Intracellular Compartmentalization of Cyclin E during the Cell Cycle: Disruption of the Nucleoplasm-Nucleolar Shuttle of Cyclin E in Bladder Cancer

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ABSTRACT

Cyclin E/cyclin-dependent kinase 2 complexes are essential during the cell cycle for entrance into S phase. Cyclin E expression starts in mid-G1, reaches a maximum at S-phase entrance, and undergoes proteolysis mediated by the ubiquitin pathway as the cell progresses through S phase. Laser scanning cytometry, a microscope-based cytofluorometer combining the advantages of both flow and image analysis, allowed the determination of subcellular localization of cyclin E, p27, and retinoblastoma protein during cell cycle progression in normal human fibroblasts and nine bladder cancer cell lines. We observed that in normal fibroblasts and most tumor cell lines, cyclin E localizes in the nucleoplasm during mid-G1 and is translocated to the nucleolus during G1-S-phase transition, and its levels are undetectable in G2-M phase. Neither levels nor subcellular localization of p27 and retinoblastoma protein was cell cycle dependent in normal or tumor cells. However, four of nine bladder cancer cell lines continued to express cyclin E in all phases of the cycle, and image analysis revealed that it was localized to nucleoli. These observations suggest that the nucleolus mediates a cyclin E “shuttling” between the nucleus and the cytoplasm that is probably involved in its regulation and that this mechanism could be disrupted in bladder cancer.

INTRODUCTION

Cell cycle progression is controlled by protein complexes composed of cyclins and Cdk3 (1–6). These complexes phosphorylate fundamental elements involved in cell cycle transitions, such as the pRB. Phosphorylation of pRB releases E2F members, which activate transcription of the components of the DNA replication machinery, thereby committing the cell to S phase (7, 8). The expression of distinct cyclins occurs at specific and well-defined periods of the cycle. Cyclins D1–D3 and E are maximally expressed during G1 phase, regulating the transition from G1 to S phase. Cyclins A, B1, and B2 reach their maximal levels during S and G2 phases and are regarded as regulators of the transition to mitosis. Cyclin D1/Cdk4 complexes govern G1 progression, whereas cyclin E/Cdk2 complexes control entry into S phase. Cyclin A/Cdk2 complexes regulate passage through S phase, and cyclin B/Cdk2 control entry into mitosis (1). Cyclins are regulated not only at the transcriptional and translational levels but also by their rate of degradation via the ubiquitin pathway (9, 10). An additional level of negative control is produced by the expression of Cdk inhibitors. Cdk inhibitors have been classified into two groups: (a) KIPs (kinase inhibitory proteins; p21CIP1, p27KIP1, and p57KIP2); and (b) INKs (inhibitors of kinases; p15INK4b, p16INK4a, p18INK4a, and p19INK4c; Refs. 1–8). KIPs are counterbalanced during the progression of the cell in the cycle (11–15). Expression studies performed in asynchronous cell populations do not perturb cell cycle progression and do not induce growth imbalance, both of which otherwise occur when cells are synchronized (16, 17). Moreover, analyses of asynchronous populations can reveal intercellular variability in cyclin expression, detect rare events and cell subpopulations with distinct features, and may determine the presence of thresholds in expression of these proteins at particular phases of the cell cycle (11, 18).

It has been reported that expression of critical cell cycle regulators is frequently altered in human cancer (1–3). To investigate the relationship between subcellular localization of cyclin E, p27, and pRB and cell cycle progression, we have used LSC to analyze normal human fibroblasts and nine bladder cancer cell lines. Similar to flow cytometry, LSC allows bivariate analysis of immunocytochemically labeled cells (19, 20). In addition, LSC allows the morphological identification of cells whose fluorescence has been measured and the intracellular distribution of such a fluorescence signal.

MATERIALS AND METHODS

Cell Lines. Human bladder cancer cell lines (RT4, T24, J82, HT1197, HT1376, 5637, UMUC3, TCCSUP, and SCABER) and human fibroblasts (CRL-1502) were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured as recommended by American Type Culture Collection. All media, supplements, and antibiotics were obtained from the Media Laboratory Core Service at Memorial Sloan-Kettering Cancer Center. The cultured cells were tested periodically for Mycoplasma infection. To maintain asynchronous exponential growth, cells were passaged by diluting them to a concentration of 1 × 10^6 cells/ml and were repassaged before approaching a density of 5 × 10^6 cells/ml. For the experiments, cell monolayers were grown directly on microscope chamber slides.

Antibodies. The following antibodies were used for the present study. A purified mouse antinucleolin mAb, clone 4E2, was obtained from Research Diagnostics Inc. (Flander, NJ). Two different anti-cyclin E mAbs were used: (a) purified cyclin E mAb clone HE-12 (PharMingen, San Diego, CA); and (b) purified cyclin E mAb clone AB-1 (Oncogene Research). A purified anti-p27 mAb, clone G173-524 (PharMingen), was also used. To assess pRB expression and phosphorylation status, we used two antibodies: (a) anti-pRB mAb clone 3C8 (PharMingen), which detected underphosphorylated and hyperphosphorylated products; and (b) anti-pRB mAb clone G99-549 (PharMingen), which detected underphosphorylated pRB.

Immunocytochemical Detection of Nucleolin, Cyclin E, p27, and pRB. The cells grown as monolayers on microscope chamber slides were washed in PBS and fixed in ice-cold 80% ethanol for up to 24 h. After fixation, the slides were washed twice with PBS containing 1% BSA and 0.1% sodium azide, followed by permeabilization with 0.1% Triton X-100 in PBS on ice for 5 min. After that, the slides were incubated with 100 µl of PBS containing 0.5 µg of the mAb and 1% BSA for 2 h at room temperature. Mouse anti-IgG served as isotype negative control. The slides were then rinsed with PBS containing 1% BSA and incubated with 100 µl of FITC-conjugated goat antimouse F(ab')2 (DAKO, Carpinteria, CA) diluted 1:30 in PBS containing 1% BSA for 30 min at room temperature in the dark. The slides were washed again, resuspended in 5 µg/ml PI (Molecular Probes) and 0.1% RNase A (Sigma) in PBS to counterstain cellular DNA, and incubated at room temperature for 20 min before measurement. The slides were then mounted under coverslips and analyzed by LSC. Additional details of the staining protocols are presented elsewhere (12, 20–25).
LSC Setting and Fluorescence Measurement. Fluorescence of individual cells was measured by LSC (CompuCyte, Cambridge, MA). The cellular fluorescence of PI and FITC was excited at 488 nm, and emission of these fluorochromes was measured using the standard long-pass (570 nm) or band-pass (530 nm) filters, respectively. At least 3000 cells were measured per slide. The relocation feature of LSC (26) was used to identify individual cells within the gated populations and to reveal their morphology. Each experiment was repeated at least three times. The samples were photographed using the LSC charge-coupled device camera and/or a Kodak DC120 Zoom Digital Camera (Eastman Kodak, Rochester, NY) adapted to the LSC microscope.

RESULTS

Detection and Quantification of Nucleoli by LSC. To detect and analyze nucleoli, we developed a novel assay using a cytometric program for fluorescence in situ hybridization. Nucleoli were identified by the immunoreactivity of antinucleolin antibodies and visualized by the signal of the secondary antibodies conjugated to FITC.

Using this approach, we were able to count the number of “spots” corresponding to nucleoli present inside the nucleus of each individual cell (Fig. 1A).

Fig. 1 illustrates the strategy used to measure the nucleolin-associated FITC fluorescence separately in the nucleoli and the cell nucleus by LSC. The triggering threshold (“threshold contour” in red) was set on red fluorescence of cells in which the DNA was counterstained with PI. The green fluorescence (integrated value) was then measured in two distinct areas. The first area, corresponding to the nuclear mass, was within the triggering threshold plus four pixels toward the outside of the threshold (“integration contour” in green). This area covered the whole cell nucleus. The second area, corresponding to “fluorescence in situ hybridization spots” and shown in white in Fig. 1, allows us to count the number of spots inside the nucleus and corresponds to nucleolar bodies. It also allows the integration of the green fluorescence signal over the nucleoli. Background fluorescence was automatically measured outside the cell (shown in blue) and subtracted.

Subcellular Localization of Cyclin E during the Cell Cycle in Bladder Cancer. To study the subcellular localization of cyclin E during cell cycle progression, we compared its pattern of expression in normal fibroblasts and nine bladder cancer cell lines (Fig. 2).

Initial fluorescence microscopic examination of asynchronous, exponentially growing fibroblasts and most cell lines revealed that the majority of cells did not express cyclin E (only a red nuclear signal corresponding to the PI counterstaining of nuclei; see Fig. 2A). However, a subpopulation of cells showed an orange nucleoplasm produced by the colocalization of cyclin E (green) and PI (red; Fig. 2A).

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Fig. 1. Immunocytochemical detection of nucleolin and quantification of nucleoli by LSC. A, J82 cells were indirectly stained with antinucleolin mAb conjugated to FITC (green), and their DNA was counterstained with PI (red), as described in “Materials and Methods.” The triggering threshold is depicted in red, whereas the total nuclear fluorescence contour is set on green. The nucleolar bodies are contoured in white inside the nucleus. See “Results” for more details. Original magnification, ×400. B, the LSC software allows counting of the number of nucleolar bodies (“spots”) present inside the nucleus of each individual cell. The bar graph on the left illustrates the number of spots (nucleoli) per cell analyzed. The numerical assessments on the right side of the figure summarize the actual counting of nucleoli (green number of spots).

The cells were categorized based on the number of spots identified per cell and given a region number (i.e., region 1 included 8.3% of cells displaying only one spot; region 5 included 3.3% of cells displaying 5 spots). Rgn. #, the number of the region in the graph; Count, the number of events in the corresponding region; Pct., the percentage of all events in the region; Mean, the mean value of the events in the region; FWHM, the coefficient of variation calculated based on full width of the distribution of events for a feature at half maximum value of the count.
Fig. 2. Immunocytochemical detection of cyclin E. A, J82 cells stained with anti-cyclin E mAb (FITC) and PI (red), as described in “Materials and Methods.” Note the heterogeneity of the pattern of cyclin E immunostaining in the field, including negative cells (nuclei stained red only, due to PI staining), yellow-green nucleoplasm-stained cells (cyclin E colocalized in the nuclei with PI), and some cells displaying nucleolar green fluorescence signal (cyclin E expression in nucleolus). Original magnification, ×400. B, cytometric analyses of cyclin E versus DNA content on J82 cells. This process allows the dynamic visualization of cyclin E expression in relation to cell cycle position. In addition, gating cells for each cycle compartment allows us to ascribe subcellular cyclin E localization. In the J82 cells, cyclin E expression is found in the nucleoplasm during mid-G1 phase of the cell cycle and is present
More interestingly, some cells showed an intense yellow-green staining in their nucleoli, representing cyclin E accumulation in this cellular compartment (Fig. 2A). Similar results were obtained by the use of the two anti-cyclin E mAbs to distinct epitopes.

We then generated cytometric data analyzing the same cells and relating their cyclin E profile with cell cycle progression (Fig. 2B). Fibroblasts and certain bladder cancer cell lines, including J82, 5637, T24, and RT4, presented the expected pattern of cyclin E expression. Maximal levels of cyclin E were in cells undergoing transition from G1 to S phase, and this expression level continuously decreased during progression through S phase and was undetectable during G2-M phase. In addition, a threshold of cyclin E expression was apparent at the G1-S-phase transition. This approach also allowed us to determine the relationship between cell cycle and intranuclear compartmentalization. To achieve this goal, several gates were created around populations in G0-early G1, mid-G1, G1-S-phase transition, and G2-M phase, and, using the relocation feature of the LSC, we identified individual cells within the gated populations. The cells in G0-early G1 were cyclin E negative, whereas mid-G1 cells expressed cyclin E in the nucleoplasm. However, cells in the G1-S-phase transition displayed a strong nucleolar cyclin E expression, with decreased nucleoplasmic signals. Finally, G2-M-phase cells had undetectable cyclin E levels. Fig. 2B illustrates such expression patterns and subcellular compartmentalization in J82 cells.

Unexpectedly, the analysis of SCABER cells, a cell line derived from a squamous cell carcinoma of the bladder, showed a different expression profile (Fig. 2C). We observed that cyclin E in these cells was present in the nucleoli at all times, regardless of the cell cycle phase, with minimal nucleoplasmic levels. Moreover, the bivariate distribution of cyclin E in relation to cell cycle position was disturbed, and cyclin E expression did not decrease during S phase, continued in G2-M phase (Fig. 2C), and was mainly nucleolar. Using the cytometric approach and color gating, we further validated this abnormal cyclin E expression profile (Fig. 2D). A gate was set around the counted nucleoli. As is evident in the histogram of J82 cells, the labeled population within this gate mostly represented cells in the nucleoli during G1-S-phase transition (for more details, see “Results”). These analyses take advantage of the cytometric quantification and the relocation features of the LSC software.

Cytometric analyses of cyclin E versus DNA content on SCABER cells. These studies were conducted as described above. Note that in these cells, cyclin E is expressed at all times in the nucleoli, following an “unscheduled” pattern of expression, and is not degraded during late S and G2-M phases. D, cytometric detection of cyclin E expression in the nucleoli during the cell cycle (left panel, J82 cells; right panel, SCABER cells). This is achieved by gating the counted spots, which correspond to nucleolar bodies, and representing such events in green on the scattergrams. Note that whereas the left scattergram and cell cycle inset (corresponding to J82 cells) show cyclin E nuclear expression only during G1-S-phase transition, the right scattergram and cell cycle inset (SCABER cells) show cyclin E nuclear expression throughout the cell cycle. Original magnifications, ×400.
Fig. 4. Immunocytochemical detection of pRB. A, normal human skin fibroblasts, CRL-1502, were stained using mAb G99-549 to detect underphosphorylated pRB. Note that there are two main phenotypes: those cells that react with this G99-549 (green fluorescent nuclei, representing underphosphorylated pRB); and those with undetectable staining (red staining due to PI, mainly expressing hyperphosphorylated pRB products). (Note: this photograph was taken using the LSC charge-coupled device camera rather than with the Kodak DC120 Zoom Digital Camera used for the other figures). The bivariate distribution of underphosphorylated pRB versus DNA content shows that most of the reactive cells are in G1 phase. B, normal human skin fibroblasts, CRL-1502, were also stained using mAb 3C8 to detect all pRB products, including hypophosphorylated and hyperphosphorylated pRB proteins. Note that all cells reacted with this antibody. The bivariate distribution of total pRB versus DNA content shows that pRB is expressed throughout the cell cycle. Original magnification, ×400.
pRB, recognized by mAb G99-549, is present in all G0-G1 cells with G1-S-phase transition, whereas in SCABER cells, cyclin E levels were observed throughout the cell cycle (Fig. 2D). These alterations in cyclin E expression and subcellular distribution were also observed in HT1197, TCCSUP, and UMUC3 cells.

Expression Patterns of p27 during Cell Cycle Progression. In cultured fibroblasts, we found high p27 levels in the nuclei of all cells, regardless of their cell cycle position (Fig. 3, A, B, and D). p27 was found to have a punctate pattern of nuclear expression in fibroblasts (Fig. 3A). However, p27 was not detected in some of the bladder cell lines analyzed. Only J82 cells displayed low to undetectable p27 nuclear levels, without cytoplasmic staining (Fig. 3C). p27 immunoreactivity in the nucleolus was not identified in either normal fibroblasts or tumor cell lines.

Patterns of Expression and Phosphorylation Status of pRB during Cell Cycle Progression. Expression levels of pRB do not change during the cell cycle; however, its activity is regulated by phosphorylation. pRB is hypophosphorylated in quiescent and early G1 cells and hyperphosphorylated throughout the rest of the cell cycle. In cultured normal fibroblasts, we observed that underphosphorylated pRB, recognized by mAb G99-549, is present in all G0-G1 cells with a diffuse nucleoplasmic pattern. Cells that progress through S phase and G2-M phase revealed low to undetectable levels of underphosphorylated pRB (Fig. 4A). This pattern is in contrast to that produced by identification of both underphosphorylated and hyperphosphorylated pRB by mAb 3C8. In bivariate analysis of hyperphosphorylated pRB versus cellular DNA content, pRB products are present throughout the cell cycle, being more prominently expressed in G2-M-phase cells (Fig. 4B). Bladder cancer cell lines displayed a very different pattern of pRB reactivity. G99-549 staining was undetectable in all cell lines, but intense nuclear signals were seen for 3C8 mAb, indicating that hyperphosphorylated pRB is the prevalent expressed product.

DISCUSSION

Cell cycle progression is regulated by a group of heterodimeric proteins composed of a cyclin acting as a regulatory subunit and a Cdk, which acts as the catalytic subunit. The critical transition from G1 to S phase is controlled by cyclin E/Cdk2 complexes. Data from the present study reveal that normal fibroblasts and most tumor cell lines analyzed had undetectable cyclin E levels during G0 and early G1, and expressed cyclin E with a nucleoplasmic pattern during mid-G1, whereas during the G1-S-phase transition, cyclin E localized to the nucleoli. Cyclin E was absent during G2-M phase (Figs. 2 and 5). Interestingly, neither levels nor subcellular localization of p27 was cell cycle dependent. Similarly, pRB levels and subcellular localization were also unchanged; however, the phosphorylation status of pRB was cell cycle dependent.

The shuttling of cyclin E between the nucleolus and the cytoplasm may serve as a titration mechanism, presenting cyclin E for enzymatic degradation and allowing progression through the cell cycle. Supporting this hypothesis, nucleoplasmic-nucleolar shuttling has been recently described as a regulatory p53 mechanism involving mdm2-p19Arf in murine models. It has been reported that nucleolar-cytoplasmic shuttling of mdm2 is essential for the ability of mdm2 to promote p53 degradation. p19Arf is encoded at the Ink4a locus and acts by attenuating mdm2-mediated degradation of p53 (27–29). mdm2 protein colocalizes with p19Arf in the nucleolus, and it has been postulated that p19 blocks the nucleolar-cytoplasmic shuttling of mdm2, thus stabilizing p53 (30, 31).

Unexpectedly, four of the nine bladder cancer cell lines (SCABER, HT1197, TCCSUP, and UMUC3) showed an altered pattern of subcellular localization of cyclin E. In these cells, cyclin E levels were constantly elevated, regardless of the cell cycle position, although we observed that these cells had a short G1 phase. In addition, cyclin E expression was identified in the nucleoli of all cells analyzed. These data suggest that cyclin E in these cells is not exported to the cytoplasm, where it undergoes enzymatic degradation. Because pRB is hyperphosphorylated in these cells at all times, it is tempting to postulate that this deregulated cyclin E profile serves as an oncogenic event.

Cyclin E has been reported to be overexpressed and to have an unscheduled pattern of expression in certain human tumors, including bladder cancer (11, 32–37). In addition, the shorter G1 phase observed in certain bladder cancer cells has also been described for human papillomavirus type 16-infected cells (38). In those cells, cyclin E expression was maintained at high levels in S phase and G2-M phase (38). Furthermore, the cyclin E promoter has an E2F1 binding motif, which appears to be the main transcription control mechanism of its expression (39). Because hyperphosphorylated pRB releases E2F1, this phenomenon could also contribute to the maintenance of high cyclin E levels. It is not understood how alterations in the expression of cyclin E contribute to tumorigenesis. A recent study by Spruck et al. (40) reported that down-regulation of cyclin E/Cdk2 kinase activity after the G1-S-phase transition may be necessary for the maintenance of karyotypic stability. Altered shuttling of cyclin E might also be involved in such a deregulated process in human tumors.

The nucleolus has distinctive involvements such as rDNA localization and rRNA synthesis. However, it appears that many other functional and regulatory events take place in this organelle, including nuclear export of rRNAs and ribosomal proteins (41–43). More recently, a more active role has been attributed to the nucleolus in the temporally ordered expression and proteolytic events that occur during cell growth (44–47). Based on data from this study, we postulate that the nucleolus mediates a "shuttling" between the nucleus and the cytoplasm to control cyclin E levels and that this mechanism is disrupted in bladder cancer. Recognition of alterations in this novel mechanism may have biological and clinical implications that deserve further analyses.

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Fig. 5. Proposed model of intranuclear compartmentalization of cyclin E during cell cycle progression. Cells have undetectable levels of cyclin E during G0 and early G1 phases. They express cyclin E with a nucleoplasmic pattern during mid-G1 phase, whereas cyclin E localizes to nucleoli during the G1-S transition. Finally, cyclin E is undetectable during G2-M phase. Dynamic changes in cyclin E affect not only the expression levels but intracellular localization as well. Shuttling of cyclin E from the nucleus to the nucleolus and finally to the cytoplasm might serve as a titration mechanism, presenting cyclin E for enzymatic degradation and thus allowing progression through the cell cycle.
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