The Zinc Fingers of Human Poly(ADP-ribose) Polymerase Are Differentially Required for the Recognition of DNA Breaks and Nicks and the Consequent Enzyme Activation

OTHER STRUCTURES RECOGNIZE INTACT DNA

The recognition of double-stranded DNA breaks and single-stranded nicks by human poly(ADP-ribose) polymerase and the consequent enzymatic activation were examined using derivatives of the enzyme expressed in Escherichia coli. The N-terminal 162 residues encompass two zinc fingers. Deletion or mutation of the first finger results in a loss of activation by DNA with either single-stranded or double-stranded damage. Destruction of the second finger reduces activation by double-stranded DNA breaks only slightly, but eliminates activation by single-stranded DNA nicks. These data suggest that activation by single-stranded DNA nicks requires two zinc fingers, but activation by double-stranded DNA breaks requires only the finger closer to the N terminus. Variant proteins that lack both zinc fingers are enzymatically inactive but still exhibit weak DNA binding, which is independent of DNA damage. Thus, other regions are also capable of binding intact DNA, but the recognition of a strand nick or break which occasions the synthesis of poly(ADP-ribose) specifically requires the zinc fingers.

Poly(ADP-ribose) polymerase is a DNA-binding protein of higher eukaryotes that synthesizes poly(ADP-ribose) when cellular DNA is damaged and is believed to be required for DNA repair (1, 2). Poly(ADP-ribose) synthesis depends on the presence of DNA nicks or breaks (3). The polymerase is highly conserved among mammals (4, 5). The human polymerase is a single polypeptide of 113 kilo daltons (1014 amino acid residues), of which the N-terminal region of 373 residues has been implicated in DNA binding and DNA dependent activation (5–8).

The N-terminal region of human polymerase contains several potential DNA-binding motifs including two potential zinc fingers (6–8). Since the polymerase molecule is known to bind two zinc ions (9, 10), the zinc coordination residues are presumed to subdivide actual zinc fingers. As a first attempt to determine how the various features contribute to DNA binding and to enzymatic activity, we have analyzed the properties of polymerase whose zinc fingers have been deleted or destroyed by altering zinc coordinating residues.

The experiments were made possible by our recent development of an expression system for human poly(ADP-ribose) polymerase in Escherichia coli (11). The expressed polymerase has the same electrophoretic mobility, turnover number, and dependence on DNA for activity as does polymerase isolated from human tissues. Some of the derivatives we have constructed are also expressed well. We find that alterations of the first zinc finger (closer to the N terminus) result in the loss of DNA-dependent enzymatic activity. In contrast, destruction of the second zinc finger reduces double-stranded DNA break-dependent enzymatic activity only slightly, but results in loss of single-stranded DNA nick-dependent enzymatic activity. These data suggest that activation by single-stranded DNA nicks requires two zinc fingers, but activation by double-stranded DNA breaks requires only the first finger. Other regions of the polymerase appear to recognize unbroken DNA in a manner that does not result in enzymatic activation. Features which may be involved in such recognition include two lysine clusters at residues 221 226 and 346 352 and a possible helix-turn-helix in the interval 220–280 (6).

EXPERIMENTAL PROCEDURES

Plasmids Encoding Variant Human Poly(ADP-ribose) Polymerase—The expression plasmid for poly(ADP-ribose) polymerase, pTP, is described in Ref. 11. It consists of the complete cDNA for human poly(ADP-ribose) polymerase under the control of a trp promoter. One base of the original cDNA, a C at position 24, was changed to a T in order to create a new HindIII site, but this did not change the amino acid sequence. Two plasmids lacking DNA-encoding zinc fingers have been constructed from pTP by deletion in trp. pTP lacks a 318-nucleotide KpnI-KpnI fragment that encodes the region between His99 and Tyr246. The resulting protein has a single hybrid finger identical with the first finger up to Tyr26 and differing only in the distal sequence His99-Pro-Gly-Cys102 derived from the second finger in place of His99-Phe-Ser-Cys102 (Ref. 1). pTPH lacks a 678-nucleotide HindIII-HindIII fragment corresponding to the protein sequence between Lys9 and Ser256 embracing both zinc fingers.

Two plasmids which encode amino acid substitutions in the zinc fingers were constructed by replacing the amino acid sequence of the zinc fingers (His44-Phe-Ser-Cys47) with His44-Pro-Gly-Cys47 (6). The amino acid substitutions was made in the interval 43–47. The resulting protein contains no zinc finger residues.
Zinc Fingers

Poly(ADP-ribose) Polymerase

Fig. 1. Amino acid sequence of zinc fingers with zinc coordinating cysteines and histidine residues circled. Arrows indicate KpnI cleavage sites in the DNA. The asterisk represents the 1st residue (Met\(^{199}\)) of P99 (the internally initiated 99-kDa protein).

Fingers were constructed by replacing a BstHII-AccIII restriction fragment in the first finger (for pTPZ1), or a KpnI-KpnI fragment including the second finger (pTPZ2), with corresponding synthetic oligonucleotides. To generate the substitute KpnI-KpnI fragment, 12 residue (Met\(^{125}\)) of P99 (the internally initiated 99-kDa protein).

The oligonucleotides also differed at a single nucleotide (Fig. 1) in the second finger (for pTPZ2), with corresponding synthetic oligonucleotides. The only coding changes were G + A transitions underlined. BstHII-AccIII fragment in pTPZ1 (codons, 18–33): GGAACCGCTGCT. KpnI-KpnI fragment in pTPZ2 (codons, 53–158): GAGTGACAGGTAAAGGCCAGGATGGAATTGGTTCGAAGGC-GATGATCAGCAGAAAGTCAAGAAGACGGCCGAAGCCGGCG-

The concentration of polymerase was estimated from the intensity of gel bands stained with Coomassie Brilliant Blue, using \(\beta\)-galactosidase as the standard. \(\beta\)-Galactosidase may not be valid as a standard for precise protein concentration measurements, but it is sufficient for comparing the protein concentrations of several different samples.

Assay for Poly(ADP-ribose) Polymerase Activity—Samples of bacterial lysate or of purified polymerase were incubated for 5 or 10 min at 25°C in assay buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM Mg(O-Co-CH\(_3\))\(_2\), 1 mM dithiothreitol, 100 \(\mu\)M \([\text{P}^32\text{P}]\)NAD (Du Pont-New England Nuclear, diluted to 0.1 Ci/mmol), 2 \(\mu\)M of fragmented pBR322 DNA at 2 \(\mu\)g of calf thymus DNA in a total volume of 0.1 ml. The fragmented DNA used (“activated DNA,” Sigma) is calf thymus DNA partially digested with DNase I in the presence of magnesium ions and thus contains both single-stranded nicks and double-stranded breaks (14). The average fragment size is 20 kilobase pairs. Poly(ADP-ribose) formation was stopped by adding 1 ml of 10% trichloroacetic acid, and acid-insoluble material was collected and counted.

**Immunoblot Assays**—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli (15). The proteins were transferred electrophoretically to an Immobilon membrane (Millipore), and immunoblots were probed with rabbit anti-bovine poly(ADP-ribose) polymerase antibody prepared by Gerald Marschik of the University of Michi (16). The antibody was detected using goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad). Blots were developed with 4-chloro-1-naphthol (Bio-Rad) and \(\text{H}_2\text{O}_2\).

**Activity Gels**—Bacterial lysates were fractionated on 7% polyacrylamide gels containing SDS, and 100 \(\mu\)g/ml sonicated salmon sperm DNA was added just before the gel was poured. The separated proteins were renatured in the gels by incubation in several changes of 50 mM Tris-HCl (pH 8.0) and 3 mM 2-mercaptoethanol for 30 min, then 6 M guanidine hydrochloride in this buffer for 30 min, and then the buffer alone for 3 h. The gel was finally incubated in 1 mM dithiothreitol, 1 \(\mu\)M \([\text{P}^32\text{P}]\)NAD (20 Ci/mmol), 10 mM Mg(O-Co-CH\(_3\))\(_2\), and 50 mM Tris-HCl (pH 8.0) at 30°C overnight as described by Scovassi et al. (16). The gels were stained with Coomassie Brilliant Blue, destained, and processed for autoradiography.

**Protein Sequencing**—The polymerase fraction purified by HPLC was separated on a 7% SDS-polyacrylamide gel electrophoresis. The protein bands were transferred to an Immobilon membrane and stained by Coomassie Brilliant Blue. The region of the polymerase band was cut out and analyzed by a gas-phase protein Sequencer (Applied Biosystems 477A) (17).

**Preparation of pBR322**—The preparations of supercoiled, HaeIII-digested, and nicked pBR322 have been described previously (3). By agarose gel analysis, the pBR322 purified by CsCl centrifugation consisted of 90% supercoiled DNA and 10% of a slower species that co-migrated with dimeric or nicked monomeric forms. pBR322 is cut into 22 blunt-ended fragments by HaeIII. The number of nicked DNA introduced by partial DNase digestion of pBR322 was estimated by extrapolating from the observed rate of disappearance of supercoiled DNA (14). The digestion was stopped at about 10 nicks/plasmid. The nicked DNA preparation contained less than 3% linear molecules, which might be inevitably formed by DNase I (14). Since the number of double-stranded breaks \((\geq 0.03 \text{ per molecule})\) is so much less than the number of single-stranded nicks \((10 \text{ per molecule})\), the contribution of double-stranded DNA breaks to the enzyme activity should be negligible. The concentration of DNA was determined by A\(_260\) and the number of the digested DNA fragments was estimated from the fluorescence intensities of bands in agarose gels stained with ethidium bromide.

**RESULTS**

Fig. 1 shows the two zinc fingers found in human poly(ADP-ribose) polymerase (6–8). Each is of the form Cys\(_1\)-X\(_2\)-Cys\(_{28–30}\)-His\(_2\)-X\(_7\)-Cys, and there is a considerable similarity of sequence between the two fingers. Finger 1 (closer to the N terminus) starts with Cys\(_{31}\), has a finger of 28 residues, and is electrically neutral (3 basic and 3 acidic residues). Finger 2 (the second finger) starts at Cys\(_{32}\), has a finger 30 residues long, and is basic (7 basic and 5 acidic residues).

We have developed an expression system for the synthesis
of human poly(ADP-ribose) polymerase in E. coli (11). pTP is a derivative of pBR322 carrying human poly(ADP-ribose) polymerase cDNA under the control of a trp promoter. The bacterially expressed protein (P113) has the same molecular size (113 kDa) and enzymic characteristics including turnover number, dependence on DNA for activity, and sensitivity to the inhibitors thymidine and benzamide as poly(ADP-ribose) polymerase purified from placenta. However, a large fraction of the expressed products appears as proteins that are shorter than full length (Fig. 2a, lanes 1 and 2). Prominent among our products are two immunoreactive proteins (P99 and P98), which correspond in size and N-terminal sequence to proteins translated from internal positions (18). The amino acid sequence of P99 was determined to be Met-(Glu)-Lys-Ile-Glu-Lys-Gly-Gln-Val, which corresponds to the region starting with Met138 in the human polymerase sequence (6-8). P98 was fractionated into two peaks by hydroxylapatite HPLC (HA-1000). Their sequences were Met-?-Glu-Val-Ala-Lys-Lys and Ala-Lys-Lys-Lys-Ser-Lys-Lys, which correspond to the regions starting with Val236 and Ala240. The substitution of Val216 by Met also suggests that P98 is the product of an internal start (19). These internal start sites are the same as those found by Herzog et al. (19), who expressed poly(ADP-ribose) polymerase lacking its first 67 amino acids. P98 proteins lack both of the zinc fingers and more than 50 additional downstream residues. P99 lacks zinc finger 1 and enough of the finger 2 sequence (Cys213-Lys-Gly-Cys218) to prevent the formation of zinc finger structure (Fig. 1).

Deletion of One or Two Zinc Fingers Reduces or Eliminates Enzymic Activity—The activity gel assay is appropriate for analyzing the internal start products since it requires no prior purification of proteins and allows the activities of fragments and intact polymerase to be compared in the same gel track. In this technique, the enzyme is fractionated on an SDS-gel containing DNA fragments, renatured in situ, and incubated with [3H]NAD. Any [3H]poly(ADP-ribose) formed remains attached to its synthetic enzyme and is detected by autoradiography.

Full length polymerase (P113) was readily detected as an active species, but none of the truncated forms were active (Fig. 2b). In particular, P99 and P98 had no DNA-dependent activity, even though they were present at about the same abundance as P113 as determined by immunoblotting of a parallel gel (Fig. 2a). Therefore, the N-terminal region of the DNA-binding domain, which contains the zinc fingers, appears to be required for DNA-dependent activity. Features beyond residue 162, as discussed below, bind DNA, but such binding is insufficient to activate the enzyme. Herzog et al. (19) were also unable to show DNA-dependent activity of the 99-kDa and 89-kDa internal start proteins.

We constructed deletion mutants in the N-terminal region by eliminating codons between paired restriction sites in frame. The larger of these deletions, pTPH, extended between two HindIII sites in the DNA and resulted in the elimination of residues 7-232 in the protein, encompassing both fingers and the first lysine cluster, Lys221-Lys-Lys-Ser-Lys-Lys226. The deletion product, P88A, is inactive. We found no activity in a total bacterial lysate expressing pTPH, no activity by activity gel analysis, and no activity of the purified protein (Fig. 2, Table I).

To construct pTPK, a smaller region was deleted, between two KpnI restriction sites, which eliminated amino acids 53-158. The derived protein, P101A, thus contains a hybrid finger that consists almost entirely of finger 1 with only the change His150-?-Pro-Gly-Cys162 in place of His150-Phe-Ser-Cys162 at the C-terminal side (Fig. 1). It had lost most of its activity and could not be demonstrated on activity gels. After purification, it was found that P101A retained negligible activity. pTPK retains the second internal start position and consequently directs the production of the inactive P89 mentioned above (Fig. 2a).

Substitutions of Cysteines in the Zinc Fingers—For a finer analysis, we constructed derivatives in finger 1 (pTPZ1) and finger 2 (pTPZ2) in which the cysteines at the start of each finger were both replaced by tyrosine residues, leaving Tyr-Tyr-His-Cys motifs which are expected not to make zinc fingers. In the yeast adrl protein, the replacement of zinc-coordinating cysteine and histidine residues by tyrosine is known to give a null phenotype (20).

Both derived plasmids encoded full length immunoreactive
proteins (113 kDa) and the internal translation start protein of 99 kDa and 89 kDa. In HB101 cells, the yield of full length product (P113-Z1-) from the finger 1 mutant was very low, suggesting proteolysis of the mutant protein. As would be expected, the internal start proteins, which were not affected by the mutations, were present in the usual amounts. To restrict proteolysis, we moved the plasmid to Len- cells (ME8417:lon::Z'nlO) and were able then to produce higher amounts of P113-Z1-. The amount of native polymerase (P113) produced from pTP was comparable in HB101 and Len- cells (11) (Fig. 2a).

Destruction of zinc finger 1 eliminated the enzymic activity of polymerase. The lysate of Lon- bacteria expressing pTPZ1 contained no active species by activity gel analysis (60 ng of polymerase. The lysate of Lon- bacteria expressing pTPZ1 contained no active species by activity gel analysis (60 ng of polymerase. The lysate of Lon- bacteria expressing pTPZ1 contained no active species by activity gel analysis (60 ng of polymerase. The lysate of Len- bacteria expressing pTPZ1 had no detectable activity although 3 ng of purified amounts of P113-Zl-. The amount of native polymerase lanes 5 and 9 in Fig. 2, polymerase from placenta was readily detectable: compare Len- cells (11) (Fig. 2a).

The activity of P113-Z2- was enhanced only by double-stranded breaks, but not by single-stranded DNA nicks; see Fig. 4e and the text.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein</th>
<th>Expected finger structures in N-terminal region</th>
<th>Activity gel</th>
<th>Specific activity of purified polymerase</th>
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<tr>
<td>pTP</td>
<td>P113</td>
<td>-</td>
<td>+</td>
<td>300</td>
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<tr>
<td>P88A</td>
<td>PTPH</td>
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<td>PTPZ2</td>
<td>-</td>
<td>210</td>
<td>2</td>
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</table>

* The N-terminal structures (1-177 residues) with the two expected zinc fingers drawn in proportion. Finger 1 and finger 2 are shown by closed and open boxes. The remainder of the molecule, indicated by the dashed line, is the same in every case.

* DNA-dependent production of poly(ADP-ribose) by gel-fractionated enzyme renatured in situ. The limit of detection is 1% of control.

* Enzymic activity measured on the purified proteins, which were at least 60% pure. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of ADP-ribose into acid-insoluble material per min. The comparable specific activities of polymerase purified from human placenta were 490 units/mg and 4 units/mg, in the presence and in the absence of DNA, respectively. The DNA used had both single-stranded nicks and double-stranded breaks.

* The nature of the minor activity in the absence of DNA is not clear. Because this DNA-independent activity is blocked by 3-aminobenzamide, an inhibitor of the polymerase, it is assumed to require at least some function of the enzyme. Polymerase-derived proteins show the same low level of DNA-independent activity (Fig. 2c) which therefore does not require the zinc finger region and cannot be ascribed, for example, to an undetected quantity of DNA fragment in the enzyme preparation.

* ND, not determined.

* The activity of P113-Z2- was enhanced only by double-stranded breaks, but not by single-stranded DNA nicks; see Fig. 4e and the text.

![FIG. 3. Purification of poly(ADP-ribose) polymerase and its derivatives expressed in E. coli. The products of pTP (P113), pTPZ2 (P113-Z2-), pTPH (P88A), and pTPK (P101A) were purified as described under "Experimental Procedures." In each case, samples were taken of crude extract and after chromatography on phosphocellulose, 3-aminobenzamide-Sepharose, and HPLC HA-1000 columns. Samples were fractionated by 7% polyacrylamide SDS-gel electrophoresis and stained with Coomassie Brilliant Blue.](http://www.jbc.org/content/280/21/21910/F1.large.jpg)
In the plasmid preparation. However, the DNA becomes substantially more effective when it contains either single-stranded nicks or double-stranded breaks. With respect to activation by different types of DNA damage, bacterially synthesized P113 behaves like polymerase purified from human placenta (Fig. 4, a and b). In contrast, the activity of P113-Z2⁻ was enhanced only by double-stranded breaks, but not by single-stranded nicks (Fig. 4c). This result implies that the second finger is required to recognize single-stranded nicks.

**Specificity of P113-Z1⁻—**We have been unable to purify the protein lacking finger 1 (P113-Z1⁻) and could not therefore estimate its response to DNA in a pure system. The activity gel containing sonicated DNA suggests that P113-Z1⁻ does not respond to double-stranded breaks (Fig. 2). To determine whether P113-Z1⁻ is activated by single-stranded nicks, we repeated the activity gel using DNase I-treated calf thymus DNA as the activator. P113-Z1⁻ was not activated although wild type polymerase (P113) was (not shown). Therefore, P113-Z1⁻ is activated neither by double-stranded breaks nor by single-stranded nicks.

**DNA Binding—**A semiquantitative gel shift assay was employed to compare the interactions of activatable (P113) and nonactivatable (P88Δ) protein with activating (HaeIII-digested) and nonactivating (supercoiled) DNA in the presence of 50 mM Tris-HCl (pH 8.0) (Fig. 5).

There was little difference between the four combinations. In all four cases, sufficient protein could move all of the DNA to the top of the gel, presumably in some multicomponent complex. Thus, it is clear that DNA binding can occur without enzyme activation, without DNA damage, and without the zinc fingers. Indeed, this generalized binding to internal, undamaged sites is the major interaction revealed by the gel-shift assay and is not obviously influenced by zinc fingers.

P88Δ shifts large HaeIII fragments preferentially, consistent with multiple internal binding.

The only apparent difference among the four combinations was that about 2-fold less P113 than P88Δ was required to give a comparable shift to HaeIII fragments. Perhaps this reflects the additional ability of P113 to recognize DNA ends.

When the gel shifts were repeated in the presence of 100 mM KCl, the binding of P88Δ to either the supercoiled or
plasmin or trypsin (10,28). Each protease releases an approximately 26-kDa (sometimes reported as 29-kDa) N-terminal fragment containing the zinc fingers and the first lysine cluster at residues 221–226. The adjacent 32-kDa plasmin fragment (apparent size 36 kDa) contains the second lysine cluster, Lys246–Lys-Leu-Lys-Val-Lys-Lys259 and possibly a helix-turn-helix (6). As we would expect, the 26-kDa and 36-kDa fragments both bind to DNA-cellulose (28). The intact polymerase protects DNA termini against exonuclease digestion (29). It has been reported that the 26-kDa fragment separated by SDS-gel electrophoresis does not protect DNA termini against DNase I digestion (30). This is unexpected in view of our data, but the 26-kDa fragment may not have recovered its DNA-binding capacity after denaturation in SDS (30). In contrast, the subterminal 32-kDa plasmin fragment does recover DNA binding after SDS-gel electrophoresis and transblotting (29), possibly indicating that it has less stringent structural requirements than does the zinc finger region for DNA interaction. In agreement with our model, the 32-kDa fragment partially protects some internal DNA regions against endonuclease (29).

We have been able to distinguish different roles of the DNA binding motifs of human poly(ADP-ribose) polymerase by estimating enzymic activities and DNA-binding properties of polymerase and derivatives of polymerase expressed in E. coli. The zinc fingers are involved in the recognition of DNA breaks and nicks and the consequent formation of poly(ADP-ribose). The region C-terminal to the zinc fingers also binds DNA weakly, but this alone is not sufficient to engender enzymic activity. Rather, we think that this region adjacent to the zinc fingers may be involved in the other action of polymerase: the generalized binding to unbroken stretches of DNA. There are three features between residues 221 and 352, which might interact with DNA, two lysine clusters, and a possible helix-turn-helix, and it will be interesting to determine whether these contribute to DNA break-independent binding.

REFERENCES


ACKNOWLEDGMENTS—We thank Dr. H. Esumi of National Cancer Center Research Institute and Dr. S. Tanuma of Teikyo University for valuable discussions and Dr. Y. Nishimura of the National Institute of Genetics for providing the Lon+ strain.
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