POLY ADP-RIBOSE SYNTHESIS AND DNA REPLICATION IN SYNCHRONIZED MOUSE L-CELLS

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Summary:
The presence and synthesis of Poly ADP-ribose in vivo has been established in synchronized mouse L-cells. The polymer is synthesized predominantly in two short-lived bursts in the S-phase of the cell cycle. Inhibition of polymer synthesis by nicotinamide (20 mM) during the S-phase inhibits the rate of DNA replication subsequent to the time of poly ADP-ribose synthesis.

Introduction:

Poly ADP-ribose is synthesized from NAD by a chromatin bound enzyme with formation of a 1'-2' glycosidic linkage between the ADP-ribose subunits of the polymer (1,2,3,4,5,). The polymer is apparently covalently attached to nuclear proteins, particularly histones (6). The consequences of poly ADP-ribosylation of chromasomal proteins is reported to repress (7,8,9), activate (10), or have no effect on (9) DNA synthesis in subcellular systems.

The synthesis and function of Poly ADP-ribose in vivo have not been extensively investigated, primarily because the immediate substrate, NAD, does not enter whole cells intact (11). Utilizing ^{3}H-adenosine, we have labeled and isolated poly ADP-ribose from intact synchronized L-cells. We also report preliminary investigations of the relationship between Poly ADP-ribose synthesis and DNA replication in whole cells.

Methods:

L-cells, strain 929, were maintained as previously reported (12). Synchrony was attained by planting 16×10^{6} randomly growing cells in 32 oz. glass bottles. After 4 days growth, without refeeding, the dense monolayers were trypsinized and transferred to shake culture with fresh media containing 3 mM hydroxyurea. Sixteen hours later the cells had accumulated at the G_1/S boundary and S-phase

ENZYME SENSITIVITY

Enzyme	Acid Precipitable CPM
NONE	8840
DNase	9180
RNase	8310
Pronase	9020
Spleen Phosphodiesterase	9600
Micrococcal Nuclease	8900
Snake Venom Phosphodiesterase	2570

Table I: Enzymatic Sensitivity of the ^{3}H-adenosine labelled polymer. 100 µg of enzyme and polymer in a total volume of 1 ml of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ was incubated for 30 minutes at 37^{o}C. Undigested polymer was quantitated by acid precipitation. Pronase and micrococcal nucleose were from Calbiochem. DNase I (electrophoretically purified) and RNase (heated to 80^{o}C for 5 min.) were from Sigma. Venom phosphodiesterase further purified according to Keller (17) and spleen phosphodiesterase were from Worthington.

was induced by centrifugation followed by resuspension of the cells in fresh medium without hydroxyurea.

Cells were pulsed with 2,8^{3}H-adenosine (New England Nuclear, 30-50 Ci/mM) to label poly ADP-ribose. For assessment of DNA synthesis, 2^{14}C-thymidine (Schwarz, 9.5 mCi/mM) was included.

After pulse labelling the cells, nuclei were isolated (13) and incubated for 60 minutes at 37^{o} C in 0.3 N NaOH to degrade all RNA. Subsequently 1.0 M Tris-HCl, pH 6.7 was added to give a final pH of 9. After cooling to 0^{o} C the sample was centrifuged at 100,000 x g for three hours. Essentially all of the poly ADP-ribose was recovered in the supernatant fluid whereas the DNA was all pelleted as shown by a lack of acid precipitable ^{14}C-thymidine in the supernatant fluid. Poly ADP-ribose was precipitated and washed three times with cold 10% CCl_3COOH after addition of serum albumin as carrier. The polymer was then solubilized by heating the pellet at 90^{o}C for 15 minutes in 0.5 M perchloric acid. Radioactivity in the extract was quantitated as described earlier (12).

For enzyme digestions, poly ADP-ribose was prepared by exhaustively dialyzing the above 100,000 xg supernatant fluid against 0.01 M HCL, then against

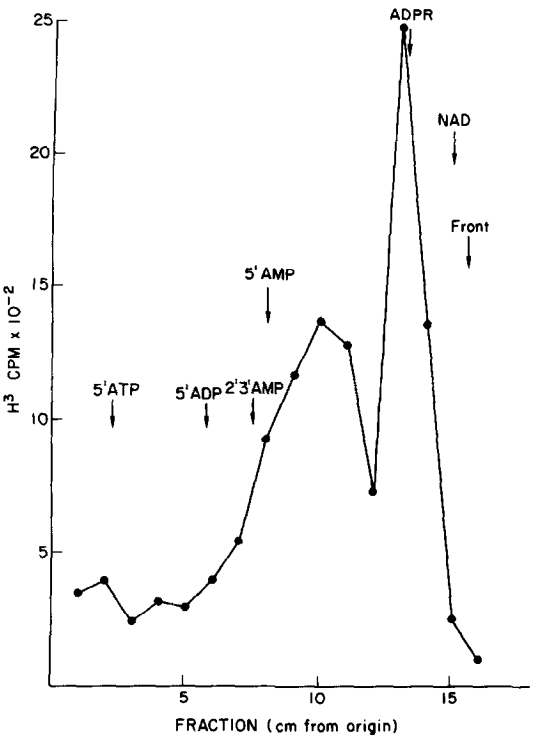

Fig. 1: Sensitivity of Poly ADP-ribose to Chromatin Glycohydrolase. The polymer was incubated as above with 100 µg of the enzyme purified to the ammonium sulfate precipitation step as described by Ueda (15). After digestion, the protein and undegraded polymer were acid precipitated with CCl_3COOH. The supernantant fluid was extracted with ether then lyophilyzed to dryness. The residue was suspended in water and applied to PEI-Cellulose (Baker) and developed in water and then with 1 M LiCl.

0.05 M Tris-HCl, pH 7.5. Following treatment with various enzymes, undigested material was quantitated by acid precipitation.

Results:

(1) Characterization of Isolated Material:

Poly ADP-Ribose was prepared as described from S-phase cells pulsed three hours with ^{3}H-adenosine. As seen in Table I only venom phosphodiesterase produced significant degradation of the ^{3}H-adenosine labeled polymer. Paper chromatography in two solvent systems (14) revealed that 80% of the venom phosphodiesterase degradation product migrated as 2'-[5"-phosphoribosyl]-5'-adenosine monophosphate. This is the major expected product (14).

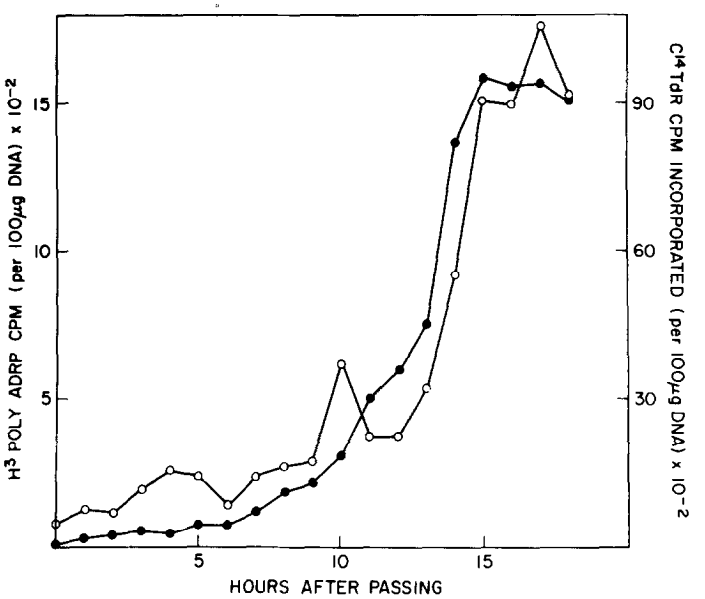

Fig. 2: Correlation of Poly-ADP-ribose and DNA Synthesis During the Cell Cycle. DNA was quantitated colorometrically (18) in the hot acid extract of the high speed pellet obtained as described in text o—o, poly ADP-ribose, ●—●, DNA.

In addition, the isolated material was degraded by a poly ADP-ribose glycohydrolase extracted from mouse L-cell chromatin according to the method of Ueda et al (15). PEI-cellulose thin layer chromatography of the degradation products is shown in Fig. 1. The main peak is ADP-ribose, the expected degradation product, and the more slowly migrating material appeared to be dimers of ADP-ribose (15). This material had a molecular weight of 1450 as determined by gel filtration on Biogel P-50 and yielded 5'-AMP on digestion with venom phosphodiesterase (15).

(2) Poly ADP-ribose synthesis in cell cycle:

High density monolayers were trypsinized and suspended in fresh media in shake culture. Consecutive one hour pulse labels of these cells with ^{3}H-adenosine (0.6 µCi/ml) and ^{14}C-thymidine (0.017 µCi/ml) revealed a correlation between the kinetics of DNA replication and poly ADP-ribose synthesis (Fig. 2).

To examine the timing of synthesis more closely, cells were accumulated at the G_1/S boundary as described. Nicotinamide (20mM) was added to one half

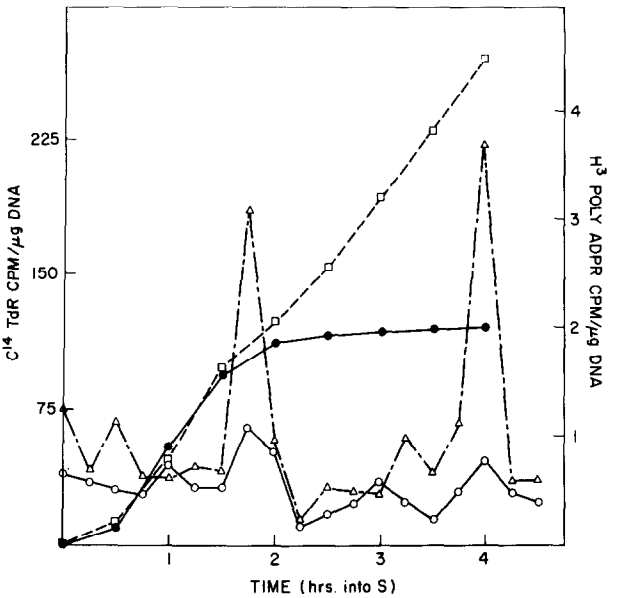

Fig. 3: Poly ADP-ribose Synthesis During the S-phase. see text for details. □—□, DNA of control. ●—●, DNA of nicotinamide treated. Δ—Δ, poly ADP-ribose of control. O—O, poly ADP-ribose of nicotinamide treated.

the culture at the time of hydroxyurea reversal. Nicotinamide is known to be a potent inhibitor of poly ADP-ribose polymerase in vitro (16). Cells were pulsed for 30 minutes with ^{3}H-adenosine and ^{14}C-thymidine at various intervals after initiating S-phase. Poly ADP-ribose was labeled maximally at two hours and again at three hours into S. Nicotinamide inhibited both bursts of polymer synthesis and reduced the rate of DNA synthesis, but only subsequent to the time of normal poly ADP-ribose synthesis (Fig. 3.)

To ascertain whether the peaks of labelling of poly ADP-ribose with ^{3}H-adenosine represented increased synthesis rather than changes in precursor specific activity the following experiment was performed. Cells were blocked with hydroxyurea and upon reversal of the block, amethopterin was added to inhibit de novo adenosine and thymidine synthesis (12). Adenosine of a known specific radioactivity and thymidine were added to allow the cells to progress through the S-phase (12). The poly ADP-ribose synthesis profile (Fig. 4) was similar to that depicted in Fig. 3, indicating that the peaks of polymer

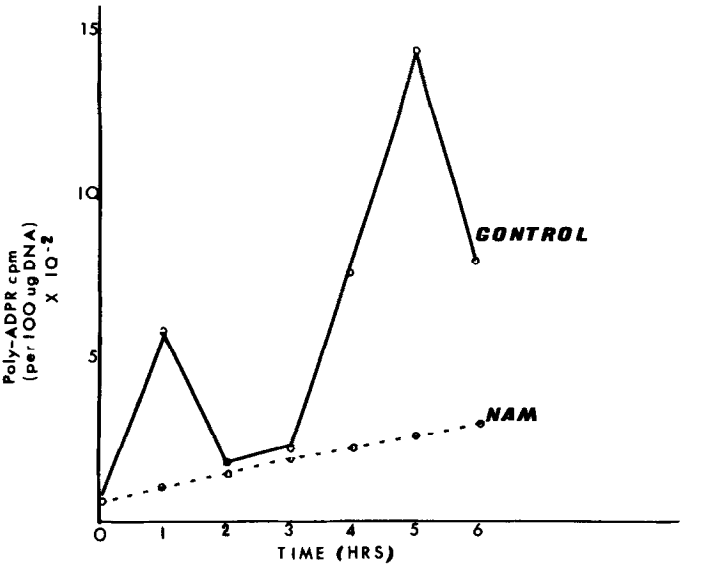

Fig. 4: Continuous Labelling of Poly ADP-ribose in Presence of Amethopterin. Cells were synchronized with hydroxyurea and 1 hour before initiating the S-phase amethopterin (10^{-6} M) and ^{3}H-adenosine (5×10^{-5} M, 1.82 mCi/10^{-4} Moles) was added. One hour later, the medium was replaced with hydroxyurea free medium containing H^{3}-adenosine and amethopterin as above plus 5×10^{-5} M thymidine. One half of the culture also received 20 mM nicotinamide (NAM).

labelling reflect increased synthesis and demonstrating that poly ADP-ribose is rapidly degraded after synthesis.

Discussion:

The present study is the first to demonstrate the kinetics of poly ADP-ribose synthesis in intact cells. We have shown the polymer is synthesized predominatly during the S-phase of mouse L-cells and further, that inhibition of polymer synthesis by nicotinamide also decreases the maximal rate of DNA synthesis. Further studies are underway to determine if poly ADP-ribose synthesis is a prerequisite for DNA synthesis during the latter part of the S-phase.

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