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TITLE: Regulatory Control of Breast Tumor Cell Poly (ADP-Ribose) Polymerase

PRINCIPAL INVESTIGATOR: Waleed Abdel-Aziz, Ph.D.
Linda Milkas, Ph.D.

CONTRACTING ORGANIZATION: Indiana University School of Medicine
Indianapolis, Indiana 46202-5254

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6. AUTHOR(S)
Waleed Abdel-Aziz, Ph.D.
Linda Milkas, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Indiana University School of Medicine
Indianapolis, Indiana 46202-5254

E-Mail: wabdelaz@iupui.edu

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13. ABSTRACT ***(Maximum 200 Words)***

We have previously isolated an intact, stable, and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant and malignant tumor cells including breast cancer cells. We have shown that poly(ADP-ribose) polymerase PARP is among the components of the DNA synthesome. The transformation of a non-malignant human breast cell to a malignant state was accompanied by a significant alteration in the 2-D PAGE profile of specific protein components of the DNA synthesome (such as PCNA) together with a 6-8 decrease in the replication fidelity of the DNA synthesome. To establish a link between the differences in the activity and alterations in the structure of PARP protein in malignant and non-malignant breast cells, PARP has been purified from breast cancer cell by phosphocellulose column and further purified and concentrated using microcon centrifugal devices. PARP has been enriched during these steps of purification. 2-D SDS-PAGE analyses have been performed using protean IEF cell. The biochemical and biophysical characteristics of PARP isolated from malignant and non-malignant breast cells will be compared to identify the molecular basis responsible for the observed error-prone DNA synthesis exhibited by breast cancer cells.

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INTRODUCTION:

The genetic damage which accompanies the development and progression of breast cancer has been linked to defects in the DNA replication and repair processes in these cells. We have previously isolated an intact, stable and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant as well as malignant tumor cells and tissues including breast cancer cells. All of the components necessary for DNA replication, including poly(ADP-ribose) polymerase (PARP) have been detected in the DNA synthesome. We have shown that the malignant breast cell DNA synthesome exhibits a 6-8-fold decrease in 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 cell epithelial cells to a malignant state is accompanied by a significant alteration in the mobility of specific protein components of the DNA synthesome (such as proliferating cell nuclear antigen; PCNA) following two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the replication complex (Bechtel et al., 1998). PARP is a nuclear enzyme of a molecular weight of 116,000. It plays an important role in DNA replication, repair, and recombination. It absolutely requires DNA single-strand or double-strand breaks for its enzymatic activity. This enzyme, once activated, catalyzes the transfer of ADP-ribose unit from nicotinamide adenine dinucleotide (NAD) to nuclear proteins such as histone and PARP itself. It has been shown that at least fifteen components of the DNA synthesome are poly(ADP-ribosylated), suggesting that PARP may play a regulatory role in controlling the activity and the fidelity of the DNA synthesome (Simbulan-Rosenthal et al., 1996 and 1998). The unique form of PCNA found exclusively in malignant breast cells lacks the poly(ADP-ribose) modification which is found in the non-malignant form of the protein. The goal of this project is to establish a link between the differences in PARP activity and the alteration(s) in structure exhibited by this protein in both malignant and non-malignant breast cells.

BODY:

1- PARP was Confirmed to be One of the Components of the DNA Synthesome:

The DNA synthesome has been isolated and purified from breast cancer MDA MB-468 cells using a series of steps, which include centrifugation, polyethylene glycol precipitation, ion exchange chromatography, and density gradient sedimentation (Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997) (Figure 1). P4 fraction (5 ml) containing approximately 30 mg of protein was loaded onto a Bio-Rad Q5 column pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1% glycerol, and 1 mM EDTA). The column was then washed with buffer A and eluted using a gradient of 50-500 mM KCl in buffer A. One-milliliter fractions were collected. The eluted fractions were then dialyzed into buffer B (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) and aliquots of each fraction were stored at –80°C.

The various protein fractions (H, S1, S2, NE, P4 and Q-sepharose peaks) were resolved using 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed using anti-human PARP antibody (BD PharMingen). PARP was found to exclusively co-purify with the DNA synthesome Figure 2A, B).

Mass spectrometric analysis of the DNA synthesome enriched protein fractions also indicated that PARP is a part of the replication complex core. Each of the protein bands from SDS-PAGE was cut and analyzed by MALDI and Q-TOF mass spectrometry. It has been shown that PARP, PCNA, replication protein A (RPA), replication protein C (RFC), DNA polymerase α, DNA methyltransferase, and FEN1 are components of the replication complex core (Dr. Suhua Han's last annual report).

2- PARP is enriched during its Purification from Breast Cancer Cells:

In order to define the kinetic and physical characteristics of PARP, we conducted chromatographic procedures to purify that protein from malignant and non-malignant breast cells. We initiated experiments to purify PARP from MCF-7, MDA MB-468 and MCF-10A cells according to the methods of Jump and Smulson (1980), Ushiro et al. (1987), and D'Amours et al. (1997). The purification procedure involves affinity chromatography with 3-aminobenzamide coupled to activated agarose gels (Affi-Gel 10 Gel, Bio-Rad).

a) Preparation of 3–aminobenzamide (3-AB) affinity column:

The Affi-Gel matrix was washed with 5x bed volume of cold isopropanol on a sintered glass funnel and transferred to a 50-ml conical tube. 15 ml of 50 mM 3-AB was added to the matrix with gentle agitation overnight at 4°C. The remaining active esters were blocked by the addition of 20 µl ethanolamine with gentle agitation for 2.5 hours. The gel was then washed thoroughly with isopropanol. The extent of coupling was

determined by measuring the decrease in absorbance of 3-AB at 280 nM in 0.1 N HCl. Approximately 50% of 3-AB has been coupled to the activated agarose matrix. The gel was transferred to the column and washed several times with buffer A containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM 2-mercaptoethanol. The column was stored in buffer A containing 0.2% sodium azide.

b) Enzyme Purification:

The first step of PARP purification involved the use of a phosphocellulose column that dissociated the synthesome and resolved PARP from other proteins. Cellulose phosphate (Sigma) was regenerated according the following procedures:

1- The resin was suspended in 5 volumes of distilled water overnight. It was stirred and allowed to settle for 45 minutes. The settled volume was measured and this was considered the column volume (CV) required to measure the volumes of the washing solution.
2- The resin was suspended in 5CV of 0.05 M NaOH containing 0.5 M NaCl for 10 minutes and the slurry was poured into a sintered glass funnel while applying gentle suction.
3- The slurry was washed with 5 CV of distilled deionized water.
4- The resin was suspended with 5 CV of 0.75 N HCl for 10 minutes and was poured back into the funnel. This was followed by passing fresh 0.75 N HCl through the bed.
5- The resin was washed with distilled deionized water using at least 9 CV until the effluent shows a pH=5.
6- The resin was packed into a column and equilibrated with buffer B containing 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, and 1 mM DTT.

10 grams of MDA MB-468 cells were fractionated to the clarified nuclear extract (NE). The cytosolic (S1), postmitochondrial (S2), or nuclear (NE) fractions were dialyzed against buffer B and applied onto the phosphocellulose column equilibrated with buffer B. PARP was eluted by a stepwise increase in NaCl concentration from 0.3 M to 0.6 M. The eluted fractions (50 μg; 5.6 μg, 1.5 μg, 0.95 μg, and 0.4 μg of the flow through, 0.3 M, 0.4 M, 0.5 M and 0.6 M phosphocellulose eluted fractions; respectively) were analyzed by 12% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody It has been found that most of the enzyme was eluted with 0.5 M NaCl (Figure 3A-C). PARP has been greatly enriched during this step of purification.

c) Concentration of PARP protein present in the phosphocellulose peak:

The phosphocellulose-eluted fractions enriched with PARP activity were concentrated using Centrifugal Filter Devices (Millipore) with a molecular weight cut-off of 50,000. PARP fraction was applied into the sample reservoir and concentrated according the instruction manual. 5 μl of concentrated fraction was then analyzed by 12% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody (Figure 4). PARP was further enriched after this step.

d) PARP Affinity Purification:

This will be performed according to Ushiro et al. (1987). The concentrated phosphocellulose PARP fraction will be applied onto the 3-AB affinity column equilibrated with buffer B and will be eluted with an increasing concentration of NaCl (0.8- 1.2 M). The active fraction will be collected and will be applied onto a size exclusion column for further purification.

3- Two-Dimensional Analyses of PARP from Breast Cancer Cells:

In order to determine the isoelectric point (pI) and the 2-D PAGE mobility of PARP isolated from breast cancer cells, the nuclear extracts from MDA MB-468 cells were prepared and analyzed using Bio-Rad Mini-Protean II tube cells (Bechtel et al., 1998). 30-50 μg protein was loaded onto the first dimension tube gel containing 9.2 M urea, 4% acrylamide, 20% Triton X-100, 1.6 % Bio-Lyte 8/10 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% TEMED. The proteins were separated along a pH gradient created using 100 mM NaOH (upper running buffer) and 10 mM H_3PO_4 (lower running buffer). The tube gel was placed onto a 12% SDS-polyacrylamide gel and the proteins were resolved by molecular weight. The proteins were transferred to a nitrocellulose membrane and PARP was detected using anti-human PARP antibody.

Since PARP is a basic protein with a pI value > 9.0, we could not be able to separate PARP by this method. In order to solve this problem, there were two alternatives:

1- Using nonequilibrium pH gradient electrophoresis (NEPHGE) according to the method of Anderson (1988) and Prasad et al. (1999). Proteins will be loaded on the top of the tube gels and overlaid with 4 M urea to protect proteins from phosphoric acid, which will be used in the upper reservoir. The lower reservoir will be filled with NaOH. The electrical leads will be reversed at the power supply and the gels will be run at 400 V for 1 hour followed by 4 hours at 800 V. The tube gels will be transferred to 12%SDS-PAGE for resolution in the second dimension.

2- Using the Bio-Rad IEF cell.
This cell performs the first dimension isoelectric focusing and can provide a maximum voltage of 10,000 V, which permits better focusing and resolution of the proteins. In addition, the precast immobilized pH gradient gel strips (Bio-Rad ReadyStrip IPG strips) were used in place of the first dimension tube gels. These strips provide reproducible gradients and eliminate the gradient drift that might occur with the tube gels.
PARP from both the nuclear and cytosolic fractions have been analyzed using the IEF cell and IPG strips.
   - Approximately 100 μg of cytosolic or nuclear fraction were desalted using the Bio-Spin 6 Tris Columns (Bio-Rad). The sample was concentrated using a SpeedVac.

- Prior isoelectric focusing, the IPG strips were rehydrated with a rehydration buffer containing 8 M urea, 1% CHAPS, 15 mM DTT, 0.1% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue.
- After SpeedVac, the protein sample was dissolved in 135 µl rehydration buffer and loaded in the sample loading well in the focusing tray. The IPG strip was placed into the channel tray by sliding it through the rehydration solution. After approximately 30 minutes, mineral oil was applied to each channel containing the IPG strip to prevent sample evaporation during the focusing step.
- Prior to running the second dimension, it was necessary to equilibrate the IPG strips. Two equilibration steps (15 minutes each) were necessary; the first step was required to saturate the strips with SDS and the reducing agent, while the second equilibration step was required to prevent protein re-oxidation during electrophoresis and alkylates the residual DTT to minimize vertical streaking.

Our results clearly demonstrated that PARP antibody recognizes one form of PARP in the nuclear fraction; however, different forms of PARP in cytosolic fractions have been detected (Figure 5A, B). These forms have similar molecular weight but they have different pI. Experiments are in progress to check the reproducibility of this pattern of migration and to detect whether these forms result from either poly(ADP-ribosylation) or phosphorylation.

PARP isolated from non-malignant breast cells will be analyzed using the same procedures and the difference in pI and/or 2D-PAGE mobility, if any, will be determined.

PARP purified from malignant and non-malignant breast cells will be digested with trypsin, chemotrypsin, lysC, and glutC. The resulting proteolytic peptides will be analyzed using SELDI mass spectroscopy and the profiles of non-malignant and malignant forms of PARP will be compared. Corresponding peptides from the non-malignant and malignant PARP differing in molecular weight will be microsequenced using the SELDI mass spectrometer in order to determine whether the modified peptide (if any) contains specific amino acids which are phosphorylated or poly(ADP-ribosylated). In addition, proteolytic peptides from malignant and non-malignant PARP will be separated using 2D-PAGE, transferred to nitrocellulose membranes, and probed sequentially with antibodies specifically recognizing phosphotyrosine, phosphothreonine, phosphoserine, and poly(ADP-ribose) polymer. Recognition of specific peptides by one or more of these antibodies will indicate that these peptides are phosphorylated and/or poly(ADP-ribosylated). Comparison of the peptides from each of the non-malignant and malignant breast cell PARP recognized by these antibodies will indicate whether non-malignant and malignant breast cells PARP undergo differential posttranslational modification.

4- PARP Activity Assay:

PARP assay was performed according to the methods of Jump and Smulson (1980), and knights and Chambers (2001).

The reaction mixture (100 μl) contains 500 mM Tris-HCl, pH 8.0, 250 mM $MgCl_2$, 1 mM NAD, 10 μg activated DNA, and 1-2 μl ^{32}P-NAD (specific activity 1000Ci/mmol) and increasing concentration of protein fractions. The reaction mixture was incubated at 25°C for 10 minutes. The radioactivity incorporated into trichloroacetic acid-insoluble materials was determined by liquid scintillation counting. Preliminary results are shown in Figure 6. An alternative way to detect PARP activity is by using biotinylated NAD (6-biotin-17-nicotinamideadenine dinucleotide; Trevigen). This provides non-isotopic alternative to radiolabeled NAD. Biotinylated NAD will allow an indirect measurement of PARP activity when biotin incorporation is detected using a conjugated-streptavidin detection system.

KEY RESEARCH ACCOMPLISHMENTS:

- Phosphocellulose purification and concentration of PARP from breast cancer MDA MB-468 cells.
- Preparation of 3-AB affinity column for further purification of PARP.
- Analysis of PARP protein from malignant breast cells by 2D-SDS PAGE using the Bio-Rad Protean IEF cell and IPG strips.
- Analysis of the DNA synthesome and detection of PARP in different steps of synthesome purification
- Setting up the *in vitro* PARP activity assay as an important step towards studying the kinetics of PARP from malignant and non-malignant breast cells

REPORTABLE OUTCOMES:

Abstracts:

- Abdel-Aziz W, Han S, Hickey RJ, Malkas LH (2002). Purification and functional characterization of breast tumor cell poly(ADP-ribose) polymerase.
  Will be presented at the *Era of Hope* Department of Defense Breast Cancer Research Meeting, Orlando, FL, September 25-28

- Abdel-Aziz W, Hoelz D, Malkas LH, Hickey RJ (2002). Purification and functional characterization of breast cancer cell poly(ADP-ribose) polymerase.
  Accepted for presentation at the 25th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 11-14

CONCLUSIONS:

- PARP is confirmed by both SDS-PAGE and mass spectrometric analyses to be one of the components of the DNA synthesome.
- PARP has been successfully purified by phosphocellulose chromatography. Most of PARP protein was eluted from the phosphocellulose column by 0.5 M NaCl
- PARP was further concentrated and purified from the 50-kDa proteins by filtration through Millipore centrifugal Filters. This step of purification resulted in further enrichment of PARP.
- Two-Dimensional PAGE analysis of PARP from MDA MB-468 cells was successfully performed using the IPG strips. Cytosolic PARP showed similar migration pattern to PARP protein present in the nuclear fraction; however, several forms of cytosolic PARP with different pI have been detected. These forms might be due to poly(ADP-ribosylation) and/or phosphorylation.

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25th Annual San Antonio Breast Cancer Symposium

Filename: 551191

Contact\Presenting Author Waleed Abdel-Aziz, Ph.D.

Department/Institution: Medicine, Hematology/Oncology Division, Indiana University School of Medicine

Address: 1044 W. Walnut Street, R4-202

City/State/Zip/Country: Indianapolis, IN, 46202, United States

Phone: 317-278-4229 **Fax:** 317-274-0396 **E-mail:** wabdelaz@iupui.edu

Abstract Category: Tumor Biology

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Scholars Program: No **Postdoctoral type:** PhD

Title: Purification and functional characterization of breast cancer cell poly(ADP-ribose) polymerase.

Waleed Abdel-Aziz [1*], Derek Hoelz [1], Linda H. Malkas [1] and Robert J. Hickey [1]. [1]Department of Medicine, Hematology/Oncology Division, Indiana University School of Medicine, Indianapolis, IN.

The genetic damage which accompanies the development and progression of breast cancer has been linked to defects in the DNA replication and repair processes in these cells. We have previously isolated an intact, stable, and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant and malignant tumor cells and tissues including breast cancer cells. All of the components necessary for DNA replication, including poly (ADP-ribose) polymerase (PARP), have been detected in the DNA synthesome. We have shown that the malignant breast cell DNA synthesome exhibits a 6-8 fold decrease in the replication fidelity relative to the non-malignant breast cell DNA syntehsome. 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 (such as proliferating cell nuclear antigen, PCNA) following 2D-PAGE of the replication complex. PARP has long been implicated in the processes of DNA replication, DNA repair, and cellular transformation. The unique form of PCNA found exclusively in malignant breast cells lacks the poly(ADP-ribose) modification which is found in the non-malignant form of the protein. In order to establish whether the malignant transformation process is accompanied by an alteration in the syntheome-associated PARP, the DNA synthesome isolated from non-malignant (MCF-10A) and malignant (MCF-7) breast cell lines was resolved by 2D-PAGE and the migration pattern as well as the isoelectric point (pI) were determined. In addition, we isolated PARP from both non-malignant and malignant breast cell lines using phosphocellulose and affinity column chromatography. PARP has been shown to be enriched during its purification. We are currently comparing the physical characteristics of PARP isolated from malignant and non-malignant breast cells in order to explain how poly(ADP-ribosylation) of DNA synthesome components contribute to the observed decrease in replication fidelity.

Signature of Presenting Author:

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Waleed Abdel-Aziz, Ph.D.

SAN ANTONIO BREAST CANCER SYMPOSIUM

August 16, 2002

ABSTRACT #579

Poster Session 1:	Wednesday 7:00-8:50 AM, Dec 11
Poster Session 2:	Wednesday 5:00-7:00 PM, Dec 11
Poster Session 3:	Thursday 7:00-9:00 AM, Dec 12
Poster Session 4:	Thursday 4:30-6:30 PM, Dec 12
Poster Session 5:	Friday 7:00-9:00 AM, Dec 13
Poster Session 6:	Friday 5:00-7:00 PM, Dec 13

Waleed Abdel-Aziz
Medicine, Hematology/Oncology Division
Indiana University School of Medicine
1044 W. Walnut Street, R4-202
Indianapolis, IN 46202

RE: Purification and functional characterization of breast cancer cell poly(ADP-ribose) polymerase.

Dear Dr. Abdel-Aziz:

Your abstract referenced above has been accepted for **poster** presentation at the 25th Annual San Antonio Breast Cancer Symposium, December 11-14, 2002. The 1st digit of your assigned abstract number indicates the poster session to which it is assigned (for example, #399 would be presented in Poster Session 3). The 2nd & 3rd digits match the board number, which remains the same for all sessions.

Instructions for preparing a poster presentation are enclosed, and also posted on the SABCS website at http://www.sabcs.org. Please follow them carefully. Also included are instructions for the **poster reproduction service** by Marathon Multimedia. Printed reproductions will be available at the symposium. Digital reproductions (CD-ROM and on line at the SABCS website) will be available shortly after the symposium.

If for any reason your presentation must be cancelled, notify the Symposium Coordinator as early as possible [E-mail Rmarkow@saci.org, FAX 210-949-5009, or phone 210-616-5912] before Friday, December 6. After that date, contact him at Marriott Rivercenter (210-223-1000). **Be sure to mention your assigned abstract number.** Failure to notify will result in activation of the No-Show Policy stated in the Abstract Submission Guidelines.

Don't forget to register to attend the meeting. You may register on line at www.sabcs.org. If you prefer fax or mail, a registration form is enclosed for your convenience. Pre-registration discount ends November 12. **REGISTRATION CLOSES NOVEMBER 27 AND RESUMES AT THE SYMPOSIUM ON DECEMBER 10.** Your symposium materials will be given to you when you check in at SABCS registration in the Tower Atrium of the Henry B. Gonzales Convention Center.

For information on reserving a hotel room, please go to the SABCS website at www.sabcs.org and click on the "Housing Information" button for instructions.

The final program booklet will be mailed in September. In the meantime, information is available on our website, which is updated periodically. Thank you for your abstract submission, and we look forward to your presentation.

Sincerely,
GARY C. CHAMNESS, PhD
Chairman, Abstract Selection Committee

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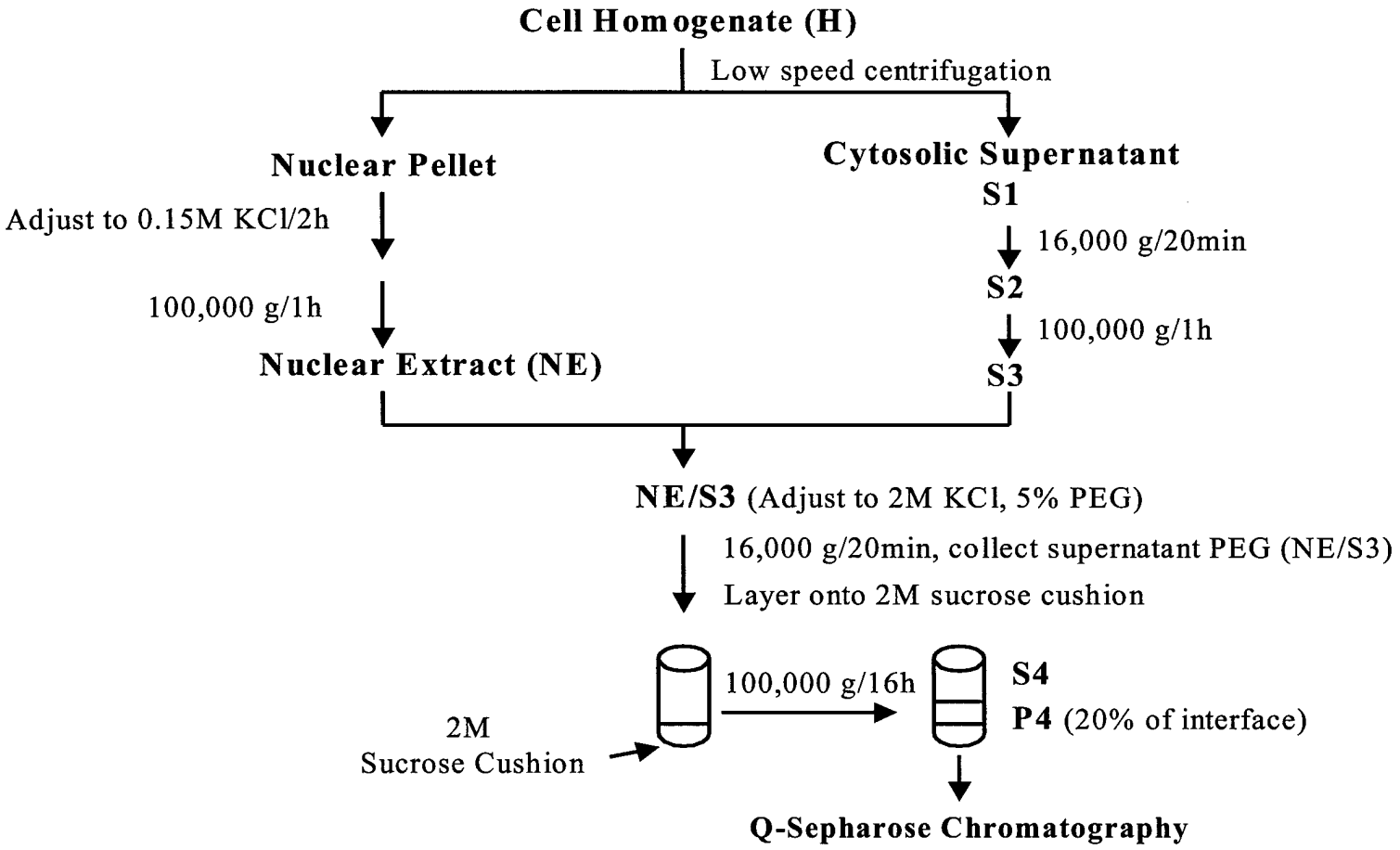

Figure 1: Flow Diagram of Subcellular Fractionation Scheme

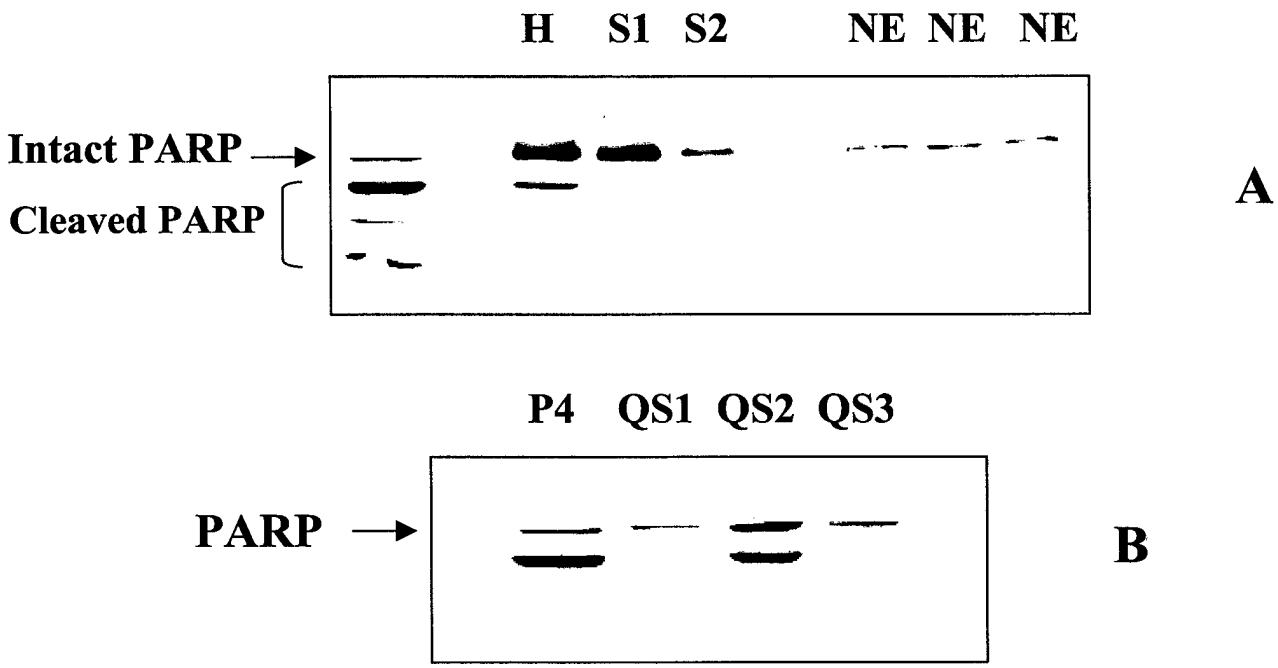

Figure 2: Immunoblot analyses for the presence of PARP in different synthesome purification fractions. Panel A; H to NE fractions; Panel B; P4 and Q-Sepharose Peaks.

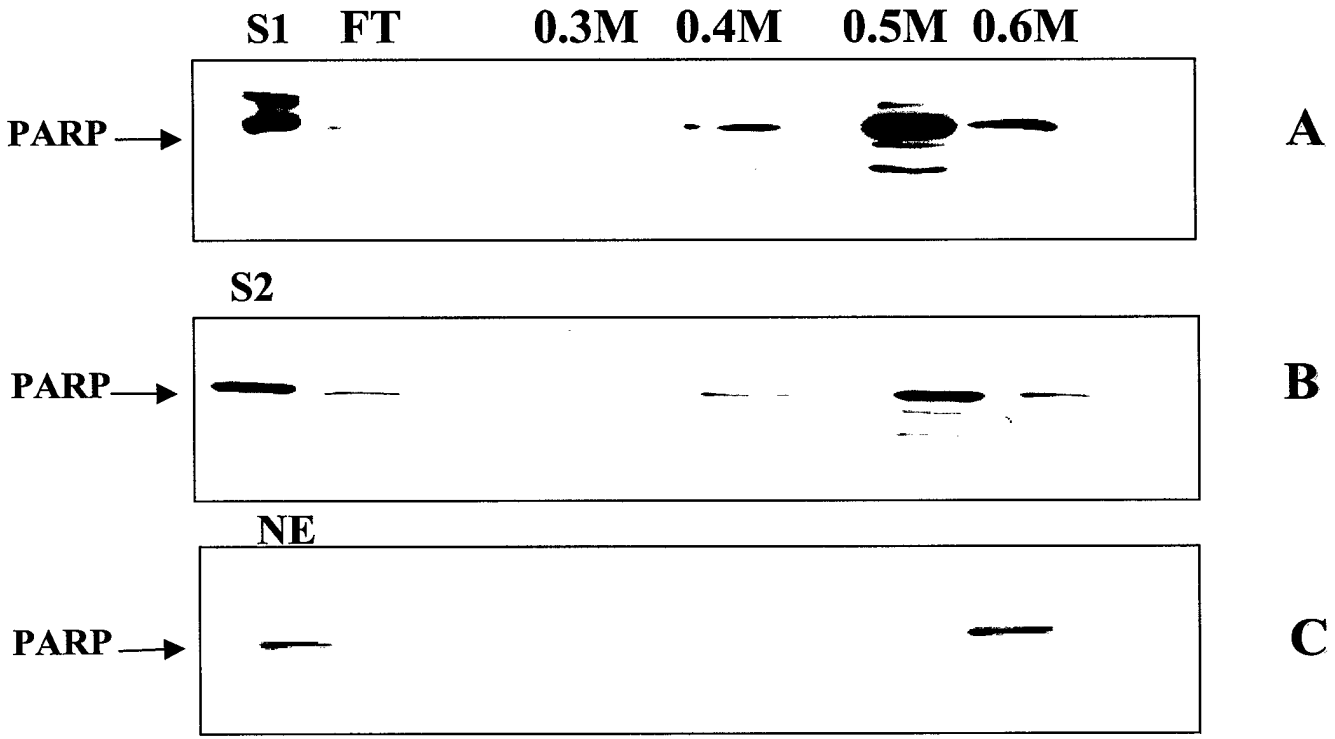

Figure 3: SDS-PAGE and Western blot analyses of PARP from phosphocellulose eluted fractions. MDA MB-468 breast cancer cells were faractionated according to Coll et al. (1996) (Figure 1). 75 μg of either the cytosolic S1 (A), the postmitochondrial S2 (B), or the nuclear NE (C) fractions were applied onto a phosphocellulose column equilibrated with 0.3 M NaCl. PARP was eluted by a stepwise increase in NaCl concentration from 0.3M to 0.6M. The eluted fractions were analyzed by 12% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody.

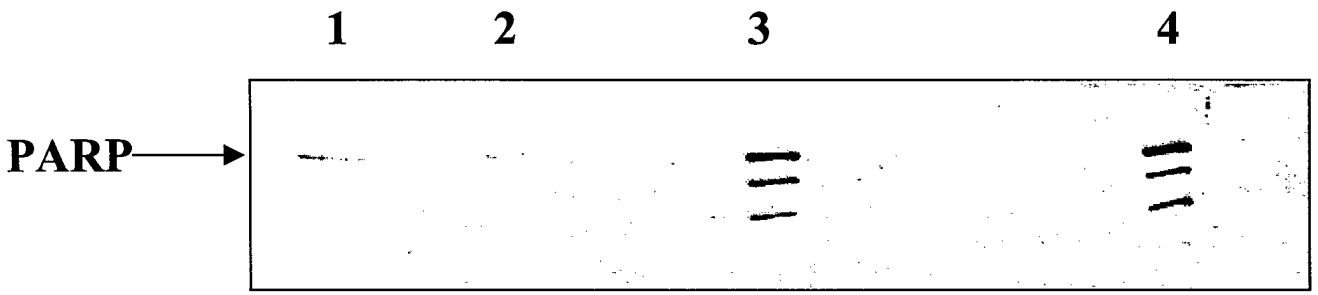

Figure 4: SDS-PAGE analysis of PARP in nuclear fraction (lane 1), the phosphocellulose peak (lane 2), and following concentration by Millipore Centrifugal Filters (lanes 3 and 4).

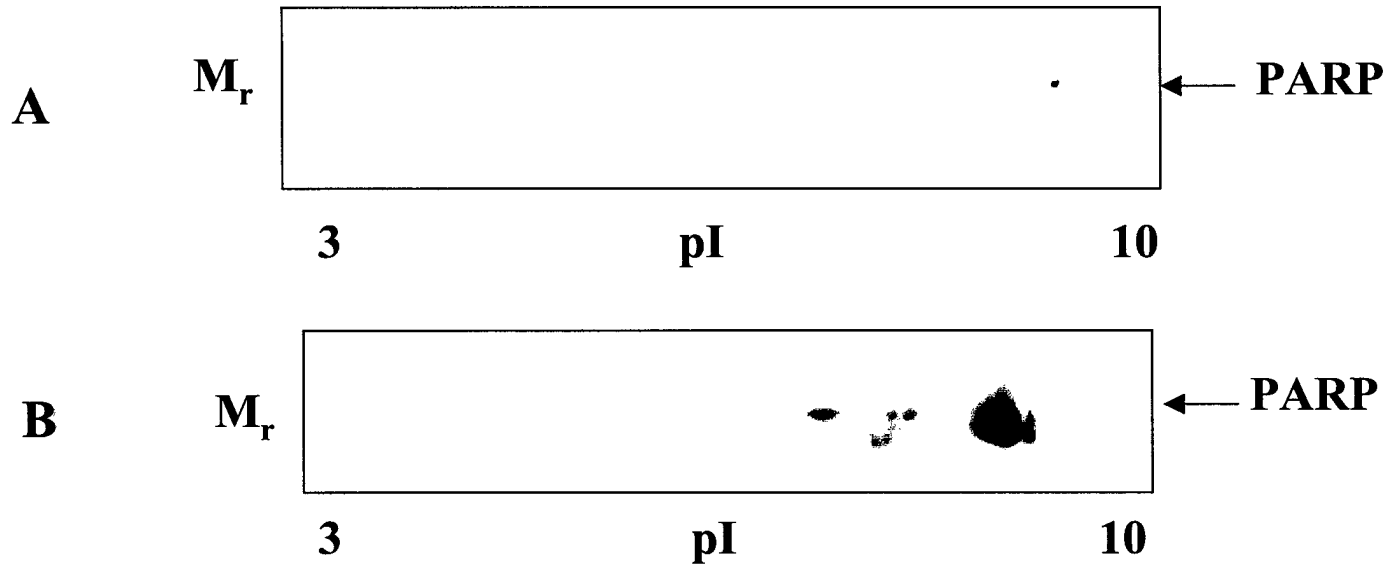

Figure 5: Protein migration of PARP from MDA MB-468 breast cancer cells. 100 µg protein of either nuclear NE (A) or cytosolic S2 (B) fractions were subject to 2D-PAGE. The resolved proteins were transferred to nitrocellulose membranes and analyzed by Western blot analysis using Antibody directed against PARP

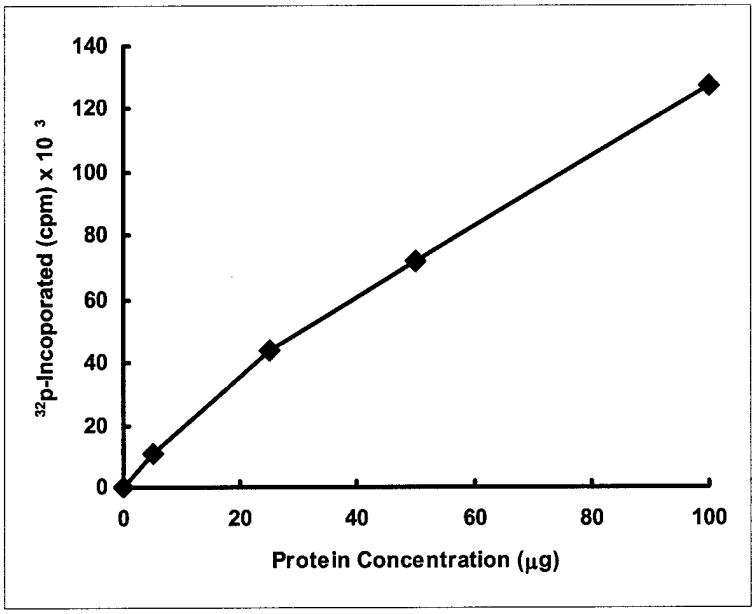

Figure 6: PARP Activity Assay. The reaction mixture (100 µl) contains 500 mM Tris-HCl, pH 8.0, 250 mM $MgCl_2$, 1 mM NAD, 10 µg activated DNA, and 1-2 µl ^{32}P-NAD (specific activity 1000Ci/mmol) and increasing concentration of protein fractions. The reaction mixture was incubated at 25°C for 10 minutes. The radioactivity incorporated into trichloroacetic acid-insoluble materials was determined by liquid scintillation counting. Control experiments were carried out in the absence of PARP.