A New Invertebrate Member of the *p53* Gene Family Is Developmentally Expressed and Responds to Polychlorinated Biphenyls

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The cell-cycle checkpoint protein p53 both directs terminal differentiation and protects embryos from DNA damage. To study invertebrate p53 during early development, we identified three differentially expressed p53 family members (p53, p97, p120) in the surf clam, *Spisula solidissima*. In these mollusks, p53 and p97 occur in both embryonic and adult tissue, whereas p120 is exclusively embryonic. We sequenced, cloned, and characterized p120 cDNA. The predicted protein, p120, resembles p53 across all evolutionarily conserved regions and contains a C-terminal extension with a sterile alpha motif (SAM) as in p63 and p73. These vertebrate forms of p53 are required for normal inflammatory, epithelial, and neuronal development. Unlike clam p53 and p97, p120 mRNA and protein levels are temporally expressed in embryos, with mRNA levels decreasing with increasing p120 protein (R^2 = 0.97). Highest surf clam p120 mRNA levels coincide with the onset of neuronal growth. In earlier work we have shown that neuronal development is altered by exposure to polychlorinated biphenyls (PCBs), a neurotoxic environmental contaminant. In this study we show that PCBs differentially affect expression of the three surf clam p53 family members. p120 mRNA and protein are reduced the most and earliest in development, p97 protein shows a smaller and later reduction, and p53 protein levels do not change. For the first time we report that unlike p53 and p97, p120 is specifically embryonic and expressed in a time-dependent manner. Furthermore, p120 responds to PCBs by 48 hr when PCB-induced suppression of the serotonergic nervous system occurs. *Key words:* neurotoxicology, p63, p73, PCBs, surf clam, *Spisula solidissima*. *Environ Health Perspect* 110:377–385 (2002). [Online 7 March 2002]

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Protein 53 (p53) is a critical regulator of cell cycle progression that is stabilized in response to DNA damage by phosphorylation at the N-terminus [reviewed in Levine (*1*)]. This stabilization enables p53 to form homotetramers that bind to the promoters of several genes involved in the cell cycle, ultimately producing either cell-cycle arrest or apoptosis. Loss of p53 function can cause unchecked cell growth and contribute to carcinogenesis. Approximately 50% of all human cancers contain mutant p53 (*2*). p53 also appears to play a role in embryonic development [reviewed by Choi and Donehower (*3*)]. During embryogenesis p53 levels positively correlate with proliferation and are relatively low in terminally differentiated cells. p53 also activates differentiation of many cell types in culture.

During murine development p53 levels and activity are highest in the nervous system (*4*), which is most sensitive to changes in p53 (*3*). A small percentage of mice lacking p53 have a neural tube defect. The remaining p53-deficient mice grow normally, but cannot survive DNA damage or environmental stress, and tend to develop spontaneous tumors. Mice may compensate for a p53-deficiency during embryogenesis by employing functionally similar genes. Two *p53*-like genes, *p63* (also known as KET, *p40*, *p51*, and *p73L*) and *p73*, have been identified and sequenced in a wide range of vertebrates, including mice and humans [reviewed by Kaelin (5), Levrero et al. (6), and Strano et al. (*7*)].

Both p63 and p73 can activate p53-regulated genes, and their overexpression induces apoptosis (*6,7*). Despite these similarities to p53, p63 and p73 perform different functions critical for early development. Whereas p63 maintains epithelial stem cells, p73 functions primarily in neurologic and inflammatory development. In addition, both p63 and p73 levels are high throughout vertebrate embryo development, whereas p53 expression decreases after the central nervous system matures. In addition, unlike p53, p63 and p73 contain a sterile alpha motif (SAM) within their C-terminal extensions. SAM is important for protein interaction and is involved in developmental regulation (*8*). Furthermore, not all DNA-damaging agents induce p73; mutations of either p73 or p63 in human cancers are infrequent; and p73-deficient mice are not prone to spontaneous tumors (*6,7*).

Thus far, two *p53* family members (*p53* and *p73*) have been sequenced in soft-shell clams (*Mya arenaria*) (*9*), and one (*p73*) in squid (*Loligo forbesi*) (*10*). Another larger homologue, p97, has also been identified in *Mya* using an antibody (*11*). One of the soft-shell clam homologues is expressed throughout gametogenesis (*12*). Both the squid and clam p73-like homologues contain a SAM domain, suggesting these proteins play a role in development.

We have previously conducted experiments linking environmental exposure to polychlorinated biphenyls (PCBs) with changes in molluscan *p53* gene family members (*13*). PCBs are lipophilic, environmentally pervasive chemicals that accumulate in plant, animal, and human tissues, promote tumor formation, can damage DNA (*14,15*), and induce neurotoxic effects during early development in clams (*16,17*) and humans (*18*). *In vitro* fertilization and subsequent developmental studies are easily conducted in the surf clam, *Spisula solidissima* (*16,17,19–21*), but to our knowledge are not successful in either soft-shell clam or squid. During investigation of these embryos we partially cloned, sequenced, and generated a multiple alignment for a new member of the *p53* gene family that codes for an exclusively embryonic 120-kD protein. Because PCBs are particularly neurotoxic during embryonic development, we asked whether this clam p120 responds to PCB exposure.

For the first time we report the differential expression of three p53 members (p53, p97, and the new p120) during embryogenesis. Throughout early development p53 and p97 protein levels are unchanged, but p120 is temporally expressed and apparently regulated at the translational level. After treatment with PCBs, p120 expression is heavily suppressed, p97 is less so, and p53 is

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unaffected. With the *Spisula* model, our data reveal that a new member of the p53 family, p120, is intimately involved in early development and is highly susceptible to environmentally relevant doses of PCBs.

Materials and Methods

Animals and* in vitro *fertilization. Juvenile *Spisula* were kindly provided by Aquaculture Research (Dennis, MA). *Spisula* oocytes, embryos, and veliger larvae were derived from ripe adults collected from Martha's Vineyard, Massachusetts, by the Marine Biological Laboratory (MBL). All animals were maintained and handled in accordance with policies of the Institutional Animal Care and Use Committee at the MBL. Standard surf clam *in vitro* fertilization and culture methods were used (*16*). At the swimming blastula stage, embryos were transferred to 4-L aerated tanks at a concentration of 100 mL. For the p53 protein analyses, larvae were cultured at a density of 1,000 mL in stirred, 250-mL containers as described in Kreiling et al. (*17*). Neither culture method caused significant stress or mortality.

RNA extraction. Whole juvenile (age 1 year) *Spisula* bodies were homogenized in Trizol (Life Technologies, Carlsbad, CA) with a tissue tearer (Biospec Products, Inc., Bartlesville, OK). Oocytes, embryos, and larvae were rinsed through cheesecloth or nitex nets, microcentrifuged at 10,000 rpm for 1 min, and placed in Trizol. Samples were snap frozen in liquid nitrogen and stored at –80°C. After thawing on ice, samples were pulled through a needle and processed according to manufacturer's guidelines.

Reverse transcriptase–polymerase chain reaction. First-strand cDNA synthesis and polymerase chain reaction (PCR) were performed with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) in a Perkin Elmer (Wellesley, MA) 9700 thermocycler according to manufacturer's instructions. Degenerate primers (DegF and DegR) (Table 1) were designed from alignments of squid (*10*) and soft-shell clam (*22*) *p53* to obtain 163 bp of *p53*-like sequence from juvenile surf clam cDNA. Touchdown cycling parameters were 10 min at 95°C; 14 cycles of 30 sec at 95°C, 45 sec at 60°C (decreasing by 1°C per cycle), 30 sec at 72°C; 21 cycles of 30 sec at 95°C, 45 sec at 46°C, 30 sec at 72°C; and 7 min at 72°C.

RACE polymerase chain reaction. Poly(A) RNA was purified from clam oocyte total RNA (1 µg) with an Oligotex mRNA kit (Qiagen, Inc., Valencia, CA). The 5′ end of the p53 gene was obtained using GspR (Table 1) and the Marathon rapid amplification of cDNA ends (RACE) cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA) as recommended by the manufacturer. To confirm the 5′ sequence and obtain the 3′ region, we used the SMART RACE cDNA Amplification kit (Clontech) and primers GspF and GspR (Table 1). New primers designed to amplify the entire consensus sequence were used to obtain a 1590 bp PCR product from 0.5 µg *Spisula* veliger larvae total RNA. The reverse transcriptase–PCR (RT–PCR) reaction conditions were the same as above except 0.5-µM primers were used. Touchdown PCR amplification conditions were 10 min at 95°C; 6 cycles of 30 sec at 95°C, 45 sec at 68°C (decreasing by 1°C per cycle), 30 sec at 72°C; 29 cycles of 30 sec at 95°C, 45 sec at 62°C, 30 sec at 72°C; and 7 min at 72°C. The last 185 bp were obtained with primer 4F (Table 1), new cDNA synthesized from oocyte poly(A) mRNA, and Clontech's SMART RACE kit. Step-down PCR conditions were modified to the following: 5 cycles of 30 sec at 94°C, 3 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, 3 min at 72°C; and 25 cycles of 30 sec at 94°C, 30 sec at 64°C, and 3 min at 72°C. The final contiguous 1774 bp sequence was obtained using the touchdown RT–PCR (above) with primers 1F and 5R (Table 1).

Cloning and sequencing. PCR products were separated on 1–2% agarose gels, excised, extracted (Quiaex II; Qiagen), and ligated into pCR2.1 (Invitrogen, Carlsbad, CA). *E. coli* INVαF′ cells (Invitrogen) were transformed with the ligation reaction and selected on Luria broth (LB) agar containing kanamycin (50 µg/mL) and Xgal (40 µg/mL). Positive colonies identified by a PCR screen using primers DegF and DegR (Table 1) were grown in LB containing ampicillin (50 µg/mL). Plasmid DNA was prepared using a Qiaprep Miniprep kit (Qiagen) and sequenced on an ABI Prism 377 automated sequencer with M13 forward and reverse primers at the Tufts University Core Facility (Boston, MA). Multiple sequences were aligned by CLUSTAL W 1.7 (*23*) and the resulting consensus identified as a p53 homologue with a basic local alignment search tool (BLAST) search (*24*).

Probes. Probe I, synthesized with primers 2F and QuantR (Table 1), corresponded to nucleotides 406–907 within the conserved DNA-binding domain. Probe II, synthesized with primers 4F and 5R (Table 1) corresponded to nucleotides 1200–1774 and was specific for *p120*. Both probes were made by PCR amplification of the target sequence from a full-length *p120* clone. The PCR products were gel purified (above) and digoxygenin-labeled with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Rotkreuz, Switzerland).

Northern blots. We isolated mRNA from total RNA (above) using the Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. mRNAs (3.5 µg/lane) were then separated on a formaldehyde agarose gel, transferred to a nitrocellulose membrane, and hybridized with the probes at 50°C overnight. Probes were detected chemiluminescently with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics) by exposure to Kodak BioMax ML film (Eastman Kodak Co., Rochester, NY) for 3.5 hr at room temperature.

Quantitative RT–PCR. We designed an RNA internal standard for the surf clam *p53*-like sequence as described by Jessen-Eller et

Table 1. Primers used to identify surf clam *p120* (degenerate, RACE, and contiguous), generate probes for Northern blot analysis, make an internal RNA standard, and perform quantitative RT–PCR.

Primers	Forward (5′ to 3′)[a]	Reverse (5′ to 3′)[a]	Size (bp)
Degenerate	DegF: CATGCIGGIWCWGARTGG	DegR: GGRCAIGCRCAKATICKWACYTC	163
RACE	GspF: CCTGTTCCAGTTCATGTGCCTTGG	GspR: CACAATCTGGAGTGGCCTTCTG	69
Contiguous	1F: GGCTTCCAGGAAAAATGTCCG	5R: GTCGGAGGGGACAGTTCTTG	1,774
Probes			
Probe I	2F: ATTACCCAGGCGATTATGGG	QuantR (see below)	501
Probe II	4F: CAGGTGGAAGTCCAAAGGC	5R (see above)	574
Internal standard			
Mutation	MutF: CCACCAACACAAGAGCCAAGGC	MutR: AGGCCACTCCAGATTGTGTTCAC	4,057
Isolation	IsF: CAGGCGGGGACTGAGTGG	IsR: GGGCAGGCGCAGATGCGTACCTC	163
T7/Poly T tail	Tprom: TAATACGACTCACTATAGGCAG GCGGGGACTCAGTGGGTC	Tpoly: T_{18}GCAGGCGCAGATGCGTACCTC	199
Quantitative RT–PCR	QuantF: CAAACCTGTTCCAGTTCATGTG	QuantR: CATCGTCTACCCAATACCTGG	114 & 102

[a]W = A/T; R = A/G; K = G/T; Y = C/T; I = inosine.

al. (*25*). The internal standard was made by ligating the 163 bp segment into pCR2.1 (Invitrogen) and using PCR with primers MutF and MutR (Table 1) to make a 12 bp deletion in the center of the fragment. We then used PCR (above) to isolate the internal standard from the vector with 0.2 µM primers (IsF and IsR; Table 1) and 200 ng plasmid DNA. PCR conditions were as follows: 10 min at 95°C, 30 cycles of 30 sec at 95°C, 45 sec at 60°C, 30 sec at 72°C; and 7 min at 72°C. The resulting 151 bp internal

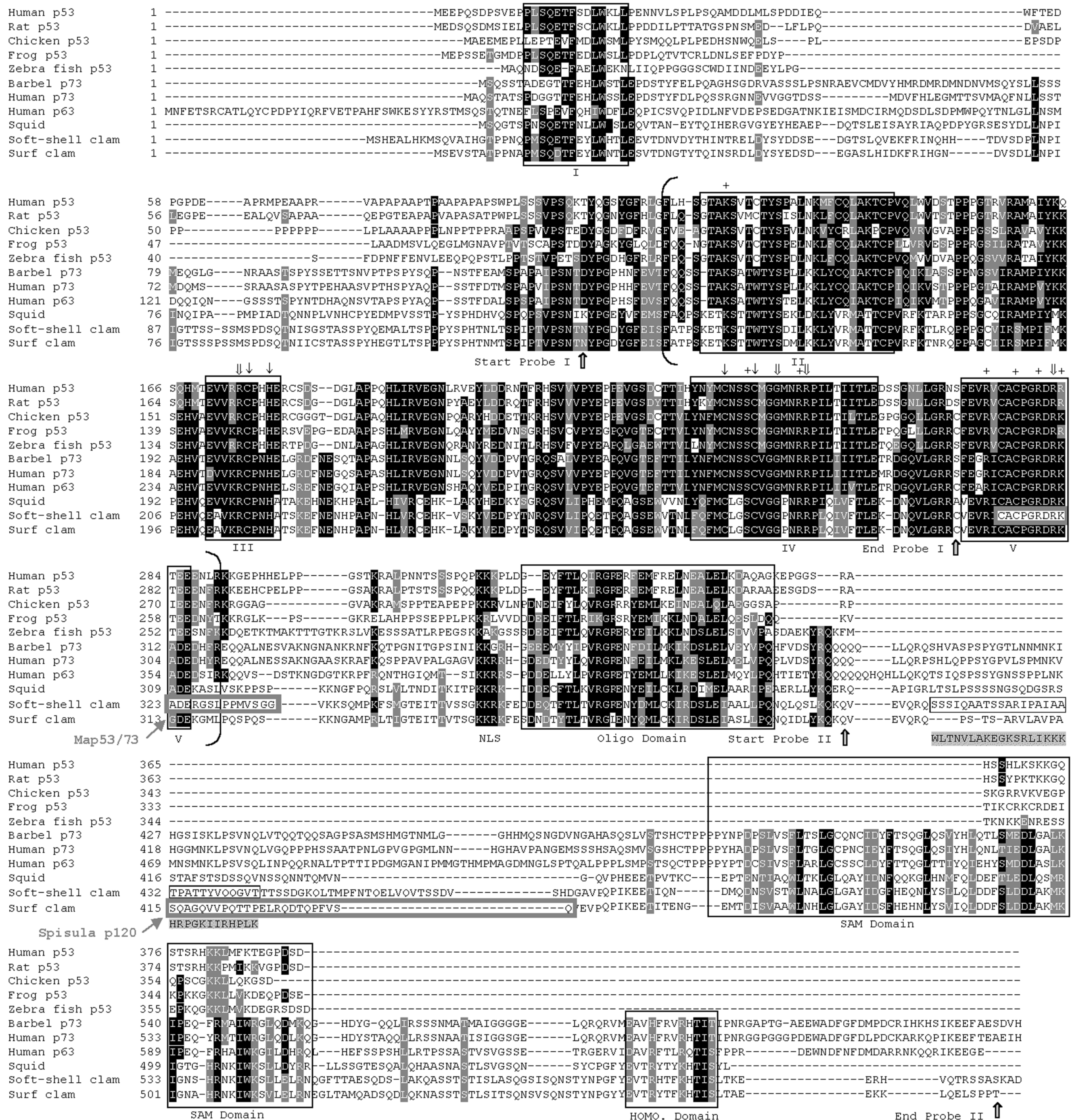

Figure 1. Comparison of deduced amino acid sequence of surf clam p120 (residues 5–588) with molluscan and vertebrate p53 family members from a composite of two multiple alignments (p53 and p53 homologues) using CLUSTAL W 1.7 (*20*). GenBank accession numbers are in Table 2. Positions at which ≥ 50% of the sequence is identical are highlighted in black, and those that are functionally similar are highlighted in gray. Sequence for the truncated splice variant of the soft-shell clam homologue, highlighted in gray, is positioned beneath the alignments as a separate line. Boxes surround the transcriptional activation (I), evolutionarily conserved (II–V), oligomerization (Oligo domain), SAM, and newly identified homologue (Homo domain) domains. The DNA-binding domain is bracketed. Symbols identify amino acids important for transcriptional activation and Mdm2 binding (*), directly contacting the DNA (+), maintaining DNA-binding or oligomerization structure (⇓), and coordinating the zinc atom (↓). Shaded arrows indicate corresponding nucleotide locations used to synthesize Northern blot probes. Locations of peptide antigen sequences used to generate antibodies (*Spisula* p120 and Map53/73) are marked with a bold gray box.

standard was gel purified, cloned, and sequenced as described above.

A T7 promoter sequence and poly(A) tail were added using PCR (above) with 4% of the internal standard PCR product, 3 mM $MgCl_2$, and 0.5 μM Tprom and Tpoly primers (Table 1) to produce a 187 bp product. Cycling proceeded for 10 min at 95°C; 14 cycles of 30 sec at 95°C, 45 sec at 74°C (decreasing by 1°C per cycle), 30 sec at 72°C; and 7 min at 72°C. The product was gel extracted, diluted 1:100, reamplified as above, eluted and gel extracted again, cloned, and sequenced. The cloned internal standard was excised from the vector with *Eco*RI (Life Technologies) and purified with a poly(A) Qiaex II kit (Qiagen). This DNA (10 ng) was transcribed to RNA using MEGAscript *In Vitro* Transcription Kit (Ambion Inc., Austin, TX) and gel purified as instructed.

We quantified p120 mRNA levels as described by Vanden Heuvel et al. (*26*). Total RNA was normalized across all samples. Aliquots (10 or 25 ng) were placed into 3–4 tubes, spiked with a range of RNA internal standard (1×10^3 to 1×10^8 molecules), and reverse transcribed into cDNA. This step controlled for tube-to-tube variability during both the RT and PCR steps. PCR proceeded with half the cDNA, 4 mM $MgCl_2$, and 0.5 μM forward (QuantF) and reverse (QuantR) primer (Table 1). Both target and internal standard were coamplified by incubating for 10 min at 95°C; 29 cycles of 30 sec at 95°C, 45 sec at 62°C, 30 sec at 72°C; and 7 min at 72°C. PCR samples (24%) were run on 6% polyacrylamide gels (NOVEX) and stained in ethidium bromide. Bands were visualized on a UV transilluminator and digitized by Scion Image 4.02 (Scion Corp, Frederick MD), and maximum optical density was determined with Gel-Pro (Media Cybernetics, Silver Spring, MD). We estimated the amount of target p120 mRNA from a simple linear regression as the point at which the ratio of target to internal standard equaled 1. Three to four replicate PCR reactions, each containing a different concentration of internal standard, were loaded on separate gels, and optical readings were taken simultaneously. We took the value of p53 mRNA as the average of this procedure repeated in triplicate.

Western blots. *Spisula* embryo samples were treated as described in Kreiling et al. (*17*), transferred to 8% Laemmli gels normalized to 2 or 4 μg total protein per lane (unless otherwise cited), and transferred with Dunn carbonate/bicarbonate/methanol buffer onto nitrocellulose (*27*). One set of blots was probed with a goat polyclonal antibody (1:500 or 1:1,000 dilutions) made by New England Peptide (Fitchburg, MA) to a 24-amino acid peptide sequence generated from surf clam *p120*. This *Spisula* sequence (SQAGQVVPQTTPELRQDTQPFVSQ) is also identified in Figure 1. These blots were visualized with an alkaline phosphatase-conjugated, donkey antigoat antibody used at a 1:5,000–10,000 dilution (Promega Corp., Madison, WI). Replicate blots were probed with an affinity-purified rabbit polyclonal antibody (Map53/73) (*9*) to a peptide sequence of *Mya* p53 (CACPGRDRKADERGSLPPMVSGG) shown in Figure 1. Map53/73 antibody was used at 1:1,000 dilution and visualized with alkaline phosphatase-coupled goat antirabbit antibody at a 1:7,500 dilution (Promega). In both cases, nonspecific binding was blocked with 5% nonfat dry milk. Immunoreactivity and sample quantification for Western blots probed with either antibody were determined as described in Kreiling et al. (*17*). The signals from four to five separate embryo cultures were integrated across each time point or treatment and normalized to the maximum signal in that culture as a percentage of the maximum (set at 100).

Experimental design for p120 expression. To characterize p120 mRNA expression using quantitative RT–PCR, we cultured surf clam larvae in three groups of 600,000.

Table 2. Comparison of the deduced surf clam p120 amino acid sequence with other invertebrate p53 homologues and vertebrate p53, p63, and p73.

Common name, species	Length	Accession number[a]	Residues 5–573[b] (*p*-values)	Residues 142–316[c] (*p*-values)
Invertebrates				
Soft-shell clam, *Mya arenaria*	621	AF253323 (G)	73% $p = 0.0$	93% $p = 9e-99$
Squid, *Loligo forbesi*	564	AAA98563.1 (G)	53% $p = 3e-161$	81% $p = 3e-84$
Mammalian p63				
Human, *Homo sapiens*	680	CAA76562 (E)	36% $p = 3e-94$	60% $p = 3e-54$
Norwegian rat, *Rattus norvegicus*	680	CAB88216 (E)	36% $p = 5e-92$	60% $p = 3e-54$
Mouse, *Mus musculus*	586	AB010152 (G)	38% $p = 1e-87$	60% $p = 3e-54$
Vertebrate p73				
Human, *H. sapiens*	636	AAD39696.1 (G)	34% $p = 2e-86$	57% $p = 1e-52$
Barbel (fish), *Barbus barbus*	641	AF043641.1 (G)	34% $p = 1e-82$	57% $p = 2e-52$
Mouse, *M. musculus*	497	AF138873 (G)	34% $p = 3e-64$	57% $p = 9e-48$
Vertebrate p53				
Barbel (fish), *B. barbus*	369	AF071570.1 (G)	43% $p = 3e-59$	50% $p = 8e-41$
Zebra fish, *Danio rerio*	373	AAB64176 (G)	40% $p = 2e-57$	47% $p = 9e-40$
Chicken, *Gallus gallus*	367	*P*10360 (S)	39% $p = 7e-52$	46% $p = 4e-40$
African clawed frog, *Xenopus laevis*	361	CAA54672.1 (E)	38% $p = 3e-50$	48% $p = 9e-48$
Mouse, *M. musculus*	389	CAA25323 (G)	36% $p = 1e-47$	45% $p = 2e-37$
Norwegian rat, *R. norvegicus*	391	*P*10361 (S)	36% $p = 3e-47$	42% $p = 1e-35$
Cow, *Bos taurus*	386	Q29628 (S)	38% $p = 7e-53$	44% $p = 6e-39$
Human, *H. sapiens*	393	AF135121 (G)	35% $p = 4e-46$	41% $p = 9e-36$

[a]Accession numbers are from GenBank (G), EMBL (E), and SwissProt (S). [b]Align with the full-length squid transcript. [c]Comprise the conserved DNA-binding domain and provide a more stringent phylogenetic comparison because the sequences were of the same length.

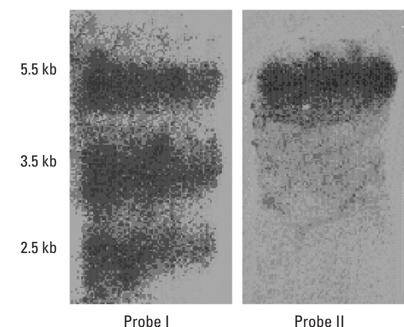

Figure 2. Northern blot analysis. Probe I, synthesized from a conserved region of the *p53* gene family, identified three mRNAs in 24-hr *Spisula* embryos, only the largest of which was recognized by a probe II, specific for p120.

Each group came from a different pair of adult surf clams and was sampled at 12, 24, 36, 48, and 72 hr after fertilization. For p120 mRNA analysis following PCB exposure, 5 million fertilized oocytes were produced by *in vitro* fertilization with two pairs of adults. Immediately after germinal vesicle breakdown, embryos (10,000/mL) were divided into glass petri dishes containing either controls (seawater or acetone) or 100 ppm PCBs (Aroclor 1254; Ultra Scientific, North Kingstown, RI) dissolved in acetone. The high concentration of PCBs was necessary to ensure these lipophilic agents partition their way through the rapidly dividing cell membranes of the embryos. In nature PCBs also accumulate in tissues to saturation and are present at concentrations greater than 4,000 ppm in contaminated sediments of Buzzard's Bay, Massachusetts (*28*). At 5 hr after fertilization, embryos were rinsed in artificial filtered seawater and placed in 4-L aerated culture tanks. Each tank was sampled up to three times per time point at 24, 48, 72, and 96 hr after fertilization. This experiment was duplicated. For the p53, p97, and p120 protein analyses, embryos were exposed to either controls (seawater or acetone) or PCBs (1, 10, or 100 ppm) after 2 hr of development, cultured in 250-mL flasks as described in Kreiling et al. (*17*), and sampled at 24, 48, 72, 96, and 120 hr after fertilization.

Results

***Characterization of surf clam* p120.** A multiple alignment of 5′ and 3′ surf clam *p120* RACE products plus the contiguous sequence produced a 1774 bp consensus sequence that had an apparent poly(A) tail (GenBank Accession No. AF285104; GenBank, National Center for Biotechnology Information, Bethesda, MD). Residues 5–573 of the deduced open reading frame (588 amino acids) aligned with the full-length squid (*Loligo forbesi*) p53 sequence (Figure 1). However, where squid has a stop codon, surf clam p120 continues for another 15 amino acids, within which there are two out-of-frame stop codons before the presumed poly(A) tail.

Residues 5–573 were used in a BLAST comparison of surf clam predicted protein sequence with several p53 family members (Table 2). Overall, surf clam p120 shares the most identity with soft-shell clam p53 (73%), followed by squid p73 (53%), vertebrate p63 (36–43%), and then p73 (34%). It is similarly homologous to vertebrate p53 (35–43%), but the BLAST rank and *p* value were lower because p53 is shorter than the surf clam sequence.

p120 aligns with several functional domains of vertebrate p53 as well as the C-terminal extension of p63, p73, and molluscan homologues (Figure 1). All evolutionarily conserved domains for p53—including the N-terminal transcriptional-activation domain (region I) and four areas within the DNA-binding domain (regions II–V)—are identified. We also located a putative nuclear localization signal (KKRK in mollusks) and an oligomerization domain. Out of 19 amino acids known to be critical for either the structure or function of each domain, 18 are identical and one is conserved.

In general, the surf clam sequence is more similar to p63 and p73 than to vertebrate p53 across all conserved regions. Within the oligomerization domain, identity is 50% with squid p73 and vertebrate p63, 31% with p73, and 25% with p53. Within the C-terminal extension, the surf clam sequence clearly aligns with the SAM identified in the squid homologue, p63, and p73 [reviewed in Levrero et al. (*6*)]. The final 24–29 amino acids of vertebrate p53 also share significant homology within this domain. We identified a new evolutionarily conserved region (surf clam residues 562–573 called "Homo. domain" in Figure 1) in which human p63 and p73 highly resemble the molluscan sequences.

We performed a more stringent phylogenic comparison by subjecting surf clam sequence that aligned with the DNA-binding domain (residues 142–316) to a BLAST search against equal-length p53 family members (Table 2). This analysis clearly shows that surf clam p120 is most similar to the soft-shell clam and squid p73 homologues (93 and 81%, respectively), followed by vertebrate p63 (60%) and p73 (57%). Among

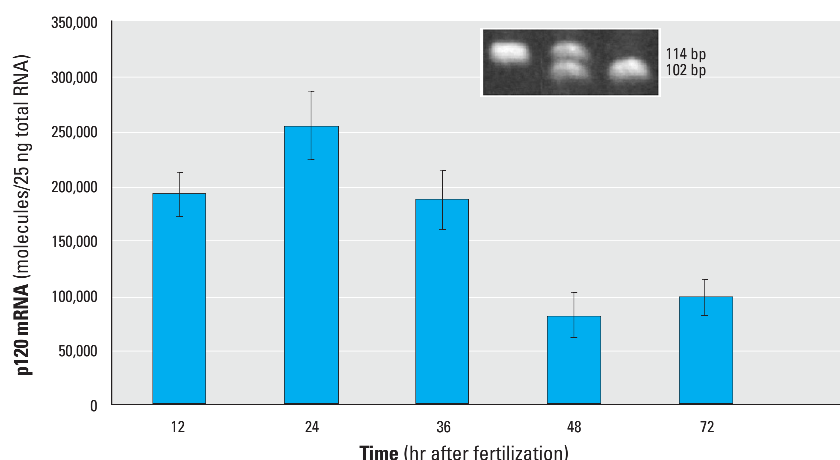

Figure 3. Quantitative RT–PCR analysis of p120 mRNA expression during normal development. p120 mRNA levels decrease from 12 to 72 hr. Scheffe pairwise comparisons revealed a 61 and 68% significantly higher level of p120 mRNA at 24 hr than at 48 or 72 hr, but no significant difference from 12 or 36 hr ($p \leq 0.05$, $n = 7–9$). Bars represent SE. Inset: 6% polyacrylamide gel stained in ethidium bromide showing separation of p120 mRNA RT–PCR product (114 bp) from that of the spiked internal standard (102 bp). Lane 1 = sample alone; lane 2 = sample plus internal standard; lane 3 = internal standard alone.

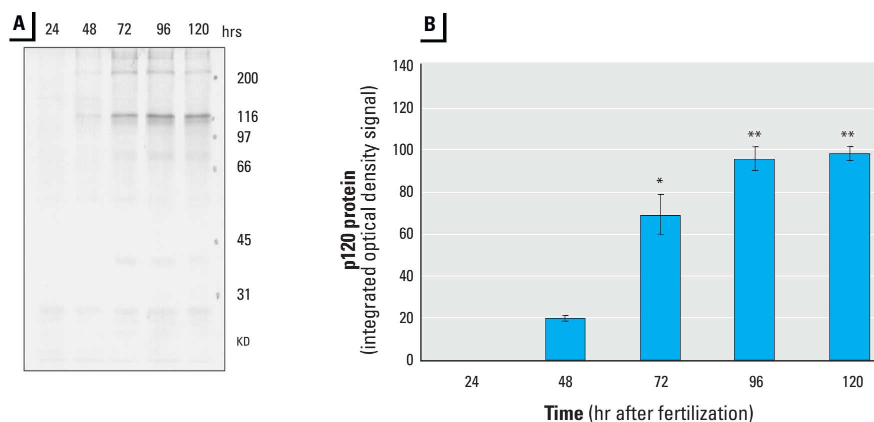

Figure 4. p120 protein levels during normal development. (*A*) Representative Western blot using the goat *Spisula* p120 antibody (1:500 antiserum, 4 µg sample/lane) showing that a 120-kD protein is the dominant band detected. No signal was detected until 48 hr after fertilization. (*B*) Graph derived from Western blot data ($n = 4$) showing significant temporal increases in p120 protein levels.
*Significantly different from 48 hr and **72 hr using S-N-K pairwise comparisons ($p \leq 0.05$, $n = 4$). Bars represent SD.

p53s, the *Spisula* sequence is most similar to fish and frog sequences, followed by chicken, mouse, cow, rat, and then human.

Northern blot analysis. Probe I, made from the conserved region of *p120*, identified three mRNAs (2.5, 3.5, and 5.5 kb) in 24-hr-old surf clam embryos (Figure 2). The largest of these mRNAs was specifically recognized by Probe II, made from the last 515 bp of the 3′ end of *p120*. The sizes of these mRNAs are consistent with the observed sizes of p53 family proteins detected by our antibodies.

Quantitative RT–PCR analysis and mRNA expression. Separate bands were easily discernible for the quantitative RT–PCR techniques (Figure 3 inset). With the stringent quantitative RT–PCR method, p120 mRNA levels varied significantly at $p \leq 0.05$ between 24 and 72 hr (Figure 3, F-ratio = 9.98). Scheffe pairwise comparisons revealed a 61% and 68% significantly lower level of p120 mRNA at 48 and 72 hr, respectively, than in the 24-hr group, but no significant difference from 12 or 36 hr ($p \leq 0.05$, n = 7–9). Thus, as embryos develop, p120 mRNA peaks at approximately 24 hr.

Western blot analysis and protein expression during normal development. The most prominent band detected with the goat polyclonal antibody to the surf clam homologue migrated to 120 kD (Figure 4A). This protein, which was not discernible at 24 hr, appeared at 48 hr as a very faint band and then increased temporally. Other minor bands recognized by the primary antibody followed the same general pattern of expression. A barely discernible band smaller than 30 kD is an artifact of the secondary developing antibody. Figure 4B illustrates the temporal increase in p120 levels based on four blots. p120 protein levels increased by 29% between 48 and 72 hr after fertilization and by 72% and 70%, respectively, from 72 hr to 96 and 120 hr (S-N-K pairwise comparisons, $p \leq 0.05$). p120 protein levels remained unchanged between 96 and 120 hr after fertilization.

Detected by the rabbit antibody Map53/73, surf clam p53 and p97 levels did not change during the developmental time course (see below). Map53/73 rabbit antibody did not detect a 73- or 120-kD band in surf clam embryos. Cross-reaction with p120 does not occur because the sequence used to generate Map53/73 differed significantly between the two clam species *Mya* and *Spisula* (Figure 1).

Western blot analysis and protein expression in adult tissue. We determined in separate experiments with the rabbit Map53/73 antibody that p73 is detected in adult surf clam striated muscle, smooth muscle, and foot, but not in embryos (Figure 5). This antibody also recognized a protein at about 51 kD in adult surf clam gill and foot as well as one migrating at an even lower molecular weight in embryos. Using the goat surf clam p120 antibody, we found that p120 is expressed in embryos but not adults (Figure 5).

p120 responsiveness to PCB exposure. Early PCB exposure (100 ppm) significantly decreased p120 mRNA levels relative to the acetone control (Figure 6A; F-ratio = 6.73) for all time points except 24 hr (Scheffe test, $p \leq 0.05$, n = 5–8). The decrease averaged 86% at 48 and 72 hr and 67% at 96 hr. The Western blot using the goat anti-*Spisula* p120

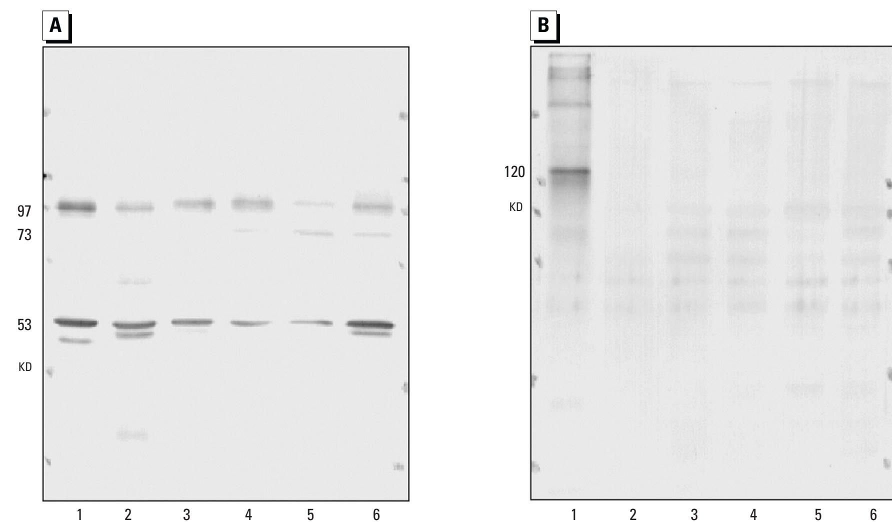

Figure 5. Representative Western blots showing embryonic (lane 1) and adult (lanes 2–6) expression of p53 family members in surf clams. (*A*) Blot using the rabbit Map53/73 antibody (1:1,000) and secondary (1:7,500) showing p53, p73, and p97. (*B*) Blot using the *Spisula* p120 antibody (1:500) and secondary (1:10,000) showing p120. For both A and B, lane 1= embryos at 96 hr, 4 µg protein loaded; lane 2 = gill filaments, 8 µg; lane 3 = mantle proper, 8 µg; lane 4 = mantle connective tissue, 8 µg; lane 5 = striated (adductor) muscle, 12 µg; lane 6 = foot, 8 µg.

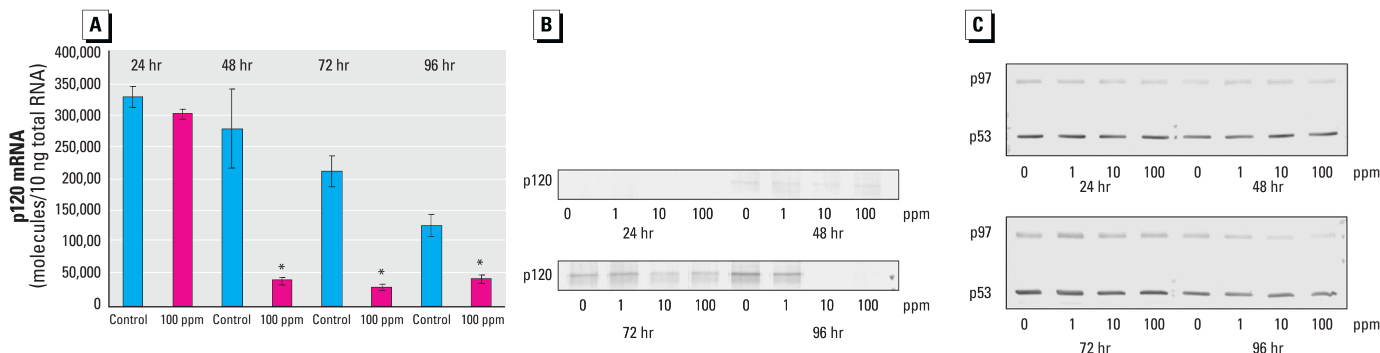

Figure 6. Time course of PCB-induced changes in levels of surf clam p53 family members. (*A*) PCB exposure (100 ppm) significantly decreased p120 mRNA levels relative to the control (F-ratio = 6.73) for all time points except 24 hr (Scheffe test, $p \leq 0.05$, n = 5–8). Bars represent standard error. (*B*) Western blots using *Spisula* p120 antibody (1:500 antiserum, 4 µg sample/lane) showing PCB-induced decreases in p120 protein at 72 and 96 hr after fertilization. p120 protein was not discernible until 48 hr, at which time there was no PCB-induced change in expression. By 72 hr, 0- and 1-ppm treatments contained significantly higher levels of protein than at 10 or 100 ppm (S-N-K analysis, $p \leq 0.05$, n = 6). At 96 hr a signal was detected at 0 and 1 ppm but not at 10 or 100 ppm. (*C*) Western blots using Map53/73 antibody (1:1,000 antiserum, 2 µg sample/lane) showing two bands migrating at 53 and 97 kD in PCB-exposed embryos sampled at the specified hours postfertilization. p53 levels remained high and constant, whereas those of p97 significantly decreased in a dose-dependent manner at 96 hr (F-ratio = 7.48, n = 5), but not at 24, 48, or 72 hr. Marks in the center of these two blots are α-tubulin and β-tubulin (nominally 59 and 53 kD, respectively).

reagent shows that the p120 protein levels were not discernible at 24 hr after fertilization and remained unchanged with PCB treatment at 48 hr (Figure 6B). At 72 hr, p120 levels were a significant 60–70% lower at 10 or 100 ppm than at 0 or 1 ppm treatment (S-N-K comparison, $p \leq .05$, $n = 6$). p120 levels did not differ between 0 and 1 ppm or between 10 and 100 ppm. At 96 hr, p120 levels did not differ between 0 and 1 ppm and were not discernible at 10 and 100 ppm.

As Figure 6C shows, p53 protein levels remained constant throughout both the normal time course and after PCB exposure. p97 levels also remained constant throughout the normal development, but showed a significant dose-dependent decrease with PCB exposure at 96 hr after fertilization (F-ratio = 7.48). Relative to the acetone control, this decrease was 3.5% (± 0.4%) at 1 ppm (insignificant), 45.2% (± 3.8%) at 10 ppm, and 45.5% (± 5.3%) at 100 ppm (S-N-K pairwise comparisons, ± standard deviation, $n = 5$). The latter two did not significantly differ from each other but both differed from the control and 1 ppm ($p \leq 0.05$).

Relationship between p120 mRNA and protein. Average mRNA and protein levels taken from control groups used in the PCB experiments were compared with each other (Figure 7). A simple regression analysis revealed a strong and significant linear inverse relationship ($y = -0.0005x + 161.97$, $R^2 = 0.97$), with mRNA decreasing as protein increased. Thus, p120 is probably regulated at the translational level.

Discussion

Differential expression of surf clam p53 family members. Based on protein expression patterns, we investigated three p53 family members (p53, p97, and p120) in surf clam embryos. We also sequenced, cloned, and characterized *p120* and followed its mRNA expression during development. p73, which is detectable in soft-shell clams (*Mya arenaria*) (*9,11*), is not found in surf clam embryos, but is present in adults. Given the molecular and protein data, surf clams appear to have at least four p53 homologues (p53, p73, p97, and p120). p53 and p97 occur in both embryos and adults, whereas p73 is found only in adults and p120 is specifically embryonic (Figure 5). Because there is quite a bit of selective translation and adenylation of mRNAs during early development in surf clams (*29,30*), p120 mRNA may translate into a smaller homologue in adults. This remains to be determined, particularly in light of the protein migrating at 51 kD in some adult tissues and at an even lower molecular weight in embryos (Figure 5). Our assumption is that this protein, at least in adult tissue, is p51 (*5*).

Unlike surf clam p53, which remains at relatively high and constant levels throughout early development and following PCB exposure, embryonic p120 is temporally expressed and responds to PCBs as early as 48 hr after fertilization. In contrast, p97, like p53 is constitutively expressed, but unlike p53, responds to PCBs at 96 hr. Thus surf clam p53 family members are not only differentially expressed during normal development, but are also differentially responsive following exposure to an environmental stressor. In addition, the reduction in p120 at 48 hr and beyond corresponds with the phenotypic expression of PCB-induced toxicity in surf clams, a reduced serotonergic nervous system (*17*).

p53, *p73*, and *p97* have been recently described in adult *Mya* tissue (*9,11*). Based on sequence information, *Mya p53* appears to be a truncated form of *p73*, suggesting both are splice variants of the same gene. This is similar to vertebrate *p63* and *p73*, both of which are alternatively spliced into several functional, differentially expressed isoforms (*6,7*). It is interesting to note that in human myeloid leukemia, only one p73 splice variant (p73 epsilon) is expressed in leukemia cells (*31*). Similarly, in soft-shell clams p73, but not p53, is upregulated in leukemia cells (*9*). We have yet to determine whether the three embryonic surf clam mRNAs identified by the Northern blots are the products of distinct genes or splice variants of one or more genes.

p120*, a novel invertebrate p53 family member specifically expressed in embryos.* This is the first study to characterize the expression of molluscan p53 homologues during embryogenesis. To do so, we cloned and partially sequenced *p120*. This surf clam homologue is the largest (5.5 kb) of three mRNAs expressed in embryos (Figure 2) and likely encodes a protein (p120) migrating at 120 kD. The significant inverse linear relationship ($R^2 = 0.97$) during development between the homologue's mRNA and p120 levels, the fact that the 5.5 kb mRNA translates into a 120-kD protein, and the significant depletion of both p120 mRNA and protein after PCB exposure provide compelling evidence that p120 is a product of the gene that we partially sequenced. Together, our data suggest that the full-length cDNA contains additional sequence at the 3′ end.

Two distinct regions characterize surf clam p120 (Figure 1): a p53-like domain (residues 5–386) that is highly conserved across all homologues, and an extended C-terminus (residues 393–573) similar to that found in other mollusks as well as vertebrate p63 and p73. All p53 family members closely resemble each other across the DNA-binding domain (Figure 1). Within this region surf clam p120 is highly homologous with other molluscan p53-like proteins and more similar to vertebrate p63 and p73 than p53 (Table 2). Among vertebrates it most resembles frog and fish p53, followed by chicken, then mammals. These data support the hypothesis that vertebrate p53 more recently evolved from a p63-/p73-like relative and also suggests that p120 is a precursor to all three homologues. Studying the regulation of p120 and its function is therefore critically important in understanding how environmental contaminants interfere with embryo development.

The DNA-binding domain of p53 includes four evolutionarily conserved regions (II, III, IV, and V; Figure 1) that contain most hot spots for p53 mutations in vertebrate tumors (*32*). These regions are similarly conserved in mollusks and all except

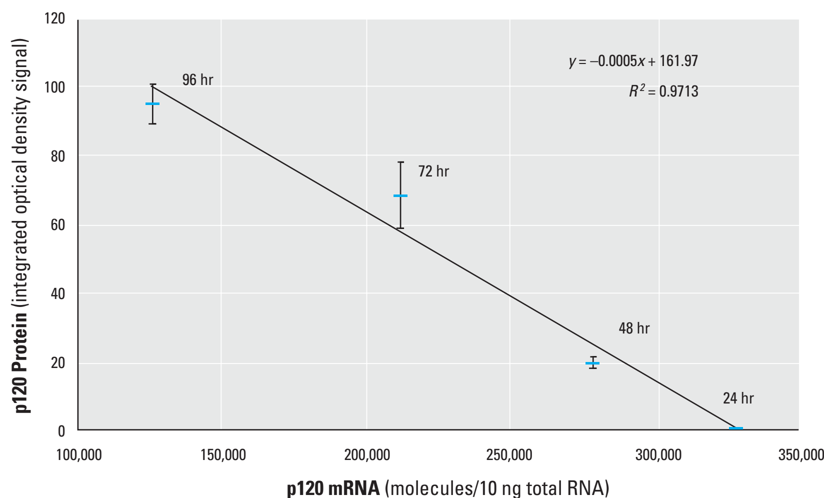

Figure 7. Linear inverse relationship between p120 mRNA and protein during normal development. Bars represent standard deviation for p120 protein levels.

one of the amino acids that contact the DNA are identical. Amino acids that help maintain the structure of the DNA-binding domain and coordinate the zinc atom (*32*) are the same in all molluscan and vertebrate p53 homologues. From this primary sequence information we predict that the p120 DNA-binding domain folds into a structure that resembles all p53 family members.

Several other domains important to p53 function are present. The N-terminal activation domain (also called region I) is highly conserved among vertebrate p53s and includes sequences that form a complex with Mdm2, a protein that negatively regulates p53 transcription (*33*). Amino acids identified as important for transcriptional activation and Mdm2 binding (*34*) are conserved across all three molluscan species and in both splice variants of the soft-shell clam homologue (Figure 1). This suggests that molluscan p120 transcription is activated and regulated similarly to vertebrate p53. However, to our knowledge an Mdm2 homologue has not yet been identified or sequenced in mollusks. In other invertebrates, a hypothetical protein that shares strong identity with mammalian Mdm2 has been sequenced in the nematode *Caenorhabditis elegans* (*35*) and in proteins that inhibit cell death, but do not share high homology with Mdm2 identified in fruit flies, *Drosophila melanogaster* (*36*).

There is also a conserved nuclear localization signal, implying that p120 is a nuclear protein. In addition, surf clam p120 contains a conserved sequence required for oligomerization of p53 (*37*). The glycine residue that stabilizes this structure is completely conserved across all analyzed molluscan and vertebrate homologues (Figure 1).

The functional divergence of p63 and p73 from p53 is most likely determined by sequences unique to the C-terminus. This hypothesis is supported by the recent discovery of a SAM within the C-terminal extensions of p63, p73, and molluscan p73 [reviewed by Levrero et al. (*6*)]. SAM is important for protein interaction and is involved in developmental regulation (*8*). Surf clam p120 and the recently described soft-shell clam p73 (*9*) also contain this motif (Figure 1). In addition, we have identified a new conserved region at the 3′ end of all vertebrate and invertebrate homologues. Taken together, the high degree of similarity between surf clam p120, p63, and p73 across the DNA-binding and oligomerization domains and the presence of a SAM motif within an extended C-terminal region suggest that the surf clam gene product plays a role in regulating development.

p120 expression during normal development. Surf clam p120 mRNA levels are highest between 12 and 36 hr after fertilization and significantly decrease at 48 and 72 hr after fertilization (Figure 3). Between 24 and 96 hr after fertilization, p120 mRNA levels decrease, whereas those of the protein increase, leading to a significant inverse relationship (Figures 4 and 7). This suggests p120 levels are translationally regulated, as has been shown for other proteins important in development [e.g., sea urchin tubulin, (*38*) and tetkin A (*39*)]. Interestingly, p120 is exclusively embryonic (Figure 5), and unlike p120, both p53 and p97 protein remain constant throughout early development (controls in Figure 6C). It is interesting to note that the p53 homologue in frogs (referred to as p53) has mRNA and protein levels that show the same inverse relationship as surf clam p120 during early development (*40*). Frog p53 mRNA levels are highest in developing oocytes and gradually decrease until they are undetectable at neurulation. Frogs rely on this oocyte mRNA to maintain constant protein levels, at least until the tadpole stage. A fruit fly p53 homologue also has a maternal component and is expressed at highest levels during early embryogenesis, but remains throughout development (*41*). A requirement for maternal p53 mRNA is not found in mice, in which p53 levels and activity are high in the nervous system until the CNS matures, after which there is a decline [reviewed by Choi and Donehower (*3*)].

High p53 mRNA levels in frog oocytes have led to the hypothesis that there is a stage early in frog development that differs from mice and requires p53 (*42*). We do not yet know whether surf clams rely on maternal p120 mRNA. However, there was a slight peak in p120 mRNA levels at 24 hr after fertilization (Figure 3B), which we have shown to be a critical stage in surf clam development. At this time the serotonergic and dopaminergic systems first appear (*17,21*) and a ryanodine response develops (*20*). There is also a primitive neural plexus that coordinates mouth and velum movement, and the shell gland begins producing material for the bivalve shell (*17,21*).

Responsiveness to an environmental stressor (PCBs). Early PCB exposure significantly decreases p120 protein and mRNA levels as well as p97 protein, whereas p53 protein levels remain high and unchanged (Figure 6). Early PCB exposure also does not alter Hsp70 levels in *Spisula* embryos (*17*), indicating that the reduction in p120 does not reflect a general stress response. No change in p120 occurs at 24 hr after fertilization, but by 48 and 72 hr after fertilization there is a significant 86% decrease in p120 mRNA followed by a 67% decrease at 96 hr (Figure 6A). PCB-induced changes in p120 protein lagged behind the mRNA and were not significant until 72 hr (Figure 6B). At both 72 and 96 hr after fertilization, there was a significant reduction in p120 levels at both the 10 and 100 ppm level of exposure. Together, these data show that p120 responds to an environmental neurotoxin early in development, within 48 hr of fertilization, and that the protein levels are not compromised until 72 hr. Interestingly, p97 protein was also significantly reduced by PCB exposure, but not until 96 hr (Figure 6C).

PCBs are globally distributed environmental contaminants that collect in sediments to concentrations of 4,000 ppm or greater (*28*). These lipophilic chemicals easily accumulate in plant and animal tissues with filter feeders such as bivalves concentrating up to 100,000 times more PCBs than are present in water (*43*). A mutant form of p53 occurs in leukemic clams taken from PCB-contaminated sites (*13*). Developing larvae are particularly at risk because up to 50% of the PCB burden of an animal can be redistributed to the gonad during oogenesis (*44*). In our study we exposed early embryos to Aroclor 1254, a defined mixture of *ortho*-substituted (noncoplanar) and coplanar PCBs. *ortho*-Substituted PCBs are thought to impact neuronal development by changing intracellular calcium levels released from either ryanodine or IP3-regulated stores, altering neurotransmitter concentrations in the brain [reviewed by Tilson and Kodavanti (*45*) and Seegal (*46*)]. Coplanar PCBs bind to the aromatic hydrocarbon receptor that can lead to a cascade of events resulting in free radical production and DNA damage (*47*). DNA damage contributes to the multifactorial process leading to cancer and may explain the high levels of leukemia in soft-shell clams from PCB-contaminated sites (*48*).

Chemical inducers of DNA damage normally increase p53 levels to induce apoptosis, whereas only a few (e.g., cisplatin, taxol) increase p73 expression [reviewed by Levrero et al. (*6*) and Strano et al. (*7*)]. However, recent evidence shows that at least one vertebrate p73 splice variant (ΔN-p73) decreases with DNA damage in nerve cells to counter p53-mediated apoptosis (*49*). Furthermore, this decrease in ΔN-p73 follows withdrawal of nerve growth factor (NGF). Molluscan nerve cells also respond to NGF (*50*). PCBs are known to alter the NGF neurotrophic system in pheochromocytoma cells (*51*), suggesting that the PCB-induced decrease in surf clam serotonergic cells involves both p120 and NGF, and possibly p97.

The PCB data show that surf clam p120 responds to environmental perturbation. We have yet to determine if the two other mRNAs identified by Northern blot analysis

arise from distinct p53 homologue genes or are shortened versions of p120. As in mammals, these homologues may perform distinct functions during development, following chemical exposure, and possibly after exposure to different types of PCB congeners. We also plan to determine whether decreased p120 levels and function are restricted to the nervous system. Because we have shown in separate experiments that PCBs target neuronal growth in surf clams (*16,17*) and that the time course of this reduction is the same for both p120 and the serotonergic system, we hypothesize that the developing nervous system is most sensitive to changes in p120.

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