Poly(ADP-ribose) polymerase at active centromeres and neocentromeres at metaphase

Elizabeth Earle*, Alka Saxena*, Andrew MacDonald†, Damien F. Hudson, Lisa G. Shaffer†, Richard Saffery, Michael R. Cancilla, Suzanne M. Cutts, Emily Howman and K. H. Andy Choo§

The Murdoch Institute, Royal Children’s Hospital, Flemington Road, Parkville 3052, Australia and †Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

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A double-stranded 9 bp GTGAAAAAG pJa sequence found in human centromeric α-satellite DNA and a 28 bp ATGTATATATGTATATAACACAAAT tandemly repeated AT28 sequence found within a cloned neocentromere DNA have each allowed the affinity purification of a nuclear protein that we have identified as poly(ADP-ribose) polymerase (PARP). Use of other related or unrelated oligonucleotide sequences as affinity substrates has indicated either significantly reduced or no detectable PARP purification, suggesting preferential but not absolute sequence-specific binding. Immunofluorescence analysis of human and sheep metaphase cells using a polyclonal anti-PARP antibody revealed centromeric localization of PARP, with diffuse signals also seen on the chromosome arms. Similar results were observed for mouse chromosomes except for a significantly enlarged PARP-binding region around the core centromere-active domain, suggesting possible ‘spreading’ of PARP into surrounding non-core centromeric domains. Enhanced PARP signals were also observed on α-satellite-negative human neocentromeres and on the active but not the inactive α-satellite-containing centromere of a human dicentric chromosome. PARP signals were absent from the q12 heterochromatin of the Y chromosome, suggesting a correlation of PARP binding with centromere function that is independent of heterochromatinic properties. Preliminary cell cycle analysis indicates detectable centromeric association of PARP during S/G2 phase and that the total proportion of PARP that is centromeric is relatively low. Strong binding of PARP to different centromere sequence motifs may offer a versatile mechanism of mammalian centromere recognition that is independent of primary DNA sequences.

INTRODUCTION

The centromere is an essential structure for the proper segregation of chromosomes in mitosis and meiosis. Centromere DNA of all higher eukaryotes studied to date has been shown to be composed of large arrays of tandemly repeated sequences (1). In humans and mouse, this DNA is the 171 bp α-satellite and 120 bp minor satellite sequences, respectively. A peculiarity of the centromeric repeats is their apparent lack of sequence homology through the phylogeny. The recent discovery of functional neocentromeres in humans (2–4) and Drosophila (5) that are devoid of the usual centromeric repeat DNA lends further support to the notion that a ‘magic’ centromere DNA sequence does not exist (1,4,6). The biological reasons for this centromere DNA plasticity and the mechanisms whereby the different species have evolved to compensate for this absence of centromere sequence conservation remain unclear.

An increasing number of centromere-binding proteins have been described (1,7–13). These proteins perform diverse roles including assembly and maintenance of centromere structure, sister chromatid cohesion, checkpoint control, chromosome movement and spindle microtubule dynamics. A number of these proteins are thought to interact with the mammalian centromere at the DNA, nucleosome or chromatin level. Four of these proteins (CENP-A, -B, -C and -G) are constitutive and specific to the centromere. CENP-A is a histone H3-related protein that probably replaces normal histone H3 to form a centromere-unique nucleosomal structure (14). A homologue of this protein, Cse4p, is found in Saccharomyces cerevisiae (15,16). CENP-B binds a 17 bp CENP-B box sequence motif found in the centromeric repeat DNA of a number of mammalian species, including those of human α-satellite and mouse minor satellites (17,18). The significance of this protein is uncertain since the protein is absent on human and mouse Y centromeres, all African green monkey centromeres (made up of α-satellite DNA) and analphoid human neocentromeres (1,4). The protein is non-essential in Cenpb-disrupted mice (19–21). CENP-C is an essential protein (22,23) that is thought to be important for centromere structure integrity and microtubule attachment (24). The highly basic nature of the protein and its significant homology with Mif2p, a centromere protein found in budding yeast that has been shown to interact with centromeric DNA in vivo (25), suggest that CENP-C may bind mammalian centromere DNA directly but its exact mode of action has not been established. CENP-G is a newly identified protein that associates with a subset of the human α-satellite DNA and is present on the centromeres of all human chromosomes (26). The role of this protein also remains to be investi-
Following washing with gel-shift buffer (see Materials and Methods), bound proteins were eluted in 0.5, 1 and 1.5 M NaCl. The 1 and 1.5 M (data not shown) fractions contained a highly purified 110 kDa protein (solid arrow). A relatively prominent 45 kDa band (open arrow) was often seen in the impure 0.5 M fraction and sometimes in the 1 M fraction. Protein size markers (mw) are shown in kDa. (b) Affinity purification of crude HeLa nuclear extract using double-stranded AT28 sequence, eluted in 1 M NaCl. (c) Affinity purification using pJ sequence variants MDR1 (i) and MDR2 (ii), eluted in 1 M NaCl. (d) South-western blot analysis of commercially purified human PARP (P) (Trevigen) and purified human protein using pJ affinity beads (i), showing strong binding to 32P-labelled pJ probe. (e) Western blot analysis of crude HeLa nuclear extract (i), human (ii) and mouse (iv) proteins purified using pJ affinity beads, human protein purified using AT28 affinity beads (iii), and commercial PARP (P), showing specific binding of the 110 kDa band to monoclonal anti-PARP antibody.

Affinity purification of a 110 kDa protein

A double-stranded 9 bp GTGAAAAAG sequence motif of the human centromeric α-satellite DNA was previously shown to bind an unknown nuclear protein, pkr, in HeLa cell nuclear extract using gel-mobility shift assay (31). Here, we employed this oligonucleotide to affinity-purify its binding protein from cultured human and mouse cells. Following binding of nuclear proteins onto the affinity resin, bound proteins were eluted stepwise with different salt gradients. Figure 1a shows results obtained at 0.5 and 1.0 M salt concentrations, indicating the presence of highly purified human and mouse proteins of 110 kDa in the 1.0 M elution fraction [also in the 1.5 M salt fraction (data not shown)]. A relatively abundant protein of 45 kDa is often seen in the 0.5 M fraction and occasionally in the 1 and 1.5 M fractions as a minor band.

We investigated further the possibility that the 110 kDa protein may bind sequences found within neocentromere DNA. This investigation was made possible by the availability of the complete sequence of an 80 kb core centromere antigen-binding region of the mar del(10) neocentromere (32). Detailed computational analysis of this sequence has demonstrated many AT-rich islands. Of particular interest was an ~600 bp variable number of tandem repeats (VNTR) sequence known as AT28, which has an A + T content of >80% and consists of 28 bp basic repeating units with a consensus sequence of 5'-ATGTATATATGTATATAGACATAAAT'. Because of the generally high A + T content of most centromeric sequences (1), we chose to test the AT-rich, double-stranded AT28 sequence as a possible substrate for affinity purification. The result demonstrated direct binding and a one-step purification of the 110 kDa protein from crude human nuclear extract (Fig. 1b).

As controls, we tested the affinity of the 110 kDa protein to bind a number of other double-stranded oligonucleotide sequences. These sequences included two variant dimeric oligonucleotides of the pJα motif (MDR1 and MDR2), a CENP-B box motif and four unrelated random oligonucleotides (R01–R04) (Table 1). Weak binding of the 110 kDa protein to MDR1 and MDR2 was observed (Fig. 1c), whereas no significant binding was detected for the CENP-B box motif and four unrelated random oligonucleotides (R01–R04) (data not shown). These results suggested preferential binding of the 110 kDa protein to the pJα and AT28 sequences, and greatly reduced or no affinity for other sequence motifs.

Table 1. Affinity of PARP for different double-stranded oligonucleotide sequences immobilized on Dynabeads

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>PARP binding</th>
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<tbody>
<tr>
<td>pJα</td>
<td>5'-GTGAAAAAG-GTGAAAAAG-3'</td>
<td>+++</td>
</tr>
<tr>
<td>AT28</td>
<td>5'-ATGTATATATGTATATAGACATAAAT-3'</td>
<td>++</td>
</tr>
<tr>
<td>MDR1</td>
<td>5'-GTGAATCAG-GTGAAATCAG-3'</td>
<td>+</td>
</tr>
<tr>
<td>MDR2</td>
<td>5'-GAGAAACAG-GAGAAACAG-3'</td>
<td>+</td>
</tr>
<tr>
<td>CENP-B box</td>
<td>5'-CTTGTTGGAACGGGA-3'</td>
<td>-</td>
</tr>
<tr>
<td>R01</td>
<td>5'-AGGCCTTGTGGAACGGGA-3'</td>
<td>-</td>
</tr>
<tr>
<td>R02</td>
<td>5'-CTTGTTGGAACGGGA-3'</td>
<td>-</td>
</tr>
<tr>
<td>R03</td>
<td>5'-CTTGTTGGAACGGGA-3'</td>
<td>-</td>
</tr>
<tr>
<td>R04</td>
<td>5'-CTTGTTGGAACGGGA-3'</td>
<td>-</td>
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</tbody>
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+ and –, positive and negative binding, respectively.
Identification of the 110 kDa protein as PARP

The 110 kDa human protein purified using the pJα-affinity resin was initially subjected to N-terminal microsequencing. The results indicated that the protein was blocked at the N-terminus, making this end inaccessible to direct sequencing. As an alternative approach, the purified protein was digested with trypsin and analysed by high-resolution HPLC. A database search of the resulting tryptic fingerprint pattern revealed a match with that of the human PARP enzyme. Microsequencing of three purified tryptic peptides confirmed their PARP identity (data not shown) and prompted further direct comparison and studies with commercially available PARP and anti-PARP antibodies. Using South-western blot analysis, the affinity-purified protein was shown to bind 32P-labelled pJα sequence identically to the commercial PARP (Fig. 1d). [32P]pJα binding was abolished when an excess of unlabelled pJα oligonucleotide was added in the probe mix as competitors (data not shown). In western blot analysis, both polyclonal and monoclonal anti-PARP antibodies cross-reacted with crude HeLa nuclear extract, mouse and human 110 kDa protein purified using the pJα oligonucleotide, as well as human 110 kDa protein purified using the AT28 oligonucleotide (Fig. 1e). These studies identified the affinity-purified 110 kDa protein as PARP.

Centromere localization of PARP

The polyclonal anti-PARP antibody was employed in immunofluorescence studies. The antibody was raised against a purified recombinant peptide corresponding to amino acids 71–329 of murine PARP (33). Figure 2a–c shows the results of immunofluorescence staining of metaphase chromosomes from cultured human, mouse and sheep cells. In order to determine clearly the position of the centromere, the slides were simultaneously stained with an anti-centromere antiserum CREST#6, which was known to cross-react with centromere proteins CENP-A and -B (2). The results indicated strong centromeric localization of PARP on all the chromosomes in the three species, with diffuse staining also seen on the chromosomal arms. Interestingly, the mouse chromosomes showed a larger area of enhanced anti-PARP staining relative to the CREST#6 antiserum staining, suggesting possible PARP association with both mouse minor and major satellite DNA.

Analysis of PARP distribution during interphase was performed. Cells in G1 phase were identified by the presence of only single CREST immunofluorescence signals (corresponding to non-replicated chromosomes) (Fig. 2d), whereas cells in S/G2 phase were recognized by the detection of paired immunofluorescence signals due to centromere replication (Fig. 2e). Staining of these interphase cells with anti-PARP antibodies revealed high background signals throughout the nucleus, with especially prominent patches of staining seen in some intranuclear structures that presumably corresponded to the nucleoli. No specific colocalization of CREST with discrete PARP signals was observed in the G1 nucleus (Fig. 2d), whereas close examination of S/G2 nuclei revealed a weak colocalization in many discernible foci despite the high background level of PARP staining (Fig. 2e). These results suggest that PARP accumulation at the centromere may begin during S/G2 phase, and continue into and become increasingly prominent at the centromere as the chromosome condenses.

Absence of PARP on human non-centromeric heterochromatin

We were interested to know whether: (i) PARP binding distinguishes between centromeric heterochromatin and other non-centromeric heterochromatic regions of the human genome;
(ii) PARP binding is specific for active centromeres only; and (iii) the protein recognizes the non-heterochromatic and alphoid neocentromeres. To address the first question, we closely examined the immunofluorescence results of human metaphases such as that shown in Figure 2a. The analysis indicated tight PARP and CREST#6 signals on all the human centromeres with no evidence of concentrated PARP staining at all non-centromeric heterochromatic regions of the genome (Fig. 2a, and data not shown). To gain further insight, we combined PARP immunofluorescence and fluorescence in situ hybridization (FISH) using a Y chromosome-specific α-satellite probe to examine the Y chromosome. This chromosome was chosen because its long-arm heterochromatic region is well separated from the centromeric heterochromatin and because its α-satellite-containing centromere is devoid of CENP-B proteins. Figure 3a indicates a distinctively enhanced binding of PARP to the centromere but not the q12 heterochromatic region of this chromosome, thus confirming preferential PARP binding for the centromeric heterochromatin.

**Presence of PARP on the active but not the inactive centromere**

In an earlier study a t(X;15) pseudo-dicentric human chromosome was shown to contain an active centromere derived from a translocated X chromosome and an inactive centromere derived from chromosome 15 (34). In that study only the active centromere bound centromere protein CENP-C, whereas both the active and inactive centromeres were shown to bind CENP-B. Here, we further characterized and confirmed the properties of the dicentric chromosome by demonstrating specific binding of the active but not the inactive centromere to another essential (35) centromere protein, INCENP, as well as establishing the presence of substantial amounts of α-satellite DNA on both the centromeres (Fig. 3b). Immunofluorescence analysis of the dicentric chromosome (Fig. 3c) indicated colocalization of the PARP signal with the anti-centromere antibody signal on the active centromere but not on the inactive centromere. The specificity of the CREST#6 anti-centromere antibody for CENP-A and -B, and the fact that CENP-B was known to bind both active and inactive α-satellite-containing centromeres (34,36,37), explained the CREST#6 immunofluorescence signal on the inactive centromere (CENP-B only), and the significantly stronger CREST#6 signal seen on the active centromere (CENP-A + CENP-B).

**PARP binding to human neocentromeres**

Two previously reported human marker chromosomes containing neocentromeres, mar del(10) and inv dup(20), derived from the q25 region of chromosome 10 (2,36,38) and p12 region of chromosome 20 (39), respectively, were used for this study. Initial immunofluorescence analysis using the anti-PARP antibody on metaphase chromosomes indicated strong signals on all the centromeres including the neocentromeres (Fig. 2a, and data not shown). Subsequent experiments employed simultaneous anti-PARP immunofluorescence and FISH using a cloned probe for the mar del(10) neocentromere. The result indicated a positive colocalization of immunofluorescence with the neocentromere DNA signal (Fig. 3d) but not on the corresponding 10q25 region of a normal chromosome 10 in the absence of neocentromere formation (data not shown), thus confirming increased direct binding of PARP to the functional neocentromere.

**DISCUSSION**

The 9 bp GTGAAAAAG pJα sequence motif used in the affinity purification procedure resulted in the isolation of a 110 kDa protein that, based on size, is different from the 15 kDa pJα protein previously shown to bind this sequence by gel-mobility shift assay (31). The reason for this discrepancy is at present unclear. Several lines of evidence indicate that the 110 k Da protein affinity-purified using the pJα or AT28 sequence is PARP. These include similar molecular weight and tryptic and sequence profiles, South-western blot comparison against commercial PARP and western blot analysis using anti-PARP antibodies. The decreased or zero binding seen when other related or unrelated oligonucleotides were used as
affinity substrates further indicates considerable, but not absolute, binding specificity for the pJα and AT28 sequences.

Immunofluorescence studies on human, mouse and sheep metaphase chromosomes establish PARP as a previously unsuspected protein of the mammalian centromere. Since PARP is a highly conserved protein in all higher eukaryotes, it is likely that the protein may also be a component of other non-mammalian centromeres. Cell cycle analysis indicates that PARP is present abundantly in the interphase nucleus. This observation is consistent with the reported role of this enzyme in many central nuclear functions (discussed below). Despite the high level of nuclear staining, making it difficult to detect PARP binding to specific substructures, some colocalization of the enzyme with centromeric antigens is discernible during S/G2 phase. Such a colocalization becomes more prominent as the chromosomes condense and the cells progress into metaphase. It is unclear whether this greater prominence is due to an increased PARP binding at the centromere or to the significantly decreased background signals following the disassembly of the nuclear membrane during the mitotic phase. Regardless, the S/G2 results suggest a possible functional role of PARP at the centromere at the time of, or shortly after, DNA replication.

Analysis of a pseudo-dicentric chromosome carrying an active and an inactive centromere, both containing α-satellite sequences, indicates that PARP binding is unique to the active centromere and that this binding can discriminate against inactive centromeric heterochromatin. That PARP binding is not preferentially associated with constitutive heterochromatin is also demonstrated by our failure to detect elevated levels of the protein on the major block of heterochromatin at the Yq12 region. Investigation of two unrelated neocentromeres derived from chromosomes 10 and 20 further indicates association of PARP with non-heterochromatic neocentromeres. These observations clearly demonstrate that enhanced PARP binding correlates directly with centromere/neocentromere activities and that binding is independent of the heterochromatic properties of a chromosomal region.

PARP has been described as a DNA-binding protein that recognizes single-strand DNA breaks in a sequence-independent manner, symptomatically covering 7–8 nucleotides on each side of the break (40,41). The protein is highly conserved through evolution and enzymatically catalyses poly(ADP-ribose)ation reactions that appear ubiquitous in higher eukaryotes (42–44). DNA binding is believed to be a prerequisite for enzymatic activity of PARP. Despite extensive studies, the physiological role of poly(ADP-ribose) metabolism in cells remains unclear. Several hypotheses suggest an involvement in central nuclear processes, such as DNA repair, apoptosis, transcriptional regulation and differentiation, anti-recombination, chromosomal stability, cell cycle control, scaffold attachment and modulation of DNA or chromatin structures (reviewed in refs 29,30). In view of these diverse possibilities, it is of interest to speculate on the mode of interaction between PARP and the centromere, and the role PARP may play in the centromere.

Our finding that PARP binds in vitro strongly to two defined DNA sequence motifs found in normal human centromeres and a functional neocentromere suggests a possible mechanism whereby PARP may be recruited to the centromere through direct DNA recognition. In the case of normal human centromeres, the high prevalence of the pJα motif in α-satellite DNA would provide a generous template for PARP binding. For the mar del(10) neocentromere, it is possible that sequences other than the AT28 DNA, such as the many AT-rich islands scattered throughout the cloned neocentromere DNA (32), may also serve as suitable substrates for PARP binding. It is now believed that there is no ‘magic’ centromere DNA sequence and that centromere DNA is, in general, not conserved throughout evolution (1,4,6). Our observation suggests the possibility that centromere/neocentromere recognition by PARP may occur in a sequence-independent manner that may offer a compensatory mechanism for the centromere DNA plasticity paradox.

Although the in vitro affinity binding studies suggest that direct interaction with centromere-specific DNA sequences may be responsible for the centromeric localization of PARP, other observations indicate that the nature of this interaction is somewhat more complex. First, the fact that centromERICally inactive α-satellite DNA on a dicentric chromosome and the DNA sequences on a normal chromosome 10 in the absence of neocentromere formation do not both show enhanced PARP binding suggests that sequence alone is insufficient and that binding is in some ways linked with centromere activity. Secondly, on mouse chromosomes, strong localization of PARP to a larger area (presumably due to major satellite DNA) than that occupied by the anti-centromere CREST antibodies (presumably due to minor satellite DNA) suggests that additional PARP binding can occur around the nucleus of the core centromere activity. This enlarged area of PARP binding is probably unlikely to be solely related to the heterochromatin-or sequence-nature of the mouse major satellite, since the human Yq12 and dicentric chromosome results suggest that heterochromatin or sequence alone is not sufficient for PARP binding. Instead, we propose a possible model for the mouse centromere where the nucleation of PARP on a preferred site (minor satellite) may promote or allow the ‘spreading’ of PARP binding to a surrounding less-preferred site (major satellite). Other workers have previously proposed a similar model for the spreading of centromere-marking factors to adjacent non-centromeric sequences as a way of imprinting a silent state of the centromere property onto neighbouring chromosomal regions (5,6).

What is the role of PARP at the centromere? Simplistically, a role related to the enzymatic function of this protein could be implicated, i.e. poly(ADP-ribose)ation modification of the protein itself (automodification) or of other protein acceptors (heteromodification) (29). The list of protein acceptors shown to be modified by PARP includes histones, topoisomerases, DNA polymerases, HMG-proteins, nuclear matrix proteins and protein kinases. Generally, modification of such potential target proteins through the addition of ADP-ribose moieties inhibits their activity, probably because the modification decreases their DNA-binding ability. It can therefore be proposed that PARP may be a negative epigenetic (6,45) regulator of the activity of one or more protein acceptors at the centromere. Two recent studies on the telomere have highlighted a role of PARP in the regulation of chromosomal structure and activity. First, Smith et al. (46) reported the identification of a PARP homologue (tankyrase) and ADP-riboseylation activity at human telomeres, and proposed a similar negative regulatory role for the protein. Secondly, d’Adda di Fagagna et al. (47) showed that PARP-deficient
mice/cells display telomere shortening and severe chromosomal instability characterized by increased chromosome fusion and aneuploidy. In addition to these studies, the observation that mice deficient in PARP appear normal (48–50) implies functional redundancy of this protein, a possibility supported by the recent report of two novel PARP homologues (51). Further studies aimed at elucidating the role of PARP and putative functional homologue(s) in the centromere should be rewarding.

MATERIALS AND METHODS

Cell lines

Human HeLa cells were used for the preparation of nuclear extracts for the affinity purification of PARP. Cell lines used for immunofluorescence and FISH analyses were: (i) two lymphoblastoid cell lines BE and JW carrying a neocentromeric marker chromosome derived from human chromosomes 10 (2) and 20 (39), respectively; (ii) a human t(X;15) pseudo-dicentric chromosome with an active X-derived centromere and an inactive 15-derived centromere (34); (iii) a mouse embryonic stem cell line R1(+/-b) derived from the R1 strain (Murdock Institute culture collection, Melbourne, Australia); and (iv) a Merino sheep fibroblast cell line S99 (Murdock Institute culture collection).

Preparation of nuclear extract

HeLa cells (10⁷) were harvested using trypsin, washed once in phosphate-buffered saline (PBS) and pelleted at 200 g. The cells were washed in 5 ml of Buffer A (10 mM HEPES–KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂) containing fresh protease inhibitors [10 mM diithiothreitol (DTT), 0.1% phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin]. After repelleting, the cells were resuspended in 5 ml of Buffer A and incubated on ice for 20 min. On immediate addition of 325 µl of 10% NP-40 the cells were vortexed for 10 s and pelleted at 300 g. The nuclear pellet was resuspended in 2.5 ml of protein extraction buffer (420 mM NaCl, 20 mM HEPES–KOH pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol) containing protease inhibitors as above and incubated on ice for 20 min, mixing occasionally. The nuclei were then centrifuged at 500 g and the supernatant, containing the nuclear proteins, collected.

Affinity purification

Affinity purification of nuclear proteins was achieved using double-stranded oligonucleotides coated onto magnetic beads. Streptavidin-coated Dynabeads (175 µl of 1 mg/ml) (Dynal, Oslo, Norway) were incubated with 3 µg (100 ng/µl) of 5'-biotinylated oligonucleotides and 60 µl of PBS (pH 7.4) on a shaker at room temperature for 30 min. Nuclear extract (1.5 ml) was pre-incubated with 125 µl of 10 mM DTT, 125 µl of 1 µg/ml poly(dI·dC) and 0.25 ml of Gel Shift Buffer (GSB) (10 mM HEPES, 40 mM KCl, 2 mM MgCl₂, 5% glycerol) and 0.5 ml of distilled water for 20 min at room temperature. The extract- and oligonucleotide-coated beads were combined for 20 min with gentle agitation at room temperature. The protein-bound beads were separated using a magnetic separator (Dynal) and washed once in GSB. Bound proteins were eluted using 300 µl of increasing concentrations of NaCl. This elution (10 µl) was analysed by polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining.

Protein sequencing

Affinity-purified protein samples were de-salted and concentrated by methanol precipitation for 3 h at −80°C followed by centrifugation at 12 000 g for 10 min. Samples were separated by PAGE and electroblotted onto PVDF membrane. The membrane was stained by a brief (<5 min) incubation in Coomassie stain (0.2% Coomassie Blue R-250, 45% methanol, 10% acetic acid) and destained in 45% ethanol, 10% acetic acid. The membrane was allowed to dry and the desired protein hand cut from the membrane for sequencing. N-terminal sequencing was performed by Edman degradation using a Perkin-Elmer (Foster City, CA) ABI Model 494 Procise sequencer with an on-line 140C Microgradient HPLC. For internal sequencing ~25 pmol were digested in trypsin and the resulting peptides were separated using the Pharmacia SMART system. Selected peptide fragments were sequenced using Edman chemistry as described above. To obtain the tryptic digest fragmentation pattern the gel slice containing sample protein was washed in 60:40 (v/v) 50 mM (NH₄)₂CO₃, acetonitrile several times to remove residual Coomassie stain. The gel slice was then dried by vacuum centrifugation for 20 min. The dried gel sample was then rehydrated in 20 µl of trypsin solution (12.5 ng/µl) in 50 mM (NH₄)₂CO₃ and incubated at 4°C for 45 min. The remaining solution was removed and the gel slice was incubated in 50 mM (NH₄)₂CO₃ for 4 h. The sample was then purified through a microcolumn filled with POROS R2 resin (PerSeptive Biosystems, Sydney, Australia) and spotted onto a MALDI plate with α-cyano-hydroxycinnamic acid matrix and subjected to MALDI-TOF mass spectrometry using a PerSep tive Biosystems Voyager DE-STR mass spectrometer fitted with a 337 nm nitrogen laser. Spectra were produced from the summed average of 256 laser shots and calibrated using internal tryptic digest fragments 842.51 Da and 2211.09 Da. The resultant peak list was used to search the Peptide program available via the ExPasy server (http://www.expasy.ch).

South-western blot analysis

Analysis was carried out in duplicate. HeLa nuclear extract (5 µg), 0.3 pmol of affinity-purified protein and 0.5 µg of commercial PARP protein were separated by PAGE and transferred to Hybond C at 80 mA for 2 h in transfer buffer (2.4 g/l Tris, 11.25 g/l glycine). Following this, the identically blotted membranes were rinsed for 5 min each in 6, 3, 1.5, 0.75, 0.375 and 0.18 M guanidine–HCl pH 7.9. The membranes were blocked overnight at 4°C in GSB containing 100 µg/ml salmon sperm DNA and 5% skimmed milk. Following this, the membranes were washed in rinse buffer pH 7.9 (GSB containing 0.25% skimmed milk, 1 mM DTT). Both membranes were incubated overnight at 4°C with 300 ng of radioactively end-labelled pK probe diluted in 10 ml of rinse buffer containing 100 µg/ml salmon sperm. A 1000-fold excess of cold pK was added to one membrane as competition. Membranes were washed by 3 x 30 min washes in rinse buffer pH 7.9 containing 100 µl of Triton X-100 at 4°C and visualized by autoradiography.
Western blot analysis

Western blot analysis was done using monoclonal anti-PARP antibody (Trevigen, Gaithersburg, MD), which recognizes full-length PARP and 85 kDa apoptosis-related cleavage fragment and polyclonal anti-PARP antibody (R&D Systems, Minneapolis, MN).

Protein samples were separated by PAGE and transferred to Hybond C as described for South-western blotting. The filter was exposed for 3 days at -70°C to visualize the bands. The bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and autoradiography.

FISH and immunofluorescence analysis

Single or simultaneous FISH and immunofluorescence detection of chromosome proteins were performed as previously described (2). Human anti-centromere antibody CREST#6 was specific for centromere proteins CEPN-A and -B (2). Rabbit anti-chicken INCENP antibody was a generous gift of W.C. Earnshaw (University of Edinburgh, Edinburgh, UK). Goat polyclonal anti-murine PARP antibody was purchased from R&D Systems.

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