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ORIGINAL PAPER

Ornella Cazzalini · Paola Perucca · Federica Valsecchi · Lucia A. Stivala · Livia Bianchi · Vanio Vannini · Ennio Prosperi

Intracellular localization of the cyclin-dependent kinase inhibitor $p21^{CDKN1A}$-GFP fusion protein during cell cycle arrest

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Abstract The cyclin-dependent kinase (CDK) inhibitor $p21^{CDKN1A}$ is known to induce cell cycle arrest by inhibiting CDK activity and by interfering with DNA replication through binding to proliferating cell nuclear antigen. Although the molecular mechanisms have been elucidated, the temporal dynamics, as well as the intracellular sites of the activity of p21 bound to cyclin/CDK complexes during cell cycle arrest, have not been fully investigated. In this study we have induced the expression of $p21^{CDKN1A}$ fused to green fluorescent protein (GFP) in HeLa cells, in order to visualize the intracellular localization of the inhibitor during the cell cycle arrest. We show that p21-GFP is preferentially expressed in association with cyclin E in cells arrested in G1 phase, and with cyclin A more than with cyclin B1 in cells arrested in the G2/M compartment. In addition, we show for the first time that p21-GFP colocalizes with cyclin E in the nucleolus of HeLa cells during the G1 phase arrest.

Keywords $p21^{CDKN1A}$ · Cell cycle arrest · Green fluorescent protein · Cyclins · Nucleolus

Introduction

The cell cycle is driven by the enzymatic activity of cyclin-dependent kinases (CDKs) active only when complexed with their binding partners, the cyclins (Roberts 1999). A group of proteins that physically interact with and inhibit the activity of CDKs, thereby mediating cell cycle arrest, are known as CDK inhibitors (Sherr and Roberts 1999). Among these proteins, $p21^{CDKN1A}$ (also known as $p21^{WAF1/CIP1/SDI1}$) is responsible for the cell cycle arrest involved in different cellular pathways, including cell cycle checkpoints, senescence, and terminal differentiation (Dotto 2000). In addition, p21 has been shown to interact directly or indirectly with proteins involved in gene expression, suggesting a role for p21 in regulation of transcription (Coqueret 2003).

Although its activity is usually associated with CDK inhibition, p21 is peculiar in that it is able to interact directly with proliferating cell nuclear antigen (PCNA), thereby inhibiting DNA replication (Ball 1997; Prosperi 1997). Since cyclins and CDKs may exhibit different localization (Prosperi et al. 1997), multiple levels of activity of p21 are supposed. However, the temporal dynamics, and the intracellular sites where p21 interacts with its targets during cell cycle arrest, have not been fully elucidated.

It is well known that p21 is responsible primarily for cell cycle arrest in the G1 phase (Deng et al. 1995); however, an involvement in the G2 phase arrest has also been demonstrated (Bunz et al. 1998; Smits et al. 2000). In a previous report, we provided evidence that expression of p21 fused to green fluorescent protein (p21-GFP) in HeLa cells induced cell cycle arrest in the G1 and in the G2/M compartments. In addition, we showed that the inhibitor was also able to colocalize with PCNA at the G1/S phase transition (Cazzalini et al. 2003). To investigate in detail the temporal and spatial dynamics of the p21-induced cell cycle arrest, we have analyzed the intracellular localization, as well as the coexpression of p21-GFP with cyclins expressed in the G1 and G2 phases of the cell cycle. In particular, the expression of cyclin E has been considered as marker of the mid-late G1 phase (Ohtsubo et al. 1995). Cyclin A, which is expressed from the G1/S phase transition up to the G2 phase (Darzynkiewicz et al. 1996), has been considered to detect cells

O. Cazzalini and P. Perucca contributed equally to this work

E. Prosperi (✉)
sez. Istochimica e Citometria,
Istituto di Genetica Molecolare del CNR,
Piazza Botta 10, 27100 Pavia, Italy
e-mail: prosperi@igm.cnr.it
Tel.: +39-0382-506267
Fax: +39-0382-506430

O. Cazzalini · P. Perucca · F. Valsecchi · L. A. Stivala · L. Bianchi · V. Vannini
Dipartimento di Medicina Sperimentale,
sez. Patologia generale "C. Golgi",
Università di Pavia,
Piazza Botta 10, 27100 Pavia, Italy

arrested in the G1/S and G2 compartments. Cyclin B1 has been taken as marker for cells located in the late G2 and M (up to metaphase) phases of the cell cycle (Chang et al. 2003).

Materials and methods

Plasmid construction, cell culture, and transfection

The wild-type human p21 cDNA obtained by RT-PCR, was inserted in the pEGFP-N1 vector (Clontech), as previously reported (Cazzalini et al. 2003). HeLa S3 cell line was grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco BRL), 4 mM L-glutamine (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin in a 5% CO_2 atmosphere. Cells (70% confluent) were transiently transfected with Effectene transfection reagent (Qiagen). The medium was changed 24 h later, and all experiments were performed 48 h after transfection.

Cell cycle analysis

The effects of wild-type p21-GFP fusion protein expression on cell cycle distribution were analyzed by flow cytometry. Transfected cells (5×10^5) were trypsinized, fixed in 4% formaldehyde for 5 min, and washed in PBS buffer. Cells were stained with propidium iodide (PI), by incubation for 30 min at room temperature (RT) in PBS containing PI (20 μg/ml) and RNase A (1 mg/ml). Cell cycle analysis was carried out with an Epics XL flow cytometer (Coulter). Twenty thousand cells were measured for each sample.

Immunofluorescence and confocal microscopy

Cells were seeded at a density of 6×10^4 cells/coverslip and transfected 24 h later with the empty vector or with the p21-GFP constructs.

To analyze the intracellular distribution of p21-GFP protein, cells grown on coverslips were transfected as above indicated. Forty-eight hours later, cells were washed twice with PBS buffer, fixed in 4% formaldehyde for 5 min at RT, and then postfixed in 70% ethanol. After rehydration in PBS, blocking of unspecific staining was performed in PBS containing 1% bovine serum albumin and 0.2% Tween 20 (PBT solution). Immunostaining for cyclins was performed by incubation with the following monoclonal antibodies: anti-cyclin A (clone CY-A1; Sigma), anti-cyclin E (clone HE12; Santa Cruz Biotechnology), and anti-cyclin B1 (clone GNS-1; Pharmingen). All primary antibodies were used at a 1:100 dilution in PBT. After extensive washing in PBT, the coverslips were incubated for 30 min with anti-mouse antibody conjugated with Alexa 594 (Molecular Probes) diluted 1:750 in PBT. After immunoreactions, cells were incubated with Hoechst 33258 dye (0.5 μg/ml) for 5 min at RT, and washed in PBS. Slides were mounted in Mowiol (Calbiochem), containing 0.25% 1,4-diazabicyclo-[2,2,2]-octane (Aldrich) as antifading agent, and visualized on a BX51 Olympus fluorescence microscope with a 100× objective (NA 1.25). For each condition, at least 500 cells were blindly scored by two independent operators, and each experiment was repeated at least three times. Fluorescence photographs of representative samples were taken with an Olympus Camedia C-4040 digital camera, and processed with Adobe Photoshop 6.0 software.

Colocalization studies of p21-GFP with chromatin-bound cyclins were performed on cells lysed in hypotonic buffer, in order to release the soluble fraction (Prosperi et al. 1994, 1997). Forty-eight hours after transfection, cells grown onto coverslips were washed twice with PBS and incubated for 10 min at 4°C in hypotonic lysis buffer: 10 mM tris HCl (pH 7.4), 2.5 mM $MgCl_2$, 0.1% Nonidet NP-40, 0.2 mM phenylmethylsulfonyl fluoride. Thereafter, coverslips were washed in physiological saline, fixed in 1% formaldehyde for 5 min (RT), and postfixed in 70% ethanol. In some experiments, cells were extracted with 0.35 M NaCl for 15 min at 4°C before fixation. Immunostaining was performed using the same primary antibodies, as above reported, followed by 30 min incubation with biotin-conjugated anti-mouse IgG (Sigma) diluted 1:100, followed by 30 min incubation with streptavidin–Texas red (Amersham) diluted 1:150. Colocalization studies were performed with a Leica TCS SP2 confocal microscope by acquiring green and red fluorescence signals at 0.3-μm intervals. Image analysis was performed using the LCS software and subsequent composition by Adobe Photoshop 6.0 software.

Results and discussion

Following the transfection of HeLa cells with a p21-GFP expression vector, we have determined by flow cytometry that the majority (86.5±4.2%) of the cells expressing the p21-GFP fusion protein were arrested with a G1 phase DNA content, while 10.9±2.9% showed a G2+M phase DNA content, and the remaining 2.6±0.2% were in S phase. HeLa cells transfected with the empty vector showed a cell cycle distribution (G1=61.9±6.3; S=21.0±5.5; G2+M=17.1±3.4) very similar to that of untransfected cells (G1=57.8±4.2; S=22.2±2.5; G2+M=19.4±2.2). These results obtained from several independent experiments (n=5) are in total agreement with previous flow cytometry and BrdU incorporation data, confirming the arrest of the cell cycle in HeLa cells expressing p21-GFP (Cazzalini et al. 2003). In parallel experiments, HeLa cells grown onto coverslips showed that p21-GFP was coexpressed, though to different extents, with cyclin E, A, and B1. Figure 1 shows an example of the expression of cyclin E which is detectable in some cells containing p21-GFP, and demonstrates that both proteins are clearly localized in the nucleus.

In order to verify that in HeLa cells cyclins E, A, and B1 followed the canonical expression pattern, their cell cycle distribution was determined by flow cytometry and found to be similar to that of untransformed cells (results not shown). To quantify the different levels of coexpression of p21-GFP with cyclins, cells immunostained for each cyclin were scored for positivity to p21-GFP. Specific immunostaining for cyclin E showed that about 58% of the cells transfected with p21-GFP were also positive for this cyclin. In untransfected cells, or in cells transfected with the empty vector (pEGFP), about 40% and 34% cyclin E-positive cells were counted, respectively (Fig. 2A). These results indicate that the majority of cells expressing p21-GFP were arrested at the mid-late G1 phase. Since HeLa cells do not express cyclin D1 at significant levels (Lukas et al. 1994), it was not possible to calculate the percentage of GFP-positive cells expressing this cyclin. However, it is possible to deduce that those p21-GFP-positive cells that were not stained by the anti-cyclin E antibody (about 28%) were arrested either at the early G1 or at the very early S phase, i.e., when cyclin E levels have not yet arisen or are declining, respectively (Ohtsubo et al. 1995).

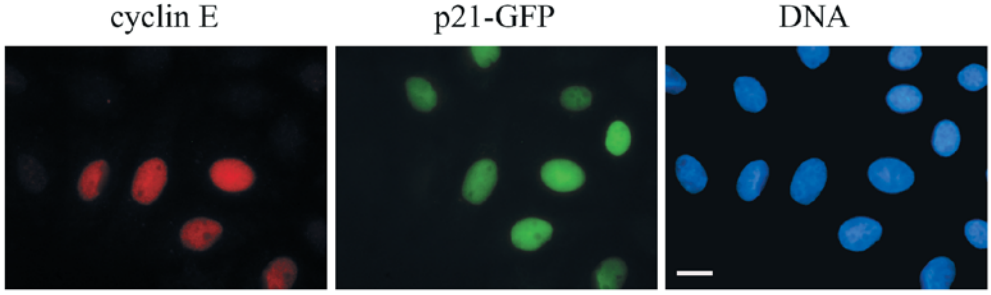

Fig. 1 Coexpression of cyclin E in HeLa cells transfected with p21-GFP vector. The *red* and *green* fluorescence signals are relative to cyclin E immunostaining and to p21-GFP fusion protein, respectively. DNA was stained in *blue* with Hoechst 33258 dye. *Scale bar* 10 μm

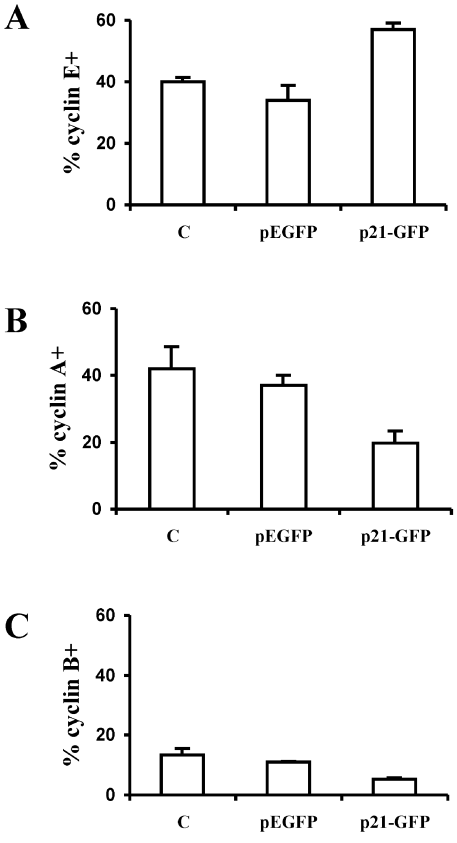

Fig. 2A–C Immunofluorescence analysis of coexpression of p21-GFP with cyclin E (**A**), cyclin A (**B**), or cyclin B1 (**C**) proteins. In untransfected control HeLa cells (*C*), the percentage of cells expressing each cyclin was calculated in the whole population. In HeLa cells transfected with empty vector (pEGFP), and in HeLa cells transfected with p21-GFP vector, only the percentage of cells showing both cyclin and GFP fluorescence was considered. Mean values ± standard deviations for at least three independent experiments are shown

The immunofluorescence analysis performed for cyclin A showed that about 20% of the cells expressing p21-GFP were also positive for this protein. In untransfected cells, and in cells transfected with the vector alone, the percentage of cyclin A-positive cells was higher because of the greater number of cells in S phase present in these samples (Fig. 2B). Flow cytometric analysis of the cell cycle distribution of p21-GFP-positive cells showed that about 10% were in the G2/M phase compartment. Thus, up to about 10% cyclin A and p21-GFP-positive cells could be arrested in the G2 phase, while the remaining cyclin A/p21-GFP-positive cells were actually blocked at the beginning of S phase (though flow cytometrically they showed a G1 phase DNA content). This conclusion is further supported by our previous results on the colocalization of p21 with PCNA recruited to DNA replication sites (Cazzalini et al. 2003).

Cyclin B1 is expressed to low levels in G2 phase and reaches maximal expression in M phase (metaphase), but it is degraded before anaphase (Chang et al. 2003). Only a small percentage (5%) of cells expressing p21-GFP showed also a clear positive staining for cyclin B1 (Fig. 2C), thus suggesting that the arrest induced by p21 at the beginning of the M phase was limited to a very low percentage of cells.

To explore further the potentiality of expressing p21-GFP for cell cycle arrest studies, the intracellular colocalization of p21-GFP with cyclins was also analyzed when these proteins are bound to chromatin in an insoluble form (Prosperi et al. 1994; Stivala et al. 1997; Kim and Kaelin 2001). For this purpose, HeLa cells were lysed in a hypotonic solution to release detergent-soluble proteins before fixation (Stivala et al. 1997).

Confocal microscopy analysis of p21-GFP and cyclin E fluorescence signals relative to the chromatin-bound forms, showed that the two proteins colocalized, while no detectable signal of GFP fluorescence was present in samples transfected with vector alone (not shown). In particular, the colocalization was also evident in the nucleolar compartment of some cells (Fig. 3A, B). To verify that this finding was not due to an incomplete extraction of p21-GFP, transfected cells were lysed in the hypotonic buffer and further extracted with a moderately high ionic strength (0.35 M NaCl), which releases loosely bound nuclear proteins. Although some spreading occurred in this condition, the majority of p21-GFP and cyclin E remained localized in the nucleolus, thus indicating their tight association with this compartment (Fig. 3C). These results are in accordance with the evidence that cyclin E is transported into the nucleolus,

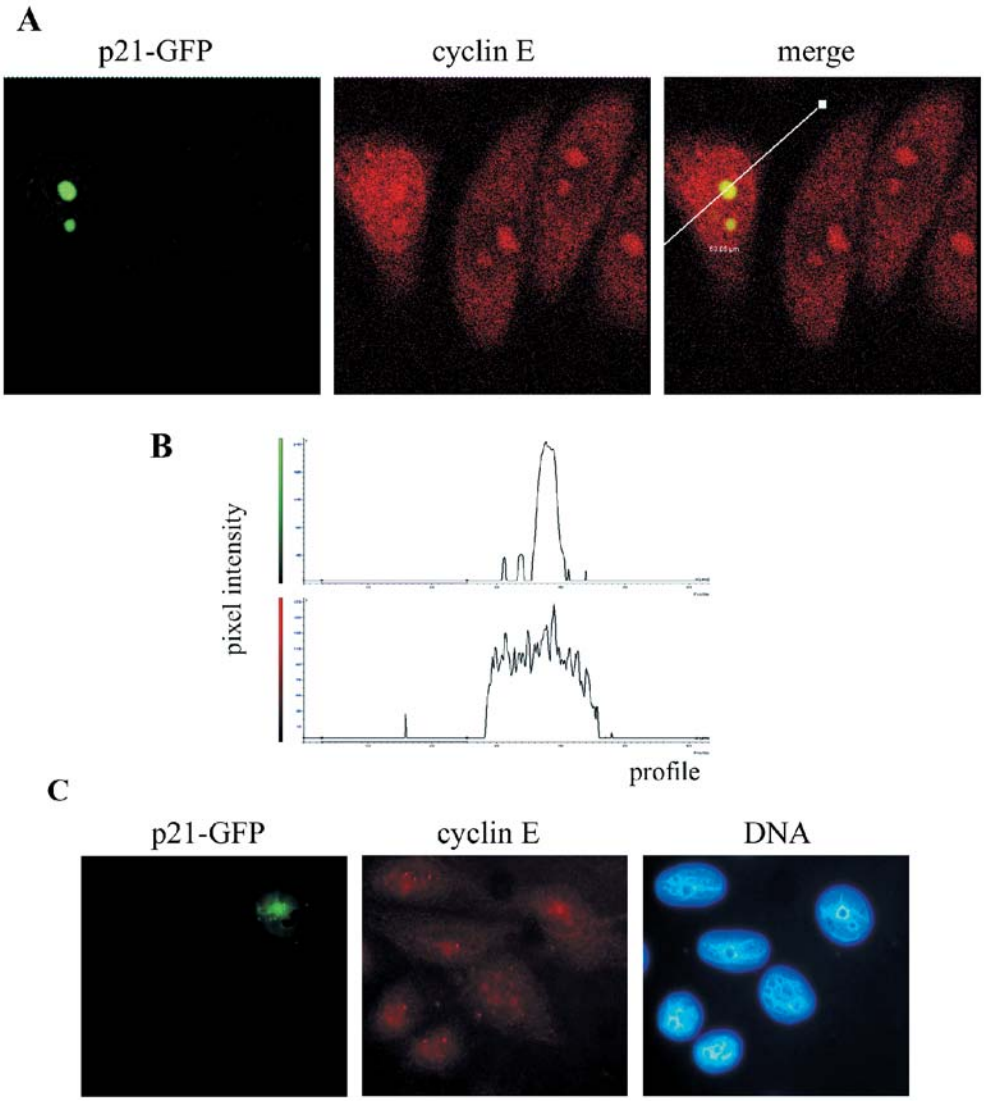

Fig. 3A–C Fluorescence confocal microscopy analysis of chromatin-bound p21-GFP and cyclin E proteins in HeLa cells. Cells transfected with p21-GFP vector were collected 48 h later and lysed in hypotonic solution to reveal only chromatin-bound proteins. After fixation, cells were immunostained with anti-cyclin E antibody, followed by biotin-conjugated secondary antibody and streptavidin–Texas red. **A** The single images of a confocal plane from the *green* (p21-GFP) and *red* (cyclin E) channels are shown together with the merged image. **B** The profiles of the pixel intensity of the green and red channels along the *white line drawn in the merged image* show the colocalization of p21-GFP with cyclin E at the nucleolar level. **C** HeLa cells transfected with p21-GFP were lysed in hypotonic solution and further extracted in 0.35 M NaCl for 15 min at 4°C to release loosely bound proteins. After fixation, cells were immunostained with anti-cyclin E antibody, as above

where it has been suggested to associate with CDK2 in Cajal bodies (Liu et al. 2000).

In conclusion, the expression of a p21-GFP fusion protein is a useful tool for studying the dynamics of cell cycle arrest in different intracellular compartments. In this study, the arrest induced by p21-GFP in HeLa cells occurred mainly in mid-late G1 phase, and only a low percentage of cells were able to reach the G1/S phase transition. In agreement with previous studies (Cayrol et al. 1998; Medema et al. 1998), the arrest in the G2 phase was restricted to a few cells, and only a residual fraction of them was probably competent to reach M phase. Interestingly, confocal microscopy analysis and in situ fractionation techniques have shown for the first time that p21-GFP was able to colocalize with cyclin E also at the nucleolar level.

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