Mutations at position 1122 in the catalytic domain of the mouse ras-specific guanine nucleotide exchange factor CDC25^{Mm} originate both loss-of-function and gain-of-function proteins

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Abstract The role of two residues within the catalytic domain of CDC25^{Mm}, a mouse ras-specific guanine nucleotide exchange factor (GEF), was investigated by site-directed mutagenesis. The function of the mutant proteins was tested in vivo in both a *Saccharomyces cerevisiae cdc25* complementation assay and in a mammalian *fos*-luciferase assay, and in in vitro assays on human and yeast Ras proteins. Mutants CDC25$^{Mm_{E1048K}}$ and CDC25$^{Mm_{S1122V}}$ were shown to be (partly) inactive proteins, similar to their yeast homologs. Mutant CDC25$^{Mm_{S1122A}}$ showed higher nucleotide exchange activity than the wild type protein on the basis of both in vitro and in vivo assays. Thus, alanine and valine substitutions at position 1122 within the GEF catalytic domain originate mutations with opposite biological properties, indicating an important role for position 1122 in GEF function.

Key words: Guanine nucleotide exchange factor; Site-directed mutagenesis; *fos*-Luciferase; Oncogene; BIAcore; Yeast

1. Introduction

Ras proteins are small guanine nucleotide binding proteins acting as molecular switches in signal transduction [1]. The level of the active, GTP-bound form results from the balance of the competing activity of GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF) [2]. The prototypes of ras-specific GEFs are the product of the yeast *CDC25* gene [3] and its close homolog SDC25 [4], the first protein of this class for which a ras-specific GEF activity was shown [5]. In mammalian cells two ras-specific GEF classes have been identified. One class – whose cDNA was originally isolated by functional complementation of a *cdc25* mutant of *Saccharomyces cerevisiae* – has been called CDC25^{Mm} and is mostly brain-specific [6,7]. Human and rat CDC25^{Mm} homologs have also been isolated [8,9]. The other class – Sos-GEF – has been isolated on the basis of homology with the *Son of Sevenless* gene of *Drosophila melanogaster* [10] and is expressed ubiquitously. Both GEFs are large, multi-domain proteins: despite their different structural organization, the two catalytic domains are approximately 70% homologous. The differences in structural organization reflect the involvement in different signal transduction pathways. Sos has been shown to work downstream of tyrosine kinase receptors [11,12], while CDC25^{Mm} may interact with trimeric G protein-coupled receptors [13,14]. More recently a role for CDC25^{Mm} in synaptic transmission and long-term memory has been proposed [15]. CDC25^{Mm} has been shown to respond to calcium levels [16,17]. Deregulated expression of both GEF classes has been shown to induce cell transformation in different experimental systems [18,19].

Understanding the mechanism by which GEFs activate ras is of paramount importance for unraveling the biochemical basis of ras-mediated signal transduction [20]. Several recent papers addressed the role played by ras residues in interaction with GEF (see for instance [21–24]), while much less information is available on GEF amino acids involved in the interaction with ras [25–27]. In order to gain information on structure-function relationships of GEF catalytic domain, we used CDC25^{Mm} as a model molecule. The properties of the mutant proteins were scored by a combination of in vitro assays on purified proteins and functional tests in both yeast and mammalian cells. We show that mutations in residue 1122 may originate either a thermolabile 'loss-of-function' protein with reduced guanine nucleotide exchange activity or a thermostable 'gain-of-function' protein with enhanced guanine nucleotide exchange activity, suggesting that this position plays a role in GEF stability and function.

2. Materials and methods

2.1. Yeast strains, media, plasmids and genetic manipulations

The yeast strain used in this study is TC7 (*MAT*α *ade lys trp1 ura3 cdc25-1*) [3]. Liquid and solid media were prepared as described [28]. Plasmid pCYM-1 [29] was used as a plasmid for expression of wild type and mutant catalytic domains in both yeast and CHO fibroblasts, because genes cloned under the control of its SV40 promoter can be expressed both in yeast and in mammalian cells. Yeast was transformed using lithium acetate [30] without carrier DNA. For the complementation assays, the cells were spread on two plates and allowed to growth at 24°C for 36 h. After this period one of the plates was shifted to 37°C and colony growth on both plates scored 72 h later. The complete CDC25^{Mm} protein was expressed in NIH3T3 cells using the pcDNA3 plasmid (Invitrogen) under the control of the cytomegalovirus promoter.

2.2. Site-directed mutagenesis

The 3′-terminal region of the wild type *CDC25*^{Mm} gene (encoding residues 974–1260 of the mature protein) was cloned in the pALTER vector (Promega). Site-directed mutagenesis was performed according to the manufacturer's instructions ('Altered sites in vitro mutagenesis system', Promega). The primers used for mutagenesis were: CDC25$^{Mm_{S1122V}}$, 5′-AGATCACCTCC**GTC**ATCAACCGCAG-

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3′; CDC25$^{Mm_{S1122A}}$, 5′-AGATCACCTCC**GCC**ATCAACCGCAG-3′; CDC25$^{Mm_{E1048K}}$, 5′-TTCCTTATGAA**AAG**TTCTTTGGCCA-3′; CDC25$^{Mm_{E1048A}}$, 5′-TTCCTTATGAA**GCG**TTCTTTGGCCA-3′. The mutant codons are shown in bold type. Each mutant gene was completely sequenced in order to confirm that only the desired single-codon change had been introduced.

2.3. Transfections and determination of fos-luciferase activity

CHO and NIH3T3 fibroblasts were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. Cells were transfected with pCYM-1- or pcDNA3-derived plasmids by the calcium phosphate method and cotransfected with 2 μg of the ras-responsive *fos*-luciferase plasmid [18,31]. In all transfection DNA was buffered to a total of 16 μg with pCYM-1 or pcDNA3 plasmid as appropriate. After transfection, cells were starved for 40 h in serum-free medium supplemented with 4 μg/ml transferrin and 10^{-8} M sodium selenite and collected. Luciferase activity was assayed essentially as described [18] and normalized to protein content.

2.4. Protein purification and enzyme assays

The catalytic domains (residues 974–1260) of all mutants were cloned into pGEX2T vector (Pharmacia) as previously described for the wild type protein [6]. Wild type and mutant fusion proteins between glutathione *S*-transferase (GST) and CDC25$^{Mm_{974-1260}}$ – ca. 58 kDa – were purified by glutathione-Sepharose chromatography (Pharmacia) as previously described [6,32]. Glutathione-Sepharose-bound fusion proteins were cleaved with thrombin (Serva) and further purified by SourceQ (Pharmacia) chromatography essentially as described [33,34]. Recombinant RAS2 and p21^{ras} proteins were obtained either from *Escherichia coli* strains harboring pGEX-derived plasmids as described by Parrini et al. [35], or as described by Lenzen et al. [34]. The dissociation rates of the Ras-labelled guanine nucleotide complexes and the cold GDP to radioactively labelled GTP exchange reactions on RAS2 or p21^{ras} were measured by a nitrocellulose binding assay at 30°C as described [32]. The synthesis of mantGDP and the charging of the p21^{ras} protein with mantGDP were performed as described by Lenzen et al. [33]. The fluorescence measurements (excitation wavelength 366 nm and emission wavelength 450 nm) were performed at 20°C. The dissociation rate of 100 nM of the p21^{ras}·mant nucleotide in 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ and 5 mM DTE was followed in time after addition of a 200-fold excess of GDP and the indicated concentration of either the wild type or the mutant CDC25^{Mm} catalytic domain.

2.5. BIAcore

The BIAcore measurements were performed basically as described elsewhere [36]. In short, the surface of a sensor chip CMS research grade (Pharmacia) was activated and charged with anti-GST serum (Pharmacia) as described by the producer. The proteins were diluted in buffer C (10 mM Na-HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ and 0.005% Igepal) to the appropriate concentration and kept at 4°C. The chip lane was charged with GST-p21^{ras} by passing 35 μl of 1 μM GST-p21^{ras}·GDP at 5 μl/min, then the protein was made nucleotide-free by passing 40 mM EDTA in buffer C for 6 min. Subsequently, GST-free CDC25^{Mm} catalytic domain was injected at the indicated concentration and its association to GST-p21^{ras} measured as a change in surface plasmon resonance in time. The dissociation of the complex was studied by passing buffer C over the lane. At the end of the measurement, the chip lane was recycled by passing 20 mM glycine, pH 2.0, and subsequently 0.05% SDS (4 min each), followed by a new charging with GST-p21^{ras}, EDTA washing and a new binding experiment. This way, the effect of at least nine different concentrations – in the range 2–150 nM – of wild type and mutant CDC25^{Mm} catalytic domains on the surface plasmon resonance were measured. The association and dissociation rate constants were calculated using the program BIAlogue 2.1 (Pharmacia).

3. Results

3.1. Effects of CDC25^{Mm} mutations in vivo

The in vivo effect of different mutations on activity of CDC25^{Mm} catalytic domains was tested in the yeast *S. cerevisiae* and mammalian fibroblasts. Positions 1048 and 1122 were chosen for mutagenesis because they correspond to positions altered in two thermosensitive yeast mutants [37]. So far no biochemical characterization of the mutant proteins is available. The same positions were also mutated to alanine, an amino acid introducing minimal conformational stress which is often used to probe the role of atoms in a given amino acid extending beyond the β-carbon [38]. All our data, with the exception of results reported in Fig. 3, refer to the catalytic domains.

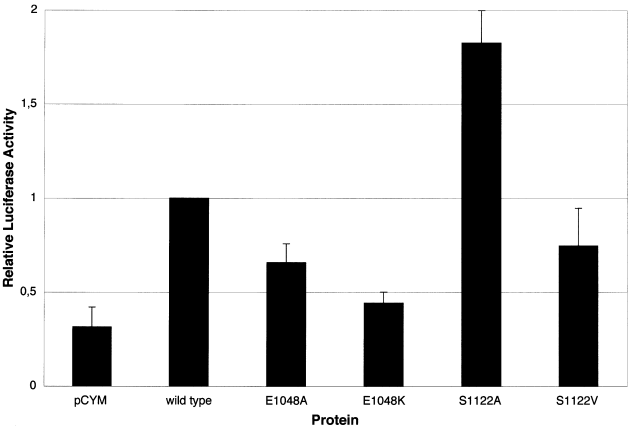

Fig. 1. Ras-dependent luciferase expression by CDC25^{Mm} in CHO cells. CHO fibroblasts were cotransfected with *fos*-luciferase plasmid (2 μg) and 3 μg of pCYM plasmid or derivatives containing the wild type or mutated CDC25^{Mm} catalytic domain. Data are expressed as percentage of luciferase activity in cells transfected with CDC25^{Mm}. Average+standard deviation of at least two independent experiments – each conducted in duplicate – is shown.

The *cdc25-1* yeast strain TC7 was transformed with expression vectors expressing the mutated *CDC25^{Mm}* catalytic domains. Mutant proteins CDC25$^{Mm_{S1122V}}$ and CDC25$^{Mm_{E1048K}}$ were unable to complement the *cdc25-1* mutation (i.e. the ratio of colonies growing at 37°C vs. 24°C was < 0.01). These results indicate that the corresponding mutations play the same role in both yeast and mammalian GEFs, despite the fact that the two positions are not universally conserved in the Ras-specific GEFs so far identified. Mutants CDC25$^{Mm_{S1122A}}$ and CDC25$^{Mm_{E1048A}}$ were functional in the yeast assay, although the latter mutant complemented the *cdc25* mutant slightly less efficiently. The ratio of colonies growing at 37°C vs. 24°C for mutants CDC25$^{Mm_{S1122A}}$, CDC25$^{Mm_{E1048A}}$ and wild type was 0.86 ± 0.03, 0.71 ± 0.07 and 0.87 ± 0.2, respectively.

The catalytic domain of CDC25^{Mm} has been shown to activate a ras-responsive *fos*-luciferase reporter gene in vivo in CHO fibroblasts [14]. We have transiently cotransfected our mutant constructs together with the *fos*-luciferase reporter plasmid into CHO cells and compared the ability of the encoded mutant proteins to trans-activate the *fos* promoter with that of the wild type molecule.

Fig. 1 indicates that mutant CDC25$^{Mm_{E1048K}}$, unable to complement the *cdc25-1* strain, was also not able to significantly activate the *fos* promoter. Mutants CDC25$^{Mm_{E1048A}}$ and CDC25$^{Mm_{S1122V}}$ were both able to weakly stimulate *fos*-luciferase activity, although they behaved differently in the yeast complementation assay. CDC25$^{Mm_{S1122A}}$ – the only mutant complementing the yeast *cdc25-1* strain as efficiently as the wild type – displayed a ras-dependent luciferase activity significantly higher than the wild type molecule.

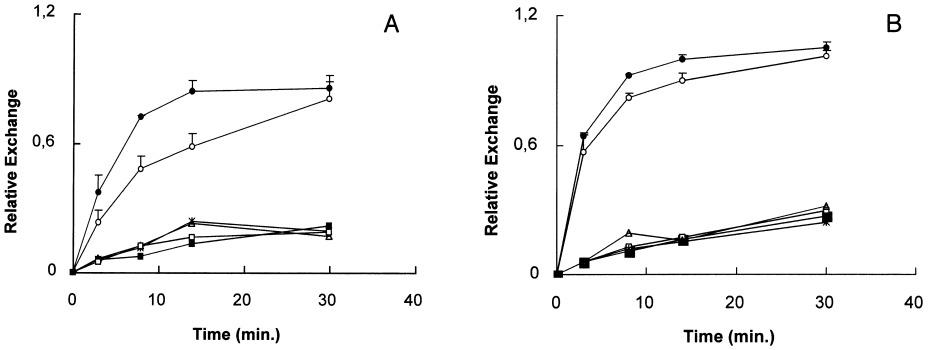

Fig. 2. Stimulation of the GDP to [^{3}H]GTP exchange reaction of p21^{ras}·GDP (A) and RAS2·GDP (B) by the catalytic domains of CDC25^{Mm} and the mutants. 0.5 μM p21ras·GDP (A) and RAS2·GDP (B) were incubated at 30°C in buffer A. The reaction was started by addition of a 25-fold excess of [^{3}H]GTP over the Ras·GDP complex as described in Section 2. Average + standard deviation of at least two independent experiments – each conducted in duplicate – is shown for wild type and CDC25 $^{Mm_{S1122A}}$. In order to avoid visual cluttering, standard deviations for inactive mutant and Ras proteins alone are not shown; at each time point they were below 10%. Symbols are as follows: (×), intrinsic exchange; (○) CDC25^{Mm} wild type; (□) CDC25 $^{Mm_{S1122V}}$; (●) CDC25 $^{Mm_{S1122A}}$; (△) CDC25 $^{Mm_{E1048K}}$; (■) CDC25 $^{Mm_{E1048A}}$.

3.2. Effects of CDC25^{Mm} mutations on GEF activity in vitro

The catalytic domain of each CDC25^{Mm} mutant was expressed in – and purified from – *E. coli* cells as GST-fusion proteins. All the mutant fusion proteins could be purified under the same conditions as the wild type and their electrophoretic mobility was indistinguishable from the wild type. Mutant GST-CDC25$^{Mm_{S1122A}}$ was more soluble than wild type, whereas the other mutant proteins, most notably GST-CDC25$^{Mm_{E1048K}}$ and GST-CDC25$^{Mm_{E1048A}}$, were less soluble than wild type (data not shown).

Addition of 40 nM CDC25$^{Mm_{974-1260}}$ strongly increased the intrinsic GDP/GTP exchange rate on the p21^{ras}·GDP complex. In keeping with results obtained in yeast, mutants CDC25$^{Mm_{S1122V}}$ and CDC25$^{Mm_{E1048K}}$ were catalytically inactive at 30°C when measured with the radioactive exchange assay on both human p21^{ras} (Fig. 2A) and yeast RAS2 proteins (Fig. 2B). Mutant CDC25$^{Mm_{E1048A}}$, which is functional in the yeast assay and gives intermediate results in the *fos*-luciferase assay, did not show any significant stimulation of guanine nucleotide exchange on either Ras protein. Only the CDC25$^{Mm_{S1122A}}$ mutant significantly stimulated the GDP to [^{3}H]GTP exchange on Ras proteins, possibly more efficiently than wild type. The reasons for the different behavior of the CDC25$^{Mm_{E1048A}}$ mutant in vivo in the yeast complementation assay and in vitro is unclear. Since equivalent results were obtained with the both the human and yeast Ras proteins, the different results obtained in vivo in yeast and in vitro with purified proteins do not depend on species-specific interaction of the mutant GEF with the Ras substrate. More likely some stabilization in vivo of the mutant protein by cytosolic yeast proteins is taking place. Similar results were obtained when measuring the stimulatory action of these mutants on ras·[^{3}H]GDP and RAS2·[^{3}H]GDP in the presence of an excess of GDP (data not shown), consistent with the observation that the nature of the associating nucleotide does not affect the exchange rate [36].

3.3. The S1122A mutation results in a 'gain-of-function' protein

Results reported in the previous sections suggested that the S1122A mutation may originate a 'gain-of-function' protein. Thus the properties of the CDC25$^{Mm_{S1122A}}$ and CDC25$^{Mm_{S1122V}}$ mutants were examined in more detail.

The full length CDC25^{Mm} molecule has been shown to activate ras-dependent luciferase activity more efficiently than the catalytic domain [14], possibly because the 1260 amino acid long molecule is more stable than the catalytic domain alone (R. Zippel, personal communication). We thus investigated whether the S1122A mutation affected similarly the full length GEF molecule. Dose-response curves were constructed with plasmids expressing both the wild type and the CDC25$^{Mm_{S1122A}}$ full length proteins. Both mutant and wild type proteins showed dose-dependent *fos*-luciferase activity, but the CDC25$^{Mm_{S1122A}}$ mutant had a significantly higher maximal activity than wild type, and reached the maximal value at much lower plasmid concentration (Fig. 3).

The wild type and mutant catalytic domains were purified free of the GST moieties by thrombin cleavage, as described in Section 2. Different preparations of the same protein showed variations in activity, most likely due to variations

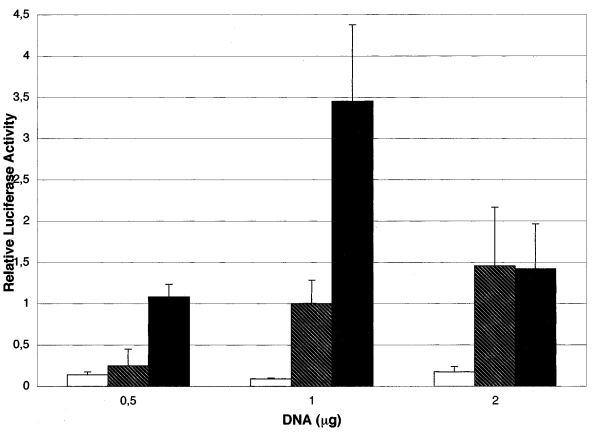

Fig. 3. Dosage dependence of wild type and S1122A mutant on *fos*-luciferase activity in transfected NIH3T3 cells. NIH3T3 cells were cotransfected with *fos*-luciferase plasmid (2 μg) and either plasmid pcDNA3 (white bars), or derivatives carrying the whole length wild type *CDC25*^{Mm} gene (hatched bars) or the corresponding *CDC25* $^{Mm_{S1122A}}$ mutant (black bars). Data are expressed as percentage of luciferase activity in cells transfected with 1 μg CDC25^{Mm}. Average+standard deviation of at least two independent experiments – each conducted in duplicate – is shown.

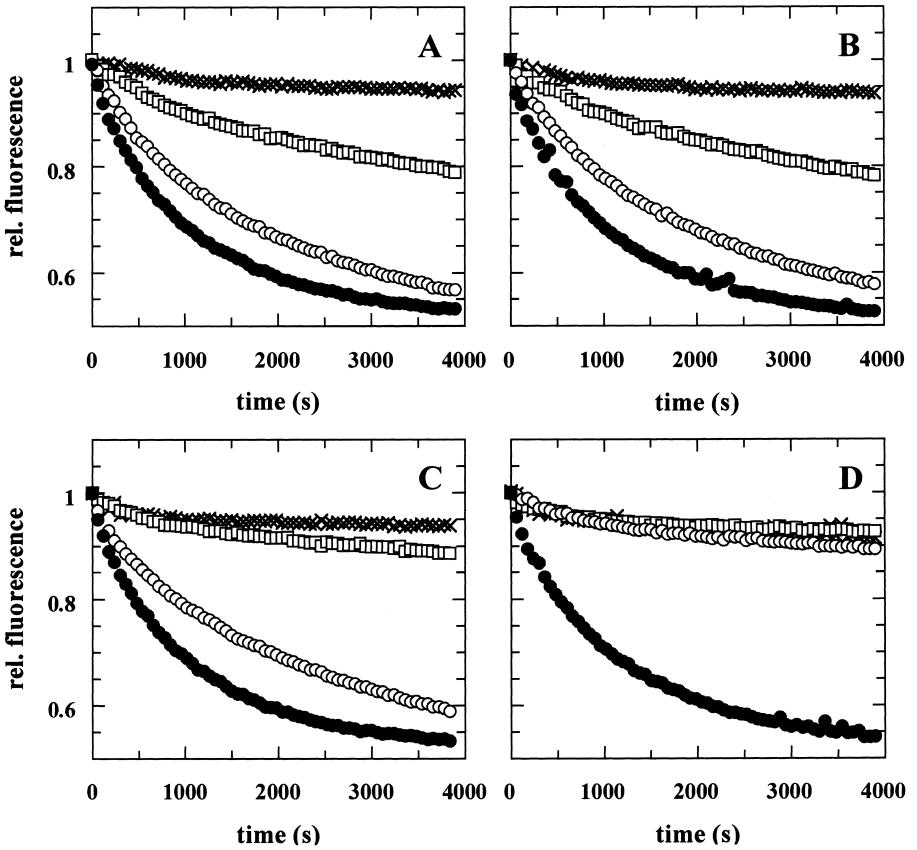

Fig. 4. Temperature dependence of CDC25^{Mm} wild type, CDC25 $^{Mm_{S1122V}}$ and CDC25 $^{Mm_{S1122A}}$ activity. CDC25^{Mm} proteins (25 nM) were incubated with Ras·mantGDP (100 nM) for 15 min at 25°C (A), 30°C (B), 35°C (C) or 40°C (D), cooled down on ice and centrifuged. The supernatant was assayed for GEF activity as described in Section 2 by adding 20 μM GDP. Depicted is the fluorescence decrease following dissociation of p21^{ras}·mantGDP in the absence (×) or presence of CDC25^{Mm} wild type (○), CDC25 $^{Mm_{S1122A}}$ (●) or CDC25 $^{Mm_{S1122V}}$ (□).

in the duration of the purification and temperature fluctuations that may occur for instance during cell extraction.

Dissociation assays were conducted at 20°C, since at this temperature the wild type does not display significant thermosensitivity [36]. Interestingly, 50 nM of the thrombin-cleaved catalytic domain of CDC25$^{Mm_{S1122A}}$ was able to stimulate the dissociation of 100 nM Ras·mGDP with a 2–4-fold higher rate than wild-type CDC25^{Mm}, whereas the CDC25$^{Mm_{S1122V}}$ was nearly two-fold less active than wild type (in the presence of CDC25^{Mm} wild type, the dissociation rate constant was 0.00104 ± 0.00020 s^{-1}).

The purified thrombin-cleaved catalytic domains were then analyzed with respect to temperature stability as depicted in Fig. 4. The proteins were pre-incubated for 15 min at the indicated temperatures and the residual GEF activity was assayed at 20°C after centrifugation. As observed earlier [36], the wild type protein is thermolabile and loses most of its activity after 15 min incubation at 40°C (Fig. 4). Mutant protein CDC25$^{Mm_{S1122V}}$ was significantly more thermosensitive than wild type, since its activity was drastically reduced after pre-incubation at 35°C, in keeping with the fact that a valine at the homologous position makes the yeast CDC25 protein thermosensitive. In this particular experiment, after pre-incubation at 25°C, CDC25$^{Mm_{S1122V}}$ is approximately four times less active than wild type, which indicates a larger difference than observed in the standard dissociation assay mentioned above. This may be caused by a sensitivity of CDC25$^{Mm_{S1122V}}$ towards pre-incubation and/or centrifugation. Interestingly, mutant CDC25$^{Mm_{S1122A}}$ was shown to be much more thermostable than wild type, since even after pre-incubation at 40°C nearly full GEF activity could be observed.

Table 1
Equilibrium and rate constants derived from BIAcore experiments for wild type and mutant CDC25^{Mm} catalytic domains

Protein	k_{on} (10^5 M^{-1} s^{-1})	k_{off} (10^{-4} s^{-1})	K_D (nM)
CDC25^{Mm}	4.4 ± 1.9	5.0 ± 0.3	1.1
CDC25 $^{Mm_{S1122A}}$	4.6 ± 2.3	3.5 ± 1.0	0.8
CDC25 $^{Mm_{S1122V}}$	2.5 ± 0.5	6.8 ± 3.5	2.7

We decided to directly investigate quantitatively the binding of CDC25$^{Mm_{S1122A}}$ and CDC25$^{Mm_{S1122V}}$ mutant proteins to nucleotide-free GST-coupled ras by BIAcore experiments. Analysis of the association and dissociation curves at different concentrations of both mutant proteins allowed us to calculate k_{on}, k_{off} and K_D. The results are summarized in Table 1. Equilibrium constants for the wild type and the CDC25$^{Mm_{S1122A}}$ mutant proteins were similar, despite the more than three times differences in GEF specific activity, while CDC25$^{Mm_{S1122V}}$, in contrast displayed an increased K_D.

4. Discussion

Up to now, the interactions between Ras proteins and their GEFs have been analyzed by mutational studies concerning

mostly the substrate Ras. We analyzed the biochemical properties of mutant GEFs mapping at positions 1048 and 1122 – corresponding to positions mutated in two yeast *cdc25* temperature sensitive mutants – using both in vivo and in vitro assays.

Mutation of glutamic acid 1048 to lysine leads to loss of function. The mutant $CDC25^{Mm_{E1048K}}$ shows no activity in the complementation assay, no significant stimulation of luciferase activity and no guanine nucleotide exchange activity in vitro. Mutating the same residue to Ala appears to affect the function of $CDC25^{Mm}$ less drastically, as demonstrated by the reduced, but significant activity of this mutant in the in vivo experiments in both yeast and CHO fibroblasts. However, the mutant does not show any in vitro guanine nucleotide exchange activity. Lack of detectable biological activity in the exchange and dissociation reaction in vitro was not the result of the production of a grossly misfolded protein, since the mutant $CDC25^{Mm_{E1048K}}$ protein was able to compete with wild type $CDC25^{Mm}$ for $p21^{ras}$ binding, leading to a severely reduced ($43 \pm 3\%$, average of two independent experiments) guanine nucleotide exchange when added in a 10-fold excess over wild type (data not shown). These seemingly contrasting results could be explained by assuming that some cell protein(s) may have a stabilizing effect on this mutant. This may result in different sensitivities of the various in vivo and in vitro assays, pointing out that careful characterization of a mutant protein requires a combination of different complementary approaches. In budding yeast a low level GEF activity appears to be sufficient to support growth as indicated by the low steady state levels and short half-life of the CDC25 protein [39]. This observation suggests that even protein with a severe reduced activity may provide enough activity to support growth.

A somehow complementary situation was observed with mutant $CDC25^{Mm_{S1122V}}$, which was inactive in the yeast complementation assay but displayed intermediate levels of *fos*-luciferase activity in transfected CHO cells, possibly reflecting different efficiencies with which the SV40 promoter of the pCYM plasmid drives transcription in the different hosts, the requirement of different levels of active GEF to give a detectable signal and/or the stabilizing effect of interacting proteins in each cell. These differences may become more important as the proteins become less stable which is the case with the $CDC25^{Mm_{S1122V}}$ mutant. In analogy to the yeast protein, mutation of serine 1122 to valine in the mammalian $CDC25^{Mm}$ protein increased the thermosensitivity of the protein (Fig. 4). Interestingly, mutation of the serine 1122 to alanine – present in the yeast wild type protein, as well as in the highly homologous mouse *GRF2*-encoded protein [40] – increases activity and thermostability of the mammalian GEF.

The opposite effects brought about by the alanine and valine mutations at position 1122 are remarkable. On the basis of the described in vitro and in vivo experiments the $CDC25^{Mm_{S1122A}}$ mutant behaves as a 'gain-of-function' mutant since it has higher *fos*-luciferase activity, is more thermostable and more efficiently catalyzes nucleotide dissociation from ras.

The latter effect is well evident at 20°C, where the purified catalytic domains of $CDC25^{Mm_{S1122A}}$ and wild type are both stable, implying that the 2–4-fold higher catalytic activity of the mutant is not a consequence of an increased thermostability. BIAcore analysis showed that mutation S1122A does not affect the interaction between nucleotide-free Ras and $CDC25^{Mm}$. Interestingly, in the recently published Ras·Sos structure [41] it was shown that residue A877 (the Sos homologue of $CDC25^{Mm}$ residue S1122) is located in an α-helix that participates in the interaction with Ras. Although its neighbor S876 interacts with Ras residue Q70 via a H-bond, A877 does not seem to be involved in direct interaction, at least in the nucleotide-free complex. Consequently, mutation S1122A is more likely to (directly or indirectly) influence the stability of the ternary complex Ras·GXP·$CDC25^{Mm}$ and/or facilitate conformational changes that occur during the exchange reaction, e.g. the putative rate-limiting isomerization from a high to a low nucleotide affinity conformation [36]. Apart from the homologous yeast CDC25 mutants, whose biochemical properties were not investigated, only one other mutant mapping close to position 1122 was described [26]. This mutant was generated in the human homolog of $CDC25^{Mm}$ and corresponds to position 1130. This Arg to Ala mutation resulted in a slightly thermosensitive protein, since it was able to complement a temperature sensitive yeast *cdc25* mutant at 37°C but not at 40°C. The mutant retained the ability to bind Ras proteins as determined by two hybrid analysis in *S. cerevisiae*. The finding that two independent mutations mapping within the same 10 amino acid region gave temperature sensitive phenotypes suggests that this region may play an important role in GEF stability. Moreover, our finding that mutation S1122A results in a gain-of-function mutant, not only more thermostable but also more catalytically active than wild type, indicates that position 1122 may play a role not only in structural stability, but in catalysis as well.

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