The isolated catalytic hairpin of the Ras-specific guanine nucleotide exchange factor Cdc25\textsuperscript{Mm} retains nucleotide dissociation activity but has impaired nucleotide exchange activity

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Abstract Cdc25\textsuperscript{Mm} is a mammalian Ras-specific guanine nucleotide exchange factor (GEF). By homology modeling we show that it shares with Sos-GEF the structure of the putative catalytic HI hairpin where the dominant negative T1184E mutation is located. Similarly to Cdc25\textsuperscript{MmT1184E}, the isolated wild-type and mutant hairpins retain the ability to displace Ras-bound nucleotide, originate a stable Ras/GEF complex and downregulate the Ras pathway in vivo. These results indicate that nucleotide re-entry and Ras/GEF dissociation – final steps in the GEF catalytic cycle – require GEF regions different from the HI hairpin. GEF down-sizing could lead to development of novel Ras inhibitors.

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1. Introduction

Ras proteins are major controllers of intracellular pathways leading to cell growth and differentiation [1]. They act as molecular switches whose activation state is set by their binding to either GDP (inactive state) or GTP (active state) [2,3]. Ras proteins are endowed with intrinsic GTPase and guanine nucleotide exchange activities. These basal low activities are stimulated by GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF), respectively [4]. Altering this fine balance by deregulation of either GAP or GEF activity may result in hypo- or hyper-activation of downstream pathway(s), so that overexpression of a GEF or inactivation of a GAP may both result in cell transformation [5].

In higher eukaryotes, three major Ras-specific GEF classes exist: Sos, RasGRF/Cdc25\textsuperscript{Mm} and GRP/Cal-DAG-GEF (reviewed in [6,7]). Despite the different domain organization and the involvement in different signal transduction pathways, they share an homologous catalytic domain [6]. In recent years, structural [8,9], mutational [10–15] and kinetic [16–18] studies have been carried out on Ras/GEF domain from the three classes. A widely accepted model for GEF function is based on crystallographic data and indicates that an helical hairpin – that we will refer to as HI hairpin – plays a major catalytic role by displacing the Ras-bound nucleotide [2]. This helical hairpin appears to be highly conserved also in all Ras relatives-specific GEF [6], namely C3G, Epac/AMP-GEF and PDZ-GEF, specific for Rap proteins [19], and RalGDS, Rlg/RalGDS-like and RalGDS-related specific for Ral proteins [20].

We previously showed that a single amino acid change within the HI hairpin turns the mammalian GEF Cdc25\textsuperscript{Mm} [21] into a dominant negative protein (Cdc25\textsuperscript{MmT1184E}) that can trap Ras molecule in the nucleotide-free state, thus effectively downregulating Ras both in vivo and in vitro [13,22]. Here we report that the Cdc25\textsuperscript{Mm}-derived HI hairpin on its own is able to dissociate the bound nucleotide from Ras and show that such a down-sized GEF is able to effectively down regulate the ras pathway in kras-transformed fibroblasts. Our data are discussed in the frame of current models of GEF action and in the perspective of targeting the Ras/GEF complex for the development of antiproliferative compounds.

2. Results

2.1. Molecular modeling of the catalytic domain of Cdc25\textsuperscript{Mm}

No 3D structure is available for the catalytic domain of Cdc25\textsuperscript{Mm}. Thus, a standard molecular modeling approach [23] was used to generate a model of the structure of the Ras GEF catalytic domain of the protein (residues 1007–1262, Fig. 1A). The structure of the Ras–Cdc25\textsuperscript{Mm} complex, obtained by homology modeling and molecular mechanics optimization, reveals that the crucial features associated with the Ras–hSos1 complex are maintained in Ras–Cdc25\textsuperscript{Mm}, as expected due to the high sequence similarity between hSos1 and Cdc25\textsuperscript{Mm} (Fig. 1A). Next, we focused on the so-called catalytic hairpin composed by helices H and I, i.e., the structure that on the basis of current models for GEF function actually displaces the nucleotide from Ras. Such an hairpin is predicted to have a very similar structure in hSos1 and Cdc25\textsuperscript{Mm} (Fig. 1B), the major difference between the two hairpins being that a short \(\beta\)-sheet present in the hSos1 structure is lacking in the Cdc25\textsuperscript{Mm} structure (Fig. 1B and C). The modeling data find support in results obtained by NMR analysis of a chemically purified Cdc25\textsuperscript{Mm}-derived HI peptide [24].
The analysis of the Ras–hSos1 structure evidenced that only the H helix from the HI hairpin directly interacts with Ras. Such helix is placed in a long cleft on Ras surface, and the tight packaging between Ras and hSos1 in this interface region is allowed by both hydrogen bonds and hydrophobic interactions. We collected evidence showing that the core of the interface between Ras and hSos1 is a cluster of three hydrophobic residues from the Switch 2 region of Ras (Y64, M67, Y71) which are buried into an hydrophobic pocket of hSos1 formed by residues M824, I825, S876, Y912, F929, F930 and G931. These residues (or their steric and electronic properties) are well conserved in Cdc25 Mm where the corresponding pocket is formed by the residues T1071, T1072, S1123, F1161, Y1178, L1179, G1180 (the identical or highly conserved residues are in bold). The residues F929, F930 (Y1178, L1179 in Cdc25 Mm) belongs to the H helix. hSos1 residues from the H helix that make hydrogen bonds with Ras are G931, T935, G943, N944; these residues are conserved in Cdc25 Mm, apart from N944 which is substituted by a threonine; however, such a substitution should result in maintenance of hydrogen bonding, thanks to the hydroxyl group included in threonine sidechain. An important consequence of the insertion of helix H into the Ras active site is that it introduces a hydrophobic side chain (L938, corresponding to V1187 in Cdc25 Mm), which blocks the magnesium binding site, an acidic side chain (E942, corresponding to E1191 in Cdc25 Mm), which forms a hydrogen bond with G60 avoiding its interaction with the alpha phosphate group of GXP. Therefore, in light of the comparative analysis of the Ras–hSos1 and Ras–Cdc25 Mm structures, it can be concluded that the interaction between the HI hairpin and Ras is very similar in the two complexes. In particular, both hydrophobic interactions and hydrogen bonds are observed along the H helix–Ras interface.

2.2. The Cdc25 Mm-derived mutant and wild-type HI hairpins in vitro induce non-catalytic dissociation of ras-bound nucleotide

In order to define the role played by the HI hairpin in nucleotide dissociation and exchange, the wild-type, HI TE and HITA hairpins were expressed in – and purified from – Escherichia coli cells as GST-fusion proteins. GST-fusion proteins were used in all in vitro experiments, while the HI hairpin is expressed in vivo in transfected fibroblasts as an un-fused protein. The hairpin-containing fusion proteins were purified under the same conditions as the GST-Cdc25 Mm wild-type protein. All GST-hairpins were unable to promote guanine nucleotide exchange even when added at a 3:1 molar ratio with His-p21 ras (Fig. 2A, closed symbols). Under the same condition, the mutant HI TE hairpin was able to promote guanine nucleotide dissociation from Ras (Fig. 2B, closed circle), as well as to compete with the wild-type catalytic domain of Cdc25 Mm (Fig. 2C, closed circle). In competition experiments 200 nM His-p21 ras was charged with labeled GDP in the presence of 20 nM GST-Cdc25 Mm wild-type. A 30-fold excess of
GST-HITE was added. Under these conditions a rapid reduction of p21 ras-bound nucleotide – induced by wild-type Cdc25Mm – was observed. After ca. 10 min, the level of p21ras-bound nucleotide reached a steady state level, which remained constant for the time course of the experiment. The catalytic domain containing the TE mutation (GST-Cdc25MmT1184E) behaved similarly (Fig. 2 B and C compare open and close circles). Purified GST could neither exchange nor dissociate Ras-bound nucleotide (Fig. 2 A and B, open squares) nor compete with wild-type GEF (Fig. 2 C, open squares), so ruling out any non-specific binding effect by the GST moiety of the fusion proteins.

Surprisingly, however, both the mutant HITA and wild-type HI hairpins display dominant-negative properties similar to those of the HI TE mutant hairpin (Fig. 2 B–C). In summary, these results indicate that when the HI TE mutant hairpin is "extracted" from the context of the intact catalytic domain, it displays a similar dominant-negative behavior as that originated by the same mutation in the entire catalytic domain. Moreover it shows that, at least in vitro, the TE mutation is dispensable for obtaining a dominant-negative hairpin, since both the mutant HITA and the wild-type HI hairpin display dominant negative properties very similar to those of the HI TE hairpin.

In order to probe whether down-sizing affected the specificity of action of the isolated HI hairpin with respect to the dominant negative catalytic domain, we tested whether isolated HITE could promote non-catalytic dissociation from two small G proteins, Ral [25] and Rac [26]. Fig. 3 A and B, show that neither the dominant-negative hairpin nor the dominant-negative catalytic domain significantly accelerate the intrinsic disso-

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**Fig. 2.** (A) GDP to [3H]GTP exchange reaction. 0.2 μM p21ram - GDP was incubated at 30°C in buffer A [13,21]. The reaction was started by addition of a 10-fold excess of [3H]GTP over the p21ram - GDP complex. (B) Stimulation of the [3H]GDP dissociation from the p21ram - [3H]GDP. Dissociation was started by addition of a 5000-fold excess of unlabeled nucleotide in the presence of appropriate concentrations of each GEF (residues 1107–1262) or peptide (resides 1175–1224) variant. Cdc25Smn1007–1262, wild-type was used at 20 nM, in ratio 1:10 with Ras, while the dominant negative Cdc25Smn1007–1262TE, as well as the peptides and GST, were used at 600 nM, in ratio 3:1 with Ras. EDTA was used at 50 mM. (C) Competition of HI peptides with wild-type Cdc25Smn. Competition assay was performed as an exchange reaction in presence of 20 nM Cdc25Smn. After 30 min, 600 nM of the test protein (peptide) were added. In all panels mean ± standard deviation of at least three independent experiments is given. Symbols are as follows: Δ, intrinsic dissociation; ○, Cdc25Smn1007–1262 wild-type; ○, Cdc25Smn1007–1262TE; ●, HITE peptide; △, HITA peptide; □, GST; *, EDTA.

**Fig. 3.** Stimulation of the [3H]GDP dissociation from Ral (panel A) or Rac (panel B). The [3H]GDP-dissociation from 200 nM GST-Ral or GST-Rac, respectively, was started by addition of a 5000-fold excess of unlabeled nucleotide in the presence of 600 nM of each GEF variant. GST was used at 600 nM and EDTA at 50 mM. In all panels mean ± standard deviation of at least two independent experiments is given. Symbols are as follows: Δ, intrinsic dissociation; ○, Cdc25Smn1007–1262, wild-type; ○, Cdc25Smn1007–1262TE; ●, HITE peptide; △, HITA peptide; □, GST; *, EDTA.

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GST-HITE was added. Under these conditions a rapid reduction of p21ram-bound nucleotide – induced by wild-type Cdc25Smn – was observed. After ca. 10 min, the level of p21ram-bound nucleotide reached a steady state level, which remained constant for the time course of the experiment. The catalytic domain containing the TE mutation (GST-Cdc25SmnT1184E) behaved similarly (Fig. 2B and C compare open and close circles). Purified GST could neither exchange nor dissociate Ras-bound nucleotide (Fig. 2A and B, open squares) nor compete with wild-type GEF (Fig. 2C, open squares), so ruling out any non-specific binding effect by the GST moiety of the fusion proteins.

Surprisingly, however, both the mutant HITA and wild-type HI hairpins display dominant-negative properties similar to those of the HI TE mutant hairpin (Fig. 2B–C). In summary, these results indicate that when the HI TE mutant hairpin is “extracted” from the context of the intact catalytic domain, it displays a similar dominant-negative behavior as that originated by the same mutation in the entire catalytic domain. Moreover it shows that, at least in vitro, the TE mutation is dispensable for obtaining a dominant-negative hairpin, since both the mutant HITA and the wild-type HI hairpin display dominant negative properties very similar to those of the HI TE hairpin.
ciliation rate of either Ras (panel A) or Rac (panel B), thus indicating that down-sizing does not alter GEF substrate specificity.

2.3. Effect of guanine nucleotide on dissociation of p21ras–GEF complex

In order to further test the specificity of the action of the dominant negative hairpin, we used mant-GDP, a fluorescent GDP derivative, to investigate binding of guanine nucleotides to mutant nucleotide-free Ras/GEF complexes (Fig. 4). We reasoned that, if the HI hairpin were acting as a non-specific denaturant, nucleotide would no longer be able to bind the complex, or at least should make it very inefficiently. Specific binding of the nucleotide to the complex between nucleotide-free Ras and each GEF or GEF derivative is detected as an increase in fluorescence due to mant-GDP/Ras binding and ensuing GEF exit [13,16]. In agreement with previously published data, higher concentrations of mant-nucleotide were required to detect specific nucleotide binding to the complex between nucleotide-free Ras and GEF1184E than to the complex between nucleotide-free Ras and wild-type GEF [13]. Mant-GDP binding to the complex between nucleotide-free Ras and the HI hairpin was superimposable to that observed with the complex between nucleotide-free Ras and GEF1184E, indicating that the dominant negative catalytic domain and the HI hairpin ablated from it form a complex with the same properties, thus ruling out that the action of the hairpins is due to non-specific denaturation of Ras.

2.4. Expression of Cdc25Mm-derived HI hairpin downregulates the Ras pathway in mammalian fibroblasts

In order to investigate the ability of wild-type and mutant HI hairpins to interfere with Ras signal transduction pathways in vivo, we transiently co-transfected pcDNA3-derived plasmids expressing Cdc25Mm-derived peptides (un-fused to any stabilizing tag) together with the fos-luciferase reporter plasmid into NIH3T3 and kras-transformed NIH3T3 (NIH-ras) fibroblasts. The ability of the transiently expressed peptides to trans-activate or downregulate the fos promoter was compared with that of full-length mutant Cdc25Mm.

Results obtained with wild-type and mutant Cdc25Mm-derived constructs (Cdc25MmW1056E, Cdc25MmT1184E, wild-type, TE and TA HI-peptides) are reported in Fig. 5. In both normal (black bars) and kras-transformed (white bars) fibroblasts, mutant and wild-type HI peptides significantly reduced the level of Ras-dependent fos-luciferase activity as compared to the level observed in cells transfected with the empty pcDNA3 vector (control level), thereby showing a good ability to attenuate Ras signaling in vivo. The inhibitory effect due to “Ras-sequestering property” of the dominant negative catalytic protein and of the derived hairpin were very similar. Indeed the inhibitory effect of the two proteins was not statistically different, although we reproducibly noticed that the effect induced by hairpin was slightly less than the one induced by a full-length dominant-negative Cdc25Mm mutant. We noticed that the HI hairpin is quite unstable in vitro when freed from the GST moiety (Sacco, Metalli and Fantinato, unpublished observations). The minor reduction in inhibitory activity of the isolated hairpin may thus be the result of an altered stability of the hairpin compared to full length molecules [24].

2.5. Stable expression of HI hairpins induces phenotypic reversion in kras-transformed cells

Results reported in the previous paragraph indicate that expression of the HI hairpin might effectively downregulate the Ras pathway in vivo. Plasmids expressing the HT and HTA hairpins were stably transfected in murine NIH3T3 fibroblasts transformed with oncogenic k-ras. Stable transformants were selected and screened for the presence of mRNA encoding the HI peptide by RT-PCR (Fig. 6A). Expression of each HI hairpin brings about a significant reduction in Ras–GTP level, compared to the level observed in the cells transformed with the empty vector, as shown by a Ras–GTP pull down assay (Fig. 6B) [27,28]. Such downregulation of the Ras pathway is reflected at the phenotypic level, as shown by the reverted morphology observed in HI-transformed cell lines (Fig. 6C) and by their incapability to acidify culture medium under confluence growth condition (Fig. 6C insert). Reversion of cell morphology and acid production indicates that hairpin expression has a similar effect as expression of a full length dominant negative GEF as previously shown [22].

3. Discussion

The most popular model for GEF action states that after interacting with nucleotide-bound Ras, a GEF molecule displaces the nucleotide, originating a nucleotide-free Ras/GEF complex. Although such a complex can be isolated under appropriate conditions [16], it would normally be extremely short-lived since a new nucleotide would rapidly bind Ras resulting in dissociation of GEF and in the start of a new cycle. An alternative model that suggests both the entering and the leaving nucleotide bind at the same time to a Ras–GEF complex has recently been proposed [17]. Results obtained with WE and TE dominant negative mutants [13] and this work indicate that binding of the entering nucleotide and exit of the leaving nucleotide are dissociable events. In fact we could show that the presence of high concentrations of external, free...
nucleotide is not enough to dissociate GEF from Ras, either when the GEF catalytic domain carries a dominant negative mutation, and/or when the “catalytic” HI hairpin (even in the wild-type version) is ablated from the core catalytic domain. Such data do not address the timing of binding of the leaving and entering nucleotide, but do indicate that while

![Graph showing luciferase activity](image)

Fig. 5. Ras-dependent luciferase activity in mouse fibroblasts transiently over-expressing Cdc25Mm mutants and Cdc25Mm-derived HI peptides. NIH3T3 and kras-transformed NIH3T3 fibroblasts were cotransfected with fos-luciferase plasmid (0.33 μg) and 1.0 μg of pcDNA3-derived plasmid. Data are expressed as percentage of luciferase activity in cells transfected with empty pcDNA3. Mean ± standard deviation of at least four independent experiments, each conducted in triplicate, is shown.

![Graph showing gene expression and Ras-GTP levels](image)

Fig. 6. (A) PCR products obtained with primers pair specific for Cdc25Mm-derived HI hairpin. The PCR was performed using as template cDNAs produced from RNA isolated from different cell lines. (B) Upper panel. Vinculin level in total extracts. (B) Central panel. Ras-GTP eluted from GST–RBD–glutathione–sepharose, preincubated with total extracts, as described under Section 4. Data are from a representative experiment. (B) Lower panel. Quantification of the Ras–GTP amount after normalization to Vinculin. The Ras–GTP/Vinculin level of kras-transformed fibroblasts transfected with the empty vector (k-ras-MOCK) was taken as 100. Data shown are mean ± standard deviation of two independent experiments. In all panels are reported the results obtained with the cell lines: (I) NIH3T3, stable kras-transformed fibroblasts transfected, respectively, with (II) the empty vector pcDNA3 (kras-MOCK), (III) pcDNA3-Cdc25MmW1056E (kras-Cdc25WE), (IV) pcDNA3-HI T1184E (kras-HI TE), and (V) pcDNA3-HI T1184A (kras-HI TA). (C) Morphological analysis of the different cell lines. The inset represent a colorimetric assay of acid produced by the same cells after 200 h of growth.
the HI hairpin is the major molecular determinant responsible
to dissociate the Ras-bound nucleotide, it is unable by itself to
respond to the incoming nucleotide. The TE mutation within a
catalytic domain (residues 1007–1262) or a full length molecule
lead to the same effect, as ascertained either in vitro and/or
in vivo [13]. The implication of these results is that “squeezing
out” GXP from Ras is a relatively simple task that can be effi-
ciently accomplished by relatively small molecules, such as the
HI hairpin. On the contrary, these molecules are insensitive to
the incoming nucleotide, originating quite stable binary com-
plexes with nucleotide-free Ras. Other portions of the GEF
molecule appear thus required to provide the ability to interact
with the incoming nucleotide, thus effectively converting a
non-catalytic nucleotide dissociator into a catalytic exchange
factor. While such a conclusion leaves unresolved the task to
identify the molecular determinant(s) involved in binding of the
entering nucleotide and leading to dissociation of the
GEF/Ras complex, it opens interesting avenues for the design of
pharmacologically active molecules able to downregulate Ras
signaling [29]. Such a pharmacologically active molecule, in fact,
would only need to help dissociation of nucleotide from Ras,
blocking nucleotide re-entry. The in vitro and in vivo behavior of
the hairpin described in this work give the proof of principle
that such molecules do work as expected in vivo, since both
wild-type and mutant HI hairpins were able to down regulate
Ras-dependent pathways inhibiting the expression of a Ras-
dependent reporter gene, in transiently transfected normal and
kras-transformed mouse fibroblasts. Additionally we could show that the HI\textsuperscript{TE} and HI\textsuperscript{TA} hairpins are able to down
regulate Ras-GTP level in kras-transformed fibroblasts and to
revert their overall growth properties to those of the parental,
untransformed cells. In conclusion, our data indicate that the
so-called catalytic hairpin of Ras-specific GEFs maintains its
nucleotide dissociating ability when ablated from the catalytic
domain. After GXP dissociation the hairpin remains tightly
bound to Ras, forming a stable binary Ras/Hairpin complex,
indicating that the second step required for a polypeptide to
act as a GEF is somehow contained in – or modulated by –
regions of the catalytic domain, different from the HI hairpin.

4. Methods

4.1. Computational methods

The structure of the catalytic domain of Cdc25\textsuperscript{Mm} was ob-
tained by homology modeling using as a scaffold the X-ray
structure of hSos1 complexed to human h-Ras [8] (PDB code
1BKD). Protein sequences were aligned according to Bor-
ack-Sjodin et al. [8] and the atomic coordinates of the back-
bone atoms inside regions featuring high similarity were
transferred from hSos1 to the Cdc25\textsuperscript{Mm} model. Fragments
connecting the scaffold elements were modeled by searching
a set of well resolved protein structures for fragments which fit-
ted in geometrically [30]. Side chain coordinates were gener-
ated using a rotamer library followed by intramolecular
energy minimization by a simulated annealing Monte Carlo
technique.

Recombinant and genetic manipulation. Recombinant DNA
manipulations were performed according to standard methods
[31]. The HI-encoding region (encoding residues 1175–1224 of
Cdc25\textsuperscript{Mm} mature protein) was amplified by PCR using the
primers 5'-CGGGATCCATGTGTGTCCTACTGAGGG-
3' and 5'-CGGAATTCAGTGTGTCCTACTGAGGG-
3'. The HI-encoding region (encoding residues 1175–1224 of
Cdc25\textsuperscript{Mm} mature protein) was amplified by PCR using the
primers 5'-CGGGATCCATGTGTGTCCTACTGAGGG-
3' and 5'-CGGAATTCAGTGTGTCCTACTGAGGG-
3'.
plasmids and cotransfected with the ras-responsive fos-luciferase plasmid as previously described [13,33,34]. After transfection, cells were starved for 24 h in serum-free medium supplemented with 4 mg/ml transferrin and 0.346 mg/ml sodium selenite and collected. Luciferase activity was assayed using the luciferase assay system with reporter lysis buffer (Promega) and normalized to protein content determined with the DC Protein Assay (Bio-Rad).

Ras–GTP pull down assay. Ras–GTP was assayed by a pull down assay using the Ras binding domain of Raf1 (RBD) bound to glutathione–sepharose essentially as described in Taylor et al. [35]. 0.4 mg of total clarified extracts were incubated with 30 ml of RBD-bound glutathione–sepharose 1 h at 4 °C under gentle shaking. After abundant washes in Shaloway buffer [35], bound Ras–GTP was eluted with reducing Laemmli sample buffer [36], loaded on 12% SDS-PAGE and detected by anti-HRas 259 antibodies (Santa Cruz Biotechnollog) after western. Ras–GTP levels were determined by densitometric scanning and quantitation with NIH-Image. Data are expressed after normalization to the level of vinculin, similarly determined by western blot analysis of total clarified extracts using anti-vinculin H-300 antibodies (Santa Cruz Biotechnollog).

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