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Nuclear recruitment of A1p145 subunit of replication factor C in the early G1 phase of the cell cycle in Faza 567 hepatoma cell line and hepatocyte primary cultures

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Abstract Using a combination of immunoprecipitation and Western blotting with Faza 567 hepatoma cell extracts revealed that the large subunit of replication factor C (A1p145; mRFC140) was in a complex with proliferating cell nuclear antigen (PCNA). Western blotting showed that A1p145 was more abundant in nuclear extracts from butyrate-treated hepatoma cells which blocks the cells in the G1 phase of the cell cycle than from routinely cultured cells. Indirect immunoperoxidase analysis of G1 blocked Faza hepatoma cells localized A1p145 protein predominantly in the nucleoli. When hepatoma cells were stimulated to progress toward the S phase, A1p145 protein was then observed in both the cytoplasm and the nucleoplasm of these cells. Studies with early cultured normal hepatocytes which are progressing from G_0 towards G_1, also showed a nucleolus distribution for A1p145. This is the first demonstration in mammalian cells that the large subunit of replication factor C is associated with PCNA in the nucleus and that its distribution within cells changes during the cell cycle.

Key words: Hepatocyte; Cell cycle; Replication factor C; Proliferating cell nuclear antigen

1. Introduction

DNA replication involves a complex multi-enzyme system whose activity is tightly regulated during the cell cycle [1]. Factors responsible for both the initiation and elongation of DNA have been extensively described [2,3]. In the first steps of DNA replication, specific proteins are involved in origin recognition and unwinding of the DNA. Additional proteins such as replication factor A (RF-A) are needed to stabilize this complex [4]. The initiation of DNA synthesis requires the interaction of DNA polymerase α with the RF-A complex. Additional replication proteins such as Activator 1 (A1) proteins, also known as replication fFactor C (RF-C) and the proliferating cell nuclear antigen (PCNA) are required for the fidelity and processivity of the polymerases [5–9].

Activator 1 (A1) consists of five different subunits of 145, 40, 38, 37 and 36.5 kDa [6]. A1 binds to the primer–template junction through the 145 kDa subunit although the 37 kDa subunit has weak DNA binding, and ATP is bound by the 40 kDa subunit [5]. Recently, cDNAs for all subunits of human A1 have been cloned and sequenced and it has been shown that each subunit has significant homology with each other and to a lesser extent with two subunits of *E. coli* DNA polymerase III [10–14]. The large subunit of activator 1, A1p145, also shows a domain with homology to DNA ligases [15,16]. Although A1p145 is required for successful DNA replication, little is known about its expression during the cell cycle. In *Saccharomyces cerevisiae* it has been shown that the levels of two RF-C gene products (RFC2 and RFC3) do not significantly fluctuate during the mitotic cell cycle [17,18]. Here, we have studied the expression of the mammalian A1p145 subunit of RF-C in rat Faza 567 hepatoma cells and cultured normal hepatocytes as a function of the cell cycle. Both hepatoma cells and hepatocytes are useful model systems to study the early steps of the cell cycle. Hepatoma cells can be synchronized and stimulated to growth and normal adult hepatocytes spontaneously progress from G_0 to the G_1 phase of the cell cycle, even in the absence of DNA synthesis, when prepared by enzymatic disruption of the liver [19,20]. After a G1 phase time-lapse of 48 h, successful DNA synthesis is achieved only when cells are stimulated with a mixture of insulin, EGF and pyruvate ([20]; Loyer et al., submitted). In this report, we show that A1p145 is recruited in the nucleus within the G_0/G_1 transition and/or early G_1 phase in both Faza 567 hepatoma cells and cultured normal hepatocytes. Furthermore, immunoprecipitation experiments demonstrate that A1p145 is in a complex with PCNA.

2. Materials and methods

2.1. Reagents

Anti-A1p145 antibodies were directed against a recombinant protein produced from a restriction fragment of the A1p145 (MSW) cDNA (amino acid residues 203–545) which was subcloned in a PQE-9 bacterial expression vector. Affinity-purified fusion protein was used as antigen to immunize a rabbit and antiserum was obtained after four injections [15].

2.2. Cells

Hepatocytes were isolated from male Sprague–Dawley rats using the two-step collagenase perfusion method [21]. Cells were collected in L-15 Leibovitz medium and then washed in 0.1 M HEPES buffer, pH 7.6. Viability of cells was estimated by the Trypan blue exclusion test. Hepatocytes were plated on plastic dishes in a mixture of 75% minimum essential medium and 25% medium-199 containing 10% fetal calf serum (FCS) and 0.02% bovine serum albumin.

The Faza 567 cell line has been subcloned from the rat hepatoma cell line H4IIEC3 isolated from the Reuber H35 hepatoma [22,23]. Hepatoma cells were routinely cultured in Williams' medium containing 10% FCS. To obtain a synchronous population of cells, Faza 567 cells were arrested in G_1 phase by addition of 10 mM butyrate for 24 h. Then, quiescent cultures were stimulated with 10% FCS. DNA synthesis was monitored by incubation of cells for 4 h with 1 mCi/ml [^{3}H]thymidine (5 Ci/mmol; Amersham, UK).

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2.3. Immunoprecipitation and Western blotting

Nuclear proteins were prepared from Faza 567 hepatoma cells as described by Dignam et al. [24]. Monoclonal anti-PCNA antibodies (Oncogene Science Inc.) were added for 4 h prior addition of protein A-Sepharose for 12 h at 4°C. After extensive washes, beads were mixed with sample buffer containing 10% SDS and 5% mercaptoethanol. Controls were obtained using nuclear extracts incubated with non-immune serum. Then, cell lysates, nuclear extracts and proteins immunoprecipitated with anti-PCNA antibodies were loaded into a 6% SDS-polyacrylamide gel. Subsequently, proteins were transferred onto nitrocellulose membranes. The filters were incubated 2 h at room temperature with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.2% NP-40 and 3% non-fat dry-milk. Affinity purified anti-Alp145 recombinant protein antibodies were then added in fresh medium containing 3% non-fat dry-milk. After 2 h, the filters were extensively washed prior incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies for a further 2 h. Subsequently, nitrocellulose sheets were washed extensively before incubation in 4-chloro-1-naphtol/H_2O_2 for 20 min.

2.4. Immunocytochemistry

Alp145 protein was localized in situ using the indirect immunoperoxidase method following paraformaldehyde fixation and treatment with saponin [25]. Briefly, cell cultures were washed with cold PBS and fixed with a 4% paraformaldehyde solution buffered with 0.1 M sodium cacodylate, pH 7.4, for 45 min at 4°C. Then, cells were incubated with 0.2% saponin in PBS for 1 h and subsequently incubated with an appropriate dilution of affinity purified anti-Alp145 recombinant protein antibodies in PBS containing 0.2% saponin. The second incubation was performed with peroxidase-labeled anti-immunoglobulins (Institut Pasteur, Paris) diluted in PBS containing 0.2% saponin. Peroxidase activity was revealed by incubating cells in a 3.3′-diaminobenzidine/H_2O_2 solution for 20 min.

3. Results

3.1. Acquisition of synchronized Faza hepatoma cells

Synchronized Faza 567 hepatoma cells were obtained by incubating cells with butyrate, thereby resulting in arrest of cells in early G_1 phase of the cell cycle [26]. Then, cells were stimulated towards the S phase of the cell cycle by addition of fresh medium containing 10% FCS. Cells were analyzed at various times for [^{3}H]thymidine incorporation (Fig. 1). Addition of butyrate resulted in minimal incorporation of [^{3}H]thymidine. Twelve hours after stimulation with FCS, [^{3}H]thymidine incorporation started and increased during the following 12 h, then decreased for 8 h.

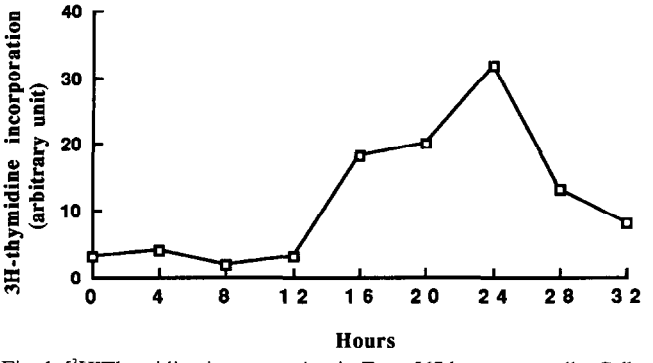

Fig. 1. [^{3}H]Thymidine incorporation in Faza 567 hepatoma cells. Cells were first incubated with 10 mM butyrate for 24 h. Then early G_1 arrested cells were stimulated with 10% FCS (time 0). [^{3}H]Thymidine incorporation was determined in synchronized cells every 4 h for 32 h in triplicate. Data were obtained in cpm/mg of protein and expressed as arbitrary unit.

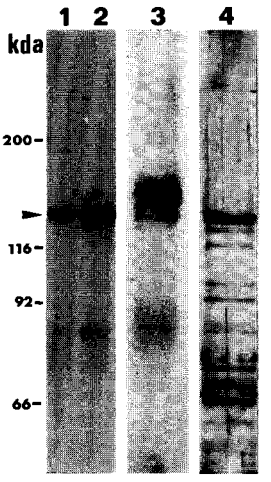

Fig. 2. Western blots of Alp145. Lanes 1 and 2: nuclear extracts from Faza 567 hepatoma cells either routinely cultured (lane 1) of treated with 10 mM butyrate for 24 h (lane 2) were resolved on a 6% SDS-polyacrylamide gel (50 μg each) and transferred onto a nitrocellulose filter which was subsequently incubated with anti-Alp145 antibodies. Lane 3: nuclear extracts from routinely cultured Faza hepatoma cells were first immunoprecipitated with monoclonal anti-PCNA antibodies. Then, protein A-Sepharose was added for 12 h and beads were mixed with sample buffer before loading into a 6% SDS-polyacrylamide gel. Then, proteins were transferred onto a nitrocellulose membrane which was incubated with anti-Alp145 antibodies. Lane 4: cell lysate from routinely cultured Faza hepatoma cells was resolved by gel electrophoresis prior Western blotting with anti-Alp145 antibodies. Migration positions of standard molecular weights (kDa) and Alp145 (arrowhead) are indicated.

3.2. Expression of Alp145 protein in Faza 567 hepatoma cell line

The expression of Alp145 protein was evaluated in Faza 567 hepatoma cells by Western blotting (Fig. 2). In nuclear extracts from both routinely cultured (lane 1) and butyrate-treated Faza hepatoma cells (lane 2), a major band of M_r = 145,000 was strongly labeled when nitrocellulose filters were incubated with specific affinity purified anti-Alp145 antibodies. Staining was more intense in nuclear extracts from butyrate-treated cells than from control Faza hepatoma cells (lane 2 vs. lane 1). To determine whether PCNA and Alp145 were in protein complex, nuclear extracts prepared from routinely cultured Faza 567 hepatoma cells were first subjected to immunoprecipitation with monoclonal anti-PCNA antibodies prior gel electrophoresis and transfer to nitrocellulose filters that were subsequently incubated with anti-Alp145 antibodies. In these conditions, the M_r = 145,000 polypeptide was strongly labeled and resolved as a doublet (lane 3). In cell lysates from routinely cultured Faza hepatoma cells the M_r = 145,000 polypeptide was also detected with additional faster migrating bands which probably correspond to degradation products (lane 4).

3.3. Immunolocalization of Alp145 protein in Faza 567 hepatoma cells

Alp145 was immunolocalized in Faza hepatoma cells using the indirect immunoperoxidase (Fig. 3). In control hepatoma cells, Alp145 was detected in both the cytoplasm and the nucleus of cells (Fig. 3A). In some cells, only the cytoplasm was labeled for Alp145. After addition of butyrate for 24 h, the nucleus of all hepatoma cells was strongly positive, particularly at the level of the nucleolus (Fig. 3B). When cells were stimu-

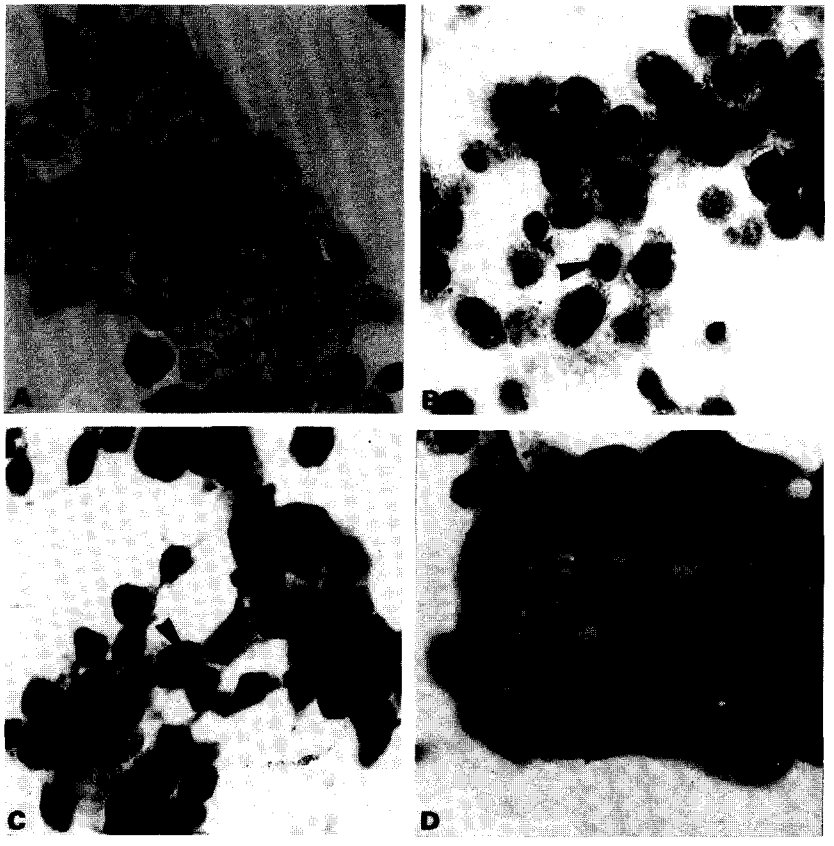

Fig. 3. Immunolocalization of A1p145 in Faza 567 hepatoma cells. Cells were first arrested in G_1 phase of the cell cycle by addition of 10 mM butyrate and stimulated with 10% FCS. A1p145 was visualized by indirect immunoperoxidase in routinely cultured Faza 567 cells (A), after incubation of butyrate for 24 h (B) and 24 h (C) and 32 h (D) after stimulation of synchronized cells with 10% FCS. A1p145 was detected in both the cytoplasm and the nucleus of cells (arrows). After addition of butyrate for 24 h, the nucleoli of hepatoma cells were strongly positive. ×480.

lated to grow, A1p145 remained associated with the nucleolus as shown 24 h after addition of FCS, i.e. at maximum of [^{3}H]thymidine incorporation (Fig. 3C). After 32 h of stimulation, A1p145 was detected predominantly in the cytoplasm of Faza 567 hepatoma cells (Fig. 3D).

3.4. Immunolocalization of A1p145 protein in normal hepatocytes

A1p145 was localized by indirect immunoperoxidase in hepatocytes prepared from normal adult rats and plated in culture. Twenty minutes after cell seeding, A1p145 was localized only in the cytoplasm of all hepatocytes (Fig. 4A). After 6 h, both the cytoplasm and the nucleus of hepatocytes were labeled, the nucleoli of several cells being strongly positive (Fig. 4B). After 24 h and thereafter, the pattern of A1p145 distribution was similar to that in 6 h hepatocyte cultures: A1p145 was detected in both the cytoplasm and nucleus of hepatocytes, with several nucleoli being strongly labeled (Fig. 4C). In controls, hepatocytes were totally negative when non-immune serum was used in the first step of indirect immunoperoxidase (Fig. 4D).

4. Discussion

In this report we show that A1p145 is abundant in the cytoplasm of Faza hepatoma cells and is associated with PCNA in nuclear extracts. This is the first demonstration in mammalian cells that the large subunit of A1 is readily associated with PCNA in the nucleus, thus indicating that A1 is a part of chromosomal replicase that includes DNA polymerase δ and PCNA. Similarly, in *E. coli*, the DNA polymerase II core is recruited after the accessory γ complex has assembled the β dimer of the clamp protein [27,28], whereas in the phage T4, the chromosomal replicase is formed by the assembly of the polymerase gp43, the accessory gp44–gp62 complex and the processivity factor gp45 [29]. Immunoprecipitation study with anti-PCNA antibodies followed by Western blotting with anti-A1p145 antibodies revealed that the M_r = 145,000 band was resolved as a doublet. Whether this particular feature corresponds to differences in processing, glycosylation or phosphorylation of A1p145 protein remains to be determined. Both immunocytochemistry and Western blotting in Faza 567 hepatoma cells showed that the distribution of A1p145 protein varies depending on the phases of the cell cycle. In routinely cultured Faza hepatoma cells, A1p145 staining was diffuse and present in both the cytoplasm and the nucleus. Surprisingly, after addition of butyrate intense staining was localized in the nucleus of all the cells, particularly at the level of nucleoli. Accordingly, Western blotting indicated that A1p145 was more abundant in nuclear extracts from butyrate-treated cells than from control Faza hepatoma cells. Since butyrate is known to block cells in early G1 phase of the cell cycle [26], this observation indicates that change in A1p145 distribution parallels that

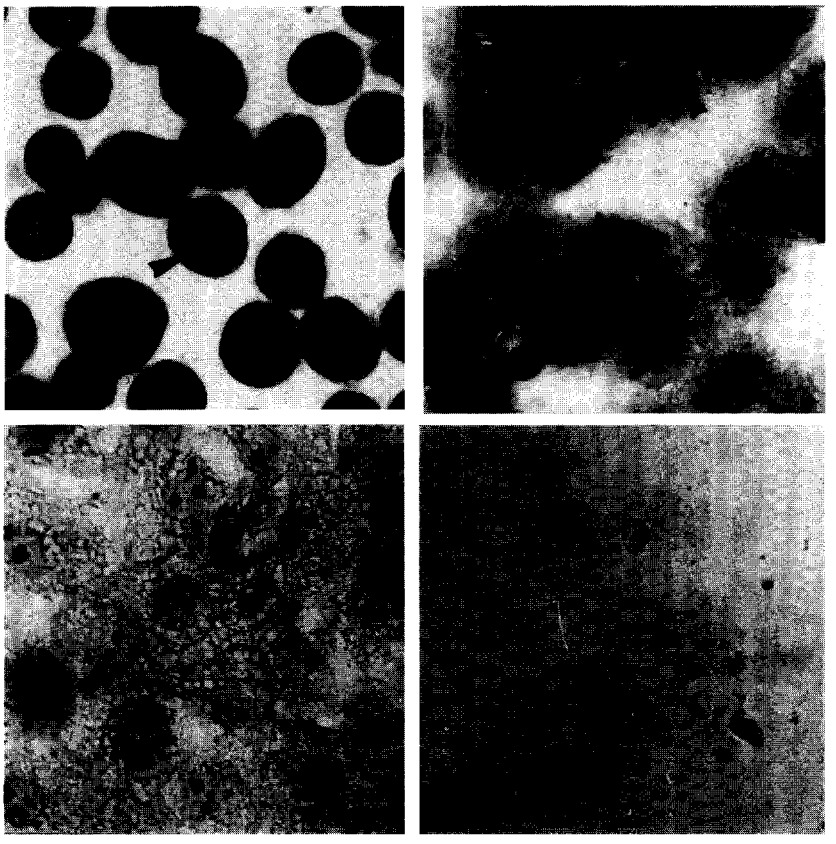

Fig. 4. Immunolocalization of A1p145 in cultured rat hepatocytes. A1p145 was visualized by indirect immunoperoxidase at 20 min (A), 6 h (B) and 24 h (C) after cell seeding. A1p145 was detected in the cytoplasm 20 min after cell seeding and in several nucleoli of hepatocytes after 6 h (arrows). (D) Staining with non-immune serum in a 24 h hepatocyte culture. ×480.

of PCNA during the cell cycle. Indeed, it has been shown that PCNA is preferentially located in the nucleoli of cells in late G_1 phase [30] or in the S phase of the cell cycle [31]. When hepatoma cells were stimulated to grow, A1p145 remained associated with the nuclei during the S phase of the cell cycle, whereas A1p145 was detected predominantly in the cytoplasm during the cell cycle progression towards G_2 and M phases. A similar pattern of distribution of PCNA has been previously demonstrated by immunofluorescence in both 3T3 cells and transformed human amnion cells [30,31]. It is unknown whether these changes in the distribution of both PCNA and A1p145 are related to a shuttle of these proteins between cytoplasm and nucleus or conformational changes leading to demasking of antigenic determinant. Nevertheless, our immunoperoxidase study indicates that A1p145 activity is associated with early events in DNA replication. This hypothesis was confirmed in hepatocyte primary cultures. Indeed, dramatic change in the distribution of A1p145 was found very early after liver disruption by collagenase perfusion and plating of hepatocytes in culture. Thus, immediately after liver perfusion, A1p145 was localized exclusively in the cytoplasm of hepatocytes, whereas as early as 6 h after cell seeding the nucleoli of numerous hepatocytes were strongly positive. Previous studies have shown that during this period of time a transient increase of c-*fos* and c-*jun* was observed following disruption of rat livers indicating an early transition from G_0 to G_1 phase of the cell cycle in isolated normal hepatocytes [19,20]. Thus, recruitement of A1p145 in the nucleoli of hepatocytes is associated with early progression of these cells in the cell cycle in vitro. Studies in regenerative liver following partial hepatectomy are required to confirm that changes in A1p145 distribution also occurs in physiological conditions [32].

In conclusion, the present study on A1p145 expression in both hepatoma cells and normal hepatocytes in vitro leads to the hypothesis that changes in the distribution of A1 occurs in the cell cycle and confirms that this protein is in complex with PCNA in the nucleus.

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