Precise mapping of the tms1 binding site on p53

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Abstract Originally identified as multicopy suppressor of a lethal growth arrest caused by expression of a tumour mutant cDNA of p53 in fission yeast the *tms1* gene product was found to form stable complexes with p53 in yeast. By using purified recombinant proteins multimeric complexes of tms1 and p53 could be demonstrated and recently the p53 binding site on the tms1 protein was established to the sequence YYITTEDFCT (aa 116–125) in the vicinity of a well conserved cell division motif. Here we report the precise mapping of the tms1 binding site on the p53 protein to the sequence LQIRGRERFE (aa 330–339) which defines a new functional domain on the p53 protein.

Key words: Tumour suppressor p53; tms1–p53 interaction; Fission yeast

1. Introduction

The growth suppressor p53 apppears to play a key role in the cellular growth control [1]. Loss of p53 activity by mutation, rearrangement or loss of both alleles results in selective growth advantage and favours cell transformation which is mirrored by a broad spectrum of p53 mutations in various human tumours [2–4]. Irradiation or DNA damaging agents lead to an increase of p53 expression resulting in G1 growth arrest [5–7] or apoptosis [8,9]. Wild type p53 binds double stranded DNA specifically [10–12] and was found to transactivate the expression of various genes somehow involved in cell growth [13–17]. There are three different functional domains of the p53 protein: an N-terminal domain which is responsable for the transactivation properties of p53 [13], a central core domain which specifically binds DNA [18] and the C-terminal domain which contains nuclear localization sequences and is required for the oligomerisation of p53 proteins [19,20]. The C-terminal domain seems to be also important for the regulation of the DNA binding ability of p53. Phosphorylation by protein kinase CK2, deletion of the last 30 amino acids or binding of the monoclonal antibody PAb421 stimulates the DNA binding of p53 [12]. The latter findings led to the proposal that some yet unknown cellular proteins might be responsible for modulating the DNA binding properties of p53 by binding to C-terminal sequences and convert p53 assembled into oligomers between latent and activated forms by allosteric transitions [21]. Since the number of p53 binding proteins which are thought to modulate p53 function is still increasing [22] it is of central importance to elucidate their functions and study complex formation in more detail. Among the candidates is the β-subunit of protein kinase CK2 [23] because it was recently demonstrated that p53 is not only phosphorylated at a C-terminal amino acid residue by protein kinase CK2, but also binds to the β-subunit of protein kinase CK2 via C-terminal sequences [24,25]. Another good candidate might be the tms1 protein. Originally identified as multicopy suppressor of a p53 induced growth arrest in fission yeast [26] the tms1 protein was found to form stable complexes with p53 in yeast [27]. By using purified recombinant proteins multimeric complexes of tms1 and p53 could be demonstrated and the binding site was localized to the C-terminal part of p53 [27]. There is evidence that the tms1 protein might be conserved during evolution. As previously reported a polyclonal antibody raised against recombinant tms1 protein was used to analyze mammalian cells for the expression of a tms1-related protein. We could identify a 42 kDa protein which forms complexes with p53 in vivo. In addition this tms1-related 42 kDa protein was localized to the nucleus of various mammalian cell lines [28]. More recently we were able to establish the p53 binding site on the tms1 protein to the sequence YITTEDFCT (aa 116–125) in the vicinity of a well conserved cell division motif [29]. Since this approach had proven sucessful we now mapped the tms1 binding site within the C-terminal part of p53 to the sequence LQIRGRERFE (aa 330–339) which defines a new functional domain on the p53 protein.

2. Experimental

2.1. Expression and purification of recombinant proteins

The p53 cDNA was modified by PCR to introduce appropiate *Bam*HI and *Hin*dIII restriction sites respectively. 5′ primers contained in addition a cAMP-dependent protein kinase site [30]. The amplified fragments were cloned into the pQE30 vector (DIAGEN) to produce plasmids pQE30-CT 264–393 (amino acids 264–393 of p53), pQE30-CT 287–393 (amino acids 287–393 of p53) and pQE30-CT 340–393 (amino acids 340–393 of p53). The plasmid for the tms1 fragment was as described [25]. Transformed *E. coli* (HB101) were grown to early log phase and induced for 3 hours with 1mM IPTG. Cells from 1 l bacterial cultures were pelleted, washed with 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl and resuspended in 6 M guanidine hydrochloride, 0.1 M sodium phosphate, pH 8.0, and lysed overnight at 4°C. After precipitating cellular debris the clearified supernatants were applied onto Ni^{2+}-NTA agarose (DIAGEN) [31]. The chelate resins were washed with 10 volumes each of lysing buffer. Bound proteins were eluted with lysing buffer, pH 4.0, and dialyzed against 20 mM Tris-HCl, pH 8.0, 300 mM KCl, 0.1% Tween 20 to allow refolding and proteins were analyzed by SDS-PAGE and checked by immuno blot analysis as described previously [32].

2.2. Labelling of recombinant proteins

According to the manufacturer's recommendations about 30 μg each of recombinant purified proteins were labelled for 30 minutes at 37°C with heart muscle cAMP dependent protein kinase (Sigma).

2.3. West-Western blot analysis

Total lysates of C-terminal p53 peptides expressing *E. coli* cells were subjected to SDS-PAGE without boiling prior to loading the gel. After renaturation in 200 ml 10 × PBS at 4°C for 1 hour proteins were transferred onto nitrocellulose filters. Filters were blocked for 1 hour at 4°C with dialysis buffer and probed with 20 μg of labelled purified tms1

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fragments for 1 hour at 4°C under gentle agitation. After washing two times with dialysis buffer filters were subjected to autoradiography.

2.4. Peptide synthesis

A set of 11 peptides of 10 amino acid long sections of amino acid 285 to 344 of the C-terminus of p53 that consecutively overlapped by five amino acids were synthesized by means of the SPOTs method (CRB, England) according to the manufacturer's recommendations.

3. Results and discussion

There is an increasing body of evidence that the carboxy-terminal domain of p53 is subject for the negative modulation of its ability to act as growth suppressor. Apart from phosphorylation of the C-terminus of p53 by cyclin-dependent kinases [33–35] and protein kinase CK2 [25,36] the complex formation with cellular proteins appears to play an important role for the conversion between latent and activated forms of p53 by allosteric transitions [21]. Recently we showed that an overexpression of the fission yeast tms1 protein is capable to abrogate the growth suppressor activity of human p53 when expressed in yeast [26]. Coexpression of tms1 and human p53 lead to stable multimeric complexes of tms1 and p53 in yeast. In vitro the interaction was mapped to the carboxy-terminal part of p53 by west-Western blot analysis using purified recombinant proteins [27]. In order to narrow down the tms1 binding region on the C-terminus of p53 we constructed expression plasmids pQE30-$CT_{264-393}$, pQE30-$CT_{287-393}$ and pQE30-$CT_{340-393}$ of human p53 respectively. The fragment of amino acids 287–393 of p53 was chosen because the fragment lacks the highly conserved box V whereas the fragment of amino acids 340–393 is known to contain the tetramerization domain of p53. Fig. 1A shows lysates of *E. coli* cells expressing the various C-terminal fragments analysed on a 15% SDS polyacrylamide gel and subsequently stained by Coomassie blue to demonstrate the expression of p53 fragments of the appropiate size. No comparable protein could be seen in non-transformed bacteria as it is shown in Fig. 1A, lane C. By probing a Western blot of the lysates with a p53 specific monoclonal antibody PAb 421 which recognizes

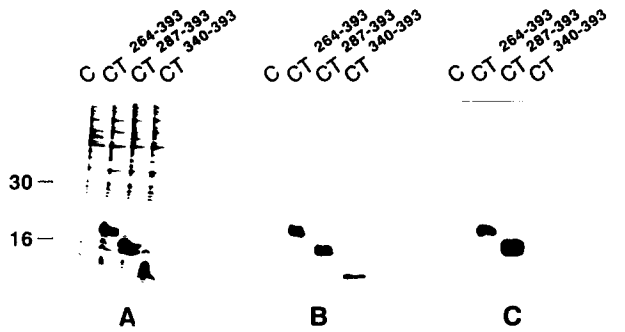

Fig. 1. Expression, immunoblot analysis and west-Western blot of C-terminal p53 fragments. *E. coli* strain HB101 was transformed with plasmids pQE30-$CT_{264-393}$, pQE30-$CT_{287-393}$ and pQE30-$CT_{340-393}$, respectively. Protein expression was induced by addition of IPTG. Aliquots of total bacterial lysates (as a control a bacterial lysates of non-transformed *E. coli* cells was added; C lanes) were analysed on 15% SDS-polyacrylamide gels and either stained with Coomassie blue (A) or proteins were transferred onto a nitrocellulose filter which was analysed by Western blot using the p53 specific monoclonal antibody PAb421 followed by ECL development (B) or proteins were renatured in the gel before transfer on nitrocellulose and subsequently incubated with purified and ^{32}P-labelled tms1 protein.

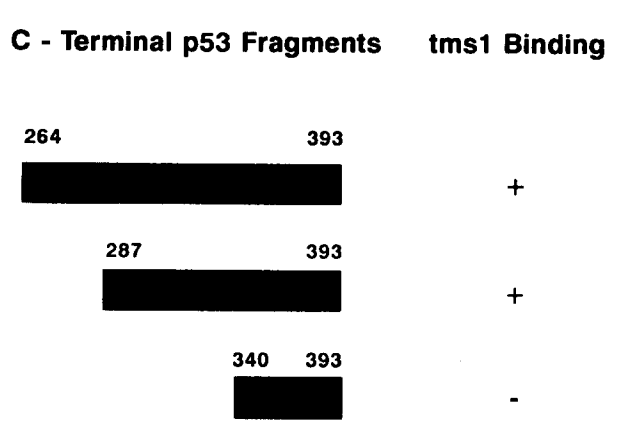

Fig. 2. Overview of C-terminal fragments. Numbers indicate amino acids on the polypeptide chain of p53.

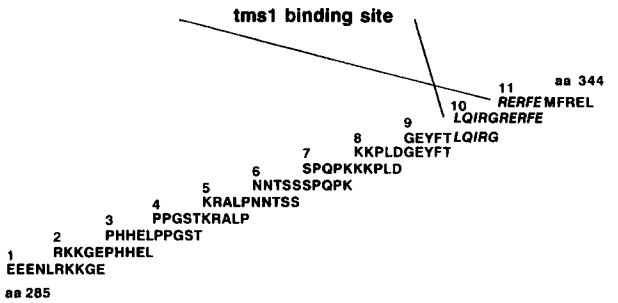

Fig. 3. Sequences of the synthetic p53-derived peptides immobilised on a membrane. Numbers indicate individual peptides ranging from amino acids 285 to 344 (aa285 to aa 344).

a C-terminal epitope on p53 spanning amino acids 370–378 [37] we verified the identity of the expressed fragments (Fig. 1B). For the expression of tms1 protein in *E. coli* we used a pQE30-tms1 plasmid introducing six histidine residues and a phosphorylation site for cAMP-dependent protein kinase to the N-terminus of the tms1 protein. This allows a rapid single step purification of recombinant tms1 protein on a Ni^{2+}-NTA agarose column and radiactive labelling of purified protein with commercially available heart muscle cAMP-dependent protein kinase (Sigma) as described previously [27]. The purified ^{32}P-labelled tms1 protein was used as probe in the west-Western blot analysis on nitrocellulose filters from total *E. coli* lysates containing the various C-terminal fragments of p53. As shown in Fig. 1C all fragments except for the C-terminal deletion fragment spanning amino acids 340–393 were capable of binding the tms1 protein indicating that the binding site lies within the region of amino acid 287 to 340 as indicated in Fig. 2. This region spanning amino acid 287 to 340 of p53 had previously been shown to be critical for the interaction of p53 with the β-subunit of protein kinase CK2 and is seemingly representing a new functional domain for modulating p53 function [25,36].

Since the systematic screening of series of short peptide sequences has become a powerful approach for characterising protein–protein interactions we used a synthetic approach to narrow down the tms1 binding domain on the C-terminus of p53 by means of the SPOTs method. The SPOTs method allows the rapid solid phase synthesis of peptides immobilised on a

cellulose membrane in a format suitable for systematic analysis of protein–protein interactions or antibody epitope mapping. This method had already proven sucessful for the mapping of the p53 binding site on the tms1 protein [29] and the precise definition of the epitopes of three different monoclonal antibodies raised against recombinant tms1 protein [38,39]. First we synthesized a set of 11 overlapping peptides of 10 amino acid long sections of the p53 primary amino acid sequence spanning the tms1 binding region from amino acid 285 to 344 as indicated in Fig. 3. After blocking the filter was probed with labelled purified tms1 protein, washed and subjected to autoradiography. As shown in Fig. 4 this resulted in binding of tms1 protein to peptides No. 9, 10 and 11 with a remarkable specificity indicating a strong interaction between the tms1 protein and the p53-derived peptides. Since these identified peptides overlapped by the sequence LQIRGRERFE (aa 330–339) we concluded that the tms1 binding domain appears to be a single site representing a new functional domain on the p53 protein.

Recently it has been established that amino acids 319–360 constitute the minimal tetramerization unit of p53 and the three dimensional structure of the tetramerization domain spanning amino acids 319–360 has been solved by multidimensional NMR [20]. The domain was found to form a 20 kDa symmetric tetramer made up from a dimer of dimers which comprise two antiparallel helices linked by an antiparallel β sheet. The turn spanning amino acids 334–337 which links the helices to the β sheets is likely to be exposed to the surface of the p53 protein [20]. Actually this is in good agreement with the mapped site for tms1 binding on the p53 protein spanning from amino acids 330–339 as it is indicated in Fig. 5.

Most of the p53 mutations detected in human tumours map to the core domain of p53 which seems to be responsible for the specific DNA binding of p53 and apart from stop and frameshift mutations only four point mutations within the C-terminus of p53 have been reported. Three out of these four point mutations are located within the mentioned turn region, namely Leu^{330}→His, Gly^{334}→Val and Arg^{335}→Cys [40–42]. It was reasoned that these point mutations are potentially destabilizing the tetrameric structure of p53 since they are located at sites nearby the helix and β sheet interactions [20]. This implies that either tms1–p53 complex formation requires the oligomerization of p53 or these mutants are still capable for oligomerisation however fail to bind proteins like the tms1

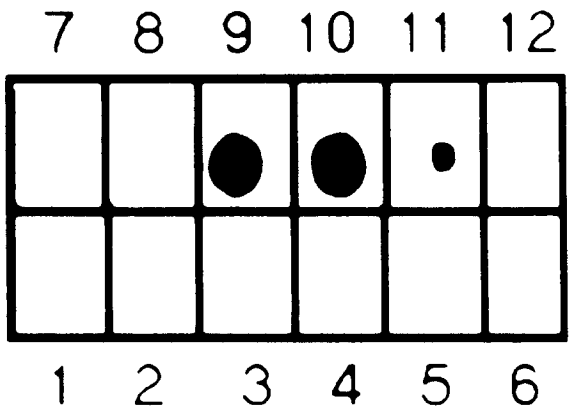

Fig. 4. tms1 binding assy on the peptide library. The membrane carrying the peptide library was probed with purified ^{32}P-labelled tms1 protein, washed and subjected to autoradiography.

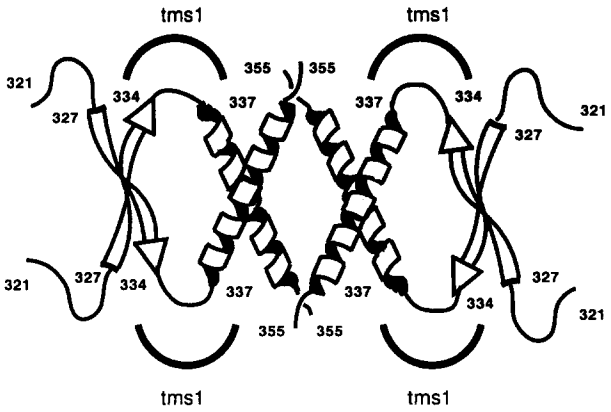

Fig. 5. Tetramerization domain of p53. The cartoon represents the p53 tetramerization domain according to the published three-dimensional structure [20] showing the mapped tms1 binding sites.

protein which are likely to modulate p53 function. These issues are currently being addressed.

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