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Characterization of cDNAs encoding p53 of *Bombyx mori* and *Spodoptera frugiperda*

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ABSTRACT

Complementary DNAs encoding homologs of the tumor suppressor gene, p53, were characterized from two lepidopteran insects, *Bombyx mori* (Bm) and *Spodoptera frugiperda* (Sf). They encoded predicted proteins of 368 (41.2 kDa) (Bm) and 374 (42.5 kDa) (Sf) amino acids. The sequences shared 44% amino acid and 60% nucleotide sequence identity with each other, but exhibited less than 20% amino acid and 46% nucleotide sequence identity to *Drosophila melanogaster* p53. Despite the sequence diversity, conserved amino acids involved in DNA and zinc binding were present in the lepidopteran sequences. Expression of Sfp53-induced apoptosis in *S. frugiperda* cells, and antiserum made against recombinant Sfp53 recognized a protein whose abundance increased after treatment with DNA damaging agents.

1. Introduction

Members of the p53 family of proteins are involved in the induction of apoptosis, the regulation of the cell cycle, and tumor suppression, and have been described from a variety of vertebrates, invertebrates, and protists (Lane et al., 2010; Lu et al., 2009; Mendoza et al., 2003). Although p53 has been widely studied because of its role in tumor suppression, this activity was an unlikely stimulus for its evolution because the abbreviated life spans of invertebrates and protists normally would preclude them from developing cancer. Furthermore, in *Drosophila*, in response to DNA damage, p53 is required for apoptosis, but not for cell cycle arrest (reviewed in Rutkowski et al. (2010)). Therefore it has been suggested that p53 may have evolved because of its role in the induction of apoptosis (Sutcliffe and Brehm, 2004), possibly as a critical element for the regulation of invertebrate development. However, recently *Drosophila* p53 has been demonstrated to be involved in cell cycle arrest in response to metabolic stress (Mandal et al., 2010).

Although a number of p53 genes have been identified in insects (see below), information from insects in the order Lepidoptera is limited. This is despite the fact that two of the major categories of anti-apoptotic genes, p35 and the inhibitor of apoptosis (iap) family were originally discovered in baculoviruses that infect lepidopteran insects (Clem et al., 1991; Crook et al., 1993; Herschberger et al., 1992). In addition, a conserved baculovirus protein encoding a sulfhydryl oxidase (Ac92) has been identified (Long et al., 2009; Nie et al., 2011; Wu et al., 2006) that binds to human p53 (Long et al., 2009; Prikhod'ko et al., 1999) suggesting a possible interaction that might influence p53 function in insect cells during viral infection. Therefore, in order to provide the basis for the examination of the role of p53 in lepidopteran and baculovirus biology, cDNAs encoding p53 were identified and characterized in two of the major model systems used for investigations of these organisms. These include *Bombyx mori* (the silk moth) that is widely used for investigation of baculovirus biology and for which a draft genome sequence is available (Mita et al., 2004; Xia et al., 2004) and *Spodoptera frugiperda* (the fall army worm) that is the source of the IPLB-Sf21AE cell line and its clonal derivative Sf9, which are important cell lines used in baculovirus research and recombinant protein expression (Vaughn et al., 1977).

2. Materials and methods

2.1. Complementary DNA isolation, RACE analysis, and sequence determination

To determine the sequence of *B. mori* p53 (Bmp53) cDNA, primers, Bmp53-2 and Bmp53-6r (Table S1) were derived from the

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sequence of two predicted ORFs of *B. mori* (BG1BMGA013185 and BG1BMGA013186). These sequences are from the *B. mori* genome database (NIAS, 2009) and were generated using the GLEAN program (Elsik et al., 2007) and encode sequences with similarity to two different regions of other p53 coding sequences. The primers were used to PCR amplify a contiguous open reading frame incorporating both of the predicted *B. mori* p53 ORF sequences using total cDNA as a template. The cDNA was produced from total RNA purified from *B. mori* larvae (Mulberry Farms, Fallbrook, CA) using a Qiagen RNAeasy and Bio-Rad iScript kits. The ORF identified did not appear to encode the complete 5′ region of Bmp53. In order to determine the upstream coding sequence, 5′ RACE (Invitrogen 5′ RACE kit) was undertaken using additional primers (Table S1) and the Invitrogen 5′ RACE primers AAP and UAP. In addition, 3′ RACE analysis was carried out using an Invitrogen 3′ RACE kit and the primers listed in Table S1.

To determine the *S. frugiperda* p53 sequence, the partial p53 sequence from *Heliothis virescens* (Database ID: BD250014) was aligned to that of *B. mori* and highly conserved regions were identified. The primers (Sfp53-1 + Sfp53-3r and Sfp53-3 + Sfp53-4r) (Table S2) were used to amplify two adjacent fragments from Sf cDNA resulting in about 315 nt of sequence. Note: although these primers are designated as Sfp53 primers, they are from the *H. virescens* sequence and, in general, may differ by a few nt from the Sfp53 sequence. The template used was a directionally cloned *S. frugiperda* cDNA library purchased from Invitrogen (Rasmussen and Rohrmann, 1994).

Based on this sequence, additional primers were synthesized and the 3′ and 5′ regions of the cDNA were determined. To determine the 3′ end, 3′ RACE analysis employing the following primers were used in conjunction with the 3′ primers from the Invitrogen 3′ RACE kit, Sfp53-3.1 + AP followed by Sfp53-3.3 + AUAP (Table S2). For determination of the 5′ region, the M13 forward primer (M13F) was used with two internal primers. These reactions were PCR 1 (M13F + Sfp53-2.2r) followed by PCR 2 (M13F + Sfp53-2.1r) (Table S2).

2.2. Construction of Sfp53 clones

Construction of the plasmid pHA-Sfp53 (pGR09-10) was done as follows. The sequence of HA-Sfp53 was synthesized by Genscript, Inc., and was designed such that it contained NcoI and NdeI sites followed by an HA tag (see 5′HA-sfp53, Table S3). This yielded the amino acid sequence MAHMYPYDVPDYA at the N-terminus (HA tag is underlined) fused to the sfp53 sequence beginning with amino acid #2 (E) of the native sequence. The restriction enzyme sites at nt 199 (NcoI), 154 (EcoRV), 228 (EcoRI), and 1026 (SacI) were eliminated by single nt changes that did not alter the amino acid sequence. An NcoI site was included at the 3′ end of the gene followed by a stop codon so that the 3′ terminal sequence was CAGTCCATGGCATAA (−QSMA). The synthesized HA-Sfp53 sequence was inserted into an expression plasmid similar to pEGFP (Pearson et al., 2000). This plasmid contained the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) ie1 promoter region (genome nucleotides 126599–127197) (Ayres et al., 1994) and a 3′ processing signal from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) (nt 112503–112796) from the p10 region (Ahrens et al., 1997). The construct (pGR09-05), was digested with NcoI and NotI and two oligomers (Nco-Not-2 and Nco-Not-comp-2) (Table S3), were annealed and inserted into the cut vector creating a polylinker. The resulting construct (pGR09-5.5) was digested with NcoI and the NcoI insert encoding HA-Sfp53 from the Genscript plasmid described above was inserted. This resulted in the construct pHA-Sfp53 (pGR09-10) expressed from the AcMNPV IE-1 promoter. Construction of the negative control plasmids expressing IE-1 (pBSIE-1HC) or GFP (pFB1-PH-GFP) have been previously described (Ribeiro et al., 1994; Wu et al., 2006). pHA-Sfp53, pBSIE-1HC and pFB1-PH-GFP all utilize the IE-1 promoter to drive expression of the inserted gene. For bacterial expression, the insert from the Genscript parent clone was removed with NdeI and HindIII and ligated into pET-28a (Novagen) yielding a His6-tagged construct, pHis6-Sfp53.

2.3. Transfection of Sf9 cells

Transfections were done using Sf9 cells, which were maintained at 27 °C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), penicillin G (60 μg/ml), streptomycin sulfate (200 μg/ml), and amphotericin B (0.5 μg/ml). Sf9 cells (10^6) were plated in 6 well plates for 2 h in TC-100 medium with 10% FBS. After 2 h, the medium was discarded and replaced with Grace's Insect Medium (Invitrogen) lacking serum. Cells were transiently transfected using 3 μg of each plasmid and 8 μl of a liposome mixture (Crouch and Passarelli, 2002). After 5 h of incubation, transfection mixtures were replaced with TC-100 containing 10% FBS. zVAD-FMK (MP Biomedicals) was added at a final concentration of 20 μM to some of the wells at the time when the transfection mixtures were replaced.

2.4. Caspase analysis

To measure caspase activity after transient expression, cells were harvested at 12 h post-transfection, and cell pellets were washed with PBS (pH 6.2). Caspase activity of transient expression of each plasmid was measured as described previously (Wang et al., 2008). To measure cell viability, Sf9 cells were transfected in the same way as above, and four random fields of view were photographed (400× magnification) at 24 h post-transfection. Viability was determined by counting the non-apoptotic cells, and comparing the numbers of viable cells obtained relative to untreated cells.

2.5. Antiserum production and western blot analysis

Recombinant N-terminally His-tagged Sfp53 was expressed in strain *Escherichia coli* strain BL21, purified using Talon resin (Clontech) and injected into rabbits for antiserum production. Animal injections and serum collection were performed by Covance, Inc. When required, cells were treated with UV (254 nm) by placing the plate on a transilluminator for the indicated amount of time, and then harvested at 4 h post-UV treatment. Camptothecin (Sigma) was added at a final concentration of 10 μM and cells were harvested 4 or 24 h later. For Western blotting, cells were washed twice with PBS (pH 6.2), mixed with equal volumes of 2× Laemmli buffer and heated at 100 °C for 5 min. The extracts from equivalent numbers of cells were subjected to 12% SDS–PAGE, transferred to PVDF membranes (Millipore, 1991), and incubated with monoclonal antibody against the HA epitope (Covance), polyclonal anti-GAPDH antibody (Abcam), or anti-Sfp53 antiserum. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (Sigma), blots were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce).

3. Results and discussion

3.1. Sequence determination and RACE analysis of B. mori p53

Sequence data from the *B. mori* genome sequencing project with homology to other p53 genes was used to design primers to PCR amplify a major region of the Bmp53 coding sequence from