyclase in vitro. The activation appears to be mediated by the Ras proteins. Moreover, data are presented indicating that glucose and its analogues activate adenylate cyclase by stimulating the exchange of guanine nucleotides at its regulatory component. Thus, it has been possible to show the action of a physiological effector on the nucleotide exchange reaction in a member of the *ras* superfamily.

Ras protein; Glucose; Yeast; Adenylate cyclase

1. INTRODUCTION

Genetic studies using different *Saccharomyces cerevisiae* mutants deficient in genes coding for proteins implicated in the cAMP pathway, indicate the key importance of this molecule in the control of both metabolism and the cell cycle [1]. The effect of glucose on yeast adenylate cyclase in vivo is well known. The addition of glucose to yeast cells collected at the stationary phase of growth causes a six- to sevenfold increase in intracellular cAMP levels [2,3]. This increase is transient, lasts for about one minute, and is followed by a stabilization of cAMP levels to values about two- to fourfold higher than those existing before glucose addition. The rise of cAMP levels is dependent on G proteins, the *RAS* products (*RAS1* and *RAS2*) [4]. In yeast, the regulatory components of adenylate cyclase are these G proteins, which structurally differ from the known heterotrimeric mammalian G proteins [5].

The intermediate steps that take place between the addition of glucose and the activation of Ras proteins remain unknown. It is logical to suppose that, mimicking what occurs in higher eukaryotic cells, the activation of the G protein by the hypothetical glucose–receptor complex is due to an increased exchange of the guanine nucleotide bound to the regulatory protein. This increase in the exchange rate will promote the binding of the activating nucleotide, GTP, since its cytoplasmic concentration is much higher than that of GDP [6], and it will activate the Ras protein [5]. The results presented in this paper show that glucose and its analogues activate adenylate cyclase in isolated membranes. The sugar does so by stimulating the exchange of the guanine nucleotide at the regulatory component of the system, further indicating the functional homology between the activation of mammalian adenylate cyclase by hormones and the stimulation of yeast adenylate cyclase by glucose.

Abbreviations: 6-deoxyglucose, 6-deoxy-D-glucose; GDP-β-S, guanosine 5′-*O*-(2-thiodiphosphate); Gpp(NH)$_p$, guanylylimidodiphosphate; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

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2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals and other reagents were obtained from the following sources: Bacto-peptone and Bacto-yeast extract (Difco) [α-^{32}P]ATP (24 Ci/mmol) and [2,8-^{3}H]cAMP (31.2 Ci/mmol) (New England Nuclear), 6 deoxy-D-glucose and 3-*O*-methyl-D-glucopyranoside (Serva), β-glucuronidase-arylsulfatase (Glusulase) (Boehringer-Mannheim). All the other reagents were from Sigma. All solutions were prepared in double-distilled-filtered Milli-Q water.

2.2. *Yeast strains and growth conditions*

Most of this work was carried out with *Saccharomyces cerevisiae* strain SMC-18, isogenic to H38-4B (*MAT*α, *leu1*, *his4*, *MAL3*, *MAL2-8C*, *SUC3*) [7] which was a gift from K.D. Entian (Physiologisch-Chemisches Institut, Tübingen University, Germany). Yeast cells were grown on YEPD medium (2% Bacto-peptone, 1% yeast extract, 2% glucose), and incubated at 30°C with shaking. Growth was monitored by determining the A_{600} and cultures were collected when A_{600} was 3.5–4.0, 5–7 h after the glucose was completely exhausted from the medium.

2.3. *Membrane preparations*

Crude membrane preparations were obtained as described by

Casperson et al. [8], except that longer incubation with Glusulase was required (3–5 h) in order to obtain spheroplasts from stationary cells. Spheroplasts were lysated as described by Toda et al. [5], and the membranes were stored in 50 mM MES/KOH buffer pH 6.0, containing 100 μM $MgCl_2$, 100 μM EGTA, 4 mM phenyl-methyl-sulfonyl-fluoride and 10% glycerol at −70°C.

2.4. *Determination of adenylate cyclase*

Unless otherwise indicated, prior to adenylate cyclase assay, membranes suspended in storage buffer were diluted threefold in the appropriate sugar (or sugar analog) solution to obtain the required concentration. Guanine nucleotides were then added as a concentrated solution to yield a final concentration of 200 μM. The membrane suspension was then preincubated for 10 min at 30°C. When determining the activity of the non-regulated catalytic subunit of adenylate cyclase, the preincubation was in the absence of guanine nucleotide and in the presence of 2 mM $MnCl_2$. Afterwards, 25 μl (containing 50–100 μg of protein) of the membranes-effectors mixture were diluted 1:1 in assay buffer and incubated for 30 min at 30°C. Assay buffer composition was: 100 mM MES/KOH pH 6.0, containing 2 mM [^{3}H]cAMP (10 000 cpm/25 μl), 0.2 mM EGTA, 4 mM β-mercaptoethanol, 0.2 mg/ml bovine serum albumin, 10 mM teophylline, 40 mM phosphocreatine, 40 U/ml creatine-phosphokinase, and 0.4 mM [α-^{32}P]ATP (1-2 μCi/25 μl), plus the indicted $MgCl_2$ concentration. The reaction was stopped by the addition of 0.9 ml of 6.25% trichloroacetic acid. After centrifugation, [α-^{32}P]cAMP was purified from the supernatant as described by others [9]. Protein concentration was determined by Bradford's method [10], using the commercial reagent from Bio-Rad.

2.5. *GppNHp stimulation of adenylate cyclase after treatment with 6-deoxyglucose and GMP*

Membrane preparations were diluted as above in a medium containing 20 mM Mg^{2+} and 200 mM of the indicated sugar in the presence or in the absence of 1 mM GMP, and preincubated for 30 min at 30°C as described [11]. Then, membranes were centrifuged at 200 000 × g for 3 min in a Beckmann Airfuge, resuspended in storage buffer and washed twice. Adenylate cyclase was assayed in the presence of 4 mM Mg^{2+}, with or without GppNHp (100 μM). Protein concentration during the enzymatic reaction was 50 μg/assay.

2.6. *Effect of different sugars on the kinetics of the activation of adenylate cyclase by GppNHp*

To determine the kinetics of the activation of adenylate cyclase by the non-hydrolysable GTP analog GppNHp, the adenylate cyclase reaction was carried out essentially as described above, except that magnesium acetate (10 mM) was used instead of magnesium chloride, the concentration of ATP was increased to 1 mM, and the reaction (1.2 ml final volume) was started by the addition of concentrated membranes (600–1200 μg of protein). At the indicated times, 100 μl samples were withdrawn, the reaction stopped and the samples processed as above. Each kinetic experiment was repeated at least three times, and all experimental points (10 for each constant) were used to calculate the rate constants. The production of cAMP is adjusted to the following equation [12].

$$cAMP_t = K_{cat}[E_{max}]t + \{(K_{cat}[E_{max}]/K_{obs})\ (\exp[-K_{obs}t]-1)\} \quad (1)$$

Were $cAMP_t$ is the amount of cAMP produced at a given time t, K_{cat} the catalytic constant of the enzyme, $[E_{max}]$ the concentration of the enzyme present during the assay, and K_{obs} the first order equation constant which determines the pseudo-linear phase of the equation. $K_{cat}[E_{max}]$ represents the V_{max} of the reaction. Thus, the equation may also be expressed as follows:

$$cAMP_t = V_{max}t + \{(V_{max}/K_{obs})\ [\exp(-K_{obs}t)-1]\} \quad (2)$$

This equation predicts a two-phase reaction. The first phase is a progressive increase in the rate of the catalysis, and is due to the fact that the activation of the enzyme is a complex process. The second one is a time-dependent, linear-shaped phase of the reaction. It only occurs when the activation of Ras proteins by the nucleotide, and hence their interaction with the catalytic component has been completed. The slope of the linear phase represents V_{max}, and the interception of the prolongation of this virtually rectilinear function at the abscissa (time) is a function of $1/K_{obs}$. Thus, higher values of K_{obs} represent shorter delays. The curve and function that best fit the experimental data and the kinetic parameters of this curve were calculated using 'Enzfitter' software application for an IBM-PC computer (Elsevier Biosoft).

2.7. *Reproducibility of data*

Each experiment was carried out in triplicate, with at least 2–3 repetitions, and with at least two different membrane preparations. The difference within triplicates was less than 5%. The specific activity of adenylate cyclase of the same membrane preparation varied by less than 10% in different assays. As others [6], we have noticed variations in the specific activity of adenylate cyclase among different preparations. These variations did not affect the findings reported here.

3. RESULTS

3.1. *Effect of glucose and glucose analogues on yeast adenylate cyclase*

To test the effect of glucose and its analogues on adenylate cyclase activity in vitro, yeast crude membrane preparations were preincubated in the presence of GTP and the sugar for 10 min at 30°C and, subsequently, adenylate cyclase was determined with Mg-ATP as substrate. Table I shows that glucose produced a modest activation of adenylate cyclase. Among the glucose analogues tested, those that are not efficiently phosphorylated, such as 6-deoxy-D-glucose (6-deoxyglucose) and 3-*O*-methyl-D-glucopyranoside [13], were more potent activators than those sugars that were hexokinase substrates, such as the proper glucose of 2-deoxy-D-glucose. The later one, in fact, caused an apparent inhibition (30%). Glucose-6-phosphate caused complete inhibition of the enzyme. The activation produced by 200 mM of 6-deoxyglucose in nine different membrane preparations varied from 50% to 200% for the adenylate cyclase determined in the presence of Mg^{2+} and GTP. This activation was higher than the one caused by 3-*O*-methyl-glucopyranoside (25% to

Table I

Effect of glucose and other sugars on adenylate cyclase activity

Sugar added (300 mM)	Adenylate cyclase activity (pmol·min^{-1}·mg protein^{-1})		
Sorbitol	14.50	± 0.70	n = 30
Glucose	14.62	± 0.78	n = 12
2-Deoxy-D-glucose	8.92	± 0.86	n = 8
6-Deoxy-D-glucose	28.10	± 2.20	n = 15
3-*O*-methylglucose	25.99	± 0.59	n = 10
Glucose-6-phosphate	0		n = 5

Membrane suspensions were preincubated for 10 min at 30°C with 0.2 mM GTP plus 300 mM of the indicated sugar. Adenylate cyclase was assayed in the presence of 2.5 mM Mg^{2+} as described in section 2.

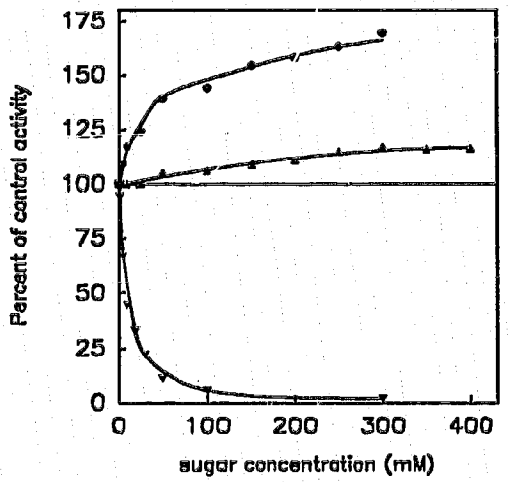

Fig. 1. Effect of different concentrations of glucose (●), 6-deoxyglucose (▲) and glucose-6-phosphate (▼) on GTP-dependent adenylate cyclase activity. The percent activation is referred to the control activity, measured in the presence of sorbitol.

110%), and than that caused by glucose (10–20%). Sorbitol (300 mM), which was used to check a possible cyclase activation due to an increase in the osmotic pressure, caused occasional activations of 5–10%. The stimulating effect of glucose and 6-deoxyglucose, as well as the inhibition caused by glucose-6-phosphate, was shown to be a function of the sugar concentration (Fig. 1). 50% of the maximum effect was obtained at concentrations of approximately 25 mM of 6-deoxyglucose, which coincides with the apparent K_m value determined for glucose as the triggering signal that causes rise in cAMP levels in whole cells [14]. The apparent K_m for the inhibiting effect of glucose-6-phosphate was 10 mM.

3.2. *Characterization of the activation of guanine nucleotide dependent adenylate cyclase by 6-deoxy-D-glucose*

As indicated above, 6-deoxyglucose caused an increase in adenylate cyclase activity. To characterize the activation process, adenylate cyclase was assayed in the presence of guanosine-5′-β-thiodiphosphate (GDPβS) to determine the basal activity. The effect of the sugar was then assayed in the presence of GTP or GppNHp to determine the guanine nucleotide-stimulated, or in the presence of Mn^{2+} to determine the activity of the non-regulated catalytic component of the system. Prior to the assay, the membranes were preincubated with the appropriate guanine nucleotides (200 μM), plus or minus 200 mM 6-deoxyglucose. The results are presented in Table II. In membranes from *S. cerevisiae* SMC-18, GTP alone produced a threefold activation over the basal activity (measured in the presence of GDPβS). Under the same conditions, the activation produced by GppNHp was nearly fourfold. In the presence of 6-deoxyglucose, an additional activation to that produced by either GTP or GppNHp was observed. This activation represented an additional 100% of the basal activity. On the other hand, 6-deoxyglucose inhibited the non-regulated catalytic subunit by 15% as determined with Mn-ATP as substrate.

These results indicate that 6-deoxyglucose acts on the guanine nucleotide-regulated adenylate cyclase. The fact that Mn^{2+}-dependent was inhibited by the glucose analog rules out that the observed effect is due to a direct activation of the catalytic subunit. This concept was further confirmed by the lack of stimulation of adenylate cyclase by 6-deoxyglucose or guanine nucleotides when the experiments were carried out with membranes from the strain T26-19C [14] (Table II). This strain lacks functional Ras proteins and contains a *bcy1*⁻ mutation to suppress the lethality of *RAS* deficiency. If Ras proteins are the only activators of the adenylate cyclase in yeast, no stimulation by GTP or GppNHp should be expected. The lack of effect of 6-deoxyglucose confirms that the sugar acts through this signal transduction pathway. As for the strain SMC18, the Mn^{2+}-dependent activity was clearly inhibited by 6-deoxyglucose.

3.3. *Effect of glucose and 6-deoxy-D-glucose on the guanine nucleotide exchange*

The ability of glucose and 6-deoxyglucose to stimulate the exchange of the guanine nucleotide at the regulatory component of the adenylate cyclase was evaluated by two different experimental approaches: (a) the stimulation of adenylate cyclase by GppNHp after treatment with 6-deoxyglucose and GMP, and (b) the effect of the sugars on the kinetics of activation of the enzyme by GppNHp.

Both are based on the fact that the exchange of the guanine nucleotide at the Ras protein is the rate-limiting step for the activation of the adenylate cyclase. Thus,

Table II

Effect of 6-deoxy-D-glucose on yeast adenylate cyclase

Addition	6-Deoxyglucose	Adenylate cyclase activity (pmol·min⁻¹·mg protein⁻¹)	
		SMC-18	T26-19C
GDPβS	–	9.6	2.7
GTP	–	25.2	2.9
GTP	+	44.5	2.2
GppNHp	–	34.2	2.7
GppNHp	+	47.6	2.3
Mn^{2+}	–	145.0	57.6
Mn^{2+}	+	123.0	39.5

Adenylate cyclase was determined in membranes derived from SMC-18 strain or in membranes derived from the mutant strain T26-19C (*MATα. leu2. his3. trp1. can1. bcy1. ras1::HIS3,ras2::LEU2*) kindly provided by T. Ishikawa (Institute of Applied Microbiology Tokyo University, Japan). Prior to adenylate cyclase assay, preincubation was carried out as described in Table I in the presence of 200 μM guanine nucleotides or 2 mM Mn^{2+}, plus or minus 200 mM 6-deoxyglucose. In samples with guanine nucleotides the Mg^{2+} concentration was 2.5 mM during the adenylate cyclase assay.

the degree and/or the rate of activation of adenylate cyclase is a reflection of the exchange of guanine nucleotides at the regulatory component [11,12], provided that the non-hydrolysable GTP analog GppNHp is used to ensure that the GTPase activity of the regulatory subunit, and hence cyclase inactivation, is blocked.

The stimulation of adenylate cyclase by GppNHp after treatment with GMP and 6-deoxyglucose was determined essentially as described [11]. The strategy consists of adding an excess of a non-activating, low-affinity guanine nucleotide (GMP), in the presence or in the absence of a possible enhancer of the exchange. This treatment is performed in the presence of 20 mM $MgCl_2$, to prevent the non-regulated exchange of guanine nucleotide at the Ras protein [16]. During the preincubation in the presence of the ligand, GMP binds to the regulatory protein while, in its absence, the endogenous ligand is not exchanged. Afterwards, GMP and the effector are washed out and adenylate cyclase activity is determined in the presence or in the absence of GppNHp. It should be expected that the Ras protein loaded with GMP would exchange the mononucleoside into GppNHp more readily than the one loaded with the endogenous ligand, which shows higher affinity. Thus, the degree of GppNHp-dependent activation of the cyclase would be directly dependent on the previous effector-induced exchange of the guanine nucleotide.

Table III shows adenylate cyclase activity determined in the presence of 20 mM $MgCl_2$, plus or minus 100 μM GppNHp, in membranes pretreated as described above. In membranes preincubated only with GMP or 6-deoxyglucose, GppNHp stimulated the adenylate cyclase activity by 30–60%. However, when both GMP and 6-deoxyglucose were present during the preincubation, the stimulation by GppNHp was nearly 300%, thus indicating that 6-deoxyglucose facilitated the guanine nucleotide exchange at the nucleotide binding site.

Table III

Effect of the preincubation of the membranes with GMP and 6-deoxyglucose on the stimulation of the adenylate cyclase by GppNHp

Pretreatment	Adenylate cyclase activity (pmol·min^{-1}·mg protein^{-1})	
	Mg^{2+}	Mg^{2+} + GppNHp
Sorbitol	15.5	19.0
Sorbitol + GMP	13.3	17.8
6-Deoxyglucose	14.0	22.0
6-Deoxyglucose + GMP	18.2	53.7

Membranes derived from derepressed cells were preincubated for 30 min at 30°C with 20 mM Mg^{2+} and the indicated sugar (200 mM) in the presence or in the absence of 1 mM GMP. Then, membranes were centrifuged at 200 000 × g for 3 min, resuspended in storage buffer and washed twice. Adenylate cyclase was then assayed in the presence of 20 mM Mg^{2+} with or without GppNHp (0.1 mM).

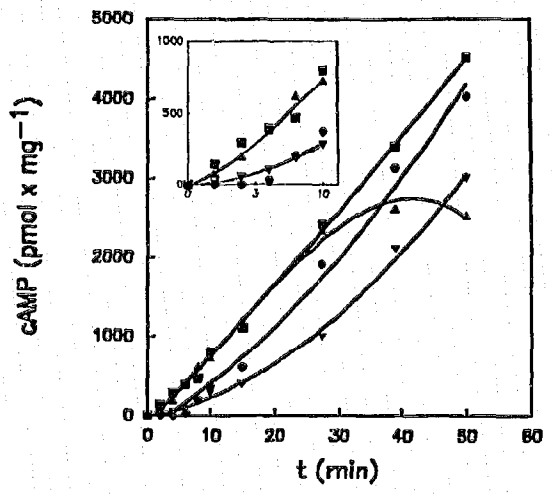

Fig. 2. Effect of glucose (▲), 6-deoxyglucose (■) and glucose-6-phosphate (▼) on the kinetics of the activation of adenylate cyclase by GppNHp. (●) Control. The inset shows the initial ten minutes of the reaction with higher resolution.

In the second experimental approach, the kinetics of that activation was determined in membranes treated with different sugars. The V_{max} and the K_{obs} of the reaction under each condition were determined as described in section 2. It is important to point out that the reaction was started by mixing the assay buffer with the concentrated membrane preparation, so that there was no preincubation.

Typical experiments are depicted in Fig. 2, where the synthesis of cAMP/mg of protein is represented as a function of time. It is clearly evident that the reaction, in the absence of effector sugars, is not linear from t=0. This delay, as previously mentioned, is a function of the K_{obs}, in such a way that the higher the K_{obs} value, the shorter the delay in cyclase activation [12]. In the absence of sugars, the determined value for K_{obs} was 0.09 ± 0.001 min^{-1}, which is consistent with the data presented by Engleberg et al. for other wild-type strains. When either 100 mM glucose or 6-deoxyglucose were present, the production of cAMP was essentially linear from the beginning. The K_{obs} values were $\gg$1 and 0.5 min^{-1} for glucose and 6-deoxyglucose, respectively, but hardly fitted equation (2), because of their approximation to a straight function. This indicates that the delay was drastically reduced by the presence of the sugar.

Once the linear phase was reached, the slope was constant and essentially the same in the presence or in the absence of 6-deoxyglucose, the apparent V_{max} values being 94 ± 1.7 pmol·min^{-1}·mg^{-1} in the absence of the sugar and 104 ± 0.005 pmol·min^{-1}·mg^{-1} in its presence. In the presence of glucose, the rate of cAMP production was linear only for 20 min, then it slowed down and the reaction eventually stopped. This negative effect was mainly due to the exhaustion of ATP, since the time during which the V_{max} remained constant decreased to 2–4 min when the concentration of the substrate was

reduced to 0.4 mM (data not shown). This phenomenon occurs even in the presence of an ATP-regenerating system, and might be due to the phosphorylation of glucose, since our membrane preparations show hexokinase activity.

When the reaction was performed in the presence of 10 mM glucose-6-phosphate, the delay in the activation of the enzyme was the same as in the control without any sugar. The K_{obs}, therefore, had a similar value. There was, however, a reduction of 50% in the value of V_{max}. This inhibitory effect, combined with the depletion of ATP mentioned above and correlated to the increase in the concentration of sugar phosphate, might explain why glucose appeared as a poor activator in the experiments depicted in Fig. 1 and Table I, while in the kinetic experiments glucose appeared to be the most potent activator.

4. DISCUSSION

The results presented here show that glucose and its non-phosphorylatable analogues can activate the adenylate cyclase in *Saccharomyces cerevisiae* crude membrane preparations. Although data presented in Table I and Fig. 1 indicate that sugars which are not substrates of the hexokinases are more potent activators than glucose itself, the kinetic analysis of the activation process (Fig. 2) clearly demonstrates that glucose is at least as potent as 6-deoxyglucose in activating yeast adenylate cyclase. The poor activation induced by glucose and 2-deoxy-glucose (Table I and Fig. 1) can be explained both by the depletion of ATP and by the production of glucose (or 2-deoxyglucose)-6-phosphate, the former appearing as a potent inhibitor of the enzyme.

The effect of glucose on adenylate cyclase appears to be mediated by Ras proteins, since 6-deoxyglucose activates that guanine nucleotide-stimulated adenylate cyclase and inhibits rather than activates the Mn^{2+}-dependent catalytic subunit of adenylate cyclase. These results indicate that the activation is produced in the regulatory system rather than via the catalytic component of the enzyme. Moreover, 6-deoxyglucose does not activate adenylate cyclase in the strain T26-19C which lacks Ras proteins. In addition, we present data demonstrating that 6-deoxyglucose as well as glucose facilitate the guanine nucleotide exchange at the regulatory component of yeast adenylate cyclase.

Our results might suggest the presence of a glucose receptor, which, by analogy with the mammalian system, would activate the adenylate cyclase by accelerating the exchange of guanine nucleotides at its regulatory component. We have determined GTP binding to membranes and observed that 6-deoxyglucose would increase the binding by 100% over the binding determined in the absence of the 6-deoxysugar (data not shown). However, since that binding could be due to GTP-binding proteins other than Ras, the results are not conclusive.

The rate-limiting step in the activation of adenylate cyclase is the exchange of the guanine nucleotide at the regulatory component. The mechanism by which stimulating hormones activate the cyclase in higher eukaryotes is by enhancing that exchange. When GDP is bound to the regulatory component, the enzyme remains inactive and becomes activated when it is exchanged by GTP. The inactivation of the cyclase is achieved by hydrolysis of the GTP at the regulatory component. Hence, when the degree or the kinetics of the activation of the adenylate cyclase are determined in the presence of the non-hydrolysable GTP analogs, the obtained values indicate the degree or the rate of exchange of the guanine nucleotide is at the regulatory component.

The data presented in Fig. 2 and Tables II and III are unequivocal evidence that the effect of glucose and 6-deoxyglucose is via the regulatory component of the adenylate cyclase, which has been demonstrated by genetic and biochemical data to be the Ras protein (see [17] for a review). Glucose and 6-deoxyglucose, do increase the nucleotide exchange rate at these proteins, as demonstrated by (i) a more effective loading of Ras proteins with GMP and (ii) the significant increment of the K_{obs} in the presence of the sugars. Altogether, these data suggest that glucose and its analogues activate adenylate cyclase upstream Ras proteins by increasing the nucleotide exchange rate. An apparently good candidate to mediate the guanine nucleotides exchange at the Ras protein binding site is the product of the *CDC25* gene [18,19]. However, our recent results indicate that the described effect of glucose is independent of *CDC25* (manuscript in preparation).

The inhibition of the adenylate cyclase by glucose-6-phosphate presented above could be of physiological importance, since the effect is observed with physiological glucose-6-phosphate concentrations [20]. The data presented in Fig. 2 indicate that its effect is neither due to the inhibition of the exchange of the guanine nucleotide nor, exclusively, to the stimulation of the GTPase activity of the Ras protein. The importance of this inhibition as well as the possible protein that could mediate it, deserve to be studied.

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