Conformational and Molecular Basis for Induction of Apoptosis by a p53 C-terminal Peptide in Human Cancer Cells*

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A p53-derived C-terminal peptide induced rapid apoptosis in breast cancer cell lines carrying endogenous p53 mutations or overexpressed wild-type (wt) p53 but was not toxic to nonmalignant human cell lines containing wt p53. Apoptosis occurred through a Fas/APO-1 signaling pathway involving increased extracellular levels of Fas/FasL in the absence of protein synthesis, as well as activation of a Fas/APO-1-specific protease, FLICE. The peptide activity was p53-dependent, and it had no effect in three tumor cell lines with null p53. Furthermore, the C-terminal peptide bound to p53 protein in cell extracts. Thus, p53-dependent, Fas/APO-1 mediated apoptosis can be induced in breast cancer cells with mutant p53 similar to the recently described Fas/APO-1 induced apoptosis by wt p53. However, mutant p53 without p53 peptide does not induce a Fas/APO-1 activation or apoptosis. Docking of the computed low energy conformations for the C-terminal peptide with those for a recently defined proline-rich regulatory region from the N-terminal domain of p53 suggests a unique low energy complex between the two peptide domains. The selective and rapid induction of apoptosis in cancer cells carrying p53 abnormalities may lead to a novel therapeutic modality.

More than 50% of human malignancies, including breast cancers, are associated with missense mutations or deletions of p53, and most of the missense mutations map to the DNA-binding domain of the protein (1, 2). p53 is a sequence-specific transcriptional factor that transactivates a number of genes whose products are involved in cell growth regulation. These include WAF1/p21/Cip1, which arrests the cell cycle, GADD45 for DNA repair, and Bax and Fas/APO-1 to modulate apoptosis (3, 4). However, p53 containing mutations in the transcriptional regulation domain of the protein also induced apoptosis (5, 6). Apoptosis is a complex process regulated by several pathways, some of which involve members of the Bcl-2 family (7–9). Fas/APO-1 also induces rapid apoptosis upon binding to Fas ligand (FasL) through the autocrine/paracrine signaling pathway (10, 11). When activated, the intracellular death domain in Fas/APO-1 binds to FADD/MORT-1, which then recruits FLICE (caspase 8/MACHa1). FLICE is a chimeric protein with an adaptor domain for binding to FADD/MORT-1 in DISC (death-inducing signaling complex) and a proteolytic domain similar to ICE (12). The formation of DISC leads to the activation of FLICE. FLICE is the first protease activated in the Fas/APO-1 signaling pathway that initiates downstream activation of caspase-3, -6, and -7 and mitochondrial damage. This in turn activates ICE family proteases, triggering a cascade of apoptotic processes (13–15).

The sequence-specific DNA-binding activity of p53 appears to be negatively regulated by its C-terminal 30-amino acid (aa) segment (aa 363–393) (16) and also by N-terminal proline-rich motifs located between aa 80–93 (17). Synthetic peptides corresponding to the C-terminal domain of p53 such as residues aa 363–393 bind directly in vitro to wild-type p53 (17). Binding experiments with p53 proteins that contain selected deletions indicate that binding of the free aa 363–393 peptide to p53 requires the presence of both C-terminal aa 363–393 and N-terminal aa 80–93 sequences in the p53 protein (17). This observation suggests either that the free peptide may interact simultaneously with both regions or that the absence of either or both of these segments in p53 results in structural changes in the protein, lowering its affinity for the free peptide.

Deletion of either or both of these regulatory regions, as well as various C-terminal modifications, stimulate specific DNA binding of p53 in vitro (3, 18, 19). Previous studies demonstrated that the addition of a chemically modified C-terminal p53 peptide restored in vitro sequence-specific DNA binding function to mutant p53-273 (Arg to His). Furthermore, intranuclear microinjection of this peptide into SW480 colon carcinoma cells carrying an endogenous p53-273 His mutation restored transcriptional activation of a p53-responsive reporter construct (20). Recently, a p53 C-terminal 22-amino acid peptide (corresponding to residues 361–382), fused to the Antennapedia (Ant) homeobox domain (17 aa) to facilitate cellular uptake, suppressed growth, and induced apoptosis of SW480 cells (21). However, the mechanism of action of the fusion peptide and effects on nonmalignant cells are not known. In this study, we report the peptide mechanisms for inducing apoptosis in breast carcinoma cell lines carrying endogenous mutant p53 or overexpressed wt p53. Using conformational energy calculations to compute the low energy conformations for the N- and C-termi-

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1 The abbreviations used are: FasL, Fas ligand; wt, wild type; aa, amino acid(s); FITC, fluorescein isothiocyanate; PARP, poly(ADP-ribose) polymerase.

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nal regulatory domains, we find that these two domains can form a unique low energy complex, suggesting that a direct interaction can exist between them.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture—**Cell lines used in this study were purchased from ATCC and maintained according to ATCC guidelines. The MDA-MB-453 cell line was subcloned, and a line was isolated that did not express detectable p53.

**Peptide Synthesis—**Peptides were synthesized by Research Genetica (Birmingham, AL). The first two arginines of Antennapedia were removed when fused to the C-terminal arginine of p53pep. Peptide stocks (4 mM) were prepared in water.

**Western Blot Analyses—**Western blot analyses were performed using standard methods and the ECL detection system (Amersham Pharmacia Biotech) with 1 μg/ml antibodies.

**Flow Cytometric Analysis for Apoptosis and Cell Surface Fas and FasL Analysis—**Propidium iodide stained cells (1 × 10^6 cells/ml) were analyzed by a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) followed by determination of the percentage of sub-G₁, cells by the CellQuest program. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal Fas/CD95 antibody and biotin-conjugated mouse anti-human FasL monoclonal antibody (PharMingen, CA) were used to determine cell surface Fas and FasL, by measuring the fluorescent intensity for 10⁴ cells using FACS-Caliber according to manufacturer's protocol.

**Docking—**The lowest energy conformation for the 369–382 segment was an a-helix and was at least 10 kcal/mol lower in energy than for any competing low energy conformation. This conformation was therefore taken as the reference structure for this sequence. The lowest energy structure for each class of conformations for the (Ala-Pro)_3 peptide was then subjected to a grid search in which the rigid body variables, but not the dihedral angles were changed systematically as described previously (40). For each representative (Ala-Pro), conformation, 3024 grid points were generated. A total of 45 representative low energy conformations for this peptide, selected as described in the preceding section, were subjected to this gridding procedure so that a total of over 136,000 conformations were evaluated. From the grid search, each low energy configuration of the two peptides was then subjected to energy minimization in which the rigid body variables, and the dihedral angles of the two peptides were allowed to change.

**RESULTS**

A number of peptides from the C-terminal region of p53 were tested for inhibition of cell growth and induction of apoptosis in various tumor cell lines. These peptides were fused to an Antennapedia-derived 17-aa domain for efficient intracellular uptake (29). Similar to the peptide reported by Selivanova et al. (21), we found peptide p53 aa 361–382 to be most effective for inducing apoptosis when fused to Antennapedia. This peptide, referred to as Ant-p53 pep hereafter, was chosen for further study. The mean IC₅₀ for induction of apoptosis by Ant-p53 pep in human breast tumor cells was found to be 30 μM, and this concentration was used throughout. The Antennapedia peptide alone displayed no cytotoxic effects when tested up to 500 μM (data not shown).

**Induction of Apoptosis by Ant-p53 pep—**Effects of Ant-p53 pep were tested using the MDA-MB-468 human breast carcinoma cell line, which contains an endogenous p53-273His mutant gene (30). This mutation has been reported as one of the two most prevalent mutated spots in the p53 gene, accounting for up to 18% of all p53 mutations (3). The effect of Ant-p53 pep on cell viability was initially examined by trypan blue exclusion. Cell viability counts showed a 10-fold decrease in viable cells after a 5-h treatment with 30 μM Ant-p53 pep in MDA-MB-468 cells. In contrast, the peptide at 30 μM for 5 h did not have any effect upon viability in the nonmalignant wt p53 breast epithelial cell line, MCF10-2A (data not shown).

To investigate the mechanism of peptide-induced cytotoxicity, we determined whether cell death was due to apoptosis and whether the effect was specific for the peptide. The DNA content and integrity were measured by flow cytometry of propidium iodide stained cells (31). A sub-G₁ peak characteristic of apoptotic cells was detected at 5 h in MDA-MB-468 cells treated with 30 μM Ant-p53 pep but not when treated with Antennapedia or p53 pep alone at the same concentration and exposure time (Fig. 1A). The percentage of sub-G₁ content cells was 30–40% in Ant-p53 pep-treated MDA-MB-468 cells. We did not detect any other significant change in the cell cycle profile. Two other human breast cancer cell lines, MDA-MB-231 carrying a p53 mutation at aa 280 (32) and MCF7 overexpressing wt p53 were also induced into apoptosis by 30 μM Ant-p53 pep (Fig. 1A). To exclude the possibility of cytotoxic effects on nonmalignant cells, we tested MCF10-2A and 27sk, a normal skin fibroblast line. Growth and viability of MCF10-2A and 27sk cells, both expressing low levels of wt p53, were not affected.
by 30 μM of Ant-p53pep when observed over a period of 72 h (Fig. 1A).

Apoptotic cell death was further confirmed by binding of Annexin V to the externalized phospholipid phosphatidylserine. In many cell types, phosphatidylserine becomes exposed to the extracellular environment because of the loss of membrane asymmetry during the early phase of apoptosis (33). MDA-MB-468 cells were treated with 30 μM of either Ant, p53pep, or Ant-p53pep for 5 h. Cell cycle distribution of 10^4 cells was analyzed by flow cytometry. Cell populations with sub-G₁ DNA content are indicated with arrows. B, detection of annexin V binding in Ant-p53pep-treated MDA-MB-468 cells. Cells were treated with Ant-p53pep for 30 min prior to binding with Annexin V. The solid line represents a background binding of annexin V in the untreated cells, and the dotted line represents the peptide-treated cells. C, measurement of sub-G₁ DNA content and annexin V binding in MDA-MB-453 cells (null p53). The experiments were performed as in A and B.

Fig. 1. Measurement of sub-G₁ DNA content in breast cancer cells by Ant-p53pep. A, three breast cancer cell lines, MDA-MB-468, MDA-MB-231, and MCF7, a nonmalignant mammary cell line, MCF10-2A (wt p53), and a normal skin fibroblast line, 27sk (wt p53), were treated with 30 μM of either Ant, p53pep, or Ant-p53pep for 5 h. Cell cycle distribution of 10^4 cells was analyzed by flow cytometry. Cell populations with sub-G₁ DNA content are indicated with arrows. B, detection of annexin V binding in Ant-p53pep-treated MDA-MB-468 cells. Cells were treated with Ant-p53pep for 30 min prior to binding with Annexin V. The solid line represents a background binding of annexin V in the untreated cells, and the dotted line represents the peptide-treated cells. C, measurement of sub-G₁ DNA content and annexin V binding in MDA-MB-453 cells (null p53). The experiments were performed as in A and B.

Mechanism of Apoptosis Induced by Ant-p53pep—We investigated possible mechanisms for peptide-induced apoptosis. The MDA-MB-468 cell line was selected for further studies because it contains a single copy of R273H mutation of p53. The R273 mutation is the most prevalent mutation in p53 that abolishes both DNA binding and transcriptional ability of p53 (2, 34). The p53-R273H mutant proteins have been shown to interact with p53-derived peptide in in vitro DNA binding studies (20). The endogenous levels of p53 were determined by
Western blot analysis in MDA-MB-468, MDA-MB-453, and MCF10-2A cells after Ant-p53pep treatment for 3 h (Fig. 2A). As expected, MDA-MB-468 cells expressed a high level of mutant p53-273H, a very low level of wt p53 in MCF10-2A, and no expression of p53 in MDA-MB-453 cells. Levels of p53 were not altered after Ant-p53pep treatment. Interestingly, cycloheximide treatment of MDA-MB-468 cells prior to addition of Ant-p53pep did not block apoptosis (Fig. 2B), suggesting that Ant-p53pep-induced apoptosis was not dependent on de novo protein synthesis. Furthermore, there were no changes in expression levels of known apoptotic regulators such as Bax, Bak, and Bcl-X<sub>L</sub> in MDA-MB-468 cells following treatment with Ant-p53pep for up to 3 h as analyzed by Western blot (Fig. 2C). These data indicated that Bax, a direct transcriptional target of p53, is not induced by Ant-p53pep. There was also no induction of p21/WAF1 level as evidenced by lack of cell cycle arrest with Ant-p53pep treatment (Fig. 1A). These data suggest that transcriptional activation function by p53, or at least induction of Bax, is not required for apoptosis of Ant-p53pep-treated MDA-MB-468 cells.

Because peptide-induced apoptosis occurred very rapidly in the absence of new protein synthesis, possible changes in cell surface expression of Fas/APO-1 in MDA-MB-468 cells were examined by flow cytometry. Cells were incubated with Ant-p53pep and controls for various times and labeled with FITC-conjugated Fas antibody prior to fluorescence-activated cell sorter analysis. In Fig. 3A, MDA-MB-468 cells showed ~40% higher levels of extracellular Fas/APO-1 protein within 30 min of Ant-p53pep treatment at 30 µM, as determined by a shift of peak (black area). Neither p53pep nor Antennapedia alone affected accumulation of extracellular Fas/APO-1 whose peaks coincided with the untreated control (white area, Fig. 3A). Similarly, extracellular localization of FasL was also increased by about 30% in MDA-MB-468 cells incubated for 30 min with 30 µM Ant-p53pep (Fig. 3A). In contrast, nonmalignant MCF10-2A and MDA-MB-453 p53 null cells did not show any increase in cell surface Fas/APO-1 by Ant-p53pep treatment (Fig. 3B and data not shown, respectively). Total cellular level of Fas/APO-1 in Westerns was not altered by Ant-p53pep treatment in both MDA-MB-468 and MCF10-2A cells (data not shown). MCF10-2A cells, however, did undergo apoptosis in the presence of agonistic, apoptosis-inducing Fas IgM antibody at 500 ng/ml within 3 h, indicating that the Fas/APO-1 signaling death pathway was functional in these cells (Fig. 3C). Furthermore, treatment of MDA-MB-468 cells with 30 µM Ant-p53pep for 2 h generated the characteristic truncated active form of FLICE (p26) by Western blot (Fig. 4). These data suggest that the apoptosis in Ant-p53pep-treated MDA-MB-468 cells was mediated through the Fas/APO-1 signaling pathway and resulted in FLICE activation.

**Effects of Ant-p53pep on CPP32 Protease Activity and PARP Cleavage**—One of the downstream components of apoptosis is caspase-3/CPP32 cysteine protease, which can be activated by multiple pathways (35). The processed, active CPP32 cleaves various substrates including PARP. The activity of CPP32 was measured in an in vitro assay using the fluorogenic tetrapeptide substrate, Ac-DEVD-AMC. Fluorescence produced from cleaved AMC is directly proportional to the amount of CPP32.
activity present in cell lysates. Fig. 5A shows measurements of CPP32 activity in lysates prepared from cells treated either with or without 30 μM Ant-p53pep for 2 h. CPP32 activity in MDA-MB-468 cells was increased by ~6-fold above baseline when treated with Ant-p53pep compared with the untreated control (Fig. 5A). In contrast, MCF 10-2A cell lysates showed no significant increase in CPP32 activity following treatment with the peptide (Fig. 5A). Studies with control p53pep or Antennapedia alone showed no effect on CPP32 activity (data not shown). Ant-p53pep treatment did not alter ICE activity as measured with an ICE-specific fluorogenic substrate (Fig. 5A).

To further assess the activation of the apoptotic cascade, a truncated 48-kDa form of PARP was translated in vitro from reticulocyte lysates and incubated with 20 μg of lysates prepared from either Ant-p53pep-treated (2 h at 30 μM) or untreated MDA-MB-468, MCF10-2A, and MDA-MB-453 (null p53) cells. Cleaved product of PARP is indicated with asterisk.

Fig. 5. A, Ant-p53pep increased CPP32 protease activity. CPP32 and ICE protease activities were measured in an in vitro assay using lysate prepared from cells treated (+) or untreated (−) with 30 μM Ant-p53pep for 2 h. The background level of fluorescence was subtracted from each measurement. B, cleavage of PARP. The [35S]methionine-labeled, truncated 48-kDa PARP was translated in vitro from reticulocyte lysates and incubated with 20 μg of lysates prepared from either Ant-p53pep-treated (2 h at 30 μM) or untreated MDA-MB-468, MCF10-2A, and MDA-MB-453 (null p53) cells. Cleaved product of PARP is indicated with asterisk.
p53. Furthermore, mutant p53-273H from extracts prepared directly from MDA-MB-468 cells also formed complexes with biotinylated Ant-p53pep (Fig. 6B). Wild-type p53 protein from MCF10-2A also formed a complex with Ant-p53pep, although the amount was markedly lower than that in MDA-MB-468 extracts, possibly because of the low expression levels of p53 in nonmalignant MCF10-2A cells (Fig. 6B), which did not undergo apoptosis when treated with Ant-p53pep (Fig. 1A).

**Interaction of N- and C-terminal Peptides**—Our finding that Ant-p53pep did not bind to the C terminus of p53, from which it was derived, suggested another possible interaction site on p53. One candidate region for the direct interaction of the C-terminal regulatory peptide is the N-terminal regulatory domain involving residues 80–93. To explore whether these two regions might interact with one another, we performed conformational energy calculations to generate the low energy conformations of each peptide. These low energy conformations of the central regions of each peptide were “ docked” against one another to explore whether stable complexes could form. Using the procedure described under “Experimental Procedures,” we found a single lowest energy conformation for the 369–382 C-terminal sequence, an all α-helix consistent with other secondary structure predictions. This structure was lower by 10 kcal/mol than the energy for the next competing structure. The lowest energy (global minimum) structure for the N-terminal (Ala-Pro)$_3$ repeat sequence was, using the single letter conformational state code (36), DADADC. This conformation was computed to have a probability of occurrence of 25%. The global energy minimum was found to have a total conformational energy that was over 5 kcal/mol lower than that of the next lowest energy structure. In this lowest energy structure, the helix axis of the DADADC conformation for (Ala-Pro)$_3$ was aligned parallel to the helix axis of the 369–382 peptide as shown in stereo view in Fig. 7. The two helices intercalate so that each of the Pro residues of (Ala-Pro)$_3$ forms favorable contacts within grooves of the α-helix of the 369–382 sequence. The two helices are aligned such that most of the side chains of Lys residues, i.e. Lys$^{372}$, Lys$^{373}$, Lys$^{381}$, and Lys$^{382}$ of the 369–382 sequence point away from the face of this peptide that makes contacts with the (Ala-Pro)$_3$ peptide. Several of the CH$_2$ groups of the side chains of Lys$^{372}$ and Arg$^{379}$ contact the Pro residues, and the side chain CH$_3$ group of Thr$^{377}$ also contacts the second Pro residue. The complex therefore has amphipathic properties in which the interface between the two peptides tends to be nonpolar, whereas the hydrophilic Lys residues point away on the opposite face of the molecule. This unique lowest energy minimum suggests the possibility that p53aa369–382 might form a binding site within p53 for the Ant-p53pep.

**DISCUSSION**

This study investigated mechanisms of apoptosis induced by p53-derived C-terminal peptide. Three human breast cancer cell lines, carrying either endogenously expressed mutant p53 or overexpressed wt p53, underwent rapid apoptosis after Ant-p53pep treatment. Peptide activity to induce apoptosis was not dependent upon de novo protein synthesis. The transcription/translation-independent induction of apoptosis has been described in various systems in which a sequence-specific transactivation-defective p53 (Gln$^{22}$/Ser$^{23}$) mutant induced apoptosis in Saos2 osteosarcoma cells (37) and, similarly, in HeLa cells with a transcription-defective, truncated p53 (38). p53-dependent apoptosis also occurred in the presence of actinomycin D or cycloheximide through yet unknown mechanisms (5). Our results indicate that induction of apoptosis is mediated through Fas/APO-1 pathway in MDA-MB-468 cells and is p53-dependent because apoptosis was not observed in p53 null cell lines (MDA-MB-453, H1299, and Saos2). Moreover, mutant p53 was found to complex with Ant-p53pep. The mechanisms by which Ant-p53pep and p53 complex initiate the Fas/APO-1 signaling pathway are not clear. Bennett et al. (39) recently reported that p53-induced apoptosis involved increased surface expression of Fas/APO-1 in untransformed human vascular smooth muscle cells carrying wt p53 that occurred within 1 h upon p53 activation. They further showed that increased cell surface Fas/APO-1 was due to redistribution of Fas/APO-1 from the Golgi complex (39). In agreement with their study, we also found that p53 peptide-induced apoptosis occurred very rapidly and independent of protein synthesis, which involved increased cell surface Fas/APO-1. Furthermore, our preliminary data by confocal microscopy showed a redistribution of Fas in MDA-MB-468 cells to the plasma membrane when exposed to Ant-
p53 peptide complex involves a similar mechanism are being addressed.

Ant-p53pep selectively induced apoptosis only in tumor cells containing mutant or overexpressed wt p53. Furthermore, the p53-273H mutant, as well as wt p53 proteins formed complexes with Ant-p53pep, and yet apoptosis was not observed in nonmalignant MCF10-2A cells. Lack of apoptosis in MCF10-2A cells could be due, in part, to instability of wt p53 under physiological conditions or to a low threshold level of p53 insufficient to initiate apoptosis in nonmalignant cells. Differential cellular levels of wt p53 were shown to affect whether Saos2 cells undergo growth arrest or apoptosis. With tetracycline-inducible expression of wt p53, low level wt p53 was shown to arrest or slow cell growth, whereas high levels of wt p53 induced apoptosis (37). Thus, apoptosis observed in MCF7 cells (Fig. 1A) may be due to the high level of wt p53, and the lack of apoptosis in the nonmalignant lines, MCF10-2A and 27sk, may be due to low expression of wt p53. Induction of apoptosis in mutant p53 breast cancer cells may be related to their increased expression/stability of mutant p53, which could serve as a target for peptide. It is tempting to speculate that Ant-p53pep binds to mutant p53 and alters mutant conformation, favoring wild-type conformation. Other post-translational modifications/alterations were shown to modulate p53 activity such as C-terminal phosphorylation or C-terminal binding with anti-p53 antibody (40). In this case, new transcription/translation may not be necessary to induce apoptosis because of a high level of p53 mutant protein present in many tumor cells. Alternatively, a low cellular level of wt p53 in nonmalignant cells may not be sufficient to induce apoptosis in the absence of other additional factors such as DNA damaging agents.

The results of the conformational energy calculations suggest that a direct interaction of the N-terminal proline-rich segment with a (Ala-Pro)₃ repeat and the C-terminal effector peptide is feasible. Interestingly, the proline-rich region of p53 may be a binding site for the SH3 domains of proteins (41), further suggesting that this region of the protein may have a role in signal transduction. The function of this region is not clear but is implicated in both p53-mediated tumor cell growth suppression (42) and apoptosis (43). It cooperates with the C-terminal regulatory region to negatively regulate p53 activity (17). It

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