The CBP/p300 TAZ1 domain in its native state is not a binding partner of MDM2

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The transcriptional co-activator CBP [CREB (cAMP-response-element-binding protein)-binding protein] and its paralogue p300 play a key role in the regulation of both activity and stability of the tumour suppressor p53. Degradation of p53 is mediated by the ubiquitin ligase MDM2 (mouse double minute protein) and is also reported to be regulated by CBP/p300. Direct protein–protein interaction between a central domain of MDM2 and the TAZ1 (transcriptional adaptor zinc-binding domain) [C/H1 (cysteine/histidine-rich region 1)] domain of p300 and subsequent formation of a ternary complex including p53 have been reported previously. We expressed and purified the proposed binding domains of HDM2 (human homologue of MDM2) and CBP, and examined their interactions using CD spectroscopy. The binding studies were extended by using natively purified GST (glutathione S-transferase)–p300 TAZ1 and GST–p53 fusion proteins, together with *in vitro* translated HDM2 fragments, under similar solution

conditions to those in previous studies, but omitting added EDTA, which causes unfolding and aggregation of the zinc-binding TAZ1 domain. Comparing the binding properties of the known TAZ1 interaction partners HIF-1 α (hypoxia-inducible factor 1), CITED2 (CBP/p300-interacting transactivator with glutamic- and aspartic-rich tail) and STAT2 (signal transducer and activator of transcription 2) with HDM2, our data suggest that TAZ1 in its native state does not serve as a specific recognition domain of HDM2. Rather, unfolded TAZ1 and HDM2 proteins have a high tendency to aggregate, and non-specific protein complexes are formed under certain conditions.

Key words: cAMP-response-element-binding-protein-binding protein (CBP), glutathione S-transferase pull down, oncoprotein, mouse double minute protein (MDM2), protein secondary structure, zinc finger.

INTRODUCTION

CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 are members of a family of transcriptional co-activators which are involved in cell growth, development, and differentiation [1–4]. Most of their known functions are mediated by protein–protein interactions through highly conserved domains; the number of described CBP/p300 interaction partners is still growing, and many different ligands have been found to interact with a given co-activator domain. It is assumed that binding proteins compete for limited amounts of CBP/p300 in the cellular environment. However, binding affinities for the interaction partners might be different and some of the described CBP/p300 protein complexes might be physiologically more significant than others. A comparison of binding affinities is rarely included in the description of new binding partners. This omission has apparently led to a number of reported interactions between ligands and CBP/p300 that may be in error or due to non-specific interactions.

CBP/p300 contains three cysteine/histidine-rich regions (C/H1–C/H3), which fold into zinc-binding modules. C/H1 corresponds to the TAZ (transcriptional adaptor zinc-binding) 1 domain; TAZ2 is part of the C/H3 region [5,6]. Although these domains comprise only approx. 100 residues each, which is small relative to the 2441 residues of the entire human CBP protein, they mediate more than half of the described protein interactions [1]. In contrast with the behaviour of other CBP domains such as KIX (CREB-

binding domain of CBP/p300), which can bind synergistically to two transcriptional activation domains in a ternary complex [7], it appears that only 1:1 complexes are formed by TAZ domains [8]. Several binding partners of CBP/p300 have been described as interacting with TAZ1, and structural data are available for complexes formed between TAZ1 and the HIF-1 α (hypoxia-inducible factor 1 α) or the CITED2 (CBP/p300-interacting transactivator with glutamic- and aspartic-rich tail) transactivation domain [9–12]. The three zinc ions bound by TAZ stabilize the fold of the domain consisting of four α -helices packed against each other. Zinc-depleted TAZ1 is incapable of binding to HIF-1 α [13].

The tumour suppressor p53 is a cellular transcription factor that is up-regulated upon various stress signals including DNA damage. It initiates DNA repair, cell-cycle arrest and/or apoptosis [14–16]. p53 contributes to the stability of the genome, and loss of its function is reported in more than 50 % of human cancers. p53 has a short half life and its cellular level is regulated by MDM2-dependent ubiquitination [17,18] and subsequent degradation by the 26 S proteasome [19,20]. Interaction of the p53 transactivation domain with the N-terminus of the E3 ubiquitin ligase MDM2 (mouse double minute protein) inhibits p53-dependent transcription [21,22]. The crystal structure of this complex has been described in [23]. p53 binding to MDM2 is weakened by phosphorylation of Thr¹⁸ and is slightly enhanced by phosphorylation of Ser¹⁵ and Ser²⁰ [24]. Both MDM2 and p53 are described interaction partners of CBP/p300. A key role in regulating p53

Abbreviations used: CBP, cAMP-response-element-binding-protein-binding protein; C/H, cysteine/histidine-rich region; CITED2, CBP/p300-interacting transactivator with glutamic- and aspartic-rich tail; CREB, cAMP-response-element-binding protein; DTT, dithiothreitol; E6-AP, E6-associated protein; GST, glutathione S-transferase; HDM2, human homologue of mouse double minute protein; HIF-1 hypoxia-inducible factor 1; HPV, human papillomavirus; IPTG, isopropyl β -D-thiogalactoside; MDM2, mouse double minute protein; STAT2, signal transducer and activator of transcription 2; TAZ, transcriptional adaptor zinc-binding domain.

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Figure 1 Schematic presentation of the HDM2 domain organization

The p53-binding region is located at the N-terminus (residues 17–125). The nuclear localization signal, the little zinc-finger motif, and a predicted double zinc finger with some similarity to a RING domain are displayed. The proposed p300-interacting region (according to [31]) is indicated with a broken line. The HDM2 fragments used in the present study are shown.

activity was assigned to a C-terminal domain of CBP/p300 [25], and a number of binding sites in CBP/p300 for p53 have been defined, e.g. the TAZ1, KIX (CREB-binding domain of CBP/p300), C/H2, ZZ (zinc-binding domain in CBP/p300 and dystrophin), TAZ2 and IBiD (interferon-regulatory factor 3-binding domain) domain [26–31].

Grossman and co-workers also proposed a role for CBP/p300 in p53 degradation [26,31,32]. They suggested that in unstressed cells, the unmodified p53 transactivation domain preferentially binds to MDM2, bound to the TAZ1 domain of CBP/p300 to form a ternary complex. Thus the TAZ1 domain of p300 has been proposed to serve as a scaffold that presents p53 for ubiquitination by MDM2. The proposed p300-binding domain is located within the first 221 residues of MDM2 and a p300 fragment comprising residues 340–406 was shown to bind full-length MDM2 [31]. This proposed MDM2-binding site on p300 contains only part of the well conserved TAZ1 or C/H1 domain.

In order to investigate the interaction between a co-activator and an oncoprotein on a structural basis, we expressed and purified various HDM2 (human homologue of MDM2) fragments based on the previously described p300–MDM2 complexes [31], and examined their binding properties for TAZ1 and p53 by CD spectroscopy. The interaction between MDM2 and p53 could be confirmed, but no specific binding was detectable between MDM2 and the CBP TAZ1 domain. Extending our interaction studies to other MDM2 domains (Figure 1) revealed that various regions of MDM2 form non-specific aggregates in solution. These results were confirmed using a GST (glutathione S-transferase)–p300 TAZ1 fusion protein and *in vitro* translated HDM2 fragments. Unfolded TAZ1 aggregates as well under certain conditions. Mixing the two proteins together leads to the formation of non-native high-molecular-mass protein complexes with low solubility. Our data clearly demonstrate that TAZ1 in its fully folded native state cannot serve as a specific binding domain for MDM2.

EXPERIMENTAL

Plasmids

For expression of recombinant proteins in *Escherichia coli*, a cDNA encoding HDM2(17–125) was cloned into a bicistronic expression vector [33], together with a fragment encoding the transactivation domain of human p53 (residues 1–61). Murine cDNA encoding CBP(340–439) (the TAZ1 domain) was inserted into pET21a. The corresponding fragment of human p300 (encoding residues 323–424) was cloned into pET42a, directing the synthesis of a GST–TAZ1 fusion protein. GST–p53 (residues 1–61) was constructed by ligation of the corresponding cDNA into a pET42a-derivative. In order to generate templates for *in vitro* translation, cDNA fragments encoding various domains of HDM2 (see Figure 1) were amplified from a human liver cDNA library by using a PCR-based mutagenesis technique and inserted into pET21a via *Nde*I/*Bam*HI sites. HIF-1 α (705–826), CITED2(220–269), STAT2(748–851) (signal transducer and activator of transcription 2), as well as full-length HDM2 cDNA (a gift from Dr Peiqing Sun, Department of Molecular Biology, The Scripps Research Institute) were subcloned into pET21a.

Protein preparation

CBP TAZ1 (residues 340–439 of mouse CBP) was prepared according to the method used for mCBP(345–439) [9], and HIF-1 α (790–826) was prepared as described in [9]. Purified refolded TAZ1 and HIF-1 α were dialysed into buffer A (10 mM Tris/HCl, pH 7 and 150 mM NaCl). HDM2(17–125) was co-expressed with the p53 transactivation domain from a bicistronic vector to enhance protein stability. A growing culture of BL21(DE3) [DNAY] cells harbouring the expression plasmid was induced with 1 mM IPTG (isopropyl β -D-thiogalactoside) at 37 °C, and protein expression was continued for 3–4 h. Proteins were purified by ion-exchange chromatography on Q or SP Sepharose respectively, and subsequent reversed-phase HPLC in a 0.1% trifluoroacetic acid water/acetonitrile gradient.

HDM2(17–221) was expressed by itself since co-expression with either p53 or TAZ1 did not enhance protein solubility or yield. The protein was purified from the insoluble fraction of *E. coli* lysates. Briefly, bacterial pellets were suspended in 40 ml of 10 mM Tris/HCl, pH 6, per litre of original culture. Bacteria were lysed by lysozyme treatment and subsequent sonication. A DNase I digest was performed and the suspension was adjusted to 0.5 M NaCl and 2% (v/v) Triton X-100. After 30 min incubation at room temperature (20 °C), the insoluble fraction was isolated by centrifugation at 20 000 g for 30 min. The pellet was washed twice in 20 mM Tris/HCl, pH 8, and the inclusion bodies were solubilized in GTD buffer [6 M guanidinium chloride, 20 mM Tris/HCl, pH 8, 20 mM DTT (dithiothreitol)] and incubated for at least 2–4 h. The pH was lowered by the addition of trifluoroacetic acid and the solution was loaded onto a reversed-phase HPLC column. Elution of the protein was performed in a 0.1% trifluoroacetic acid water/acetonitrile gradient. HDM2(17–221) containing fractions were freeze-dried and stored at –20 °C until use. For CD studies purified HDM2(17–125) or HDM2(17–221) was dissolved in GTD buffer and incubated at room temperature for 2 h. Proteins were refolded on a NAP-5 column (Amersham Biosciences) in buffer A and used for experiments within 1 day. Integrity of the purified proteins was confirmed by MALDI (matrix-assisted laser-desorption/ionization) MS.

CD spectroscopy

Protein concentration was determined by measuring the UV absorbance [34]. Spectra were recorded at 25 °C at a total protein

concentration of 10 μ M in 3 mM Tris/HCl, pH 7, and 150 mM NaCl using a 0.2 cm cell in an AVIV model 202 CD spectrometer. Proteins used for CD interaction studies were CBP TAZ1, HDM2(17–125) and HDM2(17–221), HIF-1 α (790–826) and p53(1–61). Unfolded CBP TAZ1 or GST–TAZ1 (p300) was obtained by the addition of 2 mM EDTA; for reconstitution attempts, 2.2 mM ZnSO₄ was added. The molar ellipticity for the protein solutions was calculated using the total concentration of amino acid residues present.

Preparation of GST fusion proteins and *in vitro* interaction assay

Expression plasmids encoding GST–TAZ1 or GST–p53 were transformed into *E. coli* strain BL21(DE3) or BL21(DE3) [DNAY] respectively. Cultures were grown in LB (Luria–Bertani) broth at 37 °C to a D_{600} of 0.5. For the expression of GST–TAZ1, ZnSO₄ was added to a final concentration of 150 μ M, and the culture was shifted to 25 °C. Recombinant protein synthesis was induced by the addition of IPTG. After 3–4 h of incubation, bacteria were harvested by centrifugation at 6000 *g* for 15 min. The pellets were suspended in 200 ml of 20 mM Tris/HCl, pH 7, per litre of original culture, and GST–TAZ1-expressing cultures were supplemented with 10 μ M ZnSO₄. The suspensions were stored at –20 °C until use. For purification of GST fusion proteins, 40 ml aliquots were thawed at room temperature, supplemented with protease inhibitors (CompleteTM, EDTA-free; Roche Diagnostics) and 1 mM DTT. Bacteria were lysed using a French press and GST fusion proteins were pre-purified by ion-exchange chromatography. Fractions containing the fusion protein were supplemented with protease inhibitors and stored at 4 °C for up to 3 weeks. For the *in vitro* interaction assay, approx. 5 μ g of GST fusion protein was bound to 2.5 μ l of glutathione–Sephadex and washed three times in wash buffer (20 mM Tris/HCl, pH 7, 150 mM NaCl, 1 mM DTT, 0.1% (v/v) Nonidet P40, CompleteTM protease inhibitors and additional 10 μ M ZnSO₄ for GST–TAZ1).

³⁵S-labelled proteins were *in vitro* translated using wheat germ lysates (Promega) according to the manufacturer's protocol. To minimize RNA and protein degradation RNasin (Promega) and protease inhibitors were added to the reaction mixture. The *in vitro* translation product (1.5 μ l) was mixed with protein sample buffer and loaded to the gel directly (10% input), 15 μ l of the *in vitro* translation product was diluted in 150 μ l of wash buffer and added to the Sepharose-bound fusion proteins. After 20 min of mixing at room temperature, the resins were washed three times in 150 μ l of wash buffer. To compare binding affinities of TAZ1 in its native or its unfolded state, parallel reactions were performed using wash buffer containing 2 mM EDTA instead of ZnSO₄. The washed resin was either boiled directly in protein sample buffer (unfolded TAZ1) or soluble protein complexes were eluted in 100 μ l of wash buffer containing additional 25 mM reduced glutathione (native TAZ1 or p53). Eluates were concentrated by precipitation with 10% trichloroacetic acid. Protein pellets were dissolved in sample buffer and loaded on to a 4–20% acrylamide gel for SDS/PAGE. Binding of ³⁵S-labelled proteins was detected by autoradiography.

RESULTS

Protein preparation and CD spectroscopy

To investigate the interaction between HDM2 and CBP/p300 on a structural basis, N-terminal HDM2 fragments and the CBP TAZ1 domain were expressed in *E. coli* and purified to homogeneity. Proteins were refolded and analysed for secondary-structure content and protein-binding properties by CD spectroscopy. HDM2(17–221) displays some α -helical structure as exhibited by the

typical absorption bands at 222 and 208 nm (Figure 2A). Free TAZ1 also folds into a helical structure. In the present study, the CD spectrum obtained for a mixture of HDM2 and TAZ1 did not differ from a curve calculated from data obtained for the two isolated domains (broken line). This suggests either that the formation of the protein complex is not accompanied by additional secondary structure formation or that there is no interaction between the two proteins.

In order to confirm the native fold of the HDM2 and the TAZ1 protein preparations, control experiments were performed with HDM2 and p53, as well as with TAZ1 and HIF-1 α . The transactivation domain of p53 is unfolded in solution (Figure 2B). The CD spectrum of a 1:1 mixture of this protein with HDM2(17–221) differed from the calculated average of the spectrum of the two free proteins (broken line). A similar result was obtained for a mixture of HDM2(17–125) and p53 (Figure 2D). The biological activity and the structural integrity of the refolded CBP TAZ1 domain was confirmed by its ability to interact with HIF-1 α . Free HIF-1 α (790–826) is unfolded in solution as well (Figure 2C). However, a 1:1 mixture with TAZ1 displays a higher α -helical content than the calculated average of the two free proteins (broken lines), suggesting additional secondary-structure elements in the complex. Indeed, binding of HIF-1 α (790–826) to TAZ1 is accompanied by the formation of two helical regions (termed α_B and α_C) in the 37-residue HIF-1 α peptide spanning nine and ten residues respectively [9,11]. Therefore the TAZ1–HIF complex (137 residues) gains a calculated 14% helicity compared with the isolated domains, which is reflected in our CD results. The N-terminal domain of p53 in its free state is unfolded as well (Figures 2B and 2D; and [35,36]). Within the 61 residues of the p53 peptide only nine may fold into a helix [23]. Thus a complex formed between p53(1–61) and HDM2(17–125) or HDM2(17–221) gains only 5% or 3% helicity respectively. This explains the fact that the difference between the calculated and the measured spectrum is bigger for TAZ1–HIF complexes than for HDM2–p53 complexes.

The molar ellipticity of HDM2(17–221) is approximately half of the ellipticity of HDM2(17–125), and the protein is about double in size (205 residues compared with 109). Since this property is an average over all peptide bonds, this suggests that in free HDM2(17–221), approximately the same number of residues are folded as in free HDM2(17–125). Thus the region beyond residue 125 may be unfolded, and one would expect that it forms some secondary-structure elements upon interaction with TAZ1. However, the primary sequence of the HDM2 central region displays low complexity and is rich in proline and serine residues, which is common for unstructured segments. In contrast, the amino acid sequences of the HIF-1 α and CITED2 TAZ1-interaction domains are more complex and structural prediction reveals a significant helical content for the binding region. Despite the transactivation domains of HIF-1 α and CITED2 being unfolded in their free state, they fold into helical structures upon binding to TAZ1 (Figure 2C; and [9,11,12]).

Previously, it has been shown that phosphorylation of HDM2 on Ser¹⁶⁶ and Ser¹⁸⁶ enhances its interaction with p300 [37]. We prepared HDM2(Ser166Asp), HDM2(Ser186Asp) and HDM2(Ser166Asp,186Asp) in order to mimic these modifications in our recombinant protein. Neither of the mutant HDM2 proteins displayed an interaction with TAZ1 (results not shown).

To examine the influence of zinc binding on the secondary-structure content of TAZ1, a folded CBP TAZ1 protein preparation (Figure 2E) was treated with EDTA, resulting in a spectrum of an unfolded protein. Addition of an excess of Zn²⁺ did not restore the native fold, suggesting that there is no equilibrium between the zinc-bound and the zinc-depleted form of TAZ1. A natively

Figure 2 Far UV CD spectroscopy to monitor protein-protein interaction and unfolding

Wavelength scans for the indicated proteins are displayed in solid lines, the calculated average of the molar ellipticity of two free proteins is shown in broken lines. (A) CBP-TAZ1 and HDM2(17–221) were refolded after denaturing purification. Both proteins exhibit α -helical structure, although the helix content is much higher in TAZ1 than in HDM2(17–221). The spectrum of the mixture of the two proteins is identical with the calculated average curve. (B) Recombinant purified p53(1–61) is unfolded in its free state, but interacts with refolded HDM2(17–221). The spectrum of a mixture with HDM2(17–221) differs slightly from the calculated average of the two isolated domains. (C) The native fold of the free TAZ1 protein and the ability to interact with HIF-1 α (790–826) was confirmed. (D) Interaction of HDM2(17–125) with p53(1–61) confirming the described p53-binding site in the N-terminus of HDM2. (E) Unfolding of CBP TAZ1 upon treatment with a chelating agent. The native helical structure of the zinc-binding domain is destroyed by the addition of EDTA and could not be restored by addition of an excess of zinc. (F) The p300 TAZ1 domain fused to GST (GST-TAZ1) loses helical structure after treatment with EDTA, suggesting unfolding of the zinc-binding domain.

purified GST fusion protein, which contains the p300 TAZ1 domain (GST-TAZ1), exhibited similar behaviour. The α -helical content of the protein (Figure 2F) decreased significantly after addition of EDTA. Assuming that the four helices of TAZ1 are formed in the native GST fusion protein (380 residues), it loses 16% helicity upon EDTA treatment. This is reflected by our results and indicates unfolding of the zinc-binding domain, whereas the secondary structure elements of GST are stable under these conditions.

GST *in vitro* interaction assay

CD spectroscopy might not be sensitive enough to detect small structural changes in relatively large protein complexes, and the proteins used for the studies were purified from *E. coli* lysates. The interaction between TAZ1 and HDM2 was first proposed based on pull down assays using bacterially expressed GST-p300 TAZ1 fusion proteins together with *in vitro* translated HDM2 peptides [31]. In order to enhance the sensitivity of our

Figure 3 GST *in vitro* interaction assay

For all gels, the same size standards were used, the position of the bands are indicated in the margin or labelled by dots on each panel. (A) SDS/PAGE of purified GST-TAZ1 and GST-p53 (lanes 1 and 2 respectively). M, molecular mass marker; I, 10% of the input of a typical *in vitro* reaction. Sepharose-bound fusion proteins were incubated with *in vitro* translated ³⁵S-labelled peptides. Soluble complexes formed with native GST-TAZ1 (N) or GST-p53 (P) were eluted with an excess of glutathione, whereas unfolded aggregates were solubilized in sample buffer (U). Proteins were analysed by SDS/PAGE. (B and C) Autoradiography of bound ³⁵S-labelled proteins, lanes are labelled as in (A). Exposure times varied between 16 and 48 h, but were always the same within one *in vitro* experiment. (B) Binding of ³⁵S-labelled *in vitro* translated N-terminal fragments of HDM2 to GST-p53 and GST-TAZ1 under native and denaturing conditions. The biological activity of the *in vitro* translated HDM2 protein fragments was confirmed by interaction with GST-p53 [e.g. HDM2(17-125), HDM2(17-221) and HDM2(1-228)]. As expected, addition of EDTA to GST-p53(1-61) did not affect the interaction with HDM2(1-228) (lanes P and P*). No specific HDM2-TAZ1 interaction could be detected under native conditions. (C) The known TAZ1-binding partners HIF-1 α , CITED2 and STAT2 were used as positive controls. Their interaction with TAZ1 is strong and depends on a native fold (compare lanes N and U).

interaction studies, we decided to use the same technique as an additional tool. Furthermore, *in vitro* translated HDM2 peptides may be post-translationally modified, which may be required for interaction. The TAZ1 domains of CBP and p300 share high homology; within the structured regions, 96% of the amino acid residues are identical [8]. Although some interacting proteins, e.g. HIF-1 α or CITED2, are known to bind to the TAZ1 domain of both CBP and p300 [9-12], one cannot exclude the possibility that there might be some differences in binding specificity. To answer the question of whether the described MDM2 interaction is specific for one of the paralogues, we used the TAZ1 domain of human p300 fused to GST for interaction studies together with *in vitro* translated HDM2. Control experiments for HDM2 were performed with a GST fusion protein containing the transactivation domain of p53. Both GST-TAZ1 and GST-p53 were purified from the soluble fraction of *E. coli* lysates under native conditions using ion-exchange and affinity chromatography (Figure 3A, lanes 1 and 2 respectively). Fusion proteins immobilized on glutathione-Sepharose were incubated with *in vitro* translated ³⁵S-labelled HDM2 proteins. Native wash conditions were applied for GST-TAZ1 and GST-p53 (labelled N or P respectively in Figure 3). Zinc-chelating wash conditions were applied for parallel reactions with GST-TAZ1 (labelled U in Figure 3) to distinguish between folded protein complexes and non-specific aggregates formed by unfolded (zinc-free) TAZ1. To confirm the efficiency of the zinc-chelation, a control experiment was performed. Bacterially expressed immobilized GST-HIF1 α was incubated with *in vitro* translated TAZ1. Under native conditions, TAZ1 bound to the GST-HIF protein, but the interaction was abolished when zinc-chelating wash conditions were applied (results not shown). The proteins were either eluted from the resin in a native buffer (lanes N and P) or the resin was boiled in denaturing sample buffer (lane U). These elution techniques were applied to achieve equal protein yields under native and non-native conditions as monitored by SDS/PAGE (compare lanes N and U in Figure 3A). Affinity of the various ³⁵S-labelled domains for p53 and/or native and unfolded TAZ1 was estimated by autoradiography and comparison with 10% of the input (lanes I in Figure 3) of the *in vitro* reactions.

HDM2(17-125), HDM2(17-221) and HDM2(1-228) displayed strong interaction with GST-p53 (Figure 3B) in agreement with published data [23,38]. As expected, addition of EDTA did not affect complex formation between p53 and HDM2 (Figure 3B, compare lanes P and P* in the third panel). Neither HDM2(17-221) nor HDM2(1-228) bound to GST-TAZ1, which is in contrast with results from previous mapping experiments where HDM2(1-325) and HDM2 Δ (222-350) bound to the N-terminus of p300 [31]. Control experiments with *in vitro* translated HIF-1 α (705-826), CITED2(220-269) and STAT2(748-851) confirmed the biological activity and the structural integrity of the GST-fused TAZ1 domain. All three proteins bound strongly to TAZ1 under native conditions in agreement with published data [13,39,40], but no interaction was detected with zinc-depleted, unfolded TAZ1 (Figure 3C).

In order to extend our interaction study to the entire HDM2 protein, the full-length HDM2 protein and fragments beyond the p53-binding region were examined for specific TAZ1 binding. HDM2(1-491) and HDM2(133-491) showed weak binding to native TAZ1, but exhibited at least the same or stronger affinity for the denatured zinc domain (Figure 4A). Fragments containing the central acidic region and/or the little zinc finger did not recognize TAZ1 under native conditions either (Figure 4B). However, some HDM2 constructs, e.g. HDM2(17-336), HDM2(118-336) and HDM2(133-336) bound strongly to unfolded TAZ1. The same behaviour was observed for HDM2 peptides spanning the region from the little zinc finger to the C-terminus (Figure 4C). None of these HDM2 constructs bound specifically to the folded TAZ1 domain, but some displayed weak interaction under denaturing conditions. Deletion of the five C-terminal amino acid residues (Leu-Thr-Tyr-Phe-Pro), which contain hydrophobic bulky side chains, improved the solubility of the peptides and prevented the non-specific aggregation. Similarly, large unstructured regions might contribute to the aggregation tendency of bigger HDM2 fragments, even at the low concentration used in our interaction assay. p53 interaction stabilizes only residues 17-125 of HDM2. In the present study, co-expression of these two peptides in *E. coli* yielded a soluble complex. Bacterially expressed HDM2(17-221) however was insoluble, irrespective of whether it was

Figure 4 Interaction screen of the entire HDM2 protein for TAZ1-binding

Various *in vitro* translated HDM2 protein fragments were examined for their ability to interact with GST-TAZ1 under native and denaturing conditions. Autoradiographs of the corresponding gels are shown, lanes are labelled as in Figure 3. No HDM2 interaction specific for native TAZ1 was detected. (A) Full-length HDM2 and a fragment which does not contain the p53 binding domain were incubated with GST-TAZ1. (B) The N-terminal region up to and including the little zinc finger of HDM2 was screened for TAZ1-binding. Some of the HDM2 fragments containing the entire or part of the proposed TAZ1 binding domain [e.g. HDM2(17–336), HDM2(118–336) and HDM2(133–336)] seem to bind strongly to unfolded TAZ1 (compare lanes N and U). (C) Interaction studies of the HDM2 C-terminal region with GST-TAZ1. Fragments from the little zinc finger to the C-terminus of HDM2 were analysed for interaction.

co-expressed with TAZ1 or with p53 or by itself. Nevertheless, after purification and refolding the protein was amenable for CD studies, whereas all attempts to purify and refold bacterially expressed HDM2(17–336) or full-length protein failed due to the formation of strong aggregates.

DISCUSSION

Despite a thorough search for a sequence of HDM2 that would bind specifically to natively folded TAZ1, we were unable to find evidence of a specific interaction. Fragments of HDM2 up to and including the full-length protein failed to bind to correctly folded TAZ1. Our interaction screen was performed on the basis of the sequence conservation and the domain organization of HDM2 (Figure 1). Binding properties of the indicated peptides for native and denatured GST-TAZ1 were compared: none of the HDM2 fragments examined formed a specific complex with folded TAZ1. We suggest that the weak interaction observed between some fragments of HDM2 and GST-TAZ1 may represent non-specific aggregation. The HDM2 primary sequence is rich in acidic residues. The pI of most of the fragments examined in this study ranged from 4.2 to 6.0. Electrostatic interactions with the basic TAZ1 domain (calculated pI of 9.53) may contribute to the weak

binding that was detectable in some cases by the affinity chromatography technique used.

GST-TAZ1 in its native state bound to the described interaction partners HIF-1 α , CITED2 and STAT2 (Figure 3C). In contrast, the partly unfolded fusion protein lost this ability, suggesting that these protein–protein interactions depend on a natively folded TAZ1 domain and are highly specific. This is in agreement with reports of the zinc-dependence of HIF-1 α and CITED2 TAZ1 complex formation [10,13]. The weak binding of GST-TAZ1 to some HDM2 fragments, however, was enhanced after EDTA treatment, demonstrating that the observed CBP/p300 TAZ1 MDM2 interaction [31] is independent of a native fold of the zinc domain and is therefore non-specific. Interestingly, screening for GST-C/H1 binding partners using an expression library derived from HeLa cells resulted in the isolation of clones for CITED2, HIF-1 α and STAT2 [39], but not for HDM2, although the corresponding cDNA clone was previously isolated from a cDNA library derived from the same cell line [38].

Initially, the HDM2-TAZ1 interaction was described based on some sequence similarity between two regions of the ‘high risk’ HPV (human papillomavirus) E6 protein and a C-terminal region of CBP/p300 and the first zinc cluster of the TAZ1 domain respectively [31]. HPV E6 in complex with the cellular E6-AP (E6-associated protein) also targets p53 for ubiquitin-dependent proteolysis [41,42]. However, this occurs exclusively in HPV-infected cells [43,44]. Several other differences between the two p53-degradation mechanisms have been defined [45,46]. E6 recognizes the core domain of p53, and not the N-terminus as HDM2 does [47,48]. HDM2 contains the p53 and the ubiquitin-recognition element on the same polypeptide, whereas the corresponding domains are linked by protein–protein interactions in the case of E6/E6-AP. Thus it is very unlikely that the sequence similarity between E6 and TAZ1 has functional significance in the degradation pathway of p53.

Unfortunately, many interaction studies including zinc-binding domains are performed in the presence of EDTA, because it is commonly used as an inhibitor of metalloproteases. Complex formation between MDM2 and the CBP/p300 TAZ1 domain has been reported by several other research groups [31,49–53], but their experimental conditions all include EDTA, which indicates that they are working with denatured CBP/p300 domains. When EDTA is excluded from the medium, as in the present study, no interaction is observed between the correctly folded CBP/p300 TAZ1 (C/H1) domain and HDM2. Either the interaction between CBP/p300 and HDM2 involves a different domain of the co-activator or it is mediated by other proteins. Thus the role of the central acidic domain of MDM2 in p53 ubiquitination and the mechanism by which overexpression of the TAZ1 domain enhances p53 stability still awaits clarification.

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