Oncogene (2003) 22, 2772–2781

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Random mutagenesis of PDZ_{Omi} domain and selection of mutants that specifically bind the Myc proto-oncogene and induce apoptosis

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Omi is a mammalian serine protease that is localized in the mitochondria and released to the cytoplasm in response to apoptotic stimuli. Omi induces cell death in a caspase-dependent manner by interacting with the X-chromosome linked inhibitor of apoptosis protein, as well as in a caspase-independent way that relies on its proteolytic activity. Omi is synthesized as a precursor polypeptide and is processed to an active serine protease with a unique PDZ domain. PDZ domains recognize the extreme carboxyl terminus of target proteins. Internal peptides that are able to fold into a β-finger are also reported to bind some PDZ domains. Using a modified yeast two-hybrid system, PDZ_{Omi} mutants were isolated by their ability to bind the carboxyl terminus of human Myc oncoprotein in yeast as well as in mammalian cells. One such PDZ^{m} domain (PDZ-M1), when transfected into mammalian cells, was able to bind to endogenous Myc protein and induce cell death. PDZ-M1-induced apoptosis was entirely dependent on the presence of Myc protein and was not observed when c-*myc* null fibroblasts were used. Our studies indicate that the PDZ domain of Omi can provide a prototype that could easily be exploited to target specifically and inactivate oncogenes by binding to their unique carboxyl terminus.
Oncogene (2003) **22,** 2772–2781. doi:10.1038/sj.onc.1206359

Keywords: Omi/HtrA2; PDZ domains; protein–protein interactions; protein design; Myc proto-oncogene; apoptosis; yeast two-hybrid system

Introduction

Omi, also known as HtrA2, is a human serine protease with extensive similarity to bacterial high-temperature requirement A (HtrA) protease (Pallen and Wren, 1997; Faccio *et al*., 2000b). Bacterial HtrAs act as chaperones at normal temperatures and as proteases at elevated temperatures to cleave and remove denatured or damaged proteins allowing the survival of bacteria following heat shock and other stress (Pallen and Wren, 1997). Omi was originally isolated through binding to Mxi2, an alternatively spliced form of p38 stress-activated kinase (SAPK) (Faccio *et al*., 2000a). It was subsequently isolated independently as an interactor of presenilin, a protein found mutated in some individuals with Alzheimer's disease (Gray *et al*., 2000). Omi is expressed ubiquitously and the amount of protein increases when cells are exposed to heat shock or treated with tunicamycin (Gray *et al*., 2000). The proteolytic activity of Omi is also regulated by cellular stress including kidney ischemia/reperfusion (Faccio *et al*., 2000b). Recent studies have identified Omi as a mitochondrial protein that is released to the cytoplasm following apoptotic stimuli (Suzuki *et al*., 2001; Hegde *et al*., 2002; Martins *et al*., 2002; Verhagen *et al*., 2002). In the cytoplasm, Omi interacts with X-chromosome linked inhibitor of apoptosis protein and relieves its inhibition of caspase-9 (Suzuki *et al*., 2001; Hegde *et al*., 2002). In addition, Omi can promote apoptosis in a caspase-independent mechanism through its ability to function as a serine protease (Suzuki *et al*., 2001; Hegde *et al*., 2002; Martins *et al*., 2002; Verhagen *et al*., 2002). Omi belongs to a family of mammalian proteases consisting of two members, the other being L56/HtrA1, a serine protease that is overexpressed in osteoathritic cartilage (Hu *et al*., 1998), which was also repressed in fibroblasts following SV40 infection (Zumbrunn and Trueb, 1996). The similarity between both proteins is restricted to the carboxy-terminus, which includes the proteolytic domain and a PDZ motif, while the amino-terminus of the two proteins is different (Faccio *et al*., 2000b). Furthermore, while Omi is intracellular, L56/HtrA1 is secreted (Hu *et al*., 1998; Faccio *et al*., 2000b).

Omi is made as a precursor protein and is processed by the removal of 133 amino terminal amino acids to produce the mature protein consisting of the catalytic domain and a PDZ motif (Suzuki *et al*., 2001; Hegde *et al*., 2002; Martins *et al*., 2002; Verhagen *et al*., 2002). PDZ domains are protein modules defined by a unique sequence of 80–100 amino acids able to recognize specific C-terminal sequences in target proteins (Saras and Heldin, 1996; Songyang *et al*., 1997). They are named after the three proteins where they were originally found: postsynaptic density protein-95 (PSD-95) (Cho *et al*., 1992), Drosophila discs large tumor suppressor (Dlg) (Woods and Bryant, 1991), and

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Received 11 July 2002; revised 17 December 2002; accepted 6 January 2003

the tight junction protein (ZO-1) (Itoh *et al*., 1993). PDZ proteins are commonly involved in assembling large protein complexes into signaling networks; for a recent review, see Sheng and Sala (2001). Known PDZ domains are grouped into four classes based on their binding specificity (Levchenko *et al*., 1997; Bezprozvanny and Maximov, 2001; Vaccaro *et al*., 2001). The PDZ domain of Omi (PDZ_{Omi}) is unique both in amino-acid sequence as well as binding specificity. Figure 1a compares the amino-acid sequence of PDZ_{Omi} with that of the other known PDZ domains. The only known interactor of PDZ_{Omi} is the Mxi2 protein, an alternatively spliced form of SAPK (Faccio *et al*., 2000b). PDZ_{Omi} binds the carboxy-terminus of Mxi2 protein that is different from the consensus sequences recognized by the other families of PDZ domains (Figure 1b). To further investigate the structural characteristics of the PDZ_{Omi} domain, its amino-acid sequence was randomly mutated and mutant domains (PDZ^m) with altered specificity were selected. The altered specificity in this case defines the ability of PDZ^m domains to bind the carboxy-terminus of the human c-Myc oncoprotein. Myc protein plays a major role in cell growth, differentiation, apoptosis and cell transformation (Littlewood and Evan, 1990; Evan *et al*., 1994). Therefore, interfering with its function could have a major effect on the fate of cells. Through this screening, several Myc-specific PDZ^m domains were isolated and characterized. One such Myc-specific PDZ mutant (PDZ-M1) was selected for further analysis. PDZ-M1, when expressed in mammalian cells fused to a nuclear localization signal (Kalderon *et al*., 1984), was able to bind a truncated cytoplasmic form of Myc protein ($Myc_{282–439}$) and efficiently translocate it to the cell nucleus. Furthermore, the PDZ-M1 domain colocalized with endogenous Myc protein. This specific targeting of Myc resulted in increased and significant apoptosis of the transiently transfected cells. Using c-*myc* null fibroblasts, we found the ability of PDZ-M1 domain to induce apoptosis was entirely dependent on the presence of Myc protein. In conclusion, our studies define the PDZ of Omi, originally identified by its similarity with other known PDZ domains, as a true functional domain. Furthermore, this work clearly shows that the basic structure of the PDZ_{Omi} is flexible and can be exploited to engineer PDZ^m domains with predetermined specificity. These PDZ^m binding domains can provide a novel way to target specifically oncogenes such as Myc, in an attempt to control the fate of cells. Designed PDZ^m domains linked together in tandem can also provide a biological scaffold on which to assemble specific multiprotein complexes (biological motors) that can perform specific tasks within cells.

Results

Isolation of PDZ^m with altered specificity

A population of 7.5×10^5 random *PDZ^m* mutants was constructed in the yeast pJG4-5 vector. The complexity and heterogeneity of this library was verified by sequencing several randomly isolated clones. This library of mutant PDZ domains was screened using a modified yeast two-hybrid system to isolate clones encoding domains that could bind the $Myc_{282–439}$ C-terminus. This screen yielded 21 possible interactors and further analysis showed that 15 of these were true positives. Their DNA sequence was determined and grouped into six distinct families (Figure 2).

Characterization of the PDZ^m domains

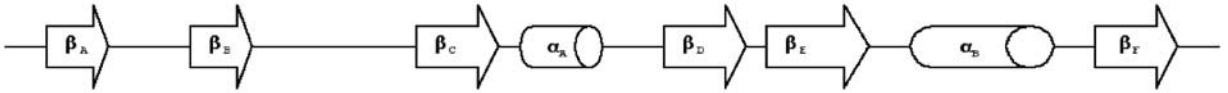
To verify the selected PDZ^m domains interacted with the C-terminus of $Myc_{282–439}$ and not somewhere else on the bait, a member from each family was tested for interaction against the $Myc_{282–437}$ bait lacking the last three amino acids (Myc Δ3). Additionally, they were tested against Mxi2 to determine if they had lost their original specificity. The results of these experiments are shown in Figure 3a. Figure 3b shows results from experiments to test the specificity of PDZ-M1 using other known members of the Myc network of proteins including N-Myc, Max and Mxi1 proteins (Zervos *et al*., 1993).

Liquid assays for β-galactosidase activity (Schneider *et al*., 1996) were used to measure the strength of the interaction between the bait and prey proteins for each yeast clone (Table 1). The PDZ-M1 domain shows the strongest and most specific interaction with $Myc_{282–439}$ and has lost the ability to bind Mxi2. PDZ-M1 has two amino-acid substitutions: $G_{390} \rightarrow V$, located between β_B and β_C strands, and $R_{408} \rightarrow W$, present between the α_A and β_D. PDZ-M5 with only one of the amino-acid substitutions, $G_{390} \rightarrow V$, shows the same strong binding with $Myc_{282–439}$ bait seen with PDZ-M1, but, unlike PDZ-M1, retains some of its binding ability for Mxi2. PDZ-M6 has two amino-acid substitutions located in the same positions as PDZ-M1 and one of them is the same, $R_{408} \rightarrow W$, whereas the other introduces a different amino acid, $G_{390} \rightarrow S$. PDZ-M6 binds strongly to $Myc_{282–439}$, but also interacts weakly with both Mxi2 and Myc Δ3. PDZ-M4 also has two amino-acid substitutions: $Q_{422} \rightarrow H$ found between β_D and β_E; $E_{458} \rightarrow G$, which is the last amino acid of PDZ_{Omi}, and shows weak binding with $Myc_{282–439}$. PDZ-M3 has four amino-acid substitutions: two of them are present in the two positions, 390 and 408, also found in other families ($G_{390} \rightarrow V$, $R_{408} \rightarrow L$), and the other two are in different locations $P_{381} \rightarrow L$ and $V_{439} \rightarrow A$. PDZ-M3 also shows weak binding to $Myc_{282–439}$. PDZ-M2 has five mutations $G_{390} \rightarrow V$, $L_{392} \rightarrow F$, $K_{395} \rightarrow T$, $T_{457} \rightarrow K$, $E_{458} \rightarrow N$ and binds weakly to both $Myc_{282–439}$ and Myc Δ3.

Interaction of PDZ-M1 with Myc$_{282–439}$ in mammalian cells

The PDZ-M1 domain that recognizes $Myc_{282–439}$ was cloned into pEGFP-C1 (Clontech) mammalian expression vector. This vector when transfected into mammalian cells expresses the PDZ-M1 domain as a fusion to the green fluorescent protein (GFP). A nuclear localiza-

a

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CLASS I
PSD95-3  --------REPRRIVIHRGS-TGLGFNIVGGED------GEGIFISFILAGGPADLSGELRKGDQILSVNGV------DLRNASHEQAAIALKNA--GQTVTIIAQYR---------
PSD95-2  --------EKVMEIKLIKGP-KGLGFSIAGGVGNQHIPGDNSIYVTKIIEGGAAHKDGRLQIGDKILAVNSV------GLEDVMHEDAVAALKNT--YDVVYLKVAKP---------
PSD95-1  --------MEYEEITLERGN-SGLGFSIAGGTDNPHIGDDPSIFITKIIPGGAAAQDGRLRVNDSILFVNEV------DVREVTHSAAVEALKEA--GSIVRLYVMRR---------
DLG      --------REPRTITIQKGP-QGLGFNIVGGED------GQGIYVSFILAGGPADLGSELKRGDQLLSVNNV------NLTHATHEEAAQALKTS--GGVVTLLAQYR---------
hDLG     ------ITREPRKVVLHRGS-TGLGFNIVGGED------GEGIFISFILAGGPADLSGELRKGDRIISVNSV------DLRAASHEQAAAALKNA--GQAVTIVAQYRPEEYSRQHA
ZO1      --------PSMKLVKFRKG--DSVGLRLAGGND-------VGIFVAGVLEDSPAAKEG-LEEGDQILRVNNV------DFTNIIREEAVLFLLDLPKGEEVTILAQKK---------
ZO2      --------PNTKMVRFKKG--DSVGLRLAGGND-------VGIFVAGIQEGTSAEQEG-LQEGDQILKVMTQ------DFRGLVREDAVLYLLEIPKGEMVTILAQSR---------
Sap97    --------REPRKVVLHRGS-TGLGFNIVGGED------GEGIFISFILAGGPADLSGELRKGDRIISVNSV------DLRAASHEQAAAALKNA--GQAVTIVAQYR---------
Lin2a    --------SRLRLVQFQKDTQEPMGITLKVNED-------GRCFVARIMHGGMIHRQATLHVGDEIREINGM------SVANRSVESLQEMLRDARGQVTFKIIPSYR---------
TorSyn   --------CKPTVRIVKQEA-GGLGISIKGGKEN-----HMPILISKIFRGLAAEQSRLLFVGDAILSVNGT------DLRDATHDQAVQALKKT--GKEVVLEVKYLKE-------

CLASS II
hCASK    -----ENVTRVRLVQFQKNTDEPMGITLKMNEL-------NHCIVARIMHGGMIHRQGTLHVGDEIREINGI------SVANQTVEQLQKMLREMRGSITFKIVPSYRTQSSSCERD
p55      --------RKVRLIQFEKVTEEPMGITLKLMEK-------QSCTVARILHGGMIHRQGSLHVGDEILEINGT------NVTNHSVDQLQKAMKETKGMISLKVIPNQQ---------

CLASS III
nNOS     --------NVISVRLFKRKV-GGLGFLVK-ERVS-----KPPVIISDLIRGGAAEQSGLIQAGDIILAVNDR------PLVDLSYDSALEVLRGI--ASETHVVLILRGP-------

CLASS IV
hINADL-3 ---------TYNVELVRKDG-QSLGIRIVGYVGTSHTGEASGIYVKSIIPGSAAYHNGHIQVNDKIVAVDGV------NIQGFANHDVVEVLRNA--GQVVHLTLVR----------

CLASS V
Omi      ISGSQRRYIGVMMLTLSPSILAELQLREPSFPD-----VQHGVLIHKVILGSPAHRAG-LRPGDVILAIGEQMVQNAEDVYEAVRTQSQLAVQIRRGRETLTLYVTPEVTE------
L56      KAITKKKYIGIRMMSLTSSKAKELKDRHRDFPD-----VISGAYIIEVIPDTPAEAGG-LKENDVIISINGQSVVSANDVSDVIKRESTLNMVVRRGNEDIMITVIPEEIDP-----
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b

PDZ CLASS	CONSENSUS TARGET SEQUENCE	EXAMPLES
I	[S/T]-X-V-COOH	Post-synaptic density 95 (PSD95)
II	[F/Y]-X-[F/V/A]-COOH	Calcium/Calmodulin-dependent serine protein kinase (hCASK)
III	D-X-V-COOH	Neuronal nitric oxide synthase (nNOS)
IV	Ψ-[D/E]-COOH	Inactivation-no-after-potential D-like protein (hINADL)
V	[L/I]-X-ϕ-[I/L/V]-COOH	Omi/HtrA2

Figure 1 (a) Sequence alignment of the different classes of known PDZ domains. Several members from each class are aligned to indicate the conservation of amino acids in particular positions. Class I has the most members. Omi is shown together with L56/HtrA1 based on the high similarity of their respective PDZ domains. α-Helices are shown as cylinders; the amino acids responsible for the secondary structures are highlighted in blue color. PSD95-3 (residues 309–393), PSD95-2 (residues 156–246), PSD95-1 (residues 61–151) are all from PSD95 (Cho *et al*., 1992); DLG is the discs large repeat 3 (residues 482–566) (Woods and Bryant, 1991); hDLG is the human homolog of the Drosophila DLG (residues 460–546) (Lue *et al*., 1994); ZO1 is repeat 2 of the tight junction protein (residues 408–491) (Willott *et al*., 1993); ZO2 is a homolog of ZO1 (residues 93–176) (Jesaitis and Goodenough, 1994); Sap97 is repeat 3 of a novel pre-synaptic protein homologous to SAP90 and the Drosophila discs large tumor suppressor protein (residues 461–545) (Muller *et al*., 1995); Lin 2a is a member of the MAGUK family of cell junction proteins (residues 542–628) (Hoskins *et al*., 1996); TorSyn is a torpedo syntrophin protein (residues 65–152) (Adams *et al*., 1993). hCASK binds to the heparan sulfate proteoglycan syndecan-2 (Daniels *et al*., 1998); p55 is the palmitoylated erythrocyte membrane protein (residues 67–153) (Ruff *et al*., 1991). nNOS is nitric oxide synthase (residues 12–100) (Bredt *et al*., 1991; Riefler *et al*., 2001). hINADL-3 is a human homolog to Drosophila INAD (Vaccaro *et al*., 2001). (b) Consensus carboxy-terminal sequences recognized by different classes of PDZ domains. PDZ_{Omi} does not belong to any of the four known classes, but rather defines a new class of PDZ domains. A representative protein for each class of PDZ domains with its corresponding consensus binding target sequence is shown. *Φ* represents hydrophobic/nonpolar, *Ψ* represents noncharged amino acids, and *X* represents any amino acid

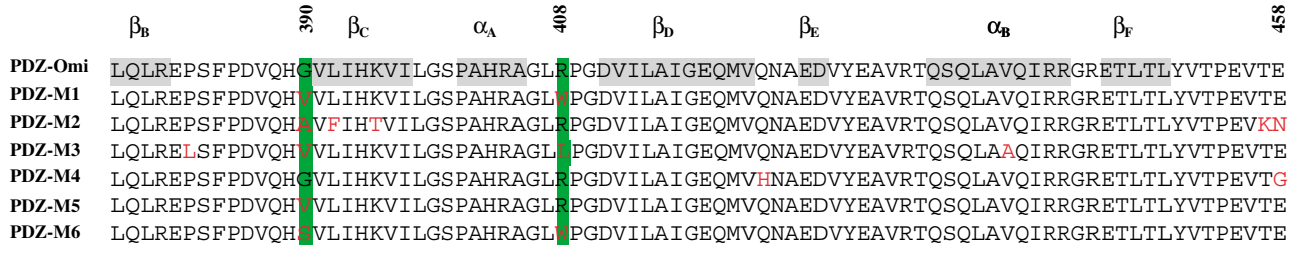


Figure 2 Alignment of the amino-acid sequence of six families of PDZ^m domains isolated for their ability to bind the C-terminus of Myc. PDZ^m domains are aligned and their amino-acid sequences compared with the wild-type PDZ domain. Amino-acid substitutions (indicated in red) that conferred altered specificity are clustered in two areas in the PDZ domain at positions 390 and 408. Amino-acids involved in secondary structures are highlighted in gray: α denotes α-helix and β denotes β-strand

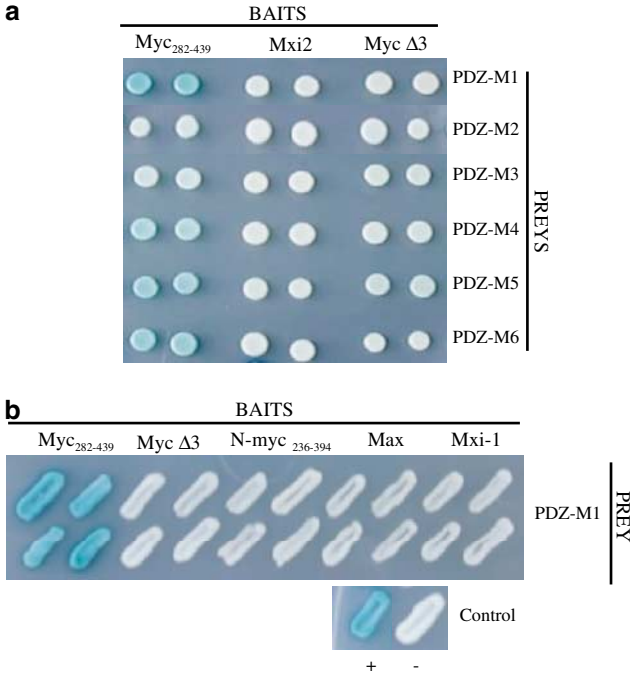


Figure 3 Yeast two-hybrid assay of different LexA-fusion proteins (baits) with various PDZ domains (preys). Yeast colonies were transformed with the indicated bait and prey; blue color results from a positive protein–protein interaction, white colonies indicate no interaction between bait and prey. The baits used in (**a**) are the carboxy-terminus of Myc (aa 282–439), the full-length Mxi2, and the carboxy-terminus of Myc lacking the last three amino acids (Myc C-term Δ3). (**b**) Specific interaction of PDZ-M1 with $Myc_{282–439}$, while no interaction was observed with N-$myc_{236–394}$, Max or Mxi-1 (Zervos *et al*., 1993)

tion signal (NLS) (Kalderon *et al*., 1984) was also added to target the PDZ-M1 fusion protein to the cell nucleus. The $Myc_{282–439}$ was instead cloned into the pDsRed1-C1 vector (Clontech), which fuses a red fluorescent protein (RFP) to the N-terminus of $Myc_{282–439}$. HeLa cells were transiently transfected with both of these constructs or the parental vectors as control. After 24 h, the subcellular localization of the fluorescent fusion proteins was observed using a confocal microscope. Figure 4, panel a1 shows the localization of the GFP-NLS-PDZ-M1 protein to be, as expected, exclusively nuclear. Panel

Table 1 Specificity and binding strength of the different PDZ domains for $Myc_{282–439}$ and Mxi2 proteins

Prey	*Bait*		
	$Myc_{282–439}$	*Mxi2*	*Myc Δ3*
PDZ-M1	+++	–	–
PDZ-M2	+	–	–/+
PDZ-M3	++	–	–
PDZ-M4	+	–	–
PDZ-M5	+++	–/+	+
PDZ-M6	+++	–/+	–

Semiquantitative representation of the strength and specificity of binding between the different PDZ^m domains and their targets. Results were compiled from several independent yeast experiments that measured β-galactosidase activity (Schneider et al., 1996).

b1 shows the DsRed-$Myc_{282–439}$ to be present in the cytoplasm of the transfected cells. Although Myc is a nuclear oncoprotein, the carboxy-terminal part of the protein ($Myc_{282–439}$) used as bait in our experiments lacks the nuclear localization signals that are present in the N-terminus of Myc (Stone *et al*., 1987). Panel c1 shows HeLa cells cotransfected with both constructs; DsRed-$Myc_{282–439}$ is now present in the cell nucleus where it colocalized with the GFP-NLS-PDZ-M1.

Interaction of PDZ-M1 with endogenous full-length Myc protein

GFP-PDZ-M1 and a specific anti-Myc antibody (Santa Cruz Biotechnology) that binds to the amino terminus of the Myc protein (aa 1–262) were used to detect interaction between PDZ-M1 and endogenous Myc protein. This antibody was selected because it cannot interfere with any protein–protein interaction that occurs at the carboxy-terminus of the protein. Figure 5, panel b3 shows that PDZ-M1 colocalizes with endogenous Myc protein in transiently transfected HeLa cells. When the PDZ_{Omi} was used instead, only coincidental co-localization was observed with endogenous Myc protein (panel a3). The expression and stability of the various fusion proteins was monitored by Western blot analysis of cell extracts derived from transfected cells (Figure 6).

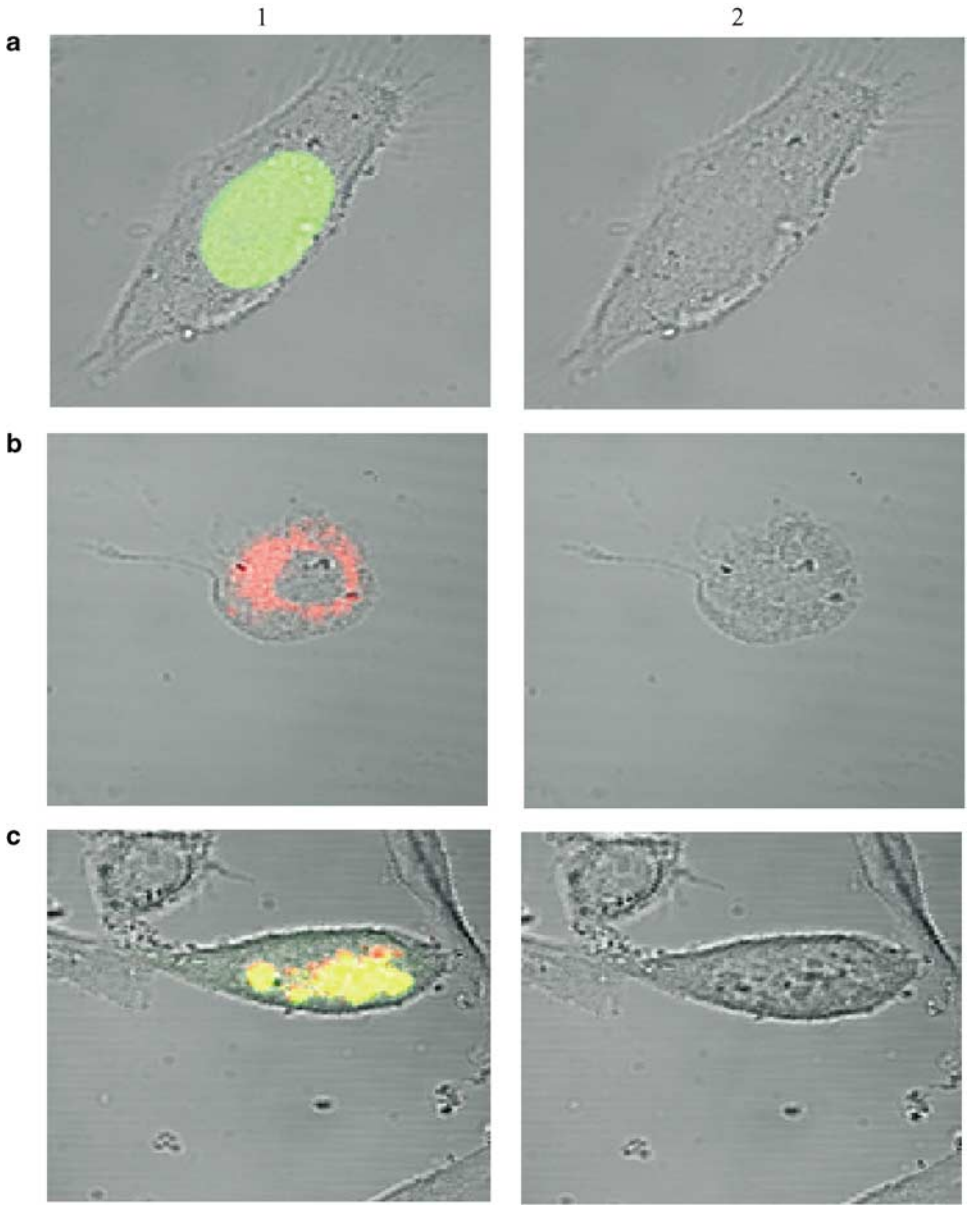


Figure 4 Nuclear translocation of RFP-Myc$_{282–439}$ through its specific interaction with GFP-NLS-PDZ-M1. (**a1**) HeLa cells transfected with GFP-NLS-PDZ-M1. This fusion protein is present exclusively in the cell nucleus because of the nuclear localization signal. (**b1**) Same cells transfected with RFP-Myc$_{282–439}$. As expected, Myc$_{282–439}$ is cytoplasmic, since it lacks the amino terminus that includes the nuclear localization signals. C1 shows HeLa cells cotransfected with RFP-Myc$_{282–439}$ and GFP-NLS-PDZ-M1. The RFP-Myc$_{282–439}$ is now present in the nucleus where it colocalizes with the GFP-NLS-PDZ-M1. a2, b2 and c2 are Normasky

The PDZ-M1 promotes apoptosis in mammalian cells

The carboxyl terminus of Myc is essential for its ability to bind to the Max protein and for these heterodimers to bind to DNA (Blackwood *et al*., 1992; Solomon *et al*., 1993; Grandori *et al*., 1996). Therefore, overexpression of the PDZ-M1 in transiently transfected cells might interfere with the normal function of Myc. Flow cytometry and annexin V probe (BD Biosciences) were used to investigate whether HeLa cells expressing the PDZ-M1 exhibited increased apoptosis. Figure 7 shows 11% of transfected cells with the EGFP vector alone were annexin V positive. Increased apoptosis was observed in cells transfected with the PDZ-M1, where 52% were annexin V positive. When the PDZ$_{Omi}$, which does not bind to the Myc protein, was used only 21% of the transfected cells were found to be annexin V positive (Figure 7).

PDZ-M1-induced apoptosis depends entirely on the presence of Myc protein

TGR-1, HO15.19. (myc −/−) and HO/myc3 (myc +/+) cells (Mateyak *et al*., 1997) were transiently transfected

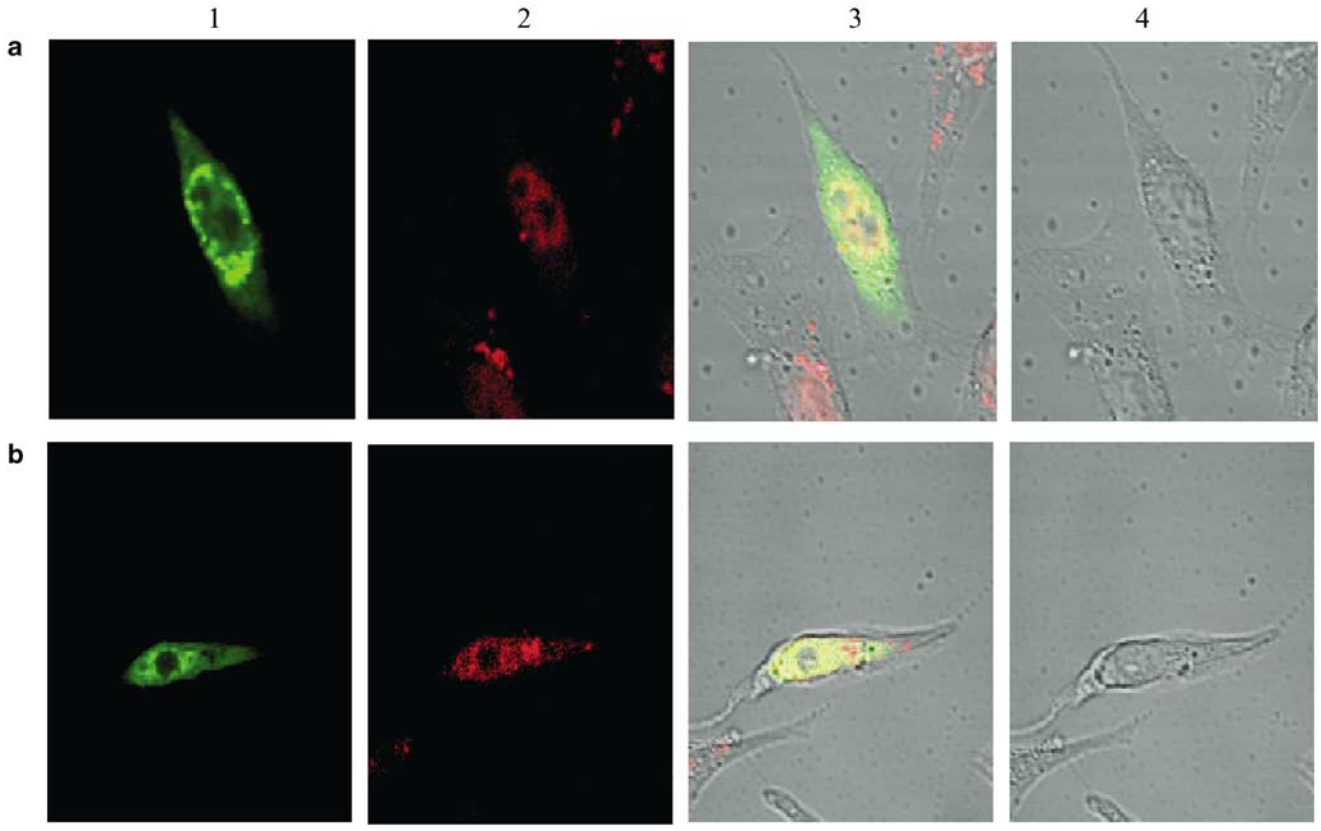


Figure 5 Colocalization of GFP-PDZ-M1 and endogenous Myc protein. HeLa cells were transiently transfected with GFP-PDZ$_{Omi}$ (**a**) or GFP-PDZ-M1 (**b**) After transfection, the cells were fixed and incubated with anti-Myc polyclonal antibody followed by PE-conjugated anti-rabbit antibody and subsequently examined by confocal microscopy. Confocal images of HeLa cells transfected with GFP-PDZ$_{Omi}$ (**a1**) or GFP-PDZ-M1 (**b1**). The same HeLa cells stained red with a Myc-specific antibody (**a2**) and (**b2**). Merged fluorescent image from 1 and 2 showing extensive colocalization of GFP-PDZ-M1 and endogenous Myc (yellow color) (**b3**) or very limited colocalization with the GFP-PDZ$_{Omi}$ (**a3**). Normasky images of the cells are also shown (**a4** and **b4**)

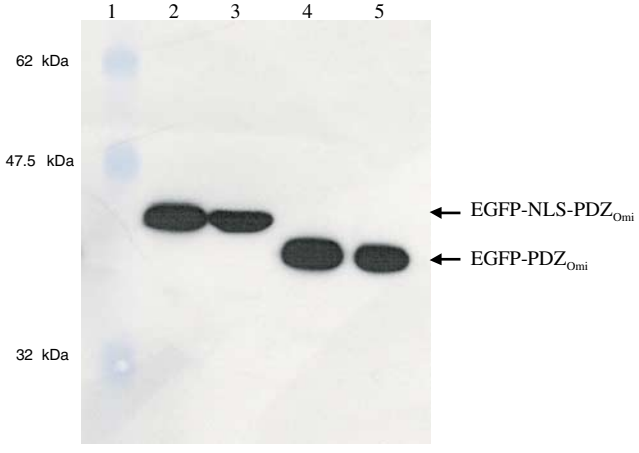


Figure 6 Expression and stability of EGFP-fusion proteins was monitored by Western blot analysis. Equal amounts of whole-cell lysates obtained 24 h post-transfection were subjected to SDS–PAGE followed by Western blot analysis using anti-GFP monoclonal antibody as described in Materials and methods. Lane 1 shows prestained protein size markers, lanes 2 and 3 represent lysates obtained after transfection of HeLa cells with EGFP-NLS-PDZ$_{Omi}$ and EGFP-NLS-PDZ-M1, including the NLS, lanes 4 and 5 show lysates obtained after transfection of HeLa cells with EGFP-PDZ$_{Omi}$ and EGFP-PDZ-M1 fusion protein without the NLS

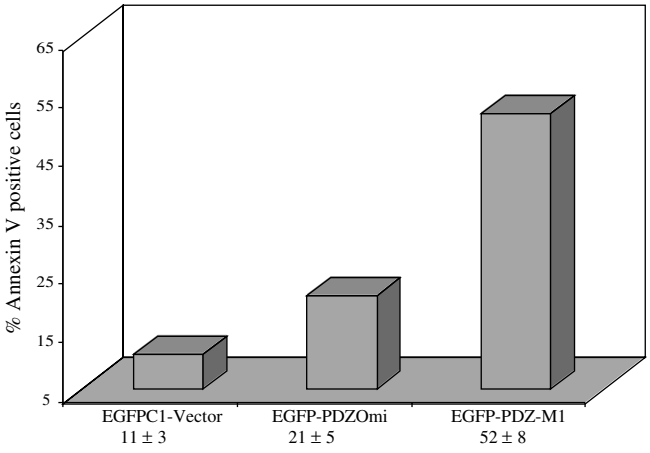
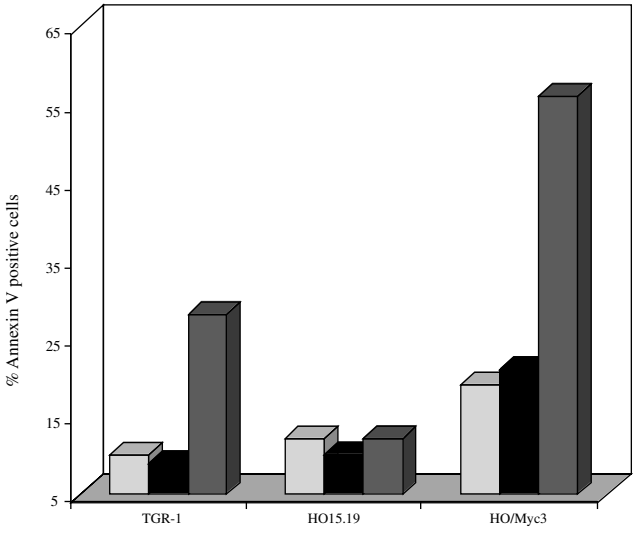


Figure 7 Expression of GFP-PDZ-M1 induces apoptosis in HeLa cells. HeLa cells were transfected using the following constructs: EGFP-C1 vector, EGFP-PDZ$_{Omi}$ and EGFP-PDZ-M1. Transfected cells were detached and stained with PE-conjugated annexin V. The population of GFP-expressing cells that was also positive for annexin V was analysed by FACSCalibur Flow cytometry. Bars represent the percentage of GFP-expressing cells that were also annexin V positive. The number shown below the abscissa is the average of three independent experiments ±s.e.

with EGFP-PDZ-M1, control vector or EGFP-PDZ_{Omi} to investigate whether PDZ-M1 induced apoptosis was dependent on the presence of Myc protein. Flow cytometry and annexin V staining were used to detect apoptosis in transfected cells. Figure 8 shows PDZ-M1 can induce apoptosis in 28% of transfected parental TGR-1 cells that express Myc. HO15.19 (myc−/−) cells were resistant to PDZ-M1-induced apoptosis, only 12% of transfected cells were apoptotic that is similar to cells transfected with the control vector or with EGFP-PDZ_{Omi}. When expression of Myc was reconstituted, these cells again became susceptible to PDZ-M1-induced apoptosis. The overall percentage of apoptotic cells was higher for the HO/myc3 reconstituted cells than the TGR-1 parental cell line independent of the construct used in the transfections. Since reconstitution of *myc* expression was achieved by retroviral infection, HO/myc3 cells have higher levels of Myc and its expression is not regulated as in the parental TGR-1 cells (Mateyak *et al*., 1997). This higher level of Myc did not affect the overall ratio of apoptotic cells expressing PDZ-M1 apoptotic cells expressing PDZ_{Omi} that was the same for both TGR-1 and HO/myc3 cell lines.



Constructs	TGR-1	HO15.19	HO/Myc3
EGFP-C1	10± 2	12± 3	19± 2
EGFP+PDZOmi	9± 1	10± 2	21± 3
EGFP+PDZ-M1	28± 2	12± 1	56± 2

Figure 8 Expression of GFP-PDZ-M1 induces apoptosis only in the presence of Myc. TGR-1, HO15.19 (c-*myc*−/−) and HO/myc3 fibroblasts were transfected using Lipofectamine 2000 (Life Technologies) with the following constructs: EGFP-C1 vector, EGFP-PDZ_{Omi} and EGFP-PDZ-M1. Transfected cells were detached and stained with PE-conjugated annexin V. The population of GFP-expressing cells that was also positive for annexin V was analysed by FACSCalibur Flow cytometry. Each bar represents the percentage of GFP-expressing cells that were also annexin V positive. Different colors indicate the different plasmid used for transfection. Results shown in the table are the average of three independent experiments for each cell lines ±s.e.

Discussion

Omi/HtrA2 is a serine protease isolated originally as an interactor of Mxi2 kinase (Faccio *et al*., 2000b). The mature Omi protein consists of a catalytic domain as well as a PDZ motif. Its amino-acid sequence has been conserved from bacteria to humans (Faccio *et al*., 2000b). There is 36% identity and 58% similarity in the amino-acid sequence between bacterial *HtrA* and mature human Omi protein (Faccio *et al*., 2000b). Bacterial *HtrA* is a chaperone at normal temperatures and an active protease at elevated temperatures that can cleave and remove denatured or damaged proteins leading to the survival of bacteria (Spiess *et al*., 1999; Krojer *et al*., 2002). There is also a mammalian homolog of Omi called L56/HtrA1 (Zumbrunn and Trueb, 1996; Hu *et al*., 1998), which has 52% identity and 72% similarity with mature Omi. The highest similarity between these two proteins is found in their catalytic domains, which share 61% identity and 80% similarity. There is less similarity in the PDZ domains of Omi and L56/HtrA1, which share 40% identity and 62% similarity. Bacterial *HtrA* has two PDZ domains that are necessary for multimerization of the enzyme as well as binding to its substrates (Fanning and Anderson, 1996; Hung and Sheng, 2001). The function of the single PDZ_{Omi} is not known, although it is assumed, as with other PDZ domains, that it is involved in protein–protein interactions. Since Omi is a serine protease, its PDZ_{Omi} domain might have a role in the recognition and binding of potential substrates. Furthermore, there is an alternatively spliced form of Omi/HtrA2 (D-Omi) that is found only in kidney, colon and thyroid tissues (Faccio *et al*., 2000c). D-Omi is an inactive protease, has a modified PDZ domain and its physiological function in these tissues is unknown (Faccio *et al*., 2000c). Figure 1 shows an alignment of the amino-acid sequence of the five classes of PDZ domains performed using the Clustal W program (Aiyar, 2000). The secondary characteristic elements defining PDZ domains, derived from X-ray crystallographic studies, are highlighted. Secondary structure motifs of the PDZ_{Omi} were predicted using homology modeling software and the PDZ-3 of PSD-95 as a template. This comparison reveals that the overall topology of the PDZ repeat is strongly conserved in all five classes. The overall structure of PDZ_{Omi} is similar to other known PDZ domains. While it has six β-strands and two α-helices, the so-called variable loop, located between putative strands β_B and β_C, is slightly longer in PDZ_{Omi} than that of the other classes of domains. As a result, the β_D strand appears distorted (Hung and Sheng, 2001). Structural studies have shown the carboxy-terminal peptide (ligand) fits in the hydrophobic pocket that includes the α-helix (α_B), the second β-strand (β_B), as well as the conserved GLGF loop (Doyle *et al*., 1996). This loop in PDZ_{Omi} is represented by a different amino-acid sequence, LAEL. The PDZ_{Omi} also differs from other families of PDZ domains, in that its variable loop between β_B and β_C is longer and, as a result, the β_D-strand is distorted. These differences suggest the

PDZ_{Omi} might have different binding specificity and it could potentially define a new class of PDZ domains. The PDZ domain of L56/HtrA1 is also included in class V together with Omi. The binding specificity of L56/HtrA1 is not known, but its PDZ domain has extensive amino-acid sequence similarity with the PDZ domain of Omi (Faccio *et al*., 2000b).

Mxi2 is the only known protein that specifically binds the PDZ domain of Omi. Mxi2 is an alternatively spliced form of SAPK and was used as bait in the original isolation of Omi (Faccio *et al*., 2000b). The carboxy-terminus of Mxi2 ends in DIELVMI amino-acid sequence and deletion of the last two amino acids completely abolish its binding with PDZ_{Omi} (Faccio *et al*., 2000b). To investigate the structural characteristics of the PDZ_{Omi}, its amino-acid sequence was randomly mutated and the pool of mutants was screened for a PDZ^m with altered binding specificity. In this case, the pool was screened for the ability to bind the carboxy-terminus of Myc ($Myc_{282–439}$). Myc is a nuclear oncoprotein involved in cell growth, apoptosis, differentiation and cell transformation (Littlewood and Evan, 1990; Evan *et al*., 1994). The carboxy-terminus of Myc ends in the amino-acid sequence LRNSCA that is substantially different from the Mxi2 carboxy-terminus that ends in DIELVMI. Myc protein does not bind the wild-type PDZ_{Omi}. Using a yeast two-hybrid screen, we were able to obtain PDZ^m domains that could bind $Myc_{282–439}$. These PDZ^m domains were grouped into six families based on their binding strength and specificity. Some of them bound to $Myc_{282–439}$ but also retained the ability to bind weakly to Mxi2. Others bound to $Myc_{282–439}$ but also bound to the MycΔ3, indicating that they recognized amino acids outside the extreme carboxy-terminus of the Myc protein. Several PDZ^m proteins showed strong and specific binding with $Myc_{282–439}$. When the amino-acid sequence of the different PDZ^m was deduced, most of the mutations were found clustered at positions 390 and 408 of the PDZ domain. Amino acids in these two positions were invariably changed to be hydrophobic. One family of PDZ^m (PDZ-M1) that exhibited strong and specific binding with $Myc_{282–439}$ was selected for further investigation. This family had a $Gly_{390} \rightarrow Val$ and $Arg_{408} \rightarrow Trp$ substitution in its PDZ domain. Both amino-acid substitutions were necessary for changing the specificity of the PDZ_{Omi} because another PDZ^m family that only differed in a single position, ($Gly_{390} \rightarrow Val$), showed binding to both Myc and Mxi2. Furthermore, the ability of PDZ-M1 to bind to the $Myc_{282–439}$ bait protein in mammalian cells was also investigated. Myc is a nuclear protein, but $Myc_{282–439}$ used in our experiments is cytoplasmic because it lacks the nuclear localization signals that are located in the amino terminus of the protein (Stone *et al*., 1987). We therefore expressed the PDZ-M1 specific for $Myc_{282–439}$ with a strong nuclear localization sequence fused to the GFP. The $Myc_{282–439}$ bait was also expressed fused to the RFP. When both PDZ-M1 and $Myc_{282–439}$ were coexpressed in mammalian cells, PDZ-M1 was able to bind to and translocate $Myc_{282–439}$ to the cell nucleus. Whether, the PDZ-M1 domain could also bind to the endogenous Myc protein and interfere with its normal function was further investigated. PDZ-M1 was expressed in transiently transfected mammalian cells and found to colocalize with endogenous Myc protein. Furthermore, the same cells transfected with PDZ-M1 exhibited increased apoptosis compared with cells transfected with a control PDZ_{Omi}, domain that does not bind to Myc. To further establish that the PDZ-M1-induced apoptosis is the result of a specific interaction with endogenous Myc protein, we used c-Myc deficient fibroblasts (HO15.19). These cells when transfected with PDZ-M1 were resistant to apoptosis. When c-*myc* expression was reconstituted in these cells (HO/myc3) they reverted back to being sensitive to PDZ-M1-induced apoptosis. These results clearly demonstrate that the ability of PDZ-M1 to induce apoptosis is entirely dependent on its interaction with Myc protein. Furthermore, PDZ-M1 by binding the carboxyl terminus of Myc interferes with the normal function of this protein driving cells to apoptosis. This is the first report of a PDZ domain used to target and modify the activity of Myc protein.

Our data indicate that the PDZ domain of Omi can be used as a prototype to develop artificial PDZ domains able to target cellular proteins. In a similar approach, the PDZ domain of the Ras-binding protein AF-6 was used as a prototype to develop PDZ mutants able to bind new protein targets (Schneider *et al*., 1999; Gee *et al*., 2000). For successful interaction to occur, the carboxy-terminus of the target proteins, recognized by the PDZ domains, must be exposed and available for binding. These artificial PDZ domains can provide scaffolds for the development of specific multi-protein complexes. They can easily be used to target, translocate, inhibit or enhance the normal function of numerous cellular proteins, thus providing information on the normal function of proteins and their potential role in human disease. Finally, artificial PDZ domains can also be used as therapeutic agents to target and modulate the activity of oncogenes such as the Myc proto-oncogene used in the experiments reported here.

Materials and methods

Yeast two-hybrid experiments

EGY48 (*MATa trp1 ura3 his3 LEU2::pLex Aop6-LEU2*) was used as the host yeast strain for all two-hybrid interaction experiments (Gyuris *et al*., 1993). This yeast strain has both an integrated LEU reporter gene with upstream LexA operators, as well as a pSH18-34 (LexAop-lacZ) 2μ plasmid that directs the synthesis of β-galactosidase (Gyuris *et al*., 1993). Positive protein–protein interactions between the bait and prey were initially detected by the ability of the yeast to grow on GAL-Ura-Trp-His-Leu-selective yeast plates. They were further tested on Ura-His-Trp-X-gal plates. Plasmids were rescued from positive clones and introduced into KC8 *E. coli* by electroporation. DNA sequence was determined by sequencing both DNA strands with a commercially available kit (PE Applied Biosystems) using an ABI Prism 310 Genetic Analyzer (Perkin–Elmer). The presence and stability of the recombinant proteins in yeast cells was monitored by Western blot analysis using either LexA (for baits) or HA-antibodies (for preys).

Generation of PDZ^m

Random mutations in the PDZ_{Omi} (between amino acids 335 and 458) were introduced using PCR reactions and the following primers: Primer A (5′-end): 5′-GATGTCCCAGATTATGCCTCTCCCGAATTCTCTGATCGTCTTCGAGAGTTT-3′. Primer B (3′-end): 5′-CTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTCATTCTGTGACCTCAGGGGT-3′

A DNA polymerase was used that is prone to errors when copying DNA (Mutazyme, Stratagene). To increase the mutation frequency, four sequential PCR reactions were performed, in which the product of the first reaction was diluted 1 : 1000 in TE buffer and used as the template for the following reaction. All four PCR reactions were combined, the DNA was digested with *Mfe*I/*Xho*I and cloned into the *Eco*RI/*Xho*I of the pJG4-5 vector (Fusco *et al*., 1999). This DNA was used to transform yeast that already contained a bait representing the C-terminus of Myc (aa 282–439) cloned in the pL202 expression vector. A population of 7.5×10^5 independent yeast clones were screened as described (Zervos *et al*., 1993).

Construction of c-MycΔ3 deletion mutants

The following primers were used in a PCR reaction: Primer C (5′-end): 5′-CCGGAATTCCCTTCTGCTGGAGGC-3′. Primer D (3′-end): 5′-GCGGGATCCTTAGTTCCGTAGCTGTTC-3′. Primer D introduces a stop codon (TAA) at position 1310 (aa 437) in the Myc sequence. This truncated form of Myc lacks the last three C-terminal amino acids. After the PCR, the DNA fragment was digested *Eco*RI/*Bam*HI and cloned into the corresponding sites of the pL202 yeast vector. The specificity of the different PDZ^m domains against LexA-MycΔ3aa, LexA-Mxi2 and LexA-$Myc_{282–439}$ was determined using standard yeast two-hybrid methodologies (Zervos *et al*., 1993; Fusco *et al*., 1999).

Construction of LexA-N-myc, Max and Mxi1 baits

The following primers were used in a PCR reaction for cloning of N-myc *c*-terminal (aa 236–394) and Max full length: Primer E (N-myc 5′-end): 5′-CCGGAATTCGCCCTGGGTCCCGGGAGGGCT. Primer F (N-myc 3′-end): 5′-CGCGGATCCCTAGCAAGTCCGAGCGTGTTCAAT. Primer G (Max 5′-end): 5′-CCGGAATTCTATGAGCGATAACGATGACATC. Primer H (Max 3′-end): 5′- CGCGGATCCTTAGCTGGCCTCCATCCGGAG.

The PCR products were digested with *Eco*RI/*Bam*HI and cloned into the corresponding sites of the pL202 yeast bait vector. LexA-Mxi1 has been previously described (Zervos *et al*., 1993).

Mammalian expression of recombinant proteins

The following primers were used to PCR the DNA fragments corresponding to wild-type PDZ_{Omi} and PDZ-M1 from yeast vectors: Primer I (5′-end): 5′-CCGCTCGAGCGCCTCCAAAAAAGAAGAGAAAGGTAGCTT CTGATCGTCTTCGAGAGTTTC-3′. Primer J (3′-end): 5′-CGGGGTACCTCATTCTGTGACCTCAGGGG-3′. Primer I includes the DNA sequence (underlined) that encodes the SV40 NLS, PPKKKRKVA (Kalderon *et al*., 1984) in frame with the PDZ domain. The PCR products were digested with *Xho*I/*Kpn*I and cloned in the corresponding sites of pEGFP-C1 vector (Clontech).

The Myc C-terminus DNA sequence corresponding to aa 282–439 was amplified using the following PCR primers: Primer K (5′-end): 5′-CGGAATTCCCCTTCTGCTGGAGGC-3′. Primer L (3′-end): 5′-CCGGGATCCTTACGCACAAGAGTTC-3′. The PCR product was digested with *Eco*RI/*Bam*HI and cloned in the corresponding sites of pDsRed1-C1 vector (Clontech).

In a different set of experiments, DNA corresponding to wild-type PDZ_{Omi} or PDZ-M1 without the NLS was amplified using the following primers: Primer M (5′end) 5′-CGGAATTCTTCTGATCGTCTTCGAGAGTTTC-3′. Primer N (3′end) 5′-CGGGGTACCTCATTCTGTGACCTCAGGGG-3′. The PCR products were precipitated, digested with *Eco*RI and *Kpn*I, and cloned to the corresponding sites of pEGFP-C1 vector (Clontech).

Cell lines and culture conditions

HeLa cells were grown using F12-Nutrient mixture (HAM) media (Life Technologies) supplemented with 10% FCS (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). TGR-1 (c-*myc*+/+), HO15.19 (c-*myc*−/− homozygous knockout) and HO/myc3 (HO15.19 cells infected with a retrovirus vector expressing c-*myc*) were grown in DMEM (GIBCO) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate (Sigma).

Transfections and immunofluorescence microscopy

For immunofluorescence, the cells were grown on cover slips. Approximately, 70% confluent cells were transfected with 2 μg of GFP-NLS-PDZ_{Omi} and RFP-$Myc_{282–439}$ using Lipofectamine Plus reagent according to the instructions provided by the manufacturer (Life Technologies). After 24 h, the transfected cells were washed three times with PBS and fixed with 4% paraformaldehyde. The coverslips were then placed on glass slides using Fluoromount-G solution (Southern Biochemical Association). The subcellular localization of GFP- and RFP-tagged proteins was monitored using a LSM510 confocal laser-scanning microscope (Carl Zeiss).

Western blot analysis

Transiently transfected cells were lysed using a Triton X-100 based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM tris pH 7.5, 2 mM EDTA) in the presence of a protease inhibitor mix (Roche). Approximately, 20 μg of whole-cell extracts were resuspended in SDS-sample buffer and boiled for 3 min. The samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto PVDF membranes (NEN Life Science products) using a Semi-Dry cell Transfer Blot (Bio-Rad). Nonfat dry milk (2%) in TBST buffer was used to block nonspecific binding of the membrane. The membrane was incubated with an anti-GFP monoclonal antibody (Santa Cruz Biotechnology), 1 : 700 dilution, followed by a secondary peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch) 1 : 15000 dilution and visualized by ECL (Pierce).

Flow cytometry

HeLa, TGR-1, HO15.19, HO/myc3 cells were grown in six-well plates to 60% confluence and were transfected with EGFPC1 vector, GFP-PDZ_{Omi} or GFP-PDZ-M1 using Lipofectamine Plus Reagent according to the instructions provided by the manufacturer (Life Technologies). Transfected cells were detached 48 h later using 0.02% EDTA (Sigma), washed twice with ice-cold PBS, resuspended

in 1X binding buffer (BD Bioscience), and stained with PE-conjugated annexin V according to BD Bioscience protocol. Analysis of annexin V, GFP positive cells was performed on a FACS Vantage Flow Cytometer (Becton Dickinson).

Acknowledgements
We are grateful to members of the Zervos' lab and L Von Kalm for comments, suggestions and critical reading of the manuscript. This work was supported by the National Institutes of Health Grant R01 DK55734-01 (to ASZ).

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