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PRINCIPAL INVESTIGATOR: Suhua Han, M.D.
Linda Mâlkas, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
Baltimore, Maryland 21201

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Suhua Han, M.D.
Linda Málkas, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland
Baltimore, Maryland 21201

E-Mail: shan002@umaryland.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Poly (ADP-ribose) polymerase (PARP) is a component of the DNA synthetic apparatus of cells, (i.e., the DNA synthesome). PARP is involved in the process of DNA replication, DNA repair, and cellular transformation. At least 15 components of the DNA synthesome are poly (ADP-ribosylated), which suggests that PARP may play a regulatory role in controlling the activity and the fidelity of the DNA synthesome of breast cancer cells. We also observed that the level of PARP activity found in breast cancer cells is below that found in non-malignant breast cells. We have also confirmed that PARP is a component of the DNA synthesome by both MALDI and quadrapole-time of flight (Q-TOF) mass spectrometry. We also observed PARP to be more highly expressed in the breast cancer cells, despite a lower overall activity. It is our goal to establish a clear link between the differences in the activity of PARP and the alteration(s) in structure exhibited by this protein in both malignant and non-malignant breast cells.

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INTRODUCTION

Our laboratory was the first to isolate and extensively purify an intact, stable and fully functional mammalian cell multi-protein DNA replication complex, termed the DNA synthesome (Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996b; Lin et al., 1997; Jiang et al., 2000). The DNA synthesome has been isolated from a variety of mammalian cells and tissues, including nonmalignant and malignant human breast cells as well as tissues. It has been shown that the DNA synthesome is fully competent to support SV-40 origin-specific and large T-antigen dependent DNA replication *in vitro*. All of the components necessary for DNA replication, including Poly (ADP-ribose) polymerase (PARP), have been detected in the synthesome. The malignant breast cell DNA synthesome exhibits a 6-8 fold decrease in the replication fidelity relative to the non-malignant breast cell DNA synthesome(Sekowski et al., 1998). In addition, the transformation of a non-malignant human breast epithelial cell to a malignant state is accompanied by a significant alteration in the mobility of specific protein components of the DNA synthesome following two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the DNA synthesome (Bechtel et al., 1998). These differences in replication fidelity and 2D-PAGE gel protein profiles between the non-malignant and cancer cell derived DNA synthesome clearly suggest critical differences in the make-up of the replication machinery of these cells. PARP has long been implicated in the process of DNA replication, DNA repair, and cellular transformation. The regulatory role of PARP in these processes is through the addition of poly (ADP-ribose) polymers to a number of nuclear proteins. PARP activity is also mediated by auto poly (ADP-ribosylation). At least fifteen components of the DNA synthesome are poly (ADP-ribosylated) (Simbulan et al., 1996), suggesting that PARP may play a regulatory role in controlling the activity (and potentially the fidelity) of the DNA synthesome of breast cells. Consistent with this suggestion is the observation that breast cancer cells exhibit decreased levels of PARP activity (Hu et al., 1997) for as yet undefined reasons. It is our goal to establish a clear link between the differences in the activity of PARP and the alteration(s) in structure exhibited by this protein in both nonmalignant and breast cancer cells. In this proposal, we will focus on defining the kinetic, biophysical and detailed structural properties of PARP in both breast cell types.

PROGRESS REPORT

Mass Spectrometry analysis of a DNA synthesome enriched protein fraction validates that PARP is part of the replication complex core

Mass spectrometry has been used as a "mining tool" for providing protein sequence information (Yates, et al. 1995; Patterson et al. 1995; Yates et al. 1998). This technique has rapidly become integrated into the filed of functional genomics, and has proven to be useful for identifying the components of multiprotein structures (Neubauer et al., 1998; Zachariae et al., 1998; Link et al., 1999), and analyzing protein-protein interactions (Shevchenko, et al. 1999). MALDI, (Matrix Assisted Laser Desorption and Ionization), peptide mapping is a recognized high-throughput and exquisitely sensitive technique that is capable of providing the identification of dozens of gel-separated proteins each day (even when these proteins are present at the low femtomole level). MALDI has nearly always been applied in time-of-flight (TOF) mass spectrometers, where the introduction of a technique known as delayed extraction (time-lag focusing) has increased the accuracy of mass measurements to the tens of parts per million (ppm) level. A hybrid MALDI/Q-TOF instrument is formed when a MALDI ion source is interfaced to an orthogonal injection time-of-flight mass spectrometer and then to a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF) (Krtutchinsky, et al., 1998; Loboda et al., 1999). Use of MALDI/Q-TOF mass spectrometry allows facile identification of proteins at the femtomole level in complex mixtures by searching against comprehensive protein and expressed sequence tag databases (Shevchenko et al, 2000). It has been used to identify proteins separated by one-dimensional or two-dimensional gel electrophoreses.

MCF-7 cells were harvested and fractionated to a clarified nuclear extract and post-microsomal (NE/S3) fraction. NE/S3 was then layered onto a 2M-sucrose cushion and centrifuged. Following centrifugation, the 2M-sucrose interface (P4 fraction) was successfully removed by aspiration. The complex of enzymes is further purified by the Q-Sepharose chromatography (Fig. 1). The Q-sepharose fraction is then resolved onto a 1-D SDS PAGE gel. Each of the protein bands on the gel was cut and analyzed by MALDI and Q-TOF mass spectrometry. Data from these analysis has shown that PARP, proliferation cell nuclear antigen (PCNA), replication protein A (RP-A), replication factor C (RF-C), DNA polymerase, DNA methyl transferase and flap endonuclease-1 (Fen1) are components of the replication complex core.

PARP is enriched during its purification from breast cancer cells

We attempted to purify PARP from MCF-7 cells according to a published procedure (Brutcher et al.). Five grams of MCF-7 cells were fractionated to a clarified nuclear extract and post-microsomal (NE/S3) fraction and shaken with 1g of solid hydroxyapatite equilibrated with ADPRT buffer [Tris-HCL (100mM), $K_2S_2O_5$ (25mM), β-mercaptoethanol (12mM), EDTA (0.5mM), and 17% glycerol at pH 7.2] containing

50mM KH_2PO_4. Subsequently, the suspension was centrifuged, the supernatant was discarded and hydroxyapatite was eluted with ADPRT buffer containing 0.5M KH_2PO_4. The presence of PARP in the eluted fractions was checked by slot blotting (Life Technologies Inc.). Data has shown most of the PARP present in the NE/S3 remains in the supernatant. Since the NE/S3 fraction is much cleaner than the crude extract from placenta used by Brutcher et al., this hydroxyapatite chromatography step was skipped.

The NE/S3 fraction was directly placed onto a phosphocellulose (P-cel) column because phosphocellulose dissociated the synthesome multiprotein complex and resolved PARP from many of the other proteins in this fraction. Then a gradient of KCl (0-2.0M) in ADPRT buffer was applied to the column. The presence of PARP in the eluted fractions was determined using a slot blotting technique. At first, PARP was not significantly enriched, and was presented in every fractions eluting from the column, (Figure 2 A). We improved the separation of PARP by altering our original activation procedure for the phosphocellulose. We stir 2g of P-cel into 50ml of 0.5N NaOH for 5minutes, wash with water until the pH was less than 11, and then we incubate the beads with 0.5N HCL for 5 minutes. Again we washed the P-cel with water until the pH was greater than 3. The key point is that the equilibration time in either acid or base should not be over 5 minutes. Then we loaded the NE/S3 onto the phosphocellulose column, and eluted the column as previously described. PARP was clearly enriched using this procedure, (Figure 2, B).

To further purify PARP, we prepared an affinity column that specifically recognizes PARP. In order to prepare the affinity column, First, a weak PARP inhibitor 4-{[3-(amino carbonyl)phenyl]amino}-4-oxybutanoic acid (California Peptide Research, Inc.) was coupled to amino-propyl-Sepharose (AP-Sepharose) according to the procedure described by (Brutcher et al.). The fractions enriched with PARP from the phosphocellulose column were collected and applied to the affinity column. PARP was later eluted from the affinity column by ADPRT buffer containing 1mM 3-methoxy benzamide. The fractions containing PARP were identified again by slot blotting (Figure 3). These fractions were then collected and dialyzed into buffer A (20mM Hepes, PH 7.5, 5mM KCL, 1.5mM MgCl2, 1mM DTT) and concentrated by speed vacuum. The degree of purification was determined by western blotting (Figure 4). Equal amounts (20ug) of NE/S3, individual fractions of the phosphocellulose eluate and the eluate from the affinity column were then subjected to a 1D-Polyacrylamide gel electrophoresis (1D-PAGE), and the resolved proteins were transferred to a nitrocellulose membrane. Western blotting analyses with an antibody specifically recognizing PARP (anti-human PARP antibody, BD PharMingen) confirmed that the phosphocellulose column greatly enriched PARP.

While the slot blot analysis demonstrated that PARP was enriched by the affinity chromatography step, (figure 3), we were unable to detect PARP following standard Western blotting of the concentrated column eluate. This result may be due to the concentrating procedure. One possibility is that PARP is lost during the concentration step which is performed in a speed vac. In order to overcome this potential problem we plan to use a High-Performance Centrifugal Concentrator (Orbital Biosciences). This

concentrator is equipped to remove glycerol and concentrate protein without lyophilizing it. A user's guide is attached to extensively explain the procedure.

Characterization of PARP in non-malignant and malignant breast cells

In order to analysis the isoelectric point and 2-D PAGE mobility of PARP expressed by the non-malignant (MCF-10A) and malignant (MCF7) breast cells, (we found that it was necessary to obtain the MCF-10A cells from the Barbara Ann Karmanos Cancer Institute, Cell Lines Resources, Detroit, MI), we fractionated pellets of each of the two cell types to the level of a clarified nuclear extract and a post-microsomal (NE/S3) supernatant. The resulting NE/S3 was then layered onto a 2M sucrose cushion and centrifuged at 100,000 xg for 18 hours. Following centrifugation, the 2M sucrose interface (P4 fraction) was collected by aspiration. P4 fractions were dialyzed into buffer A, and applied to a CHROMA SPIN+STE-10 Column (CLONTECH INC.), which had been washed three times with de-ionized water in order to desalt the P4 fractions. Equal amounts of MCF-7 and MCF-10A P4 fraction (30ug) were then analyzed by 2-D PAGE and the proteins in the P4 were resolved from one another. Two gels were run for each cell line, one of the gels was silver stained, while the other gel was transferred to a nitrocellulose membrane and western blotting was done using anti-human PARP antibody (BD PharMingen). Since the theoretical PI of PARP is 8.99 (Swiss Institute of Bioinformatics), it was difficult to resolve PARP in our regular tube gel preparations (5.5g urea, 1.33ml polyacrylamide, 2.00ml 10 Triton-100, 0.4ml 5/7 ampholyte, 0.1ml 3/10 ampholyte, 1.97ml H_2O). Our initial 2D gel analyses have been difficult. Different conditions have been tried to improve resolution of the protein. As an example, I substituted 0.4ml of pH 8/10 ampholyte for the pH 5/7 ampholyte used to prepare the first dimension isoelectric focusing gel. This alteration in the protocol resulted in the protein resolution becoming better, but the focusing of the 2D PAGE was still not ideal. The true isoelectric point of PARP was found to be difficult to calculate or determine presumably because of post-translational modifications that altered the pI from its theoretical value.

To improve the resolution of PARP we have decided to explore a new method. A ReadyPrep 2-D gel Starter Kit (BioRad Laboratories, Inc.) which substitues a linear pH test strip for the first dimension tube gel. These strips produce a true linear gradient of pH whereas the gradient formed in the tube gels is somewhat variable and difficult to repeat. I will use the ReadyStrip IPG strips, 11cm, pH 7-10 in place of the first dimension gel, and run them with PROTEAN IEF cell (BioRad Laboratories, Inc.). The focusing electrical voltage is 8000 volts. We expect, the resolution to be better than that obtained with the non-linear first dimension tube gels. The P4 fractions from each cell line will be resolved into two gels each time. One of the gels will be silver stained, the other will be transferred to a nitrocellulose membrane and western blotting will be done. The gel that was silver stained will be analyzed using the Melanie II software (Bio-Rad). The analysis will identify the location of the PARP in the gel, and empirically predict the isoelectric point(s) (pIs) of PARP expressed by malignant and the non-malignant breast cells.

During the first phase of the investigation, we observed that PARP is more highly expressed in breast cancer cells than in non-malignant breast cells. In order to understand why despite a higher level of protein expression, there is a lower activity of PARP in malignant breast cells we have designed an experiment to measure PARP mRNA levels in both MCF-7 and MCF-10A cells. Exponentially growing MCF-7 and MCF-10A cells will be harvested and quantified using a dye exclusion assay. RNA will be isolated from equal numbers of cells using Trizol reagent. The same amount of RNA will be loaded onto a nitrocellulose membrane and a [^{32} P] labeled PARP cDNA will be hybridized to the slot blot. The blot will be used to charge a PhosphoImager screen, and the amount of mRNA expressed in both cell types will be quantified. This experiment should be able to reveal whether the level of PARP mRNA is greater in the MCF-7 than the MCF-10A cells, and if not will point toward the PARP protein being more highly expressed because of either increases in its level of synthesis or a decrease in its rate of degradation in the MCF-7 cells.

Key Research Accomplishments

- Analyzed DNA synthesome by MALDI and Q-TOF mass spectrometry
- Purified PARP from breast cancer cells
- Performed 2D PAGE and analyzed PARP in malignant and non-malignant breast cancer cells

Reportable Outcomes

Papers

Han, S.H., Malkas, L.H., Hoelz, D.J., Hickey, R.J. (2001): Purification of Poly (ADP-ribose) Polymerase from Breast Cancer Cells by Chromatography. Manuscript in preparation.

Conclusions:

- PARP is confirmed to be one of the components of the DNA synthesome
- PARP is enriched by phosphocellulose chromatography.
- PARP is more highly expressed in breast cancer cells
- Purified PARP protein is lost during the concentrating process

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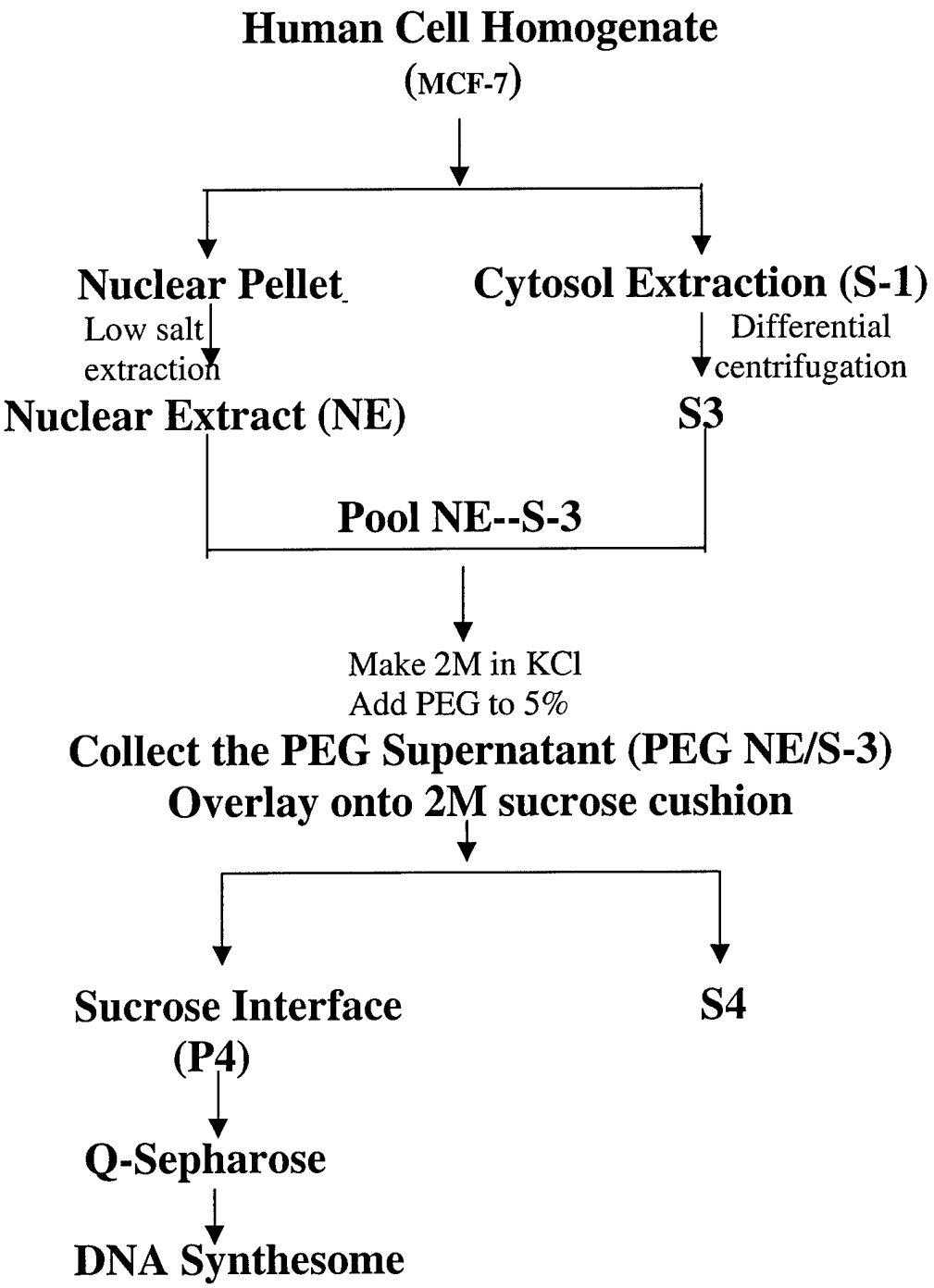
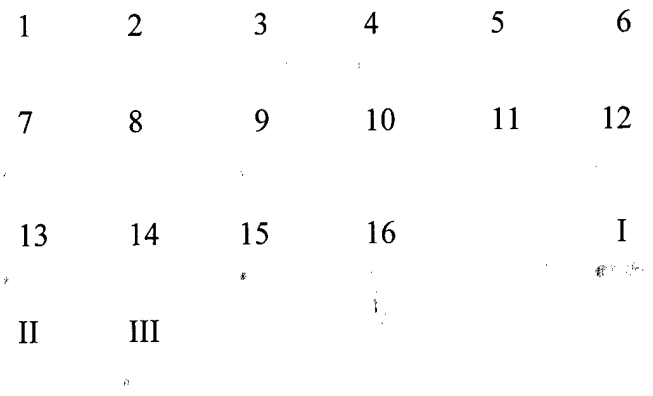


Figure 1. The purification of DNA synthesome from human cells.



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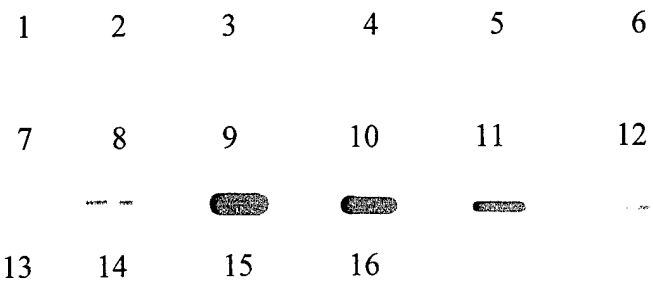

B

Figure 2. The Presence of PARP in the Eluted Fractions of a Phosphocellulose Column. MCF-7 cells were fractionated to NE/S3. NE/S3 was loaded onto a phosphocellulose column, washed with ADPRT buffer, and eluted with ADPRT buffer containing KCL (0.01M-2M). The presence of PARP in the eluted fractions was determined by slot blotting (Life Technologies, Inc.). Slot #1.) 0.01M KCL; 2.) 0.025M; 3.) 0.05M; 4.) 0.075M; 5.) 0.1M; 6.) 0.2M; 7.)0.3M; 8.) 0.4M; 9.) 0.5M; 10.) 0.75M; 11.) 1.0M; 12.) 1.2M; 13.) 1.4M: 14.) 1.6M; 15.) 1.8M; 16.) 2.0M; I) Flow through; II) First wash; III) Second wash.

A. PARP distribution in the initial purification
B. PARP distribution after changing the phosphocellulose activation conditions

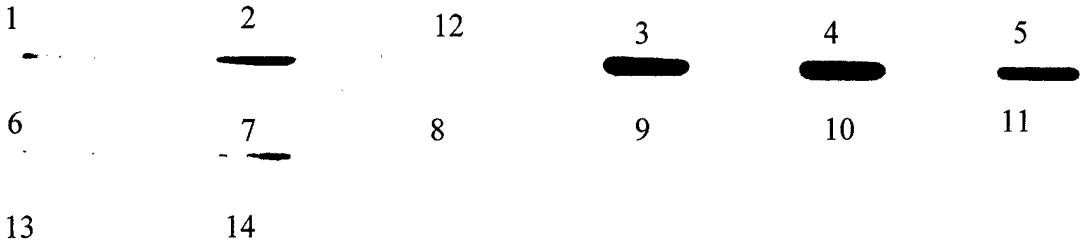

Figure 3. The Presence of PARP in the Eluted Fractions from the Affinity Column. The Eluted fractions from the phosphocellulose column that was enriched with PARP were collected, loaded onto affinity column and eluted with ADPRT buffer containing 1mM 3-methoxy benzamide (Step 1-14). The presence of PARP in the eluted fractions was determined by slot blotting.

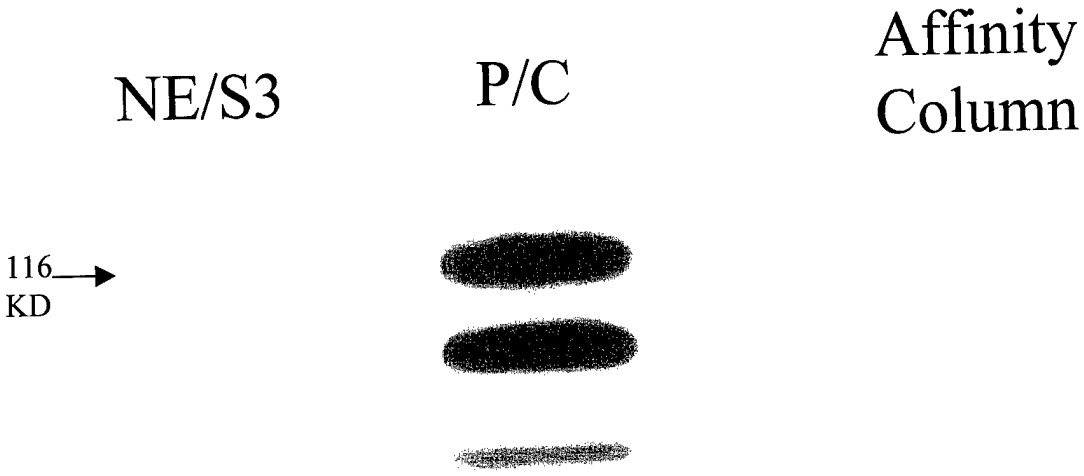

Figure 4. PARP was Enriched by the Phosphocellolose Column. Equal amounts of protein from NE/S3, and the eluted fractions of the phosphocellulose and the affinity columns (enriched with PARP), were resolved by 1D PAGE and transferred to a nitrocellulose membrane. The presence of PARP was determined by Western blotting.

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Advances in Brief

A Unique Form of Proliferating Cell Nuclear Antigen Is Present in Malignant Breast Cells[1]

Pamela E. Bechtel, Robert J. Hickey, Lauren Schnaper, Jennifer W. Sekowski, Brian J. Long, Robert Freund, Ni Liu, Carla Rodriguez-Valenzuela, and Linda H. Malkas[2]

Departments of Pharmacology and Experimental Therapeutics [P. E. B., R. J. H., J. W. S., B. J. L., N. L., C. R-V., L. H. M.] and Microbiology and Immunology [R. F.], University of Maryland School of Medicine; Greenebaum Cancer Center [R. J. H.]; Program in Oncology, Greenebaum Cancer Center [R. F., L. H. M.]; Programs in Molecular Cellular Biology [R. J. H., R. F., L. H. M.] and Toxicology [R. J. H., L. H. M.], University of Maryland; and Greater Baltimore Medical Center, Comprehensive Breast Care Center [L. S.], Baltimore, Maryland 21201

Abstract

Despite extensive research efforts to identify unique molecular alterations in breast cancer, to date, no characteristic has emerged that correlates exclusively with malignancy. Recently, it has been shown that the multiprotein DNA replication complex (synthesome) from breast cancer cells has a significantly decreased replication fidelity compared to that of nonmalignant breast cells. Proliferating cell nuclear antigen (PCNA) functions in DNA replication and DNA repair and is a component of the synthesome. Using two-dimensional PAGE analysis, we have identified a novel form of PCNA in malignant breast cells. This unique form is not the result of a genetic alteration, as demonstrated by DNA sequence analysis of the PCNA gene from malignant and nonmalignant breast cells. This novel form is most likely the result of an alteration in the post-translational modification of PCNA in malignant breast cells. These findings are significant in that it is now possible to link changes in the fidelity of DNA replication with a specific alteration of a component of the DNA synthetic apparatus of breast cancer cells. The novel form of PCNA may prove to be a new signature for malignant breast cells.

Introduction

Breast cancer is a leading cause of death among women. In 1996, there were ~44,000 deaths attributed to breast cancer (1). Breast tumors show a strong correlation between high rates of DNA synthesis and poor overall patient prognosis. Data suggest that high levels of breast cancer cell DNA synthesis are associated with an increased probability of lymph node metastases (2–7). Extensive genetic damage in mammary cancer cells is also associated with increased levels of DNA synthesis. An evaluation of thousands of clinical cases indicated that ~65% of mammary cancer cells are aneuploid (8). Therefore, the observed high levels of DNA synthesis and extensive genetic damage in breast tumors strongly suggest that an alteration in the DNA replication machinery of these cells may contribute to uncontrolled and error-prone DNA synthesis.

We have recently found that human breast cells mediate DNA synthesis using a multiprotein replication complex, designated the DNA synthesome (9). The human breast cell DNA synthesome is fully competent to support SV40 *in vitro* DNA replication. Recently, it was found that the fidelity of DNA synthesis mediated by the synthesome isolated from the malignant breast cells is several-fold lower than the synthesome isolated from the nonmalignant breast cells (9, 10). The decreased replication fidelity of the malignant breast cell DNA synthesome clearly suggests a critical alteration(s) in the composition of the replication machinery of these cells.

PCNA[3] is associated with the human cell DNA synthesome (11) and is required for synthesome-mediated *in vitro* DNA replication (12). PCNA has been demonstrated to function in mammalian cell DNA replication (reviewed in Ref. 13) and DNA repair (14, 15). In DNA replication, PCNA serves as an accessory factor to polymerase δ. PCNA is also commonly used as a proliferation marker in breast cancers. Unfortunately, there has been no conclusive evidence that increased levels of PCNA correlate with tumor progression or patient prognosis (16–18). Here, the DNA synthesome-associated PCNA isolated from nonmalignant and malignant breast epithelial cells was analyzed by 2D PAGE. Here, we describe, for the first time, a unique form of PCNA that is found in all malignant breast cells. This is an exciting finding that may prove to be a new signature for malignant breast cells.

Materials and Methods

Cell Culture. MCF7, early-passage MCF10A, MBA-MD468, and Hs578T cells were cultured according to protocols provided by American Type Culture Collection. Primary cells were cultured in Mammary Epithelial Growth Medium (Clonetics, San Diego, CA) supplemented with 2× penicillin/streptomycin, 2.5 mg/ml amphotericin B, 50 units/ml polymixin B sulfate, 50 mg/ml gentamicin sulfate, 10 ng/ml epidermal growth factor, 5 mg/ml insulin, 0.5 mg/ml hydrocortisone, and 52 mg/ml bovine pituitary extract and grown as described previously (19). To stimulate MCF7 cells with estrogen, we grew cells in medium containing charcoal-treated serum. Cells were treated for 48 h with 1 nM 17-β-estradiol or an equivalent volume of vehicle. Cells were harvested, and the DNA synthesome isolated as described previously (9).

Isolation and Purification of the DNA Synthesome from Breast Tumor Tissue and Breast Cell Lines. The mammary mouse tumor was induced using polyoma virus as described previously (20). The isolation and purification of the DNA synthesome were performed as described previously (9).

2D PAGE. DNA synthesome protein (20–40 μg) was loaded onto the first-dimension tube gel [9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3–10), and 20% Triton X-100]. The polypeptides were separated along a pH gradient created using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide-SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western Blot Analysis. The antibody directed against PCNA (PC 10; Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light-enhanced chemiluminescence system (Amersham).

Immunoprecipitation of PCNA. One hundred μg of isolated DNA synthesome were incubated overnight with a PC10 antibody directed against PCNA. Thirty μl of protein A-conjugated agarose beads were added to the

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[2] To whom requests for reprints should be addressed, at The Department of Pharmacology and Experimental Therapeutics, University of Maryland, 655 West Baltimore Street, BRB Room 4-002, Baltimore, MD 21201. Phone: (410) 706-2313; Fax: (410) 706-0032; E-mail: lmalkas@umaryland.edu.

[3] The abbreviations used are: PCNA, proliferating cell nuclear antigen; 2D PAGE, two-dimensional PAGE.

reactions for 1 h. The reaction mixtures were washed twice with buffer A [0.154 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.05% Triton X-100, and 0.05% SDS] and three times with buffer B [0.154 M NaCl, 50 mM Tris-HCl (pH 7.4), 2.5 M KCl, and 0.5% Triton X-100]. The protein was removed from the beads by incubation at 100°C for 30 s and analyzed by 2D PAGE.

Mutational Analysis of the PCNA Gene. The cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand cDNA Synthesis (Life Technologies, Inc.) followed by cloning of the amplified cDNA into the vector pCR2.1 (InVitrogen) according to the manufacturer's instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Second-strand cDNA synthesis was carried out by priming the first-strand cDNA with oligonucleotide 5′-GCGTTGTTGCCACTCCGC-3′ on the 5′ end of the cDNA and 5′-GCAGTTCTCAAAGAGCTTAG-3′ on the 3′ end of the cDNA and amplifying the primed first strand using reverse transcriptase PCR.

Results

Unique Form of PCNA Identified in Malignant Breast Cell Cultures. Studies were performed to determine whether DNA synthesome components are structurally altered in malignant breast cells compared to nonmalignant cells. The DNA synthesome was isolated from four established breast cells lines (MCF7, MDA-MB468, Hs578T, and early passage MCF10A), as well as from nonmalignant primary breast epithelial cells using our published procedures (9). The malignant breast cells lines (MCF7, MDA-MB468, and Hs578T) produce tumors in animal breast cancer models (21), whereas the nonmalignant breast cell line (MCF10A) does not (22). The nonmalignant primary breast cells were prepared from a human breast reduction sample as described by Stampfer (19). Thirty μg of DNA synthesome isolated from MCF10A, MCF7, MDA-MB468, Hs578T and nonmalignant primary breast cells were each subjected to individual 2D PAGEs (23, 24). These gels, containing the resolved synthesome polypeptides, were transferred to nitrocellulose membranes. Western blot analyses of the membranes were performed using an antibody directed against the 36 kDa PCNA polypeptide. A comparison of the mobility of the PCNA component of the MCF10A, MCF7, MDA-MB468, Hs578T, and primary cell-derived DNA synthesome (Fig. 1) indicated a clear and significant difference in this protein's 2D PAGE profile for the nonmalignant and malignant cells. The PCNA associated with the synthesome isolated from malignant MCF7 and MDA-MB468 cells was present in two forms, a basic form and an acidic form (Fig. 1, *A* and *E*,, respectively). The PCNA isolated from the malignant Hs578T cells exhibited PCNA with an acidic pI (Fig. 1*C*) and barely detectable levels of PCNA with a basic pI. PCNA in nonmalignant MCF10A and primary breast cells was present in a

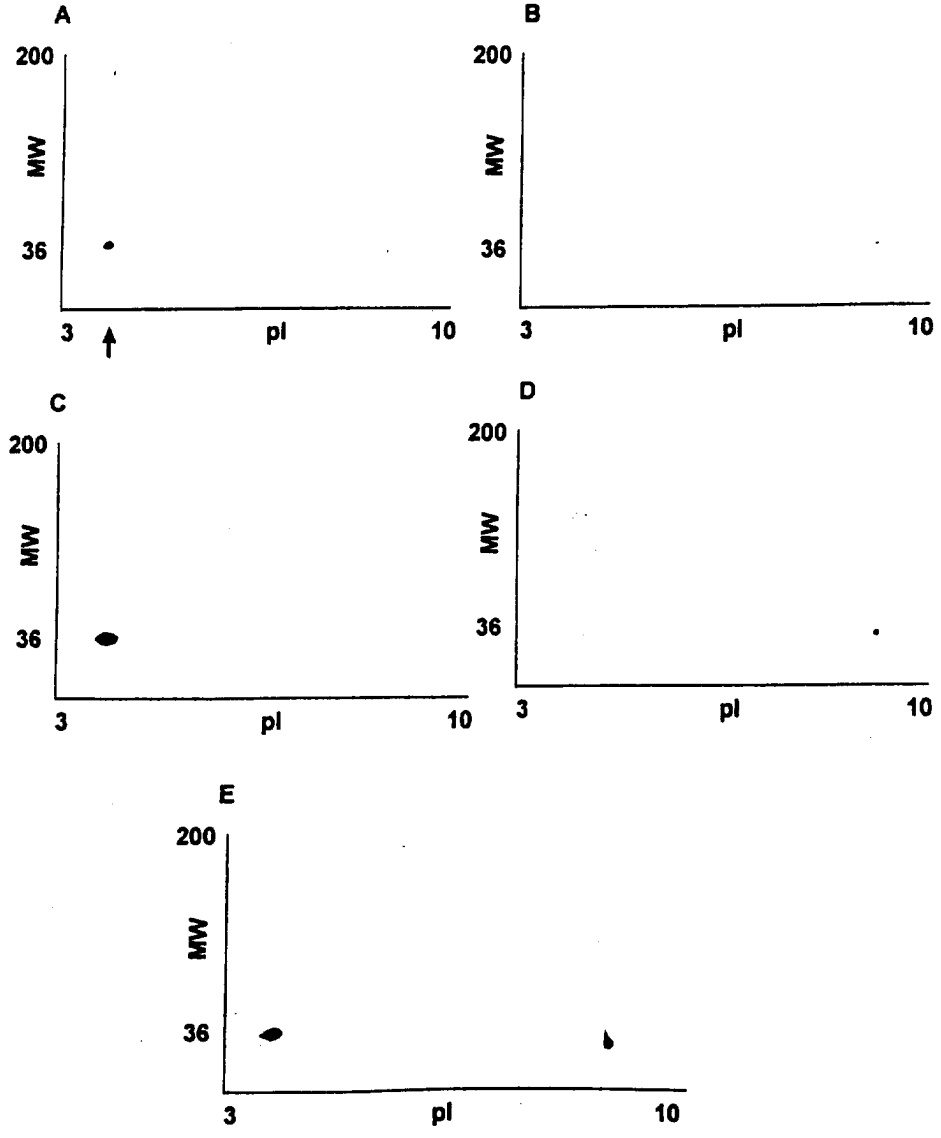

Fig. 1. Protein migration of PCNA from human breast cell lines. Thirty μg of DNA synthesome protein isolated from four human breast cell lines (MCF7, MDA-MB468, Hs578T, and MCF10A) and nonmalignant primary breast cells were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis with an antibody directed against PCNA. The protein migration patterns shown are: *A*, MCF7; *B*, MCF10A; *C*, Hs578T; *D*, nonmalignant primary breast cells; and *E*, MDA-MB468. *Arrow*, form of PCNA that is unique to malignant breast cells.

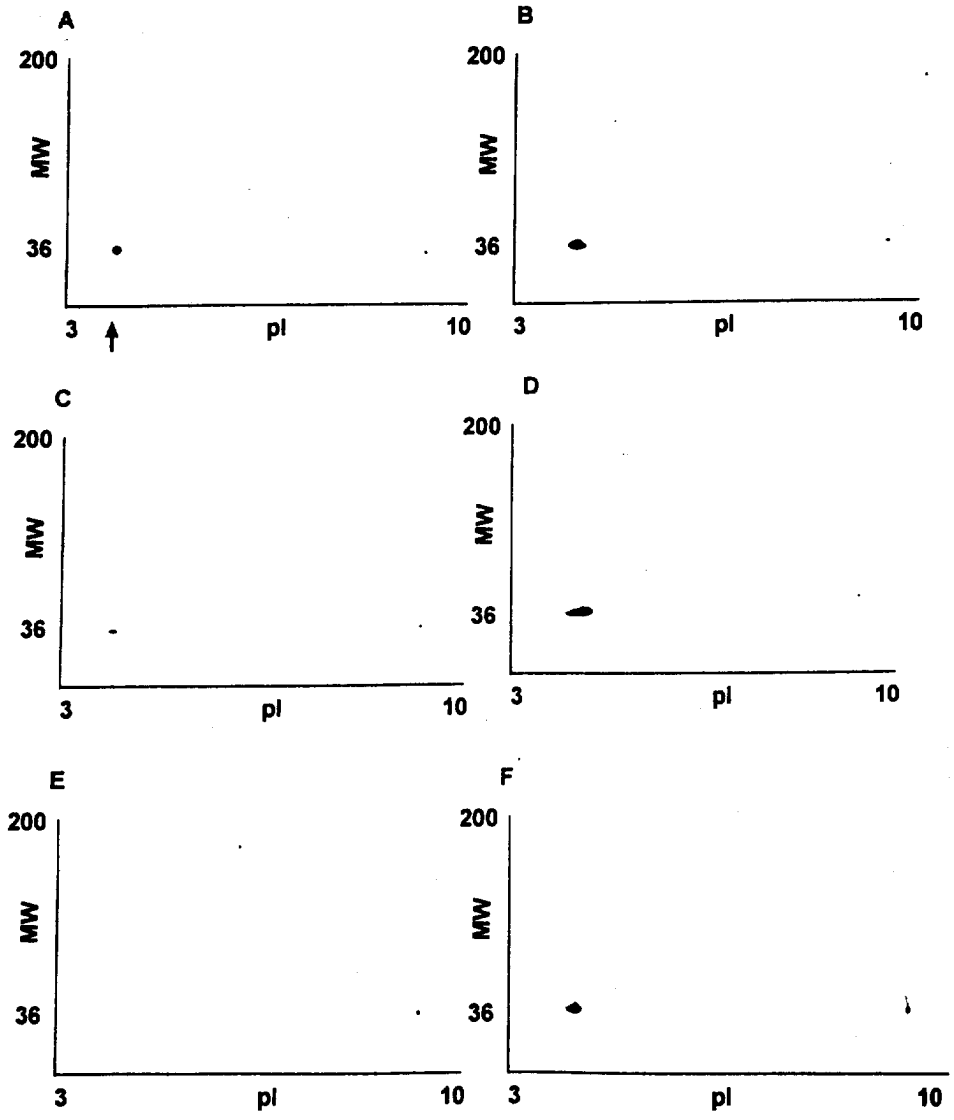

Fig. 2. Protein migration of PCNA from malignant human and mouse breast tissue and nonmalignant human breast tissue. Thirty μg of isolated DNA synthesome from malignant human and mouse breast tumors and nonmalignant human breast tissue were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis using an antibody directed against PCNA. The resulting protein migration patterns are shown: *A* and *B*, human ductal tumor; *C* and *D*, human lobular tumor; *E*, nonmalignant human breast tissue; and *F*, mouse tumor. The nonmalignant breast tissue (*E*) is derived from the same source as the human lobular tumor in *D*. *Arrow*, form of PCNA that is unique to malignant breast cells.

single form that exhibited a basic pI (Fig. 1, *B* and *D*, respectively). The PCNA form with the acidic pI was not detectable in the nonmalignant cells.

In a separate experiment, 2D PAGE analysis was performed using a sample containing isolated DNA synthesome from both MCF7 and MCF10A cells. The resulting protein migration pattern showed only one basic form and one acidic form of PCNA (data not shown). This result indicated that the basic form of PCNA was identical in both the malignant and nonmalignant cells, whereas the acidic form of PCNA was unique to the malignant cells.

Breast Tumors Contain the Unique Form of PCNA. Studies were initiated to determine whether the DNA synthesome derived from nonmalignant and malignant breast tissue exhibited the same 2D PAGE profile for PCNA as that observed in the non-malignant and malignant breast cell cultures. The DNA synthesome was isolated from a virally induced mouse breast tumor (20). The DNA synthesome isolated from six human lobular breast cancer tissues and from four ductal breast cancer tissues was also analyzed. For comparison, the DNA synthesome-associated PCNA isolated from nonmalignant breast tissue from two sources (breast reduction tissue and genetically matched nonmalignant tissue taken from the patients with malignant breast tumors) was examined. The purified DNA synthesome derived from these tissues was resolved by 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and probed with an antibody directed against PCNA. PCNA derived from malignant mouse and human tumor tissue had a 2D PAGE profile consistent with that of the malignant breast cell lines (Fig. 2, *A–D* and *F*). There were two forms of PCNA present, an acidic form and a basic form. The PCNA from the nonmalignant breast tissue was in the basic form (Fig. 2*E*). These findings were consistent with the 2D PAGE profile of PCNA from the MCF10A cells.

The Unique Form of PCNA Is Not Proliferation Dependent. To demonstrate that the abundant levels of the form of PCNA with a acidic pI was a property unique to malignant breast cells as opposed to a proliferation response, we analyzed the 2D PAGE profile of PCNA isolated from benign proliferative breast tumors and estrogen-stimulated MCF7 cells. Estrogen has a stimulatory effect on cellular proliferation (25). We found that the estrogen-stimulated cells had an increased rate of proliferation compared to control cells, as demonstrated by several parameters (Table 1). Similar findings have been reported by other investigators (25–27). The DNA synthesome was isolated from these cells, and the components were resolved by 2D PAGE followed by Western analyses for PCNA. There was an overall increase in the level of PCNA in estrogen-stimulated cells; however, there was no effect on the 2D PAGE profile for PCNA (Fig. 3, *A* and *B*).

Table 1 *Stimulation of cell proliferation following treatment with 17-β-estradiol*

Parameter	Control cells[a]	17-β-Estradiol (E_2)-treated cells[b]
[^{3}H] Thymidine uptake[c]	1,548 cpm/10^5 cells	10,564 cpm/10^5 cells
DNA polymerase α activity[d]	496 ± 80 cpm/mg	1,359 ± 118 cpm/mg
Cells in S phase[e]	10.7%	60.1%

[a] Control cells are MCF7 cells that were grown in phenol red-free DMEM, which was supplemented with 10% dextran-coated, charcoal-treated fetal bovine serum, 1% penicillin/streptomycin, and nonessential amino acids.
[b] 17-β-estradiol (E_2)-treated cells were grown for 48 h under essentially the same conditions as the control cells along with the addition of 1 mM 17-β-estradiol to the medium.
[c] [^{3}H]-Thymidine uptake, according to the procedure described by Malkas *et al.* (11).
[d] DNA polymerase α activity was measured as described by Malkas *et al.* (11).
[e] Cell cycle distribution analyses of the cultured cells grown in the presence or absence of 17-β-estradiol were performed as described by Lin *et al.* (34).

The DNA synthesome was also isolated from several benign breast tumors. In general, the 2D PAGE profile for PCNA from the benign tumors was identical to that of nonmalignant cells in culture and in nonmalignant breast tissue (Fig. 3*C*). These data provide compelling evidence that the acidic form of PCNA is characteristic of only malignant breast cells.

Genetic Mutation Is Not Responsible for the Acidic Form of PCNA in Malignant Breast Cells. Total cellular RNA isolated from MCF7 and MCF10A cells was used to clone the cDNA encoding the entire PCNA translation unit from each cell line. Four independent clones encoding the PCNA gene derived from MCF7 cells and four independent clones from MCF10A cells were sequenced. Sequence analysis indicated that these eight independent clones have an identical nucleotide sequence (Fig. 4). Furthermore, this nucleotide sequence does not differ from that of the sequence for the PCNA gene cloned from the human lymphoma cell line MOLT-4 (Ref. 28; Fi. 4).

Discussion

Here, it was demonstrated that a unique form of PCNA with an acidic pI is present in malignant breast cells. This unique form is found in malignant breast cell cultures, as well as malignant breast tumors, but not in nonmalignant breast cell cultures, nonmalignant tissue, or a sampling of benign breast tumors. It was shown that the cancer-specific form of PCNA was not due to a proliferation response or genetic mutation. This novel form of PCNA is most likely a result of an altered posttranslational modification in the malignant breast cells.

One of the hallmarks of breast cancer is the accumulation of genetic mutations that lead to genomic instability (29). These mutations may contribute to uncontrolled cellular proliferation, resistance to antiproliferative processes, and metastasis (4, 7, 30). The accumulation of mutations in breast cancers is high, considering that normally mutations occur infrequently. This has lead to the hypothesis of a "mutator phenotype" (31). It was proposed that a cell's progression to malignancy is accompanied by the accumulation of multiple genetic mutations created by error-prone DNA replication and a reduction in the efficiency of the DNA repair processes in the cell. Sekowski *et al.* (10) have recently found that the DNA replication apparatus from malignant breast cells is mutagenic, resulting in a decreased replication fidelity. Due to the essential role PCNA plays in both DNA replication and DNA repair, the unique form of PCNA in malignant cells is a likely contributor to the accumulation of genetic mutations and genomic instability.

An implication of the described findings is that posttranslational modification of PCNA is most likely responsible for the development of the acidic form of the protein found in the cancer cells. Bravo and Celis (32) demonstrated that PCNA from HeLa cell extracts was not posttranslationally modified by phosphorylation and that acetylation, glycosylation and sialylation are not likely contributors to the migration pattern of PCNA. Simbulan *et al.* (33) have recently shown that PCNA is modified by poly(ADP) ribosylation. Further studies to determine how the posttranslational modification of PCNA affects its ability to interact with proteins involved in DNA replication and DNA repair are underway and should provide crucial insights into the role

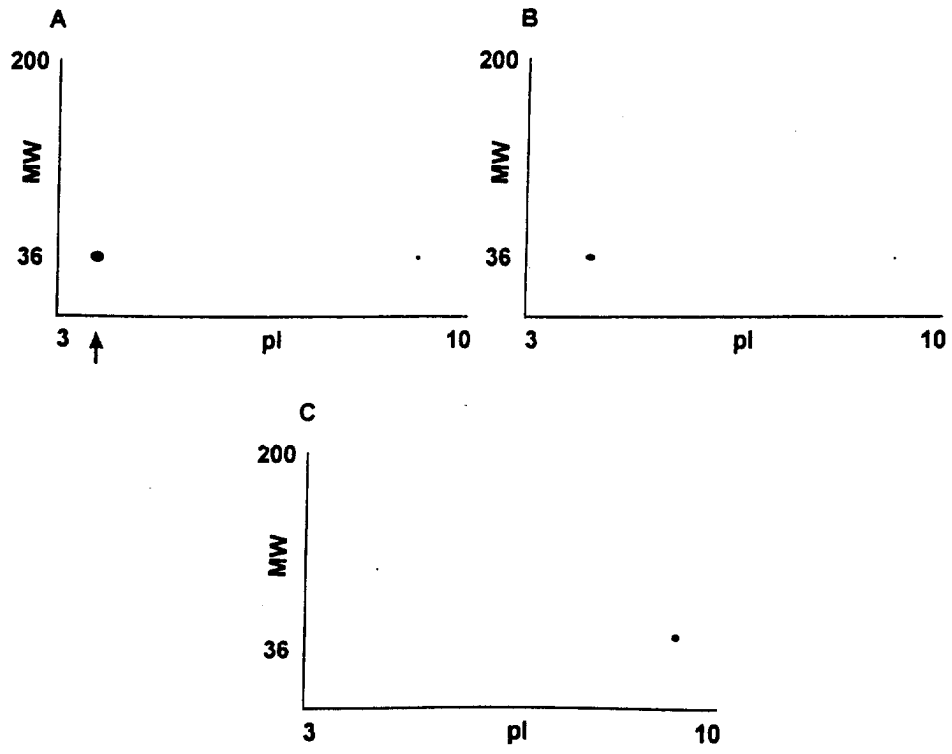

Fig. 3. Protein migration of PCNA from estrogen-treated MCF7 cells, control MCF7 cells, and a benign breast tumor. Thirty to 60 μg of DNA synthesome isolated from MCF7 cells treated with 17-β-estradiol, MCF7 (*A*), control cells (*B*), and a benign breast tumor (*C*), were analyzed by 2D PAGE and Western blot analysis. *Arrow*, form of PCNA that is unique to malignant breast cells.

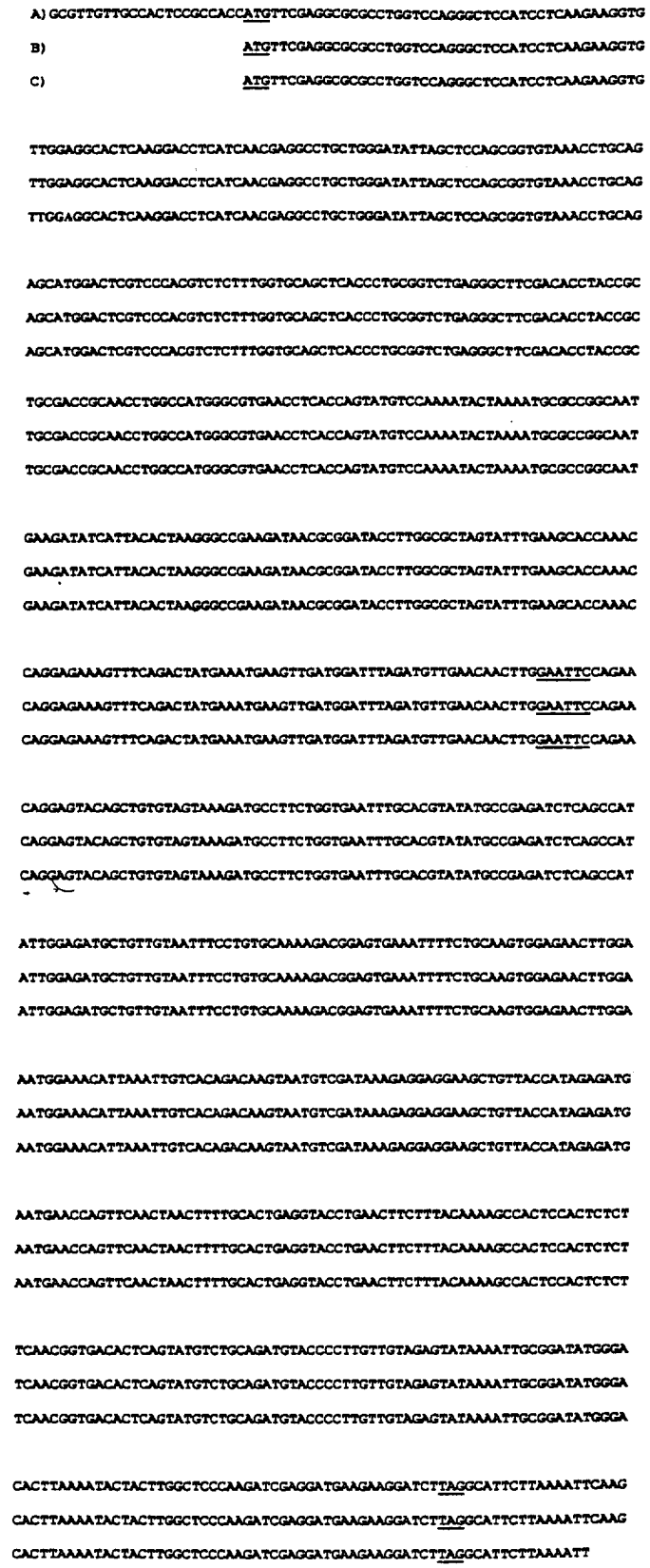

Fig. 4. Nucleotide sequence of the PCNA cDNA cloned from MCF7 and MCF10A cells. The nucleotide sequence of the PCNA cDNA cloned from the two breast cell lines is aligned with the sequence reported for an acute lymphoblastic leukemia cell. The PCNA nucleotide sequences shown are those of MOLT-4 (*A*), MCF7 (*B*), and MCF10A (*C*). *Underlined sequences*, positions of the ATG start codon and the internal EcoRI restriction endonuclease cleavage site. The cDNA was cloned from total cellular RNA isolated from exponentially growing MCF7 and MCF10A cells using reverse transcriptase PCR and the pCR2.1 vector. Ampicillin-resistant colonies containing the cDNA were chosen using the blue/white assay, and Miniprep DNA was isolated from the selected colonies and given to our Biopolymer Core Facility for nucleotide sequence analysis. The nucleotide sequences of the analyzed clones (four from MCF7 and four from MCF10A) were identical to the sequence depicted here.

of PCNA in the maintenance of genomic stability and control of cellular proliferation.

Another implication of this work is that the malignant cell-specific form of PCNA may be a more fundamental characteristic of cancer cells than any single type of genetic mutation. This supposition is based on the observation that the cancer-specific form of PCNA was found in 100% of malignant breast cells examined to date. This correlation is higher than that observed for other molecular markers for malignancy currently examined for in breast tumors (i.e., p53, BRCA, *her2/neu*, and progesterone and estrogen receptor status), which are only found in a fraction of all breast tumors.

The findings described here are novel in that this is the first time it has become possible to link the decreased fidelity of cellular DNA replication with a specific alteration to a component of the DNA synthetic apparatus of breast cancer cells. PCNA is currently used as a marker for cellular proliferation. The novel form of PCNA that we have identified may prove to be a new signature for malignant breast cells. Our findings provide the first evidence that the unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of malignancy.

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Isolation of ADP-Ribosyltransferase by Affinity Chromatography[1]

HELMUT J. BURTSCHER,[2] BERNHARD AUER, HELMUT KLOCKER, MANFRED SCHWEIGER, AND MONICA HIRSCH-KAUFFMANN

Institut für Biochemie (Naturwissenschaftliche Fakultät), Universität Innsbruck, A-6020 Innsbruck, Austria

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An affinity adsorbent for ADP-ribosyltransferase (EC 2.4.2.30) has been synthesized by coupling 3-aminobenzamide to Sepharose 4B. Using this material, ADP-ribosyltransferase from human placenta has been purified from crude extract to homogeneity within a few hours. The enzyme has an apparent K_m for NAD^+ of 52 μM. Its molecular mass is 115,000 as determined by gel electrophoresis. The enzyme is DNA dependent and stimulated by histone, its temperature optimum is at 25°C, and its pH optimum is around pH 9. α-NAD^+, thymidine, caffeine, theophylline, theobromine, 3-methoxybenzamide, and nicotinamide inhibit the enzyme. Purification of ADP-ribosyltransferases from horse, rat, and chicken liver was also achieved with the method described.

KEY WORDS: ADP-ribosyltransferase; affinity chromatography; enzyme purification; 3-aminobenzamide.

ADP-ribosyltransferases (EC 2.4.2.30) are enzymes that modify proteins covalently by attaching one or many ADP-ribosyl residues using NAD^+. ADP-ribosylation seems to be involved in a number of biological processes, for instance, regulation of gene expression, differentiation, and DNA repair (1–9).

NAD^+ analogs are among the most potent inhibitors of ADP-ribosylation (10) and immobilization of one of these analogs should result in a material for affinity chromatography. This should lead to a highly specific purification method for ADP-ribosyltransferases, avoiding time-consuming steps such as gel filtration.

Here we describe the preparation of *O*-[15-[3-(aminocarbonyl)phenyl]-1-imino-11,14-dioxo-2,6,10,15-tetraazapentadecyl]-Sepharose (3-aminobenzamide-Sepharose)[3] and its use in the purification of ADP-ribosyltransferase from human placenta.

MATERIALS AND METHODS

Phosphocellulose P11 was obtained from Whatman and Sepharose 4B from Pharmacia. Glass fiber filters were from Schleicher and Schüll. Nicotinamide adenine dinucleotide ([2,8-^{3}H]adenine) was obtained from New England Nuclear. Unlabeled NAD^+, calf thymus DNA, histone (type V-S from calf thymus), α-NAD^+, thymidine, caffeine, theophylline, theobromine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, nicotinamide, and gel markers (*Escherichia coli* β-galactosidase, phosphorylase *b* from rabbit muscle, bovine serum albumin, and ovalbumin) were obtained from Sigma Chemical Company; 3-methoxybenzamide was from Aldrich Chemical Company Inc.; and other chemicals used

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[2] To whom requests for reprints should be addressed.

[3] Abbreviations used: AP-Sepharose, *O*-[[3-[(3-aminopropyl)amino]propyl]guanyl]-Sepharose; 3-aminobenzamide-Sepharose, O-[15-[3-(aminocarbonyl)phenyl]-1-imino-11,14-dioxo-2,6,10,15-tetraazapentadecyl]-Sepharose; ADPRT buffer, Tris·HCl (100 mM), $K_2S_2O_5$ (25 mM), β-mercaptoethanol (12 mM), EDTA (0.5 mM), and 17% glycerol at pH 7.2; CH-Sepharose, *O*-(6-carboxyhexyl)guanyl-Sepharose; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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were of analytical grade. Hydroxyapatite was synthesized according to the method of Bernardi (11).

3-Aminobenzamide-Sepharose. 3-Aminobenzamide was synthesized by standard organic chemistry: 3-nitrobenzoic acid was refluxed in thionyl chloride and the resulting acid chloride was converted to 3-nitrobenzamide by ammonolysis. 3-Nitrobenzamide was reduced with hydrazine/Raney nickel to 3-aminobenzamide. Intermediates were identified by melting point and thin-layer chromatography. (Alternatively, commercially available 3-aminobenzamide may be used.)

Equimolar amounts of succinic acid anhydride and 3-aminobenzamide were heated in anhydrous pyridine for 2 h in a boiling-water bath, pyridine was removed by evaporation, and the resulting 4-[[3-(aminocarbonyl)phenyl]amino]-4-oxobutanoic acid was crystallized from ethanol.

O-[[3-[(3-Aminopropyl)amino]propyl]-guanyl]-Sepharose (AP-Sepharose) was prepared from cyanogen bromide-activated Sepharose and 3,3'-iminodipropaneamine according to Cuatrecasas (12) and contained about 5 μmol of spacer arms per gram of Sepharose, as measured with the fluorescamine method (13) in the corresponding hydrochloric acid hydrolysate.

4-[[3-(Aminocarbonyl)phenyl]amino]-4-oxobutanoic acid (0.5 g) was dissolved in 20 ml dimethylformamide and added to 20 g (wet wt) of AP-Sepharose. The coupling reaction was performed by the addition of 0.5 g 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 3 ml distilled water. After shaking in a rotatory water bath at room temperature for 12 h the material was washed first with 3 vol of dimethylformamide/water (1:1) and then extensively with water alone. The amount of 3-aminobenzamide bound was estimated by measuring the increase in uv-absorption at 250 nm in a 10% suspension of Sepharose in 20% glycerol.

Polyacrylamide gel electrophoresis. For electrophoresis 10% polyacrylamide gels were used according to the method of Davis (14) modified by Laemmli (15). Gels were
for 30 min in 300 ml of a 50% trichlo
acid solution containing 1 mg/ml Co
brilliant blue R. Destaining was done
acetic acid containing 1% glycerol.

Protein determination was perform
cording to the method of Bradford (16),
bovine serum albumin as standard.

Placentas. Human placentas from n
full-term pregnancies were obtained fro
Universitäts-Frauenklinik Innsbruck i
diately after delivery, cut in pieces, was
0.15 M KCl solution to remove most
blood, pressed into thin slices (about 3–5
thick), and stored at −80°C until used.

ADP-ribosyltransferase assay. The
was carried out in glass tubes (37 × 7 mm
25°C for 5 min in a total volume of 10
Amounts of 10 μl of enzyme solution
added to 90 μl of 100 mM Tris·HCl (pH
1 mM DTT, 2 μg DNA, 1 μl [^{3}H]NAD
Ci/mmol; 0.0028 mg/ml), 10 μM NAD^+,
2 μg histone. DNA used was calf thymus
II activated by the procedure of Loeb (17).
reaction was stopped by adding 100 μl of
trichloroacetic acid. Aliquots of 160 μl fr
each tube were pipetted onto a glass fiber fi
(2.5-cm diameter), which was then was
with 5% trichloroacetic acid followed by
1:1 solution of ethanol/ether and dried
100°C. Acid-precipitable radioactivity w
measured in a liquid scintillation counter.

Extraction. All procedures, unless otherw
stated, were carried out between 0 and 4°
Two human placentas (approximately 800
were used for the preparation. Four liters
ADPRT buffer (100 mM Tris·HCl; 17% gly
erol; 25 mM $K_2S_2O_5$; 12 mM β-mercaptoet
anol; 0.5 mM EDTA; buffer pH was adjust
to 7.2 at room temperature) containing 50 m
KH_2PO_4 and 0.5 M KCl was added to the fr
zen tissue and left for 30 min at room tem
perature (buffer temperature does not ri
above 0°C during this time). The tissue w
homogenized in a Waring Blendor (5 liter) f
four periods of 45 s. The homogenate w
centrifuged at 14,000*g* for 15 min.

Hydroxyapatite chromatography. Cru

extract was filtered through a double layer of cheesecloth and then shaken with 200 g of solid hydroxyapatite (equilibrated in ADPRT buffer containing 50 mM KH_2PO_4) for 30 min. Subsequently the suspension was centrifuged at 1600*g* for 5 min. The supernatant was discarded and the hydroxyapatite was resuspended in 2 liters of ADPRT buffer containing 50 mM KH_2PO_4. This suspension was centrifuged again at 1600*g* for 5 min. The supernatant was discarded and the hydroxyapatite was suspended in 500 ml of ADPRT buffer containing 0.5 M KH_2PO_4. The suspension was shaken for 15 min and then centrifuged as before.

Phosphocellulose chromatography. Dry phosphocellulose (20 g) equilibrated in ADPRT buffer was used. Phosphocellulose was added to the eluate of hydroxyapatite, the suspension was agitated for 60 min and then centrifuged (1600*g*, 5 min), and the supernatant was discarded. The phosphocellulose pellets were washed out of the tubes with 250 ml of ADPRT buffer and poured into a 5-cm-diameter column. Then a linear gradient in ADPRT buffer from 0 to 2 M KCl was applied.

Affinity chromatography. 3-Aminobenzamide-Sepharose (in a column of 2.5 × 8 cm) was equilibrated in ADPRT buffer containing 0.3 M KCl. After the sample had been applied, the column was washed with ADPRT buffer containing 0.3 M KCl. The enzyme was eluted with ADPRT buffer containing 0.3 M KCl and 1 mM 3-methoxybenzamide.

RESULTS

Preparation of 3-Aminobenzamide-Sepharose

3-Aminobenzamide was coupled to Sepharose 4B as described (see Materials and Methods). The resulting 3-aminobenzamide-Sepharose contained 4.7 μmol of 3-aminobenzamide per gram of swollen gel. Compared to the content of spacer groups (5 μmol per gram of Sepharose) coupling was almost quantitative. Other coupling methods (e.g., carbodiimide coupling of 3-aminobenzamide to *O*-[(6-carboxyhexyl)guanyl]-Sepharose (CH-Sepharose) or succinylation of AP-Sepharose with subsequent carbodiimide coupling to 3-aminobenzamide) led to adsorbents with a much lower binding capacity for ADP-ribosyltransferase. 3-Aminobenzamide-Sepharose is stable for at least 2 years at 4°C and can be reused many times after regeneration with 2 M KCl in ADPRT buffer with no loss of binding capacity.

Purification of Human ADP-ribosyltransferase

A crude extract of human placenta was prepared and then mixed with hydroxyapatite for 30 min. After the hydroxyapatite was washed, enzyme was eluted in one step with 0.5 M KH_2PO_4 in ADPRT buffer. The enzyme solution was diluted 10-fold with ADPRT buffer and then phosphocellulose was added. After 60 min of gentle shaking, the phosphocellulose was packed into a column (5-cm diameter). A linear gradient from 0 to 2 M KCl in ADPRT buffer was applied (Fig. 1). The active fractions (eluting at 0.3 M KCl) were pooled and applied directly onto the 3-aminobenzamide-Sephar-

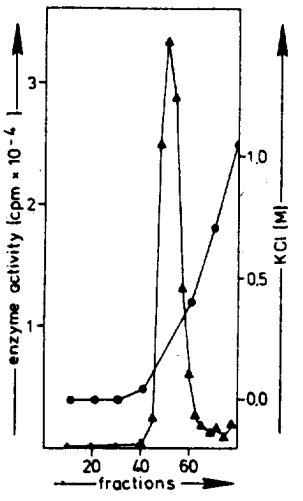

FIG. 1. Elution profile of human ADP-ribosyltransferase from phosphocellulose. Chromatography was performed as described under Materials and Methods. (▲) Enzyme activity, (●) concentration of KCl.

TABLE 1

PURIFICATION OF HUMAN ADP-RIBOSYLTRANSFERASE FROM PLACENTA

Step	Total protein (mg)	Specific activity (nmol min^{-1} mg^{-1})	Purification factor
Crude extract	20,000.0	0.07	1
Hydroxyapatite	325.0	1.65	24
Phosphocellulose	60.0	4.16	61
3-Aminobenzamide-Sepharose	0.3	335.00	4900

ose column. This column was washed with ADPRT buffer containing 0.3 M KCl and the enzyme was eluted with the same buffer (ADPRT + 0.3 M KCl) containing 1 mM 3-methoxybenzamide, an inhibitor of ADP-ribosyltransferases (10). 3-Methoxybenzamide was removed from the enzyme solution by use of a small hydroxyapatite column (0.7 × 0.5 cm). Data of a representative purification are shown in Table 1.

Characterization of the Enzyme

The purified enzyme migrates as a single band in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Fig. 2) corresponding to a molecular mass of approximately 115,000. The enzyme remains stable at 0°C for several days and at −80°C for several months, but it is very sensitive to repeated freezing and thawing. The purified enzyme is DNA dependent. Histone stimulates the activity by a factor of about 7 (data not shown). Maximum stimulation by histone is at a DNA/histone ratio (w/w) of 1. The enzyme has a temperature optimum at 25°C. The optimal pH for the enzyme reaction is about 9. The time course of the enzyme reaction is linear only during the first minutes. After 10 min the activity begins to level off and after 30 min there is no further increase in acid-precipitable radioactivity. An apparent K_m for NAD^+ of 52 μM was determined out of a double reciprocal plot.

Several NAD^+ competitors were tested for inhibition of the enzyme (Table 2). ADP-ribosyltransferase from human placenta is sensitive to α-NAD^+, nicotinamide, and [illegible] dine. Methylxanthines inhibit the en[illegible] caffeine being markedly less inhibitor[illegible] theophylline and theobromine. 3-Me[illegible] benzamide inhibits the enzyme strongl[illegible] ble 2).

Application of the Purification Scheme [illegible] Other Tissues

Using the method described we pu[illegible] ADP-ribosyltransferases from horse, ra[illegible] chicken liver. A crude extract was prepar[illegible]

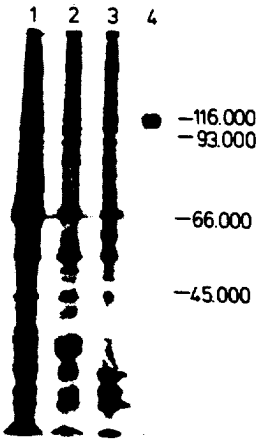

FIG. 2. 10% SDS–polyacrylamide gel showing the [illegible] of the purification. Molecular weight markers were [illegible] lactosidase (*E. coli*), 116,000; phosphorylase *b* (r[illegible] muscle), 94,000; bovine serum albumin, 66,000; ovalbumin, 45,000. The gel was run at 45 V for 14 h, then stained with Coomassie brilliant blue (see Mat[illegible] and Methods). Lane 1, crude extract; lane 2, hydrox[illegible] atite pool; lane 3, phosphocellulose pool; lane 4, 3-[illegible] nobenzamide-Sepharose pool.

TABLE 2

INHIBITORS OF HUMAN ADP-RIBOSYLTRANSFERASE

Inhibitor	Concn (mM)	Percentage of control
Control	—	100
Theophylline	1	12
Caffeine	1	72
Theobromine	1	19
3-Methoxybenzamide	1	10
2-Methoxybenzamide	0.1	7
Nicotinamide	1	0

[illegible] Reaction conditions were according to the standard assay, and inhibitors were added in 10 μl of a 10-fold concentrated solution.

homogenizing the tissues in ADPRT buffer and the enzymes were isolated via hydroxyapatite, phosphocellulose, and 3-aminobenzamide-Sepharose. Elution profiles from phosphocellulose were identical. In all cases after affinity chromatography a protein with a molecular mass of 115,000 was found.

DISCUSSION

ADP-ribosyltransferases have been isolated by a variety of methods, but most of them were rather time consuming. This prompted us to look for a more effective purification method based on affinity chromatography. 3-Aminobenzamide is one of the strongest inhibitors of ADP-ribosyltransferases. Since acylation of the amino group does not affect the inhibitory potential (10), we used this group for immobilization of 3-aminobenzamide. Using 3-aminobenzamide-Sepharose we have purified an ADP-ribosyltransferase from human placenta. Both the hydroxyapatite and the phosphocellulose step are necessary for this purification method. Applying the crude extract directly onto 3-aminobenzamide-Sepharose did not yield a homogeneous enzyme (data not shown). Elution of the enzyme from the affinity material with 3-methoxybenzamide proved to be superior to gradient elution with KCl, which led to loss of activity and less purity.

The method described here allows rapid purification of the enzyme from crude extract to homogeneity (within hours) and yields up to 500 μg of enzyme per kilogram of placenta. The enzyme purified by this procedure is identical to an enzyme purified by conventional methods alone (18,19) in terms of K_m, molecular mass, inhibitors, and pH and temperature dependence.

The enzyme is rather unstable during the purification procedure and considerable loss of activity occurs at all stages of the purification, but it is stable during the last step. We repeatedly observed that at least part of the inactivation is accompanied by disappearance of the 115,000 band in the gel, starting from an apparently homogeneous preparation (data not shown). Whether this effect is due to the enzyme itself or to traces of a $K_2S_2O_5$-resistant protease remains to be resolved. Proteolytic degradation, for instance, was seen with calf thymus ADP-ribosyltransferase (20).

A decisive advantage of this purification procedure is that it is rapid and allows easy purification of the enzyme from different tissues, which opens the way to the elucidation of the enzyme's many roles. We have so far successfully applied this method to horse, rat, and chicken liver without any modifications.

ACKNOWLEDGMENTS

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Note: This product is offered for research use only. Not for clinical use, diagnostic procedures, or for preparation of fluids to be used for human injection.

Apollo 7 ml concentrators are disposable ultrafiltration devices made from premium hydrophilic nonadsorptive regenerated cellulose membrane for the concentration or purification of protein solutions. They are far superior to alternatives in efficiency, operating speed, and recovery. This is due to their unique conical design (patents pending), increasing both membrane area and sample size that, in turn, provide higher degrees of concentration possible in a single spin as well as better control of protein polarization and fouling at the membrane surface, and reduction in adsorptive loss to plastic walls.

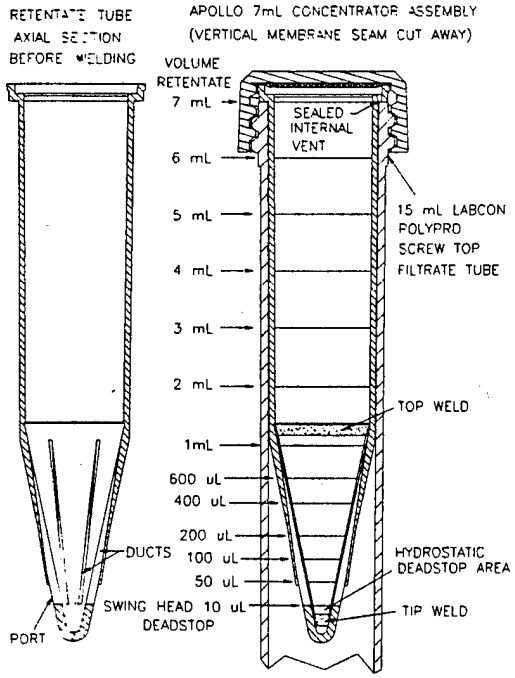
SPECIFICATIONS

Volumes

	Maximum Initial Sample	Deadstop
With 35° angle rotor:	5 ml	3 µL
With swing-head rotor:	7 ml by decanting filtrate	10 µL
	6 ml without decanting	10 µL

Total Volume Added to Sample + Filtrate Tubes

Swing Head	35°	Deadstop
NA	<= 5.7 mL	3 µL
<= 6.2 mL	6.1 mL	10 µL
6.45 mL	6.25 mL	20 µL
6.85 mL	6.6 mL	50 µL
7.2 mL	6.9 mL	100 µL
7.6 mL	7.35 mL	200 µL
8.25 mL	8.05 mL	500 µL
9.0 mL	8.75 mL	1000 µL



Maximum Centrifugal Force

With 35° angle rotor: 8,500 rcf (200 psi with 5 ml)
With swing-head rotor: 4,500 rcf typical rotor maximum, not to exceed 8,500 rcf

Materials

Membrane: Regenerated cellulose skin on polyethylene microporous support
Sample reservoir, vial and cap: Polypropylene copolymer

Dimensions

Active membrane area: 5.2 cm^2
Collection tube: Diameter, OD: 16.8 mm, 0.66 in (typical)
Length incl. cap): 123.4 mm, 4.86 in (typical)
Filter: Length (filter tip to top flange): 73.4 mm, 2.89 in (typical)
Diameter: 14.3 mm, 0.56 in (typical)

Environmental Resistance

Temperature: 34.7 °C, 120° F, max. Do not autoclave
Limit of pH: 1 to 14

Chemical Compatibility

Common chemicals (√ = acceptable; ***X = not recommended***)

Acids and Bases

Acetic acid (10%)	√	Hydrochloric acid (1.0N)	√	Sodium hydroxide (0.1N)	√
Ammonium hydroxide (10%)	√	Lactic acid (50%)	√	Sodium hydroxide (2.5N)	X
Formic acid (70%)	√	Perchloric acid (5%)	√	Trichloroacetic acid (10%)	√
		Phosphoric acid (30%)	√		

Organic Solvents, Miscellaneous Chemicals

Acetone	X	Dithiothreitol ((0.1 M)	√	Propanol (70%)	√
Acetonitrile (40% in 1% TFA)	√	Ethanol (70%)	√	Pyridine	√
Acetonitrile	√	Ethyl acetate	√	PyroCLEAN™	√
Alconox™ (1%)	√	Formaldehyde (5%)	√	Sodium carbonate (20%)	√
Ammonium sulfate (50%)	√	Formamide	√	Sodium chloride (2M)	√
Benzene	X	Glycerin	√	Sodium deoxycholate (5%)	√
n-Butanol	√	Guanidine HCl (6M)	√	Sodium dodecyl sulfate (0.1M)	√
CAPS (250 mM, pH 11.0)	√	Guanidine thiocyanate	√	Sodium thiocyanate (3M)	√
Carbon Tetrachloride	X	Imidazole (1M)	√	Terg-A-Zymne™ (1%)	√
CHAPS (100 mM)	√	Lubrol PX (0.1%)	√	Tetrahydrofuran	X
Chloroform	X	Mercaptoethanol (0.1M)	√	Toluene	X
Diethyl pyrocarbonate (0.2%)	√	Methanol	√	Tris buffer (1M, pH 8.2)	√
Dimethyl formamide	√	Nonidet P-40® (2%)	√	Triton X-100™ (0.002M)	√
Dimethyl sulfoxide	√	Phenol (1%)	√	Tween-20™	√
Dioxane	√	Phosphate buffer (1M, pH 8.2)	√	Urea (8M)	√
		Polyethylene glycol (PEG400,10%)	√		

Some of the recommended chemicals listed above may affect membrane performance, thereby altering the recoveries, passage, and /or spin times. Alconox is a registered trademark of Fabric Chemicals, Co. Nonidet P-40 is a registered trademark of Shell Oil Co. Terg-A-Zyme is a registered trademark of Rohm and Haas Co. Tween is a registered trademark of Atlas Powder Co.

HOW TO USE THIS PRODUCT

Preparations

Make sure it will fit in your centrifuge

Prepare a 15 to 17 ml carrier accepting a 124 mm length tube in centrifuge, either fixed angle or swing head rotors can be used. Check clearance of tube to both swing mechanism and rotor cover or centrifuge lid.

Make sure you have chosen the right device for your application

Select a device with a retention rating equal to or smaller than the MW of the macromolecule to be concentrated (see Table I). The membrane Quantitative Molecular Weight Limit (QMWL) rating is engraved near the top lip. Insert the device into the filtrate collection tube.

If glycerin removal is required

Add 5 ml clean water or buffer. Place device assembly into the rotor and counterbalance with a similar device or tube of the same weight. Spin at 8500 or maximal permitted rotor rcf to produce 2-3 ml filtrate. Shake water out of device and collection tube, and then replace the device in the tube.

Operation

1. ***Add sample and cap tube snugly.***
   There is no need to leave cap loose. An internal vent hole near the lip permits air from the collection tube to pass into the filter to maintain maximal flow without release of aerosols.
2. ***Place assembly into rotor.***
   Counterbalance with a similar device or tube of the same weight and spin. Note specified centrifugal force limits and observe max. relative centrifugal force rating for the rotor.

3. ***Spin for the required time (see Table II)***
Spin at the suggested speed to achieve the desired concentration factor. To exchange microsolute by diafiltration, decant filtrate, and refill with buffer, mixing retentate by repeated aspiration with the pipette tip held near the top of the cone to avoid scraping the membrane. Concentrate and dilute until desired solute removal is achieved. If your application will allow a concentration factor of greater than 500x, 100% salt or solute removal is possible in a single spin.

4. ***Harvest retentate***
Use a pipette tip small enough to reach the bottom of the device. Remove Apollo from the collection tube and hold it up to a light. The meniscus may be seen in the conical section through the viewing port formed between the vertical membrane edges. The tip of the recovery pipette is easily seen when it touches the bottom of the device retentate chamber.

Precautions

- ***Avoid scraping membrane skin*** with pipette tip when adding or decanting. Exceeding the maximum centrifugal force limits specified above may cause retentate leakage. With linear nucleic acids, maximal selectivity is obtained at filtration velocities <1mm/min. In Apollo 7 ml, this corresponds to filtration rates <0.5 ml/min. For most selective retention of nucleic acids and removal of primers and oligonucleotide when concentrating and diafiltering DNA and RNA, reduced rcf should be used.

- For best recovery, ***remove retentate in <10 min***. Upon standing, wicking can cause it to spread upward and continue to filter, further reducing retentate volume. For retentate volumes <10 µl, mass recovery is improved by adjusting volume with buffer to about 10 µl before recovery, and/or by subsequently adding 20-100 µl of buffer to the device, mixing into and out of the tip several times and recovering the wash as well.

- ***To clean devices, vortex or sonicate them*** with 2 – 3 ml of surfactant. Discard. Vortex then rinse several times with water or buffer. Refrigerate, filled with several ml of buffer, water, or alcohol and tightly capped to avoid drying of the membrane skin and permanent loss in flow rate.

TYPICAL PERFORMANCE

Table I: Membrane Retention

		Retention of diafiltered solute				
QMWL (Quantitative Molecular Weight Limit) >95% retention of globular proteins, Daltons		**5,000**	**10,000**	**30,000**	**70,000**	**150,000**
Equivalent Nominal Molecular Weight Limit ("Cutoff") >90% Rating		***5,000***	***8,000***	***10,000***	***30,000***	***100,000***
Challenge Solute	**Molecular Wt.**					
1.0 A_{270} $d(pC)_{21}$ oligonucleotide	10k Da, linear	n.a.	77%	15%	3%	2%
1.0 A_{270} $d(pC)_{24-34}$ oligonucleotide	11-16k Da, linear	n.a.	93%	71%	50%	14%
200bp DNA	130k Da, linear	99%	99%	98%	n.a.	n.a.
0.25 mg/ml bovine cytochrome-c	12k Da, globular	99%	99%	10%	n.a.	n.a.
1 mg/ml alpha-chymotrypsinogen	25k Da, globular	99%	99%	94%	4%	n.a.
1 mg/ml bovine carbonic anhydrase	29k Da, globular	99%	99%	98%	n.a.	n.a.
1 mg/ml ovalbumin	46k Da, globular	99%	99%	98%	17%	n.a.
1 mg/ml bovine serum albumin	67k Da, globular	99%	99%	99%	98%	29%
1 mg/ml alcohol dehydrogenase, yeast	150k Da, globular	n.a.	n.a.	n.a.	97%	n.a.
1 mg/ml apoferritin, horse serum	443k Da, globular	n.a.	n.a.	n.a.	>99%	n.a.
0.5 mg/mL bovine thyroglobulin	669k Da, globular	n.a.	n.a.	n.a.	n.a.	97-99%

All protein dissolved in pH 7.4, 0.01M Phosphate buffered saline solution (PBS).

Table II: Time to Concentrate

Actual conditions will vary with details of initial solution temperature, concentration, and protein characteristics, but the table below can be used to provide an estimate of spin time (more detailed data is available at www.orbio.com).

Device	Solution	Vol.	Rotor	RCF	Time (min)	Conc. factor
5k Da	0.25 mg/ml bovine cytochrome-c, 0.01M TrisCl, pH 8	5 ml	35 ° fixed	8,500	35	550x
5k Da	0.25 mg/ml bovine cytochrome-c, 0.01M PBS	6 ml	Swing head	4,500	35	180x
5k Da	0.25 mg/ml bovine cytochrome-c, 0.01M PBS	6 ml	Swing head	4,500	52	250x
5k Da	0.01M PBS only	6 ml	Swing head	4,000	29	180x
10k Da	0.25 mg/ml bovine cytochrome-c, 0.01M TrisCl, pH 8	5 ml	35 ° fixed	8,500	25	450x
10k Da	0.25 mg/ml bovine cytochrome-c, 0.01M PBS	6 ml	Swing head	4,500	20	220x
10k Da	0.01M PBS only	6 ml	Swing head	4,500	29	550x
30k Da	0.5 mg/ml bovine serum albumin, 0.01M PBS	6 ml	Swing head	4,500	15	300x
30k Da	0.01M PBS only	6 ml	Swing head	4,500	15	550x
70k Da	0.5 mg/ml bovine serum albumin, 0.01M PBS	6 ml	Swing head	4,500	16	600x
70k Da	0.01M PBS only	6 ml	Swing head	4,500	16	600x
150k Da	0.5 mg/mL bovine thyroglobulin, 0.01M PBS	5 mL	Swing head	2.000	20	500x

Ordering Information

Product Name	QMWL*	Identification	Qty/Pk	CLP Order No.
5k Apollo 7 ml	5k Da	Sample pack	2ea	3491.1
5k Apollo 7 ml	5k Da	Rack of filters in capped tubes	25 ea.	3491.2
5k Apollo 7 ml	5k Da	10 racks, bulk bags of filters, tubes, caps	250 ea.	3491.3
10k Apollo 7 ml	10k Da	Sample pack	2ea	3492.1
10k Apollo 7 ml	10k Da	Rack of filters in capped tubes	25 ea.	3492.2
10k Apollo 7 ml	10k Da	10 racks, bulk bags of filters, tubes, caps	250 ea.	3492.3
30k Apollo 7 ml	30k Da	Sample pack	2ea	3493.1
30k Apollo 7 ml	30k Da	Rack of filters in capped tubes	25 ea.	3493.2
30k Apollo 7 ml	30k Da	10 racks, bulk bags of filters, tubes, caps	250 ea.	3493.3
70k Apollo 7 ml	70k Da	Sample pack	2ea	3494.1
70k Apollo 7 ml	70k Da	Rack of filters in capped tubes	25 ea.	3494.2
70k Apollo 7 ml	70k Da	10 racks, bulk bags of filters, tubes, caps	250 ea.	3494.3
150k Apollo 7 ml	150k Da	Sample pack	2ea	3495.1
150k Apollo 7 ml	150k Da	Rack of filters in capped tubes	25 ea.	3495.2
150k Apollo 7 ml	150k Da	10 racks, bulk bags of filters, tubes, caps	250 ea.	3495.3

*Minimum protein molecular weight that has been found to be quantitatively (>95%) retained by the membrane when tested in an Apollo device, as determined by filtrate optical density.

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Orbital Biosciences, LLC

4 Winsor Lane • P.O. Box 304 • Topsfield, MA 01983 • USA
Tel. 978-887-5077• FAX 978-887-2906 • e-mail: orbio@shore.net .