The role of primer recognition proteins in DNA replication: association with nuclear matrix in HeLa cells

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Summary

Primer recognition proteins (PRP) enable DNA polymerase \(\alpha\) to utilize efficiently DNA substrates with low primer to template ratios. We have previously identified the protein-tyrosine kinase substrate annexin II, and the glycolytic enzyme 3-phosphoglycerate kinase as components of PRP. As a step towards elucidation of the role of PRP in the process of DNA replication, we have investigated the subcellular distribution and specific association of these proteins with the nuclear matrix in HeLa cells. Nuclear extracts prepared from HeLa cells in S phase contain the enzymatic activity of 3-phosphoglycerate kinase (PGK) and phospholipase A2 inhibitory activity of annexin II. Monomer annexin II is approximately equally distributed between the nuclear and cytoplasmic fractions, while a majority of PGK is in the cytoplasm. Immunoblot analyses reveal the presence of these two proteins in nuclei, specifically associated with the nuclear matrix. This is further confirmed by observation of the presence of annexin II and PGK in isolated nuclear matrices by immunoelectron microscopy. The phospholipase A2 inhibitory activity of annexin II colocalizes with the nuclear matrix-bound annexin II. A related protein, annexin I, is not detectable in the nuclear extracts and nuclear matrix. Attempts to dissociate PGK and annexin II from the nuclear matrix with octyl-\(\beta\)-glucoside, high salt or metal ion chelators were unsuccessful, suggesting that the interaction is very strong.

Key words: DNA replication, primer recognition proteins, nuclear matrix.

Introduction

DNA replication involves various protein-protein and protein-DNA interactions (Thommes and Hubscher, 1990). DNA polymerase \(\alpha\) (Pol \(\alpha\)) plays a major role in mammalian DNA replication (DePamphilis and Wasse, 1980; Thommes and Hubscher, 1990). Pol \(\alpha\) exists in multiple forms; a multiprotein Pol \(\alpha\) complex from HeLa cells (Vishwanatha et al., 1986) was shown to be associated with DNA primase, primer recognition proteins, deoxyribonuclease and Ap4A binding protein. Primer recognition proteins (PRP) enable Pol \(\alpha\) to utilize efficiently DNA substrates with low primer to template ratios (Pritchard and DePamphilis, 1983; Vishwanatha et al., 1986), a situation found in lagging-strand DNA replication. PRP has been purified to homogeneity from HeLa cells and human placenta, and is composed of two polypeptides of 36 and 41 kilodaltons (Jindal and Vishwanatha, 1990a). By amino acid sequence analysis of tryptic peptides derived from the two proteins, we have identified the 41 kilodalton polypeptide as the glycolytic enzyme, 3-phosphoglycerate kinase (PGK) (Jindal and Vishwanatha, 1990b). The 36 kilodalton protein has been identified as the protein-tyrosine kinase substrate, annexin II (Jindal et al., 1991). The complex of PGK and annexin II functions as PRP; neither of them alone can act as PRP.

Annexins are major in vivo substrates of oncogene and growth-factor receptor tyrosine kinases (reviewed by Klee, 1988). The annexin family is composed of at least six different members including annexin II (also called p36, lipocortin II or calpactin I; Pepinsky et al., 1988). The annexin family has been implicated in mitogenic signal transduction (Pepinsky et al., 1988) and regulation of inflammation (Flower et al., 1984; DiRosa, 1985). Annexin II exists in two forms: the monomer (representing 20-30% of the total cellular annexin II) is mainly cytosolic and the heteromer, in association with a 10 kilodalton annexin II light chain, is mainly cytoskeletal (Klee, 1988). The physiological role of such a distribution is not clear and may indicate involvement of annexin II in more than one cellular function. The involvement of annexin II in DNA synthesis is supported by the following observations: (a)
annexin II is part of the primer recognition protein complex (Jindal et al., 1991); (b) the growth-regulated gene 1B6 is identical to annexin II (Keutzer and Hirschhorn, 1990); (c) annexin II expression is basal in normal hepatocytes and significantly enhanced in human hepatocellular carcinoma (Frohlich et al., 1990); (d) enhanced expression of annexin II in hamster pancreatic cancers is specifically associated with proliferating and metastatic areas (Kumble et al., 1992); and (e) immunodepletion of Xenopus egg extracts with anti-annexin II monoclonal antibodies results in loss of replicative ability of the extracts (S. Kumble and J. K. Vishwanatha, in preparation).

The identification of annexin II and PGK as subunits of PRP activity led us to examine the existence of these proteins in nuclei and nuclear matrix. We have previously demonstrated, using immunoelectron microscopy, that annexin II monomer and PGK are present in the nuclei of normal and transformed cells (Kumble and Vishwanatha, 1991). Nuclear matrix (also called nuclear cage or nuclear scaffold) is a dynamic proteinaceous nuclear framework obtained by treatment of isolated nuclei with nuclease, salt and detergent (Berezney, 1984). Replication forks are associated with the nuclear matrix (Vaughn et al., 1990), and association of replicative enzyme activities with the nuclear matrix has been demonstrated in HeLa cells and regenerating rat liver (Smith and Berezney, 1983; Foster and Collins, 1985; Tubo and Berezney, 1987; Collins and Chu, 1987). Isolated nuclear matrices are capable of in vitro DNA synthesis (Smith and Berezney, 1983; Foster and Collins, 1985). Nuclear matrix is also implicated in transcription (Jackson et al., 1984) and RNA processing (Ciejek et al., 1982). Data presented here show the association of PGK and annexin II with the nuclear matrix, indicating that the complex of PGK and annexin II like PRP may have a role in DNA replication.

Materials and methods

Cell fractionation

HeLa S3 cells were maintained in suspension cultures in Joklik's modified Eagle's medium supplemented with 5% defined iron-supplemented calf serum (HyClone Laboratories, Utah, USA) at 37°C. Cells were synchronized by the double thymidine block technique (Collins and Chu, 1987) and harvested 5 hours after release from the second thymidine block. The cell pellet was rinsed twice with Hank's balanced salt solution and once with hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl and 1 mM MgCl2). Cells were resuspended and held in hypotonic buffer for 2 hours at 4°C. The cells were disrupted with a Dounce homogenizer (30 strokes with pestle B) and disruption was monitored by light microscopy. No intact cells were observed by microscopy after homogenization. The homogenate was subjected to centrifugation in a Sorvall SS-34 rotor at 2,000 revs/min for 30 minutes at 4°C. The supernatant was designated as "cytoplasmic extract". The nuclear pellet was further rinsed three times with the hypotonic buffer and the supernatant from each washing was added to the cytoplasmic extract. The nuclei were resuspended in 0.4 M potassium phosphate, pH 7.2, 1 mM EDTA, 1 mM DTT (dithiothreitol) and 10% glycerol and extracted for 1 hour at 4°C. Following centrifugation in a Sorvall SS-34 rotor at 10,000 revs/min for 30 minutes at 4°C, the supernatant was collected as "nuclear extract". The cytoplasmic and nuclear extracts were dialyzed against 50 mM potassium phosphate, pH 7.2, 1 mM DTT, 1 mM EDTA and 10% glycerol, and stored at —80°C until further use.

Antibodies against annexins

The polyclonal rabbit anti-chicken annexin II antiserum (Cooper and Hunter, 1983) was generously provided by Dr. Tony Hunter, Salk Institute, San Diego, California, USA. In immunoblot analysis, we used a 1:200 dilution of this antiserum. The polyclonal rabbit anti-human annexin II antiserum (no. 774), the rabbit anti-human annexin III antiserum (no. 646) (Pepinsky et al., 1988) and the mouse monoclonal anti-recombinant human annexin III (Lipo-103) (Pepinsky et al., 1990) were generously provided by Dr. Blake Pepinsky, Biogen, Massachusetts, USA. Antisera 646 and 774 were used at 1:2000 dilution and the lipo-103 antibody was used at 20 µg/ml in our immunoblot analysis.

Polyclonal antiserum against human PGK

New Zealand white rabbits were immunized by multiple injections (intradermal and intraperitoneal) with 200 µg of human placental PGK purified to homogeneity by published procedures (Kuntz and Krietsch, 1982). The Ribi adjuvant for rabbits (Ribi Immunochem., Montana, USA) was used as an adjuvant in immunization. Immunization was repeated two weeks later with 100 µg of purified PGK. Antisera were obtained weekly for four weeks after the boost and antibody titers were determined by enzyme-linked immunosorbent assay.

Preparation of the nuclear matrix

Nuclear matrices were prepared from freshly isolated nuclei of S phase HeLa cells. Briefly, nuclei prepared as described by Collins and Chu (1987) were digested with 80 µg/ml DNase I for 30 minutes at 37°C. The cytosolic fraction was kept separately and called "soluble fraction". Digested nuclei were washed 3 times with PBS and 3 times with TMP buffer (10 mM Tris-HCl, pH 7.5, 0.2 M MgCl2, 1 mM PMSF). Supernatants from each of these washings were combined and this fraction was designated as "low-salt nuclear extract". Low-salt extracted nuclei were resuspended in TMP buffer, and TMP buffer containing 5 M NaCl was added slowly over a 1 hour period to a final concentration of 2 M NaCl. Following centrifugation, the nuclear pellet was washed 3 times with PBS, 4 times with low-salt phosphate buffer (20 mM potassium phosphate, pH 7.5, 2 mM β-mercaptoethanol), three times with high-salt phosphate buffer (0.5 M potassium phosphate, pH 7.5, 2 mM β-mercaptoethanol) and finally, 3 times with the low-salt phosphate buffer. Supernatants from the above washes were combined and designated as "high-salt nuclear extract". The low-salt and high-salt nuclear extracts were concentrated with membrane concentrators (Omega Cell, Pharmacia LKB, California, USA), dialyzed against 50 mM potassium phosphate, pH 7.2, 1 mM DTT, 1 mM EDTA, 10% glycerol, and stored at —80°C. The final nuclear matrix was resuspended in the same buffer and stored in aliquots at —80°C.

Electron microscopy of cells, nuclei and nuclear matrix

HeLa cells, isolated nuclei and the nuclear matrix (prepared as described above) were fixed in 1.5% paraformaldehyde, 1% glutaraldehyde, 0.13% picric acid, 0.1 M phosphate...
buffer, pH 7.4. The fixed cells, nuclei and matrix were then washed with 0.1 M phosphate buffer, pH 7.4, and dehydrated through a series of increasing ethyl alcohol concentrations. Following dealkoholization in the presence of propylene oxide, cells, nuclei and matrix were embedded in Araldite resin. Thin sections were cut using the Portex-Blum MT 2 ultramicrotome and then mounted on nickel grids (200 mesh) for electron microscopy. The nickel grids were double stained with uranyl acetate and Reynolds's lead citrate, and viewed through a Philips 410 LS electron microscope at 60-80 kV.

**Immunoelectron microscopy of nuclear matrix**

Ultrathin sections of nuclear matrices mounted on nickel grids were prepared as described above and used for immunolabeling. The grids were initially fixed with 3% hydrogen peroxide and after rinsing in PBS and blocking buffer (PBS containing 1% BSA) for 1 min each, incubated with the primary antibody (diluted in PBS) for 2 h. The grids were then washed with blocking buffer and incubated with the Protein A-gold conjugate (30 nm particle size) for 60 min at room temperature. At the end of this incubation, the grids were washed successively with blocking buffer, PBS and distilled water, and finally dried. The tissue sections were double stained with uranyl acetate and Reynolds's lead citrate. The sections were viewed using a Phillips 410 LS electron microscope at 60-80 kV.

**Solubilization of nuclear matrix**

Extensively washed nuclear matrix preparation was sonicated 5 times for 15 seconds each at 20 second intervals and allowed to remain on ice for 2 hours. Following this, an aliquot of the homogenate was incubated with 22 mM octyl-β-glucoside and 0.5 M KC1 for 1 hour at 4°C. Another aliquot of the homogenate was adjusted to 5 mM final concentration of ethyleneglycol-bis(β-amino-ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) and incubated for 1 hour at 4°C. Following centrifugation at 40,000 rev/min in a Beckman type 65 rotor for 1 hour at 4°C, the supernatants were saved as "dissociated fraction". The pellet was resuspended in PBS, washed three times with PBS and the "residual matrix" was stored in 50 mM potassium phosphate, pH 7.2, 1 mM DTT, 1 mM EDTA, 10% glycerol at -80°C. The "dissociated fraction" was dialyzed against the same phosphate buffer and stored at -80°C.

**Enzyme assays, SDS-PAGE and immunoblot analysis**

Previously published procedures were used for assays of DNA polymerase α activity (Vishwanatha et al., 1986) and PGK activity (Jindal and Vishwanatha, 1990a,b). Phospholipase A2 inhibition assay (Haigler et al., 1987) was conducted with modifications as previously described (Jindal et al., 1991). Proteins were resolved on 4% to 15% gradient polyacrylamide gels containing SDS and immunoblotting was performed as previously described (Jindal et al., 1991).

**Results**

**Presence of annexin II and PGK in nuclear extracts**

PGK in association with the protein-tyrosine kinase substrate, annexin II, forms the primer recognition protein complex that stimulates DNA polymerase α activity on substrates with long single-stranded DNA templates. Annexin II is abundant in cells mainly in association with plasma membrane in a complex with calpain light chain. Monomer annexin II is cytosolic. PGK is abundant in the cytoplasm. In order for these proteins to be involved in DNA replication, they must be present in the nucleus during S phase of the cell cycle. We investigated the presence of these proteins in nuclear extracts of HeLa cells in S phase. Nuclei were prepared by Dounce homogenization of cells incubated in hypotonic buffer and extensively washed to remove cytoplasmic contamination. Nuclear extracts from such washed nuclei contain significant amounts of PRP (Jindal and Vishwanatha, 1990a,b; Jindal et al., 1991). The activities of PGK, annexin II and Pol α in cytosol and nuclear extracts of HeLa cells are presented in Table 1. As expected, nuclear extracts have significantly higher activity of Pol α than is found in the cytosol. There is a tenfold higher activity of PGK in cytosol compared to nuclear extracts. The monomer annexin II activity (as judged by the inhibition of PLA2 activity) is present in both nuclear and cytosolic extracts to the same extent. We were unable to determine the stoichiometry of Pol α, PGK and annexin II in the nuclear extract and in the DNA Pol α complex, due to the unavailability of antisera or monoclonal antibodies to DNA Pol α that would react quantitatively on Western blots. Fig. 1 shows the activities of annexin II (A) and PGK (B) as a function of protein concentration in the reaction. At the various protein concentrations used, annexin II activity in the cytosol and nuclear extract are nearly equal. No activity is seen with various concentrations of a non-specific protein gelatin. Immunoblot analysis using polyconal antibodies against PGK and annexin II demonstrates the presence of these proteins in the nuclear extracts (Fig. 2). The immunoblot from Fig. 2 was analyzed on a Betascope 603 (Betagen, Inc.) radioanalytical imager and the radioactivity corresponding to the PGK and annexin II bands was quantitated. A correlation of 0.996 is seen between imaging data obtained from the Betascope 603 and radioactivity determined with a gamma counter (R. G. MacDonald and J. K. Vishwanatha, unpublished). We

**Table 1. Pol α, annexin II and PGK activities in cytosolic and nuclear extracts of HeLa cells**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Pol α (units/mg)</th>
<th>PGK (units/µg)</th>
<th>Annexin II (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>90</td>
<td>324</td>
<td>1300</td>
<td>31.0</td>
<td>68</td>
</tr>
<tr>
<td>Nuclear extract</td>
<td>59</td>
<td>183</td>
<td>3230</td>
<td>3.4</td>
<td>67</td>
</tr>
</tbody>
</table>

The starting material was 20 g wet weight of HeLa cells collected in early S phase of cell cycle. The preparation of extracts and assays for Pol α with activated DNA template, PGK and annexin II are described under Materials and methods. One unit of Pol α activity corresponds to 1 pmol of dNTP incorporated per hour at 35°C; one unit of PGK activity corresponds to oxidation of 1 µmol of NADH/min, using a molar extinction coefficient for NADH of $3.4 \times 10^3$ cm$^{-1}$. Annexin II activity is expressed as % inhibition of phospholipase A2 activity at a protein concentration of 5 µg/ml.
Fig. 1. Annexin II and PGK activities of cytosolic and nuclear extracts. HeLa cell cytosolic and nuclear extracts were prepared and assayed for PGK and PLA₂ inhibitory activities, as described under Materials and methods. (A) PLA₂ inhibitory activity profiles of cytosolic extract (filled squares), nuclear extract (open squares), crude lipocortin preparation (filled circles) and gelatin (open circles) at various indicated protein concentrations in the reaction; 100% activity represents PLA₂ activity in the absence of added inhibitory or control protein. (B) PGK activity of nuclear extract (hatched bars) and cytosolic extract (filled bars) at various indicated protein concentrations.

Fig. 2. Annexin II and PGK are present in HeLa cell nuclear extracts. HeLa cells collected in early S phase were fractionated into cytoplasmic and nuclear extracts as described under Materials and methods. Samples (10 μg) of protein from cytoplasmic extract (lane C) and nuclear extract (lane N) from S phase HeLa cells were subjected to electrophoresis on a 4% to 15% gradient polyacrylamide gel containing SDS. Proteins were stained with silver reagent (A), or transferred to Immobilon-P membrane, which was then incubated either with polyclonal rabbit anti-annexin II antiserum or polyclonal rabbit anti-human PGK antiserum (B). Reactivity was then assessed using [¹²⁵I]protein A followed by autoradiography. The positions of ¹⁴C-labeled marker proteins run on the same gel (lane M) are indicated (in kilodaltons). The blots were exposed for 8 h at −70°C using Kodak X-Omat AR film and a Cronex-plus intensifying screen.

find that PGK is predominantly cytosolic (94%) and only a small amount exists in the nucleus (6%). Annexin II, on the other hand, is distributed between nucleus (40%) and cytosol (60%). The nuclear annexin II represents that part of the monomer annexin II that is not associated with the light chain. Monomer annexin II has been variously estimated to represent 20-30% of the total annexin II in cells. Thus, the amount of annexin II...
in the HeLa nucleus represents 8-12% of the total annexin II in cells. These results are in agreement with the activities shown in Table 1. The presence of annexin II and PGK in the nuclear extracts is further supported by the demonstration of the presence of these proteins in the nucleus by immunoelectron microscopy using colloidal gold-conjugated secondary antibody (Kumble and Vishwanatha, 1991). Previously, we have shown that the cytoplasmic protein, annexin I (also called p35, calpactin II or lipocortin I) is not present in nuclear extracts prepared by this procedure (Jindal et al., 1991).

**Preparation of the HeLa cell nuclear matrix**

A number of proteins and enzymes implicated in DNA replication are found associated with the nuclear matrix (Smith and Berezney, 1983; Foster and Collins, 1985; Tubo and Berezney, 1987; Collins and Chu, 1987) and recently the association of replication forks with the nuclear matrix was demonstrated (Vaughn et al., 1990). We investigated the association of PRP with the nuclear matrix in HeLa cells. We compared different procedures for isolation of the nuclear matrix, since differences in these procedures may influence the composition of the matrix. The final procedure that we routinely used in all of the following experiments is essentially that of Collins and Chu (1987) and is described under Materials and methods. Electron microscopic pictures of the prepared nuclear matrices, isolated nuclei and the original cells are shown in Fig. 3. The nuclear membrane maintains its integrity throughout the matrix preparation procedures (Fig. 3B). The matrices exhibit well-preserved internal structure, with the perichromatin granules and interchromatin in the matrix (Fig. 3A and B) being very similar to the isolated nuclei or the intact nucleus of the whole cell (Fig. 3C). Some debris present on the outside of the matrix (Fig. 3A, B) possibly represents lysis of some nuclei, since the isolated nuclei are free of any cytoplasmic debris (Fig. 3D). The nuclear matrices that we prepared are very similar in appearance to the nuclear matrices from chicken erythroblast cells (Farache et al., 1990) and the yeast nuclear scaffolds (Cardenas et al., 1990). No differences were seen in the quality of matrices obtained when nuclei were extracted with or without reducing agents in the extraction buffer.

**Annexin II activity in nuclear matrix**

The soluble fraction, low-salt and high-salt extracts of nuclei and the nuclear matrix were prepared as described under Materials and methods. The phospholipase A2 inhibitory activities of these fractions are shown in Fig. 4. Significant amounts of the nuclear annexin II activity are released by low-salt washing of nuclei (bar 3). Further washing of nuclei with high-salt buffers (bar 4) does not release any PLA2 inhibitory activity. Nuclear matrices prepared after these salt washes contain significant levels of PLA2 inhibitory activity (bars 5,6,7). Increasing PLA2 inhibitory activity as a function of increasing protein concentration is observed with the nuclear matrix preparation (bars 5,6,7). Although PGK is present in the nuclear matrix (see below), measurable activities are not observed.

**Immunoblot and immunoelectron microscopic analyses of annexin II and PGK in nuclear matrix**

The presence of annexin II and PGK in the nuclear matrix was demonstrated by immunoblot and immunoelectron microscopic analyses using various antibodies.
that recognize the two proteins. Analyses of the polypeptide composition of various fractions during nuclear matrix preparation were presented in Fig. 5 (A). The complex nature of nuclear matrix seen (Fig. 5A) is similar to that reported earlier for nuclear matrix and chromosome scaffolds (Berezney and Coffey, 1974; Earnshaw et al., 1985). We examined different fractions obtained during nuclear matrix preparation for their reactivity against a polyclonal rabbit antiserum raised against chicken annexin II (Cooper and Hunter, 1983), and the data are presented in Fig. 5B. The antiserum recognizes a 36 kilodalton polypeptide in all of the fractions analyzed. In addition, the antiserum reacts with a protein of 39 kilodaltons in the nuclear matrix. We have not seen a cross-reaction between a 39 kilodalton protein and the anti-annexin II antiserum in any other fraction tested so far. The 39 kilodalton protein appears to be a modified form of annexin II and is recognized by the anti-annexin II antiserum as shown in Fig. 7A). However, the anti-annexin I antiserum does not recognize the 39 kilodalton protein that cross-reacts with the anti-annexin II antiserum. In the soluble fraction (Lane 1), the anti-annexin I antiserum reacts with a 32 kilodalton protein. The 32 kilodalton protein is a proteolytic product of annexin I. While the annexin I in the soluble fraction is recognized by this antibody (lane 1), no cross-reactivity is observed with the proteins in the salt extracts of nuclei (lanes 2 and 3) or the nuclear matrix (lane 4). Prolonged overexposure of the blot did not result in appearance of any other bands. Thus, annexin II is specifically associated with the nuclear matrix, while annexin I is not present in either nuclear extracts or the nuclear matrix.

Strong association of annexin II and PGK with nuclear matrix

DNA replication proteins tightly associated with the nuclear matrix are dissociated by treatment of nuclear matrices with the detergent octyl $\beta$-D-glucoside under high-salt conditions (Collins and Chu, 1987). We
Association of PRP with nuclear matrix

Fig. 5. Immunoblot analysis of annexin II and PGK in nuclear extracts and nuclear matrix. The soluble fraction (lane 1), low-salt nuclear extract (lane 2), high-salt nuclear extract (lane 3) and nuclear matrix (lane 4) from S phase HeLa cells, and crude lipocortin from human placenta (lane 5), were electrophoresed on 4% to 15% gradient SDS-PAGE gels. Proteins were stained with silver reagent (A), or transferred to Immobilon-P membranes and incubated with polyclonal rabbit anti-annexin II antiserum (B) or polyclonal anti-human PGK antiserum (C). Reactivity was assessed using [125I]protein A and autoradiography. Positions of 14C-labeled marker proteins (lane M) run on the same gels are indicated with their molecular masses in kilodaltons.

Discussion

The complex of annexin II with the glycolytic enzyme, PGK, functions as PRP in stimulating Pol α activity on templates with low primer to template ratios (Jindal and Vishwanatha, 1990a,b; Jindal et al., 1991). The majority of annexin II in cells exists as a heteromer with calpactin light chain (p10). However, the monomeric annexin II is cytosolic (Klee, 1988). The physiological role of annexins or the significance of two pools of annexin II in cells is not yet understood. Presumably,

attempted to dissociate the nuclear matrix-bound PGK and annexin II under a variety of conditions. The results are shown in Fig. 8. Treatment of nuclear matrices with 22 mM octyl-β-D-glucoside in the presence of 0.5 M KCl or incubation of the matrix with 5 mM EGTA fails to dissociate either the annexin II (Fig. 8A) or PGK (Fig. 8B) proteins. Interestingly, the 39 kilodalton protein, presumably modified annexin II, is also tightly bound and is not dissociated. However, these proteins are dissociated from the matrix by strong denaturing agents such as SDS.
annexin II is involved in different cellular processes. The identity of growth-regulated gene, 1B6, as annexin II (Keutzer and Hirschhorn, 1990) and enhanced expression of annexin II in human hepatocellular carcinoma (Frohlich et al., 1990) and in proliferating and metastatic areas of pancreatic cancer (Kumble et al., 1992) further support our findings that annexin II is involved in DNA synthesis (Jindal et al., 1991). We have previously demonstrated that the complex of PGK and annexin II is functional as PRP and that inhibition of one of the components reduces the ability of the complex to function as PRP (Jindal and Vishwanatha, 1990b; Jindal et al., 1991). We do not know the physiological role of PRP in the DNA replication process. It has been proposed that they interact with Pol α in lagging-strand DNA replication (Vishwanatha et al., 1986).

For PRP to be involved in DNA replication, its components must be present in the cell nucleus during active DNA synthesis. Published reports on the intracellular localization of annexin II indicate that it is cytoskeletal (reviewed by Klee, 1988). However, it is known that the monomer (approximately 20-30% of total annexin II in cells) is cytosolic. Further, there are no reports of intracellular localization of annexin II during different phases of the cell cycle. In our attempts to elucidate the physiological role of annexin II and PGK in DNA replication, we examined the presence of annexin II and PGK in nuclei and nuclear matrix in cells collected at early S phase during active DNA synthesis. Nuclear extracts prepared from HeLa cells in S phase contain PGK and annexin II. The presence of these proteins in nuclear extracts is not due to cytoplasmic contamination as evidenced by specific association of these proteins with nuclear matrix, immunoelectron microscopy and the differential distribution of PGK (only 6% in nuclear extracts) and annexin II (40%) in the nuclear and cytosolic extracts. Further, a monoclonal antibody to annexin I, a cytosolic protein, does not detect its antigen in the nuclear extracts. Immunoelectron microscopic studies have demonstrated specific association of PGK and annexin II with nuclei in normal and transformed cells (Kumble and Vishwanatha, 1991). Thus, our observations of PGK and annexin II being present in nuclei during S phase support a possible role of these proteins in DNA replication.

A number of recent reports suggest the involvement of nuclear matrix in DNA replication in vivo (Berezney, 1984; Carri et al., 1986; Tubo and Berezney, 1987). Replication forks are associated with the nuclear matrices prepared either by the high-salt method or by hypotonic or isotonic LIS extraction (Vaughn et al., 1990). Replication-dependent association of Pol α, DNA primase, ribonuclease H, DNA methylase and other replicative enzymes with the nuclear matrix (Smith and Berezney, 1982, 1983; Foster and Collins, 1985; Tubo and Berezney, 1987) further suggests the involvement of nuclear matrix in DNA replication.

Strong association of PGK and annexin II with the

Fig. 6. Immunoelectron microscopy of ultrathin sections of nuclear matrices using protein A-colloidal gold particles. Ultrathin sections of HeLa cell nuclear matrices were prepared on nickel grids as described in Materials and methods. Grids were incubated with anti-annexin II polyclonal antiserum (A and B) at 1:150 dilution or with anti-PGK polyclonal antiserum (C and D) at 1:50 dilution. After washing with the blocking buffer, the grids were incubated with Protein A-colloidal gold conjugate (30 nm particle size). The grids were then double stained with uranyl acetate and Reynolds's lead citrate, and viewed using a Philips 410 LS electron microscope. Locations of a few of the gold particles are indicated with arrowheads.
nuclear matrices prepared from S phase cells has been shown in our studies. This is the first identification of these two activities being associated with the nuclear matrix. The nuclear matrix-associated annexin II exists in two forms as distinguished by their apparent molecular masses. Calpactin I is known to be phosphorylated (Powell and Glenney, 1987) and myristylated (Soric and Gordon, 1985). The biochemical consequences or physiological role of such modifications are not known. Preliminary studies indicate that phosphorylated annexin II is specifically associated with the nuclei (K. D. Kumble and J. K. Vishwanatha, unpublished). The mechanisms involved in the translocation of PGK and annexin II to the nuclei and their association with the matrix are not known.

Fig. 7. Reactivity of nuclear matrix with antisera and monoclonal anti-human annexin I antibodies. Protein fractions were subjected to SDS-PAGE and transferred to Immobilon-P as described in the legend to Fig. 5. The membrane was incubated with either polyclonal anti-human annexin I antiserum (no. 646) (A) or Lipo-103 monoclonal antibody (B). The blots were subsequently incubated with [\(^{125}\)I]protein A, followed by autoradiography, as in legend to Fig. 5.

Fig. 8. Strong association of PGK and annexin II with nuclear matrix. HeLa cell nuclear matrix (lane 1) was incubated with 22 mM octyl-\(\beta\)-D-glucoside and 0.5 M KCl as described under Materials and methods. Following incubation, the matrix was subjected to centrifugation. The supernatant (lane 2) and resuspended pellet (lane 3) were subjected to electrophoresis. In another experiment, the matrix was incubated with 5 mM EGTA and, following centrifugation, the supernatant (lane 4) and resuspended pellet (lane 5) were subjected to electrophoresis. Electrophoresis, transfer and Western blotting using \(^{125}\)I-labeled Protein A were performed as described under Materials and methods. (A) Results using anti-chicken annexin II antiserum; (B) an immunoblot using anti-human PGK antiserum. Lane M denotes \(^{14}\)C-labeled marker proteins run on the same gel.
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