p202 self-associates through a sequence conserved among the members of the 200-family proteins

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Received 6 August 1998; received in revised form 25 September 1998

Abstract Murine p202 is an interferon-inducible primarily nuclear phosphoprotein (52 kDa) whose expression in transfected cells inhibits colony formation. p202-binding proteins include the pocket proteins (pRb, p107 and p130), a p53-binding protein (sm53BP1), and transcription factors (e.g. NF-κB (p50 and p65), AP-1 (c-Fos and c-Jun), E2F-1, E2F-4, MyoD, and myogenin). p202 modulates the transcriptional activity of these factors in transfected cells. Here we demonstrate that p202 self-associates directly and a sequence in p202, which is conserved among the members of the 200-family proteins, was sufficient for self-association in vitro. Our observations reported herein raise the possibility that self-association of p202 may provide a mechanism for the regulation of its activity.

Key words: p202; Self-association; Protein-protein interaction motif; Conserved sequence; 200-family proteins

1. Introduction

p202 is a member of the 200-family proteins [1,2]. The proteins (i.e. murine p202, p203, p204, and D3; human MNDA and IFI16) [3–6] in the family share partially conserved 200-amino acid-long segments. p202, p204, and IFI16 proteins include two partially conserved 200-amino acid segments, one **a**-type and one **b**-type [1,2]. The other proteins contain a single **a**-type or **b**-type segment.

p202 is primarily nuclear phosphoprotein (52 kDa) whose levels are increased in cultured cells in response to treatment with interferon (IFN) [7] and during differentiation of murine skeletal muscle cells in vitro [8]. Moreover, constitutive overexpression of p202 in transfected cell lines inhibits colony formation ([3,7,9]; Choubey, unpublished data) and inhibition of p202 expression in murine fibroblasts increases their susceptibility to cell death by apoptosis [10]. p202-binding proteins include the pocket proteins (pRb [11], p107 [12], p130 [12]), a p53-binding protein (sm53BP1 [13]), and transcription factors (NF-κB (p50 and p65) [9], AP-1 (c-Fos and c-Jun) [9], E2F-1 [14], E2F-4 [12], MyoD [8], and myogenin [8]). Moreover, p202 modulates the transcriptional activity of these factors in transfected cells [9,12–14]. p202 also binds to single- and double-stranded DNA non-specifically in vitro and the N-terminal segment in p202 comprising a non-conserved sequence was found to be sufficient for this binding [15].

The partially conserved 200-amino acid segments present among the members of the 200-family proteins are unique [1] and appear to have a transcriptional repression domain [16]. The longest continuous sequence conserved in the 200-amino acid-long segments found in the 200-family proteins is M(F/L)HATVA(T/S) [1]. The sequence MFHATVAT is present in p202, p203, p204, D3 and IFI16 proteins [1]. Moreover, in p202 amino acid substitution of His by Phe in both conserved MFHATVAT sequences (i.e. in **a**-type segment amino acids 82–89 and in **b**-type segment amino acids 281–288, see Fig. 1 in [3]) results in loss of interaction of p202 with sm53BP1 in yeast two-hybrid assays [13].

The study reported here was started by examining interactions of in vitro-translated labeled p202 in affinity chromatography with various bacterially expressed glutathione-*S*-transferase (GST) fusion proteins, including the GST-p202. These experiments revealed that p202 has the ability to self-associate. This prompted us to identify a segment/motif in p202 which is involved in self-association. Here we report that p202 directly binds to itself and two segments in p202, each containing the conserved sequence MFHATVAT found in the members of the 200-family proteins, are able to associate with p202. Moreover, we demonstrate that this conserved sequence is sufficient for self-association of p202 in vitro.

2. Materials and methods

2.1. Plasmids

Plasmids to express GST, GST-p202(19–445), GST-p202(58–291), GST-p202(255–445), GST-p202(255–293) and GST-p202(295–445) proteins have been described previously [11]. To express GST-p202(82–89), a double-stranded deoxyoligonucleotide encoding the amino acid sequence MFHATVAT in p202 was synthesized and ligated in frame into the *Sma*I site of pGEX expression vector (Pharmacia). The resulting plasmid was sequenced to confirm the open reading frame. To express GST-p204(31–640) *Msc*I-*Bam*HI fragment of pBluescript-204 (see Fig. 3 in [3]) was ligated in frame into pGEX expression vector (Pharmacia). The resulting plasmid was sequenced to confirm the open reading frame.

For generating labeled p202, mRNA-encoding p202 was transcribed in vitro from plasmid pBluescript-202 and translated in vitro in rabbit reticulocyte lysate supplemented with [^{35}S]methionine as described previously [11].

2.2. Expression of GST fusion proteins and loading of glutathione-Sepharose beads

The GST fusion proteins were expressed in *Escherichia coli* BL21 and affinity purified as described by Kaeline et al. [17]. The beads were loaded with ~0.5 mg of the indicated GST fusion protein, blocked in blocking buffer (0.25% gelatin, 50 mM KCl, 50 mM HEPES, pH 7.5) at room temperature for 30 min, washed in binding buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 0.1% NP-40), and used for affinity chromatography.

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PII: S0014-5793(98)01263-0

2.3. Affinity chromatography of proteins

Aliquots (10 μl) from the reaction mixtures in which [^{35}S]methionine-labeled proteins had been translated in vitro were incubated with washed glutathione-Sepharose beads (loaded with the indicated GST fusion protein) in binding buffer at room temperature for 30 min. The beads were washed four times in binding buffer, and the bound proteins were released by boiling in sample buffer [18] and subjected to SDS-PAGE followed by fluorography.

2.4. Far-Western blotting

Far-Western blotting was performed using in vitro-translated (IVT) [^{35}S]methionine-labeled p202 as described previously [11].

2.5. Chemical cross-linking with dimethylsuberimidate (DMS)

In vitro-translated labeled p202 reaction mixture (10 μl) was diluted 1:20 in cross-linking buffer (20 mM HEPES, pH 8.0, and 100 mM NaCl) and cross-linked with dimethylsuberimidate (final concentration 1 mM) at room temperature. Aliquots were removed at the indicated times and the reaction was stopped by adding 1 M glycine to a concentration of 100 mM. Proteins were then analyzed by SDS-PAGE on a 7% gel followed by fluorography.

2.6. Peptide synthesis and characterization

Peptides containing the amino acid sequence MFHATVAT and MFGATVAT were synthesized on Polymer Laboratory's (Amherst, MA, USA) PL-DMA resin using 4-hydroxymethyl phenoxyacetic acid as the linking agent. The peptides were synthesiszed by sequential addition of *t*-butyl protected Fmoc amino acids using standard solid phase techniques [19]. The peptides were cleaved and deblocked from the resin using 5% phenol in trifluoroacetic acid. The cleaved peptides were purified to homogeneity by reverse phase HPLC using a gradient of acetonitrile in water with 0.1% trifluoroacetic acid. The molecular masses of the peptides were confirmed by FAB-mass spectrometry.

3. Results and discussion

3.1. Self-association of p202

To test whether p202 self-associates, we incubated in vitro-translated labeled p202 with glutathione-Sepharose beads bound to GST alone, GST-p202(19–445) or GST-p204(31–640). As seen in Fig. 1A, p202 did not bind to beads bound by GST alone but bound selectively to beads bound to GST-p202(19–445). p202 also bound to GST-p204(31–640) (compare lane 2 with lane 4), albeit weakly (p204 protein contains one **a**-type and one **b**-type 200-amino acid segment found in p202 protein [3]). This experiment revealed that in vitro p202, directly or indirectly, bound to GST-p202(19–445).

To test if p202 could bind to p202 in the absence of GST fusion protein, we performed a far-Western assay. As seen in Fig. 1B, in this assay labeled p202 did not bind to GST or several protein markers but did bind selectively to GST-p202(19–445), suggesting that p202 can directly bind to p202.

To support our conclusion further, we incubated in vitro-translated [^{35}S]methionine-labeled p202 with a chemical cross-linker and the reaction products were analyzed by SDS-PAGE followed by fluorography. As seen in Fig. 2, protein bands with molecular weights of about 105 and 150 kDa equivalent to a dimer and possibly trimer of p202 were observed. These observations are consistent with dimerization/oligomerization accounting for the self-association of p202.

3.2. Localization of self-association segment in p202

To identify a segment in p202 to which p202 associates, we utilized a series of C- and N-terminally truncated p202 proteins fused to the GST protein. The fusion proteins bound to glutathione-Sepharose beads were incubated with labeled p202 and the bound proteins were analyzed by SDS-PAGE. As shown in Fig. 3 (upper panel), p202 did not bind to GST or GST-p202(295–445) but it selectively bound to GST-p202(19–445), GST-p202(58–291), GST-p202(255–445), and GST-p202(255–293).

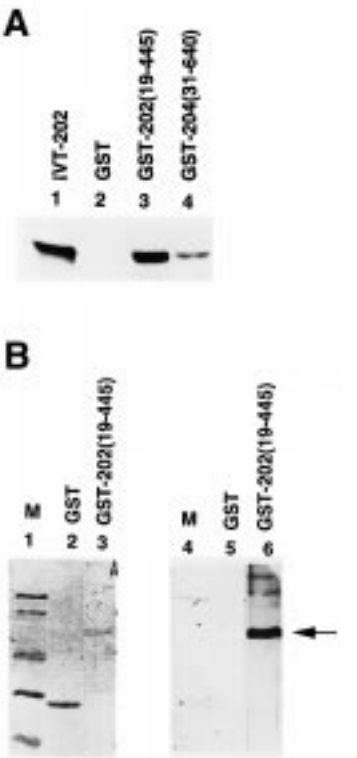

Fig. 1. Self-association of p202. A: Affinity chromatography. In vitro-translated p202 (10 μl of reaction) was incubated with the glutathione-Sepharose beads loaded with glutathione-*S*-transferase (GST) (lane 2), GST-p202(19–445) (lane 3) or GST-p204(31–640) (lane 4). The bound proteins were analyzed by SDS-PAGE followed by fluorography. As a control, 5 μl of total rabbit reticulocyte lysate reaction containing p202 was also run in lane 1. The p202 protein band is indicated. B: Far-Western assay. Affinity-purified GST-p202(19–445) (0.5 μg) (lanes 3 and 6), GST (2 μg) (lanes 2 and 5), and unstained protein markers (M) (myosin heavy chain, 200 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 68 kDa; and ovalbumin, 43 kDa) (lanes 1 and 4) were subjected to SDS-PAGE, blotted to a membrane, and stained for proteins with Ponceau S (lanes 1–3) or processed for far-Western blotting using labeled p202 protein translated in vitro (lanes 4–6). The p202 protein band is indicated.

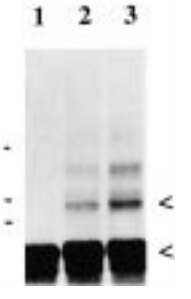

Fig. 2. Chemical cross-linking of in vitro-translated p202 protein. [^{35}S]Methionine-labeled p202 was left without any treatment (lane 1) or cross-linked with dimethylsuberimidate (1 mM final) at room temperature for 30 min (lane 2) or 60 min (lane 3) as described in Section 2. The reaction products were analyzed by SDS-PAGE (7%) followed by fluorography. The prestained molecular weight markers (indicated towards left side) from top to bottom were: 175 kDa, 83 kDa and 63 kDa. The p202 bands equivalent to monomer and dimers are indicated by arrowheads.

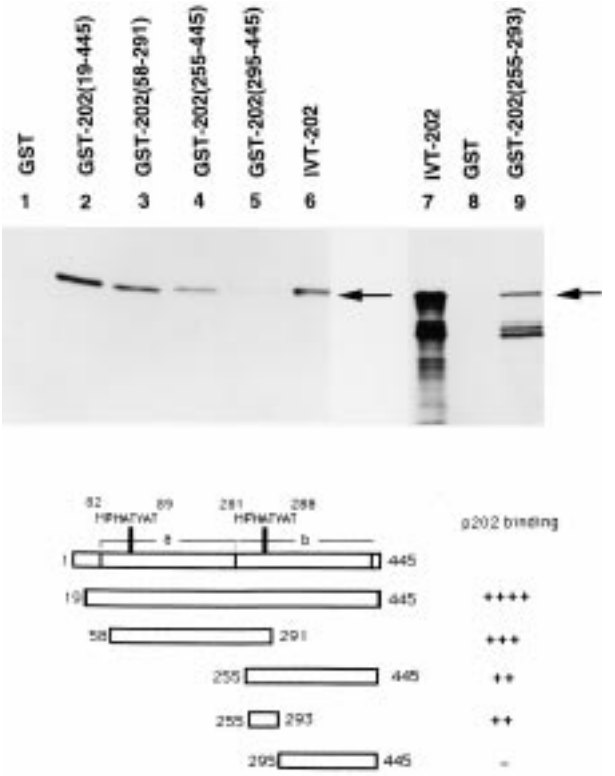

Fig. 3. Localization of self-association segments in p202. Upper panel: Affinity chromatography. In vitro-translated labeled p202 was incubated with glutathione-Sepharose beads loaded with GST (lanes 1 and 8), GST-p202(19–445) (lane 2), GST-p202(58–291) (lane 3), GST-p202(255–445) (lane 4), GST-p202(295–445) (lane 5) or GST-p202(255–293) (lane 9). The bound proteins were analyzed by SDS-PAGE followed by fluorography. As a control, aliquots of total reactions were run in lanes 6 and 7. The full-length p202 protein band is indicated. (It should be noted that the multiple p202 protein bands seen in lane 9 result from binding of the truncated p202 proteins to GST-p202(255–293). These truncated p202 proteins result from the initiation of translation of p202 protein at the internal protein initiation sites in 202 mRNA and/or premature termination of translation of p202 protein in vitro, see [3].) Lower panel: Schematic representation of p202, its segments, and their abilities to bind p202 in affinity chromatography. The thin vertical lines indicate the borders of the 200 amino acid repeat regions **a** and **b**. The thick vertical lines indicate the positions of the conserved MFHATVAT sequences. The numbers of the N- and C-terminal aminoacyl residues of these sequences are indicated. +++, Strong binding; ++, weak binding; −, no detectable binding.

3.3. The sequence MFHATVAT in p202 is sufficient for self-association

Since the minimal GST-p202(58–291) and GST-p202(255–293) fusion proteins, which bound to p202, both contained the common amino acid sequence MFHATVAT (see Fig. 3, lower panel) conserved among the members of the 200-family proteins [1], we tested binding of p202 to GST-p202(82–89) containing the sequence MFHATVAT. As shown in Fig. 4 (left panel, lane 3), IVT-p202 selectively bound to GST-p202(82–89). Moreover, incubation of GST-p202(82–89) with increasing concentrations of a peptide containing the wild-type amino acid sequence MFHATVAT reduced binding of p202 to GST-p202(82–89) (left panel, compare lane 3 with lane 6). However, incubation of GST-p202(82–89) with a peptide containing mutated sequence (MFGATVAT), at the concentrations tested, did not appreciably reduce binding of labeled p202 to GST-p202(82–89) (right panel, compare lane 3 with 4 or 5). These observations demonstrate that the conserved sequence MFHATVAT is sufficient for self-association of p202 in vitro.

We earlier reported that almost full-length p202(19–445) weakly associated with in vitro-translated retinoblastoma protein (pRb) [11] and transcription factor E2F-1 [14] in affinity chromatography. In contrast, segments of p202, containing only one conserved MFHATVAT sequence, showed a greater degree of association with pRb [11] and E2F-1 [14]. In our present study, almost full-length GST-p202(19–445) bound p202 more efficiently than either of the p202 segments containing a single MFHATVAT motif (Fig. 3, lower panel). Thus the presence of two MFHATVAT motifs may result in cooperative binding and increased homodimerization of p202. This homodimerization in turn might impair the ability of p202 to associate with pRb and E2F-1.

MNDA, a member of the 200-family proteins, was reported to dimerize in vitro through a non-conserved segment [20]. However, it remains conceivable that in addition to this segment MNDA protein also dimerizes through the conserved MFHATVAS sequence. It is possible that this segment of MNDA protein, in contrast to p202 protein, did not renature appropriately following denaturation, thus making it difficult to detect dimerization using the far-Western technique used [20].

Since the MFHATVAT motif is conserved in the 200-amino acid-long repeats found in other members of the 200-family proteins (i.e. p204 **b**-type segment and IFI16 segments), it is conceivable that these proteins could also associate with p202 through this motif. Consistent with this prediction, we found that p204 associated with p202, albeit weakly (Fig. 1), and IFI16 protein from extracts prepared from Daudi cells also associated with GST-p202 in affinity chromatography (data not shown). As noted previously [13], mutation of the conserved His in the MFHATVAT motif in p202 abrogates the interaction of p202 with sm53BP1 and, intriguingly, expres-

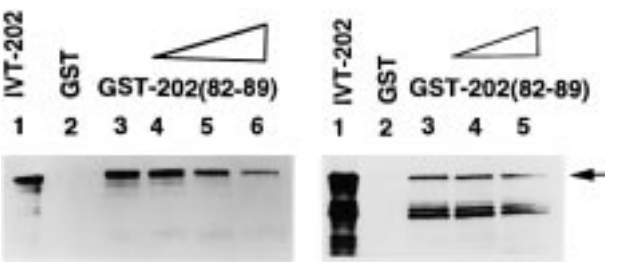

Fig. 4. Binding of labeled p202 to GST-p202(82–89) in the presence of a peptide containing the wild-type (MFHATVAT) (left panel) or mutated (MFGATVAT) (right panel) amino acid residues. Left panel: Glutathione-Sepharose beads loaded with GST (lane 2) or GST-p202(82–89) (lanes 3–6) were incubated with labeled p202 protein in the absence (lanes 2 and 3) or presence of a peptide containing the wild-type (MFHATVAT) amino acid sequence (lanes 4–6; lane 4: 20 mole excess of peptide; lane 5: 50 mole excess of peptide; lane 6: 100 mole excess of peptide) in binding buffer. Right panel: Glutathione-Sepharose beads loaded with GST (lane 2) or GST-p202(82–89) (lanes 3–5) were incubated with labeled p202 protein in the absence (lane 2) or presence of a peptide containing the mutated (MFGATVAT) amino acid sequence (lanes 4 and 5; lane 4: 50 mole excess of peptide; lane 5: 100 mole excess of peptide) in binding buffer. The bound proteins were analyzed by SDS-PAGE followed by fluorography. The full-length p202 protein band is indicated.

sion of sm53BP1, which appears to bind p202 through the conserved MFHATVAT sequence, overcomes p202-mediated inhibition of cell growth in yeast [13].

Taken together, our observations reported herein raise the possibility that self-association of p202 and/or its association with other members of the 200-family proteins could alter its interactions with other proteins. Moreover, our observations reported herein will facilitate studies to examine whether self-association of p202 alters its ability to repress transcription [16] and/or cell growth regulation [3,7,9,10].

Acknowledgements: This research was supported by a grant (CA69031) from the National Institutes of Health to D.C.

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