Characterization of Mammalian C-CDC25^{Mm} Exchange Factor and Kinetic Properties of the Exchange Reaction Intermediate p21•C-CDC25^{Mm}†

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ABSTRACT: This work characterizes several aspects of the interaction between H-ras p21 and the catalytic domain of the mammalian GDP/GTP exchange factor (GEF) CDC25^{Mm} (C-CDC25^{Mm}), isolated in pure form as recombinant protein from *Escherichia coli*. Formation of a complex with nucleotide-free H-ras p21 could be analyzed on native gel electrophoresis by combining C-CDC25^{Mm} and p21•GDP, as the result of the fast separation of GDP from p21. Therefore, in order to obtain highly purified heterodimer in preparative amounts, p21•GDP and C-CDC25^{Mm} were exposed to an electric field and the complex purified by anionic chromatography. The rate of the C-CDC25^{Mm}-mediated nucleotide exchange on p21 depended on the nature of the bound nucleotide (6 times faster for p21•GDP than p21•GTP) but was uninfluenced by the nature of the free nucleotide acting as second substrate. No saturating concentration of p21•GDP could be determined by measuring the C-CDC25^{Mm}-mediated increase in the nucleotide exchange rate with a nitrocellulose binding assay. On this basis the K_m and the k_{cat} of the reaction were calculated to be >11 μM and >0.8 s^{-1}, respectively. The dramatic increase in the nucleotide exchange rate was found to be almost exclusively based on the strong stimulation of the "off-rate". The "on-rate" of the nucleotide on the p21•C-CDC25^{Mm} complex was similar for GDP and GTP and was little increased vs that on p21 alone. The observation that the apparent affinity of both nucleotides for the p21•C-CDC25^{Mm} complex was lower than for p21 alone stressed the great affinity of this complex, whose K_d was calculated to be ~3 pM. These results are discussed and compared with the properties of other GEFs, from which C-CDC25^{Mm} differs for a number of specific properties.

The mouse CDC25^{Mm} [1] protein, whose gene was identified by Martegani et al. (1992), Cen et al. (1992), and Wei et al. (1992), has been proposed to be a tissue-specific GDP/GTP exchange factor (GEF) of ras-p21 protein. Unlike the ubiquitous SOS-like GEF, CDC25^{Mm} has been detected so far only in the central nervous system, in which it is apparently restricted to the gray substance (Martegani et al., 1993). Its upstream connection in the ras signaling pathway is still unknown, differently from the SOS-like GEF, which is related to hormone-receptor-bound tyrosine kinases via the adaptor protein GRB2 (Chardin et al., 1993; Egan et al., 1993, Gale et al., 1993). The purification of the entire CDC25^{Mm} (1260 aa) has yet to be achieved, and so far only the isolation of two C-terminal catalytic fragments of 285 amino acid residues (C-CDC25^{Mm}) (Jacquet et al., 1992) and 256 residues (Coccetti et al., 1995) has been reported. From the homologous product from rat (Shou et al., 1992) and human (Schweighoffer et al., 1993; Wei et al., 1994), catalytically active fragments of around 400 residues were obtained. Only a few *in vitro* properties of the catalytic domain of mammalian GEFs have been studied (Jacquet et al., 1992; Schweighoffer et al., 1993; Jung et al., 1994), whereas the characterization of the catalytic domains of *Saccharomyces cerevisiae* Cdc25p (Lai et al., 1993; Haney & Broach, 1994; Jacquet et al., 1994) and Sdc25p (Créchet et al., 1990, 1993; Poullet et al., 1995) has progressed more. In the yeast system, the existence of stable complexes with wild-type or mutated ras proteins was reported by several authors, using partially purified preparations (Mistou et al., 1992a; Lai et al., 1993; Hwang et al., 1993; Mosteller et al., 1994; Poullet et al., 1995).

With the aim at deepening our knowledge of the mechanism activating the ras protein, we have studied the interaction of human H-ras p21 with highly purified C-CDC25^{Mm}, using kinetic and electrophoretic methods. Enzymatic properties of the intermediate state of the exchange reaction, the H-ras p21•C-CDC25^{Mm} complex isolated in pure form, are reported and discussed.

MATERIALS AND METHODS

Isolation of Pure C-CDC25^{Mm}. For the production of the catalytic domain of CDC25^{Mm}, the *Nsi*I−*Ava*I fragment of plasmid pCDC25/12 was cloned in pGEX2T and expressed in *Escherichia coli* strain JM101 (Martegani et al., 1992). This vector encodes the last 285 C-terminal residues (aa 976−1260) of CDC25^{Mm} fused downstream to the GST. In this construction, three amino acids (Gly-Ser-Pro) were added between the thrombin-specific site of cleavage and the amino terminus of C-CDC25^{Mm}. Ten-liter cultures were grown at

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[1] Abbreviations: C-CDC25^{Mm}, catalytic domain of the murine CDC25^{Mm} gene product, comprising the last 285 amino acid residues; GST, glutathione-*S*-transferase; ME, 2-mercaptoethanol; DTT, dithiothreitol; BSA, bovine serum albumine; EDTA, ethylenediaminetetraacetic acid; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; IPTG, isopropyl β-D-thiogalactopyranoside.

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28 °C in LB medium containing 50 μg/mL ampicillin. The induction was started with 0.1 mM IPTG at a cell density of 0.2 A_{600} units and continued overnight to a density of 3.0 A_{600} units. The resuspended pellet was sonicated five times for 30 s in 80 mL of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 7 mM ME, and 10% glycerol) in the presence of 0.5 mM Pefablock-SC [4-(2-aminoethyl)-benzene sulfonyl fluoride] and centrifuged for 20 min at 17000*g*. The resulting supernatant was divided in four portions, each of which was mixed batchwise with 5 mL of glutathione−agarose resin (Sigma) and gently shaken at 4 °C for 40 min. After low-speed centrifugation the supernatant was discarded and the resin washed five times with buffer A plus 3.5 mM $CaCl_2$. The GST-C-CDC25^{Mm} fusion protein, bound to 5 mL of the resin, was cleaved by 150 units of human thrombin (Sigma T7009) in 5 mL of buffer A plus 3.5 mM $CaCl_2$ for 45 min at 30 °C. Pefablock-SC (0.5 mM) was then added, and the tubes were cooled for 15 min at 0 °C. The supernatant was recovered and the resin was washed with 5 mL of buffer A. After filtration on Minisart filter (Sartorius), the protein solution was loaded on a 6 mL ResourceQ column (FPLC system, Pharmacia). The nonfused C-CDC25^{Mm} was eluted at about 120−150 mM NaCl using a linear NaCl gradient (80−220 mM) in 50 mM Tris-HCl, pH 7.5, and 7 mM ME at a flow rate of 6 mL/min. A final chromatographic step was carried out on Superdex 75 (FPLC system, Pharmacia) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 7 mM ME, and 10% glycerol at a flow rate of 0.5 mL/min. The purest fractions identified on SDS−PAGE were concentrated with Aquacide 2 (Calbiochem), dialyzed against 50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 7 mM ME, and 50% glycerol, and stored at −20 °C.

Preparation and Purification of the p21·C-CDC25^{Mm} Complex. As will be reported elsewhere (Parrini et al., 1995), human H-ras p21 protein was produced as recombinant protein in *E. coli*, by expressing its gene cloned on a pGEX vector. The p21 was purified on glutathione−Sepharose resine and, after the thrombin-dependent cleavage of the fused GST, on MonoQ HR5/5 (Pharmacia). The p21 (M_r = 21 000), purified to homogeneity, was stored at −20 °C in 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM $MgCl_2$, 7 mM ME, 50% glycerol, and 1 μM GDP. The two purified proteins C-CDC25^{Mm} and GDP-bound p21 (2.7 and 2.2 mg, respectively) were mixed in 2 mL of 25 mM Tris-HCl, pH 7.5, 7 mM ME, and 20 mM NaCl in a dialysis bag that was submitted to a current intensity of 50 mA for 30 min in a small agarose gel apparatus filled with the same buffer (50 mL). The electrophoresis was performed at 4 °C and the buffer changed three times, every 10 min. The separation of the p21·C-CDC25^{Mm} complex from the free forms was carried out by gel filtration on Superdex 75 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 7 mM ME, and 10% glycerol at a flow-rate of 0.5 mL/min or alternatively by anionic chromatography on ResourceQ column. In the latter case the fractions containing pure p21·C-CDC25^{Mm} complex were obtained at 200−230 mM NaCl using a linear 20−300 mM NaCl gradient in 25 mM Tris-HCl, pH 7.5, and 7 mM ME at a flow rate of 3 mL/min. The complex isolated by this method was stored at −20 °C in 50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 7 mM ME, and 50% glycerol.

Native Gel Electrophoresis. To study the p21-GEF interaction on native polyacrylamide gel electrophoresis (in Tris-glycine buffer, pH 8.9, Laboratory manual LKB), a Hoeffer SE 250 apparatus refrigerated by cryostat to 12−15 °C was used. The stock buffer solution contained 90.12 g of glycine and 3 g of sodium azide adjusted to pH 8.9 with Trizma base in 6 L. The sample buffer, containing bromophenol blue, and the electrode buffer were prepared by dilution in H_2O of stock buffer (1:1 and 1:20, respectively). The gel composition was 10% acrylamide/0.27% bisacrylamide in stock buffer diluted twice and polymerized using ammonium persulfate and TEMED. When indicated, 1 mM $MgCl_2$ and/or 20 μM GDP were added in the acrylamide gel, electrode, and sample buffers. A current of 20 mA per gel was applied during 1 h for the electrophoresis. The proteins were visualized by Coomassie Blue staining.

p21-Guanine Nucleotide Interaction and GEF Activity. The C-CDC25^{Mm}-dependent guanine nucleotide release activity was determined by the nitrocellulose binding method. The dissociation rate of the p21·guanine nucleotide complexes and the GDP/GTP exchange reaction were measured at 30 °C. The labeled complexes were prepared by incubating, for 5 min at 30 °C, 2 μM p21 in buffer B (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, and 0.5 mg·mL^{-1} BSA) with 3 mM EDTA and 6 μM [^{3}H]GDP (350 Bq·pmol^{-1}, DuPont/NEN) or [^{3}H]GTP (270 Bq·pmol^{-1}, DuPont/NEN), followed by the addition of $MgCl_2$ (3 mM). The dissociation rate of the complex (final concentration: 0.1−0.2 μM in buffer B) was kinetically followed after the addition of at least a 500-fold excess of unlabeled nucleotides. To determine the GDP/GDP or GDP/GTP exchange reactions, 0.2 μM p21·GDP was incubated at 30 °C in buffer B, and the reaction was started by the addition of 2 μM [^{3}H]-GDP or [^{3}H]GTP. The p21-bound radioactivity was determined on 20 μL samples filtered through nitrocellulose discs (Sartorius 11306, 0.45 μm) that were then washed twice with 3 mL of ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 100 mM NH_4Cl. The retained radioactivity was measured in a Wallac 1410 (LKB) liquid scintillation spectrometer.

For the determination of the K_m and k_{cat} of the exchange reaction, the GDP-dissociation experiments were performed in the absence and in the presence of the GEF, using a constant amount (10 pmol) of p21 preloaded with radiolabeled GDP that was mixed with increasing amount (0−1050 pmol) of p21 bound to cold GDP. The dissociation reaction was followed kinetically up to 8 min, with p21 concentrations varying from 0.1 to 10.6 μM, in the presence of 0.1 μM C-CDC25^{Mm} and 1 mM free GDP at 30 °C. The apparent GEF-stimulated dissociation rate constants of the radiolabeled complexes obtained from the dissociation curves were used to calculate the initial rate of the exchange reaction, expressed as the amount of p21·GDP complex dissociated per minute, referred to the total amount of the radiolabeled and cold p21·GDP complexes initially present as substrate of the GEF activity. During the period of measurement, several cycles of nucleotide exchange take place. For these experiments Millipore filters (type HA, 0.45 μm) were used.

Determination of the Association Rate of Guanine Nucleotides to Nucleotide-free p21 and p21·C-CDC25^{Mm} Complex. Nucleotide-free p21 and purified p21·C-CDC25^{Mm} complex were used to determine the association rate of GDP and GTP on p21. The p21·C-CDC25^{Mm} complex was prepared as described in the previous section, and nucleotide-free p21 was prepared by gel filtration chromatography (Mistou et al., 1992b). The gel filtration was performed at room temperature on Sephadex G-25 fine resin. The column was

loaded with 500 pmol of p21·GDP which had been preincubated for 5 min at 30 °C in 50 mM Tris-HCl, pH 7.5, 500 mM $(NH_4)_2SO_4$, 5 mM EDTA, and 7 mM ME and eluted with the same buffer. Fractions containing the nucleotide-free p21 were collected and supplemented with 10 mM $MgCl_2$. Second-order association rate constants of p21 with GDP or GTP were measured at 30 °C in buffer C (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl) by mixing 0.25−1 nM nucleotide-free p21 or p21·C-CDC25^{Mm} complex with 3−12 nM [^{3}H]GDP (350 Bq·pmol^{-1}, DuPont/NEN) or [^{3}H]GTP (270 Bq·pmol^{-1}, DuPont/NEN). Five milliliter aliquots of the reaction mixture were withdrawn every 7 s for a 2 min period and filtered on nitrocellulose discs; the filters were washed twice with 5 mL of cold buffer. The association rate constants were calculated using the equation $P = 1/(b - a)\ \ln[a(b - x)/(b(a - x)] = k_{+1}t$ (Fasano et al., 1978) where a is the initial concentration of nucleotide, b the initial concentration of free p21 or p21·C-CDC25^{Mm} complex, and x the concentration of p21·nucleotide complex formed at the different times t.

Determination of the Dissociation Constant of p21·C-CDC25^{Mm} Complex. To determine the dissociation constant of p21·C-CDC25^{Mm}, the displacement of C-CDC25^{Mm} by GDP or GTP was measured. For this purpose, a constant amount of p21·C-CDC25^{Mm} was incubated with various concentrations of [^{3}H]GDP or [^{3}H]GTP. The p21·[^{3}H]GDP or p21·[^{3}H]GTP complex formed at equilibrium allowed the calculation of the apparent affinity of the guanine nucleotide for p21 as part of the p21·C-CDC25^{Mm} complex. From these data, the procedures described by Arai et al. (1974) were used to calculate the dissociation constant of the p21·C-CDC25^{Mm} complex. The p21·C-CDC25^{Mm} complex (0.15 nM) was incubated at 30 °C in buffer C with 0.5−100 nM [^{3}H]GDP or [^{3}H]GTP. After 4 and 8 min incubation, aliquots (5 mL) of the reaction mixtures were filtered on nitrocellulose filters that were then washed twice with 5 mL of cold buffer. From the values obtained for the nucleotide-bound p21 as a function of the nucleotide concentration, a Scatchard plot allowed the calculation of the relative affinity of the nucleotide for p21 in the presence of C-CDC25^{Mm}. To calculate the dissociation constant of p21·C-CDC25^{Mm}, the equation $x/(b - x) = (a/K)[(1/x) - (1/a)]$ was used (Arai et al., 1974), where x is the amount of p21·nucleotide formed, a the amount of p21·C-CDC25^{Mm}, and b the amount of GDP or GTP initially added to the reaction mixture. K is the equilibrium constant of the reaction

$$\text{p21·nucleotide} + \text{C-CDC25}^{Mm} \leftrightarrow \text{p21·C-CDC25}^{Mm} + \text{nucleotide}$$

The dissociation constant of p21·C-CDC25^{Mm} ($K_{\text{p21·CDC25}}$) was derived from the dissociation constant of p21·GDP ($K_{\text{p21·GDP}}$) using the equation $K_{\text{p21·CDC25}} = K_{\text{p21·GDP}}/K$.

Other Materials and Methods. Protein concentration was determined by the Bio-Rad protein assay, using BSA as standard. In the case of p21, the obtained values were checked by GDP binding. The procedure described by Harlow and Lane (1988) was used to produce rabbit polyclonal antibodies anti-C-CDC25^{Mm} for Western-Blot analysis. SDS−PAGE was carried out using a 12% acrylamide separating gel; the samples were prepared by dilution in 50 mM Tris-HCl, pH 6.5, 1 % SDS, 2 mM EDTA, 5% ME, 10% glycerol, and 0.01% bromophenol blue and boiled for 3 min when the samples contained total cell extracts.

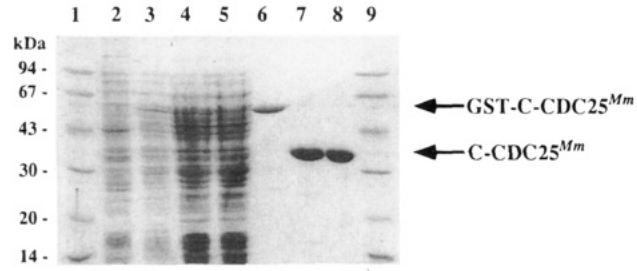

FIGURE 1: SDS−PAGE analysis of the C-CDC25^{Mm} purification steps. Molecular weight markers (lanes 1 and 9). Total cell extract before (lane 2) and after overnight induction (lane 3) of a strain expressing GST-fused C-CDC25^{Mm}. Protein extract prior to mixing with glutathione-sepharose resin (lane 4) and fraction nonretained (lane 5). Glutathione-eluted GST-C-CDC25^{Mm} fusion after purification on MonoQ chromatography (lane 6). Purified preparations of nonfused C-CDC25^{Mm}, obtained after the thrombin-mediated cut of the glutathione-sepharose-bound GST-C-CDC25^{Mm} and chromatographic steps on MonoQ (8 μg, lane 7), followed by Superdex 75 gel filtration (8 μg, lane 8). The gel was stained with Coomassie Blue.

RESULTS

Isolation of Pure C-CDC25^{Mm}. The use of the pGEX system made it possible to obtain pure recombinant C-CDC25^{Mm} (Figure 1) in high yield (2 mg/L of cell culture). The incubation temperature for cell growth was crucial for the solubility of the product. Incubation at 28−30 °C gave a much higher yield of soluble protein than at 37 °C, despite a weaker production. Although purified nonfused C-CDC25^{Mm} could be obtained by thrombin cleavage of the protein fusion after elution with glutathione, the thrombin treatement was more efficient when carried out on the Sepharose-bound GST-C-CDC25^{Mm} fusion. It was important to perform all the purification steps from cell extract to chromatographic separation at 4 °C within the same day to obtain reproducible results. As shown in Figure 1, no proteolytic degradation was observed after chromatography on glutathione-Sepharose and MonoQ. After these two steps, the C-CDC25^{Mm} cleaved from GST displayed the expected apparent molecular mass of 34 kDa and was at least 95% pure as judged on SDS gel by Coomassie Blue staining. An additional chromatography carried out on Superdex 75 was needed to obtain homogeneous C-CDC25^{Mm}. The presence of 10% glycerol in the elution buffer during the gel filtration chromatography was essential to prevent aggregation phenomena causing the appearance of a second peak of C-CDC25^{Mm}. The C-CDC25^{Mm} purified by this method was stable for at least 1 year in storage buffer at −20 °C.

Formation of the p21·C-CDC25^{Mm} Complex on Native Gel Electrophoresis. We have used purified p21·GDP and C-CDC25^{Mm} (Figure 2A) to examine the ability of the p21 and GEF to form a stable complex on native gel. As shown in Figure 2B, the lanes containing both products displayed a slower migrating band, whose staining intensity increased with increasing the concentration of GEF. This band was not visible if the two proteins were separately loaded on the gel or denaturated by heating prior to loading the gel. Thus, it represents the heterodimer resulting from a stable interaction between p21 and C-CDC25^{Mm}, as also confirmed by western-blot analysis using monoclonal antibodies Y13-259 specific for p21 and rabbit polyclonal antibodies anti-C-CDC25^{Mm} (not shown). The isolation of purified C-CDC25^{Mm} cleaved from GST was a necessary step to study the complex formation on native gel, since the GST fusion could not enter the gel. The amount of p21·C-CDC25^{Mm} formed represented

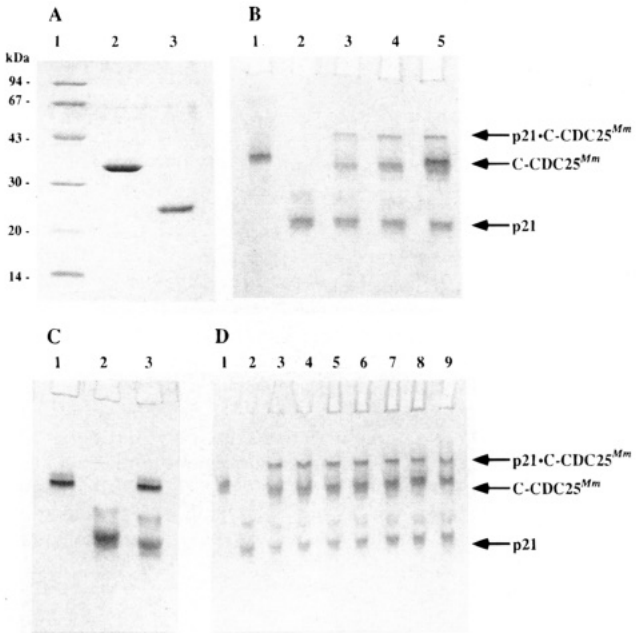

FIGURE 2: SDS−PAGE of purified proteins and formation of the p21•C-CDC25^{Mm} complex on native gel electrophoresis. (Panel A) SDS−PAGE loaded with molecular weight markers (lane 1), C-CDC25^{Mm} (42 pmol, lane 2), and p21•GDP (42 pmol, lane 3). (Panel B) Native PAGE loaded with C-CDC25^{Mm} (30 pmol, lane 1) and p21•GDP (42 pmol, lane 2 to 5) without (lane 2) or with increasing amounts of C-CDC25^{Mm}: 14 (lane 3), 30 (lane 4), and 59 pmol (lane 5). (Panel C) Native PAGE in the presence of 20 μM GDP and 1 mM $MgCl_2$, in gel, electrode, and sample buffer. The gel was loaded with C-CDC25^{Mm} (lane 1), p21•GDP (lane 2), and C-CDC25^{Mm} plus p21•GDP (lane 3). The amount of p21•GDP was 42 pmol and that of C-CDC25^{Mm} 30 pmol. (Panel D) Native PAGE loaded with C-CDC25^{Mm} (lane 1), p21•GDP (lane 2), C-CDC25^{Mm} plus p21•GDP (lanes 3−9). The amount of each protein was 42 pmol. Lanes 4−9 include increasing amount of GDP added only in the sample buffer: 1 (lane 4), 10 (lane 5), 100 (lane 6), 1000 (lane 7), 10 000 (lane 8), and 92 600 pmol (lane 9). The gels were stained with Coomassie Blue.

maximally 50% of the products initially added and depended of the quantity of protein loaded, the ras/GEF ratio, and the migration conditions.

Our p21 preparations, stored as GDP-bound form, were able to form a stable complex with C-CDC25^{Mm} without pretreatment to eliminate the bound GDP prior to the gel loading, despite the fact that the presence of GDP is known to prevent a stable interaction between GTPases and exchange factors. Complete inhibition of this stable interaction was obtained by the addition of 20 μM GDP and 1 mM $MgCl_2$ in the PAGE buffer (Figure 2C). The presence of either GDP or $MgCl_2$ in the polyacrylamide gel and the electrophoretic buffer was sufficient to inhibit the formation of the p21•C-CDC25^{Mm} complex, even at concentration of GDP 100 times lower (not shown). On the contrary, the experiment in Figure 2D shows the low inhibitory effect on the complex formation in the absence of $MgCl_2$ by a 2200 times excess of free GDP (90 000 pmol) over p21, when the nucleotide was only present in the sample loaded on the gel and not in the running buffer. Taken together these results suggest that the formation of a stable complex starting from p21•GDP on electrophoresis is due to (i) the fast dissociation rate of the p21-bound GDP due to the absence of magnesium in the electrode buffer and to the presence of a high amount of GEF and (ii) the fast separation of GDP from p21 due to the high difference in their electrophoretic mobilities.

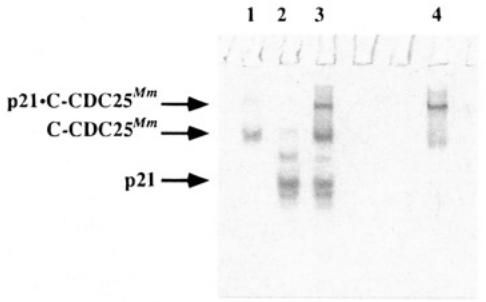

FIGURE 3: Migration of purified p21•C-CDC25^{Mm} complex on native gel electrophoresis. The native PAGE was loaded with C-CDC25^{Mm} (lane 1), p21•GDP (lane 2), C-CDC25^{Mm} plus p21•GDP (lane 3), and p21•C-CDC25^{Mm} complex purified on Resource Q (8 pmol, lane 4). The amount of p21•GDP was 35 pmol and that of C-CDC25^{Mm} 25 pmol. The gel was stained with Coomassie Blue.

Purification of the p21•C-CDC25^{Mm} Complex. The results of the above experiments have been exploited for the purification of the p21•C-CDC25^{Mm} complex. Pure GDP-bound p21 and C-CDC25^{Mm} were exposed to an electric field as described in Materials and Methods in order to favor the separation from the nucleotide and induce the formation of a stable complex. The effect of this treatment, as analyzed on Superdex 75 gel-filtration, where the p21•C-CDC25^{Mm} complex is eluted as a peak distinct from the noncomplexed p21 and C-CDC25^{Mm}, greatly increased the amount of heterodimer (not shown). Pure p21•C-CDC25^{Mm} complex could be obtained in milligram amounts by anionic chromatography on ResourceQ column. In these conditions the complex was eluted at higher salt concentration than p21 and C-CDC25^{Mm}. The migration velocity on native gel electrophoresis of the purified p21•C-CDC25^{Mm} complex (Figure 3, lane 4) corresponded to the band observed by the formation of the complex during electrophoresis (Figure 3, lane 3). This band was dissociated into p21 and C-CDC25^{Mm} by the addition of GDP in the PAGE buffer or by loading on SDS−PAGE (not shown).

Guanine Nucleotide Exchange Activity of C-CDC25^{Mm} on H-ras p21. According to its physiological function, i.e., the regeneration of the active p21•GTP complex, the inactive GDP-bound state of p21 should be the privileged target for the catalytic domain of the exchange factor. The results presented in Figure 4 describe the differential effect induced by the nature of the nucleotide on the C-CDC25^{Mm}-dependent stimulation of the exchange rate. These reactions were performed using an excess of free radiolabeled nucleotide in the reaction mixture (p21•GNP+GNP*) or radiolabeled p21-bound nucleotide with an excess of cold free nucleotide (p21•GNP*+GNP). The C-CDC25^{Mm}-dependent exchange rate of p21•GDP*+GTP was 11 times faster than with p21•GTP*+GTP (Figure 4A). This was calculated from the ratio of the slopes of the linearized curves of the respective experiments. However, after correction for the intrinsic GTPase activity and for the dissociation rate in absence of exchange factor, the real C-CDC25^{Mm} effect on the GDP-bound p21 was 6 times stronger than on the GTP-bound form. Interestingly, the rates of the p21•GDP+GDP* or p21•GDP+GTP* exchange kinetics were not differently affected by the presence of the C-CDC25^{Mm} (Figure 4B). This shows that the C-CDC25^{Mm} activity is conditioned by the nature of the nucleotide prebound to p21 and not by the free nucleotide acting as second substrate in the exchange reaction.

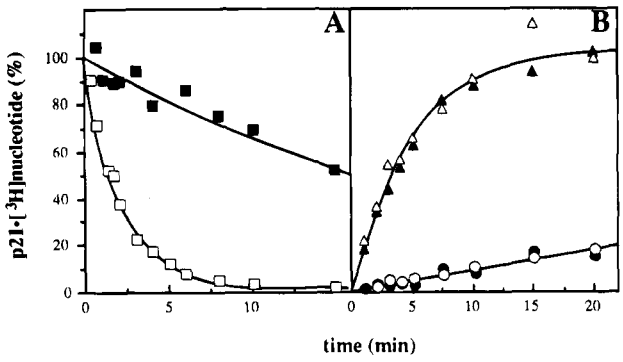

FIGURE 4: Free GDP or GTP does not affect the rate of the C-CDC25^{Mm} stimulated nucleotide exchange rate reaction. The exchange reactions were measured under conditions described in Materials and Methods. In panel A, 0.2 μM p21•[^{3}H]GDP (□) or p21•[^{3}H]GTP (■) were incubated with 0.1 μM C-CDC25^{Mm} at 30 °C in presence of 600 μM cold GTP. In panel B, 0.2 μM p21•GDP were incubated without (○, ●) or with (△, ▲) 0.05 μM C-CDC25^{Mm} at 30 °C in presence of 2 μM [^{3}H]GDP (○, △) or 2 μM [^{3}H]GTP (●, ▲).

In order to evaluate the properties of our *in vitro* system, the effects of temperature, pH, and salt concentrations on the C-CDC25^{Mm} activity were also determined. In standard buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, and 0.5 mg•mL^{-1} BSA), the GEF activity was stable for at least 2 h at 30 °C, but a 50% irreversible inactivation was observed at 42.5 °C after only 8 min of incubation time (not illustrated). The thermal inactivation of CDC25^{Mm} was found not to be influenced by the presence of p21 whether free or nucleotide bound. As shown in Figure 5A, the concentration of monovalent salt in the reaction buffer did not markedly affect the C-CDC25^{Mm}-stimulated dissociation rate of the p21•GDP complex, unlike the yeast GEF C-Sdc25p, whose activity is strongly inhibited by increasing the concentration of monovalent salts (Poullet et al., 1995). However, also with C-CDC25^{Mm} the optimum exchange activity was obtained in the absence of monovalent cations. The optimum pH value for GEF activity in a pH range between 6 and 10 was determined by following the variation of the dissociation rate constant (k'_{-1}) of p21•GDP in the absence and the presence of C-CDC25^{Mm}. The dissociation rate in the presence of C-CDC25^{Mm} was maximal at pH 6 and progressively decreased from pH 6 to 10 by a factor 7 (Figure 5B), whereas the intrinsic k'_{-1} only varied in the pH 6−7.5 range (Figure 5C). The ratio of the k'_{-1} value in the presence of C-CDC25^{Mm} to the intrinsic k'_{-1} value showed that the optimal stimulatory effect was obtained at pH 7.5 (Figure 5D).

The activity of C-CDC25^{Mm} was also investigated as a function of the concentration of p21•GDP. The amount of p21•[^{3}H]GDP dissociated was strictly proportional to the concentration of p21•[^{3}H]GDP. The highest concentration of p21 used in the experiment of Figure 6 was 10.6 μM and corresponded to the upper limit measurable with the nitrocellulose binding assay. This substrate concentration allowed us to calculate an initial rate of ~250 pmol of p21•GDP dissociated per min in the presence of 100 nM of C-CDC25^{Mm} in 100 μL at 30 °C. From these experiments one can deduce that the K_m of the C-CDC25^{Mm} -dependent exchange reaction is higher than 11 μM and the k_{cat} faster than 0.85 s^{-1}.

Comparison of the GDP/GTP Association Rates on p21 and p21•C-CDC25^{Mm}. The p21•C-CDC25^{Mm} complex was used as an important tool to characterize the interaction between these two proteins. The purity of this complex, isolated by anionic chromatography, was checked on native- and SDS−PAGE, and the concentration was determined by the Bradford method on the basis of a molecular mass of 54 kDa. The concentration of the complex obtained with this method was in agreement with the biochemical activities of the two components. The GEF activity was verified by the stimulation of the GDP dissociation of exogenous p21•[^{3}H]GDP, using noncomplexed C-CDC25^{Mm} as control, and the p21 concentration was tested by GDP binding at 0 and 30 °C with saturating amount of free [^{3}H]GDP.

In order to determine whether the presence of the C-CDC25^{Mm} tightly bound to p21 could influence the interaction between the nucleotide and p21, we have compared the association rate of GDP with p21•C-CDC25^{Mm} to the association rate of GDP with nucleotide free p21. A typical experiment of GDP binding to p21 in complex with C-CDC25^{Mm} is illustrated in Figure 7. The maximal binding was obtained within a few minutes; the linearization of the binding kinetics allows the calculation of the association rate constant. As indicated in Table 1 the k'_{+1} of GDP with p21•C-CDC25^{Mm} is somewhat stimulated by the GEF (35%). This effect is more pronounced with GTP, where the k'_{+1} is increased twice. The addition to the reaction mixture of a 200-fold excess of free C-CDC25^{Mm} (50 nM) over p21•C-CDC25^{Mm} did not modify the association rate constant of the nucleotide with p21. The association rate values of Table 1 are mean values of independent experiments that showed virtually the same association rate using different concentrations of the p21•C-CDC25^{Mm} complex from 0.25 to 1 nM.

Competition between Guanine Nucleotides and C-CDC25^{Mm} for Binding to p21 and Dissociation Constant of p21•C-CDC25^{Mm}. To determine the apparent affinity of the nucleotide for the binary complex p21•C-CDC25^{Mm}, experiments were carried out by incubating various amounts of GDP or GTP in the presence of a constant amount of p21•C-CDC25^{Mm} complex. In preliminary experiments, the displacement of the p21-bound exchange factor by the nucleotide was followed kinetically to determine the time needed for reaching equilibrium. As shown in Figure 8, a high molar excess of GTP over the p21•C-CDC25^{Mm} complex is required for the complete binding of the guanine nucleotide to p21. Half-saturation is achieved with ~12 times excess of nucleotide. Such a concentration (2 nM) is 2 orders of magnitude higher than the dissociation constants of the p21•nucleotide complexes. A Scatchard plot of the data was used to calculate the apparent affinity of the nucleotide for p21 as a part of the p21•C-CDC25^{Mm} complex. The values obtained lay between 1 and 2.5 nM for both GDP or GTP. From these data it was possible to calculate the equilibrium constant K of the reaction (Arai et al., 1974; see also Materials and Methods):

$$\text{p21•nucleotide} + \text{C-CDC25}^{Mm} \leftrightarrow \text{p21•C-CDC25}^{Mm} + \text{nucleotide}$$

The dissociation constant of p21 for the exchange factor C-CDC25^{Mm} ($K_{p21•CDC25}$) can be obtained by the ratio of the dissociation constant of p21 for the nucleotide ($K_{p21•GDP}$ or $K_{p21•GTP}$) to the respective equilibrium constant of the reaction (K) calculated from the p21•nucleotide binding kinetics in the presence of GDP or GTP. As reported in Table 2, the dissociation constant for the p21-C-CDC25^{Mm} interaction obtained with this method lies in the picomolar range

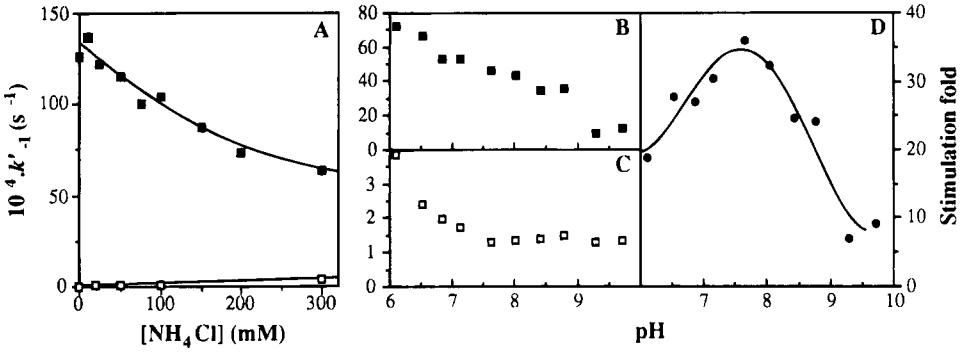

FIGURE 5: Effect of NH_4Cl concentration (panel A) and pH (panels B−D) on the C-CDC25^{Mm}-stimulated exchange activity. In panel A the dissociation rate constants of p21•[^{3}H]GDP complexes in the absence (□) and in the presence (■) of 0.1 μM of C-CDC25^{Mm} were calculated from 90 s kinetic experiments. The dissociation experiments were performed as described in Materials and Methods except for the NH_4Cl added to the dissociation buffer. The dissociation rate constant k_{-1} was calculated using the equation $\ln (C_t/C_o) = -k_{-1}t$, in which C_o represents the initial concentration of the p21•[^{3}H]GDP complex and C_t the concentration at the time t. (Panels B−D) The dissociation rate of p21•[^{3}H]GDP complexes was measured in a buffer solution containing 100 mM NH_4Cl, 1 mM $MgCl_2$, 0.5 mg•mL^{-1} BSA in ATE (100 mM ACES, 52 mM Tris, 52 mM ethanolamine) to adjust the pH. Each k'_{-1} value was determined by kinetic experiments over a period of 90 s using 0.1 μM C-CDC25^{Mm} (panel B) or 90 min if C-CDC25^{Mm} was absent (panel C). The stimulation fold representing the C-CDC25^{Mm} activity (panel D) was obtained by the ratio of the dissociation rate constant k'_{-1} of p21•GDP complexes in the presence of C-CDC25^{Mm} to the k'_{-1} in the absence of the exchange factor.

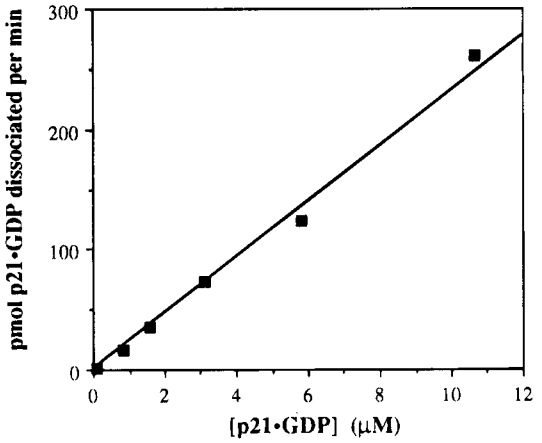

FIGURE 6: C-CDC25^{Mm}-mediated exchange rate activity as a function of the p21•GDP concentration. The kinetics of the p21•[^{3}H]GDP dissociation were performed in 100 μL at 30 °C in the presence of 0.1 μM C-CDC25^{Mm} for a period of 8 min. Diverse kinetics using p21•GDP concentrations from 0.1 to 10.6 μM were carried out to calculate the initial rate of the exchange reaction in pmol of p21•GDP complex dissociated per minute (see Materials and Methods).

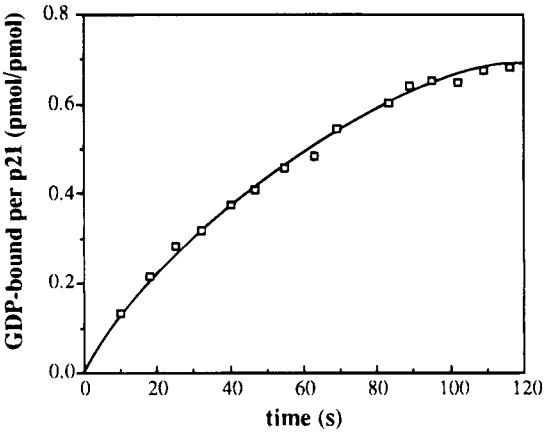

FIGURE 7: Kinetics of the association of GDP to the p21•C-CDC25^{Mm} complex. The binding of free nucleotide to the p21•C-CDC25^{Mm} complex was measured as described in Materials and Methods, using 3 nM [^{3}H]GDP (350 Bq•pmol^{-1}, DuPont/NEN) and 0.26 nM p21•C-CDC25^{Mm} complex. The reaction was started by mixing the two solutions of each component prepared at twice the final concentration in 45 mL each. At the indicated time intervals, the amount of GDP bound was determined in 5 mL aliquots filtered through nitrocellulose filters. The blank values, subtracted in the figure, were obtained under the same conditions in the absence of the p21•C-CDC25^{Mm} complex and corresponded to 2−3% of the maximal values observed in the presence of the complex.

($K_{p21•CDC25} = 3.4 \times 10^{-12}$ M), showing that p21 has a higher affinity for the C-CDC25^{Mm} than for the nucleotide.

DISCUSSION

In this report several aspects of the interaction between H-ras p21 and the catalytic domain of the exchange factor C-CDC25^{Mm} were examined with highly purified components. Particular attention was given to the isolation and characterization of the stable complex formed by these two factors, as a way to obtain information on the mechanism of the exchange reaction.

The analytical method used to follow the formation of the p21•C-CDC25^{Mm} complex by simply mixing GDP-bound p21 and C-CDC25^{Mm} prior to native gel loading is based on the rapid electrophoretic separation of GDP from p21. This method can be applied to microquantities of materials (0.5−1 μg of protein) and allows the rapid visualisation of the ras•GEF complex avoiding immunocoprecipitation or affinity chromatography. Moreover, the native gel electrophoresis can reveal conformational changes, as in the case of purifications carried out over several days, where a second band of C-CDC25^{Mm} may appear, whose presence is undetectable on SDS−PAGE (E. Jacquet, unpublished results). As an additional development, the method to obtain preparative amounts of the p21•C-CDC25^{Mm} complex in pure form, using an electric field followed by a chromatographic separation, presents the advantage of avoiding the technically difficult preparation of large amounts of nucleotide-free H-ras p21 (Mistou et al., 1992b; John et al., 1990) that due to the instability of nucleotide-free p21 at low magnesium ion concentrations can influence irreversibly its native conforma-

Table 1: Association Rates of Guanine Nucleotides to Nucleotide-free p21 and p21•C-CDC25^{Mm} Complex

	p21	p21•C-CDC25^{Mm}
$k'_{+1(GDP)}$ (10^6 M^{-1} s^{-1})	2.8 ± 0.4	3.8 ± 0.4
$k'_{+1(GTP)}$ (10^6 M^{-1} s^{-1})	1.5 ± 0.4	3.1 ± 0.3

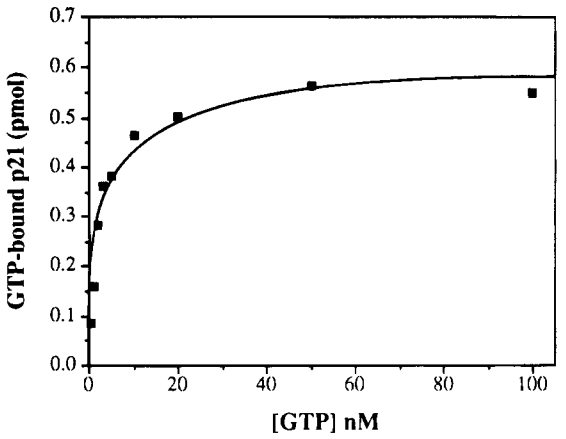

FIGURE 8: Dissociation of p21•C-CDC25^{Mm} and formation of the p21•GTP complex as a function of the GTP concentration. The GTP binding to p21 was measured as described in Materials and Methods, using concentrations of [^{3}H]GTP (270 Bq•pmol^{-1}, DuPont/NEN) from 0.5 to 100 nM and 0.14 nM p21•C-CDC25^{Mm} complex. For each experimental point the reaction was started by mixing 500 μL of a 10 times concentrated GTP solution in 4.5 mL of complex solution. In this experiment, the incubation time was 8 min. The amount of GTP bound to p21 was determined on aliquots passed through nitrocellulose filters. Blank values increased with increasing GTP concentration up to a maximum of 5−10% of the values obtained in the presence of the p21•C-CDC25^{Mm} complex.

Table 2: Dissociation Constants of p21•GDP and p21•C-CD25^{Mm} and Enzymatic Parameters of the C-CDC25^{Mm}-Catalyzed Guanine Nucleotide Exchange Reaction on p21

$K'_{d(p21•GDP)}$	48 pM
$K'_{d(p21•C\text{-}CD25^{Mm})}$	3.4 ± 2.1 pM
K_m	>11 μM
k_{cat}	>0.85 s^{-1}

tion. The purified p21•C-CDC25^{Mm} complex can be used for structural studies.

Differential activity of GEF in the Exchange Reaction. As shown for EF-Tu (Romero et al., 1985; Hwang & Miller, 1985), all the different steps of the GEF-stimulated exchange reaction are reversible and can use both GDP and GTP. The observation that p21•GDP is 6 times more responsive to C-CDC25^{Mm} than p21•GTP with respect to the first step of the exchange reaction, the dissociation of the GDP and GTP complexes with ras proteins, favors the increase in "activated" GTP-bound p21 over "inactivated" p21 bound to GDP. This differential value, somewhat lower than reported for experiments using partially purified GST-C-CDC25^{Mm} (Jacquet et al., 1992), is twice stronger than for the *S. cerevisiae* catalytic domain of Cdc25p (Haney & Broach, 1994) but lower than for Sdc25p that was 15 times more active on Ras2p•GDP than on Ras2p•GTP (Poullet et al., 1995). In contrast to this, the GEF of the nuclear GTP-binding protein Ran, the RCC1 factor, was found to have nearly the same stimulation on the dissociation of both GDP• and GTP•Ran complexes (Klebe et al., 1995), whereas the differential effect of EF-Ts on the EF-Tu•nucleotide complexes (Fasano et al., 1978) was close to that of C-CDC25^{Mm}. The existence of considerable variations between the various GEFs with respect to their interaction with the nucleotide-specific conformations of these GTP-binding proteins strongly suggests that other factors can influence the kinetic direction of the exchange reaction. Haney and Broach (1994) proposed as major regulatory mechanism the fluctuation of the cellular pools of GDP and GTP, whose levels are determined by diverse metabolic pathways and oscillate depending on the functional state of the cell. Under normal conditions, the higher concentration of GTP vs GDP would kinetically favor the regeneration of the GTP complex by the GEF. Another important regulatory factor could be represented by the competition between GEF and GAP for binding to ras•GTP, for which GAP has a much higher affinity than for ras•GDP. Unfortunately, too little is still known about competition between GAP and GEF for an evaluation of this possibility. It is likely that various mechanisms act simultaneously in the cell to determine a productive direction of the exchange reaction. A differential regulation is an absolute requirement, considering that all single steps of the exchange reaction are reversible.

Rate-Limiting Step in the ras-GEF Interaction. From the activity of C-CDC25^{Mm} obtained in exchange reaction using p21•GDP, we calculated that the K_m must be greater than 11 μM and the k_{cat} than 0.85 s^{-1}. These values stress a substantial difference for the K_m values between C-CDC25^{Mm} and yeast GEFs, of at least 1−2 orders of magnitude, whereas k_{cat} seems to be less divergent. Indeed, the $K_{m(Ras2p•GDP)}$ and k_{cat} values of *S. cerevisiae* C-Cdc25p were calculated to be 0.16 μM and 0.22 s^{-1}, respectively (Haney & Broach, 1994); the $K_{m(p21•GDP)}$ of C-Sdc25p is 2.3 μM and the k_{cat} 0.24 s^{-1} (Mistou et al., 1992a). In essential agreement with these values, the specific activity of yeast C-Cdc25p, as determined by the stimulation of the exchange reaction with 0.1−0.2 μM Ras2p, was found to be 8−10 times higher than Sdc25p and 15−20 times higher than C-CDC25^{Mm} (Jacquet et al., 1994). For comparison, the ras GRF stimulated nucleotide exchange reaction on the homologous Ki-ras p21 displayed a K_m close to that of Sdc25p (2.5 μM) but a considerably lower k_{cat} (0.006 s^{-1}) (Orita et al., 1993). For Ran it was calculated that the intrinsic nucleotide exchange rate could be enhanced by its GEF by 5 orders of magnitude (Klebe et al., 1995).

By the use of the purified p21•C-CDC25^{Mm} complex, we could evaluate the rate of the second step of the exchange reaction: the association of the nucleotide to the nucleotide-free ras•GEF. The rate of this reaction revealed only a modest increase as compared to the association of the nucleotide with p21 in the absence of GEF (see Table 1), showing a similar k_{+1} values for GDP and GTP. Different results were obtained with other systems: Sdc25p-C in a 1:1 molar ratio with Ras2p induced a 9-fold increase in the association rate constant of both GTP and GDP (Poullet et al., 1995). It is important to note that, in the absence of GEF, the k_{+1} for the nucleotide association to Ras2p is lower (~0.08 × 10^6 M^{-1} s^{-1}) as compared to p21 (2 × 10^6 M^{-1} s^{-1}). The stimulatory effect on the nucleotide "on-rate" by EF-Ts in equimolar amount to EF-Tu was different for the two nucleotides and practically significant only for GDP [5-fold for GDP and 1.2-fold for GTP (Fasano et al., 1978)].

The analysis of the effects of C-CDC25^{Mm} makes evident that the effect on the association rate of GDP to p21 is very modest vs that on the dissociation rate. With the nitrocellulose binding assay, we could observe linear stimulations of the dissociation rate of p21•GDP by CDC25^{Mm} up to 1400 times, but we were unable to determine the saturating concentration of C-CDC25^{Mm} for the exchange reaction. One should stress that the association rate in the absence of the GEF is already *per se* a very fast step, 1−2 orders of magnitude below the diffusion constant in aqueous solvents, whereas the intrinsic "off-rate" of the p21•nucleotide complex is an extremely slow step. This strongly suggests that the

nucleotide dissociation rate represents the rate-limiting step of the ras·GTP regenerating system. There may be, however, differences depending on the system. Indeed, in the yeast system Mosteller et al. (1994) and Haney and Broach (1994) presented evidence that the nucleotide exchange on Ras2p depends on the nature of the free nucleotide, GTP, being more efficient than GDP. Haney and Broach show an effect of the free nucleotide concentration on the k_{cat} of the exchange reaction and conclude that the rate-limiting step is the nucleotide association step and not the binding of Cdc25p to Ras2p. In our system we were so far unable to observe a similar phenomenon. The rate of the reaction was dependent on the nature of the bound nucleotide and was not influenced by the free nucleotide. In our opinion, this discrepancy is likely to depend on the specific properties of the yeast and mammalian systems.

Affinity of the Nucleotide-free ras·GEF Complex. We have used a competition method to evaluate the dissociation constant of the ras·GEF complex, a parameter that was unknown so far. The association of C-CDC25^{Mm} in a stable complex with p21 was found to reduce markedly the affinity of p21 for the nucleotide. The excess of nucleotide over the complex required for displacing C-CDC25^{Mm} from p21 indicates that this GEF binds to p21 with a higher affinity than the nucleotide. Our experiments show a very high affinity for nucleotide-free p21·C-CDC25^{Mm} ($K'_d = \sim 3$ pM) that is 3 orders of magnitude lower than for the procaryotic EF-Tu·EF-Ts complex. Noteworthy, the K_d for EF-Tu·EF-Ts is close to that for EF-Tu·GDP (~3 nM) but also much lower (~80 times) than that for EF-Tu·GTP (Arai et al., 1974). The high affinity of p21·C-CDC25^{Mm} is indicative for a complex interaction likely to involve several contact sites and multiple residues, in agreement with recent observations suggesting that multiple structures of p21 are likely to be involved in the interaction with GEF (Verrotti et al., 1992; Mosteller et al., 1994; Segal et al., 1995). The fact that a factor of at least 10^7 exists between the K_m of the exchange reaction and the K'_d of the nucleotide-free p21·GEF complex suggests that the nucleotide-free p21 has a structure different from the GDP- and GTP-bound forms causing specific differences on the ability to interact with GEF.

Conclusions. This work characterizes for the first time several partial steps of the interaction between the catalytic domain of a mammalian GEF and p21, emphasizing specific differences and similarities between GEFs from different organisms. Although we have obtained interesting results with the catalytic activity of CDC25^{Mm} expressed by a domain that comprises only one-fifth of the mass of the intact molecule (Cen et al., 1992), we hope that it will soon be possible to isolate the intact GEF, which has escaped any successful attempt of purification so far, in order to study the influence of the N-terminal moiety on the activity of the catalytic domain. Moreover, evidence exists suggesting that the N-terminal moiety mediates the action of other elements possibly located in the cell membrane, as part of the upstream ras pathway or other regulatory pathways.

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