On the Concentrations of Cyclins and Cyclin-Dependent Kinases in Extracts of Cultured Human Cells†

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ABSTRACT: Cyclins and cyclin-dependent kinases (CDKs) are key regulators of the human cell cycle. Here we have directly measured the concentrations of the G_1 and G_2 cyclins and their CDK partners in highly synchronized human cervical carcinoma cells (HeLa). To determine the exact concentrations of cyclins and CDKs in the cell extracts, we developed a relatively simple method that combined the use of ^{35}S-labeled standards produced in rabbit reticulocyte lysates and immunoblotting with specific antibodies. Using this approach, we formally demonstrated that CDC2 and CDK2 are in excess of their cyclin partners. We found that the concentrations of cyclin A2 and cyclin B1 (at their peak levels in the G_2 phase) were about 30-fold less than that of their partner CDC2. The peak levels of cyclin A2 and cyclin E1, at the G_2 phase and G_1 phase, respectively, were only about 8-fold less than that of their partner CDK2. These ratios are in good agreement with size fractionation analysis of the relative amount of monomeric and complexed forms of CDC2 and CDK2 in the cell. All the cyclin A2 and cyclin E1 are in complexes with CDC2 and CDK2, but there are some indications that a significant portion of cyclin B1 may not be in complex with CDC2. Furthermore, we also demonstrated that the concentration of the CDK inhibitor $p21^{CIP1/WAF1}$ induced after DNA damage is sufficient to overcome the cyclin−CDK2 complexes in MCF-7 cells. These direct quantitations formally confirmed the long-held presumption that CDKs are in excess of the cyclins in the cell. Moreover, similar approaches can be used to measure the concentration of any protein in cell-free extracts.

Cyclin-dependent kinases (CDKs)[1] and their regulatory subunits, cyclins, are key regulators of the eukaryotic cell cycle (*1*, *2*). By definition, the activity of CDKs is dependent on binding to a cyclin subunit. The levels of most CDKs are relatively constant in the cell cycle, but the levels of their cyclin partners may fluctuate and represent a key determinant of the CDK activity. Apart from binding to cyclins, CDKs are also regulated by phosphorylation and inhibitor proteins (*3*).

Cyclins are defined as proteins that are related in sequence to the originally isolated mitotic cyclins, especially in the cyclin box region. In mammalian cells, cyclin B−CDC2 is the principal mitotic cyclin−CDK complex that drives the G_2−M transition (*4*). CDC2 also binds to cyclin A2 (cyclin A1 in embryonic cells), and the activity of cyclin A2−CDC2 peaks before that of cyclin B−CDC2 (*5*). The second member of the CDK family, CDK2, associates with cyclin E1 and cyclin A2 during the G_1 phase and S phase, respectively (*6*). CDK4 and CDK6 associate with the D-type cyclins and are important for G_1 progression (*7*). Cyclin F was isolated as a suppressor of a yeast *cdc4* mutant (*8*), suggesting that cyclin F may be involved in the degradation of other cell cycle regulators. The function of cyclin G is not known, but it can be transcriptionally activated by the tumor suppressor p53 (*9*). Cyclin I was identified by sequence homology, and it may have noncell cycle regulatory functions (*10*). CDK5 was identified as one of the CDK partners of cyclin D in human normal fibroblasts. However, there is no other indication that CDK5 has a function in normal cell cycle control, but instead CDK5 was found to have essential functions in neuronal development. CDK5 is activated in postmitotic neurons by binding to a protein called p35, which shares no sequence homology to cyclin. Cyclin C−CDK8 (*11*), cyclin H−CDK7 (*12*), cyclin K (*13*), and cyclin T−CDK9 (*14*, *15*) appear to have roles in regulating basal transcription through their association and phosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II.

Cyclin expression is controlled at both the transcriptional and posttranslational levels. The posttranslational regulation of cyclins occurs mainly through ubiquitin-mediated proteolysis. The mitotic cyclins contain a sequence motif known as the destruction box that is required for their destruction at the end of mitosis. Cyclin proteolysis involves a nonspecific ubiquitin-activating enzyme (E1), which transfers ubiquitin to an ubiquitin-carrier protein (E2). Ubiquitin is then transferred to the cyclin (to a lysine residue at the C-terminus of the destruction box) by a cyclin-specific ubiquitin ligase (E3) known as the anaphase-promoting complex (APC) (or the cyclosome). The ubiquitinated cyclins are then rapidly degraded by a constitutively active protea-

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[1] Abbreviations: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase.

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some complex (*16*). The activity of APC is highly regulated, turning on in late mitosis and persisting until late G_1 (*17*−*19*). Unlike the mitotic cyclins, the G_1 cyclins contain PEST sequences at the C-terminal portion of the protein that are partly responsible for the relatively short half-life of these cyclins.

The changes in the expression and activities of cyclins and CDKs in the cell cycle have been studied extensively. However, little is known about the quantitative changes of these key proteins in the cell. Direct quantitation of proteins in cell-free extracts or in the cell is important and revealing, but it is uncommon (and technically difficult) to obtain quantitative data on the concentration of proteins in cell extracts. Here we investigate the relative levels of cyclins and CDKs in HeLa cells. We describe a relatively simple procedure that combines the use of rabbit reticulocyte lysate produced standards and immunoblotting to obtain measurements of the absolute concentrations of cyclins and CDKs in cell lysates.

EXPERIMENTAL PROCEDURES

DNA Constructs. Cyclin A2 in pGEM4, cyclin B1 in pGEM4, and cyclin E1 in pBluescript were from Dr. Tony Hunter (Salk Institute, La Jolla). Human cyclin F in pET11d was from Dr. Tim Hunt (ICRF, South Mimms). Cyclin H in pBluescript was from Dr. David Morgan (UCSF, San Francisco). CDK2 in pBluescriptII was from Dr. Ed Harlow (Massachusetts General Hospital Cancer Center). CDC2 in pBluescriptII was from Dr. John Newport (UCSD, La Jolla). GST−CDK2 (*20*) and CDK5−H6 (*21*) were as described.

Cell Culture and Synchronization. AG1523 normal human foreskin diploid fibroblasts were obtained from the NIA Aging Cell Repository, Institute for Medical Research (Camden, NJ) (used between passage 8 and passage 25). A chemical carcinogen-transformed human fibroblast cell line, HUT12, was a gift from Dr. Gertrud Orend (Burnham Institute, La Jolla). MCF-7 cells (human mammary gland adenocarcinoma cells) were gifts from Dr. Yong Xie (HKUST, Hong Kong). HeLa (human cervical carcinoma cells), HaCaT (immortalized human keratinocytes), 293 (transformed human embryonic kidney cells), HepG2 (human hepatocellular carcinoma cells), H4 (human neuroglioma cells), HLB100 (human breast epithelial cell), MG63 (human osteosarcoma cells), K562 (human chronic myelogenous leukemia cells), and U2OS (human osteosarcoma cells) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% v/v calf serum or 10% v/v fetal bovine serum (GIBCO-BRL) in a humidified incubator at 37 °C in 10% CO_2. Cell-free extracts (*22*), flow cytometry analysis, and adriamycin treatment were as described (*23*). Cell cycle synchronization by mitotic block (nocodazole) was as described (*22*). For double thymidine synchronization, cells were incubated in medium containing 2 mM thymidine for 16 h. The cells were released from the first block by washing twice with PBS and incubated in medium containing 24 μM deoxycytidine. After 9 h, 2 mM thymidine was added for a second block. After 16 h, the cells were released from the second block as above.

Quantitation of Proteins in Cell Extracts. Cyclins and CDK standards were produced in rabbit reticulocyte lysates. Coupled transcription−translation reactions in the presence of [^{35}S]methionine in TNT rabbit reticulocyte lysate were performed according to the manufacturer's instructions (Promega, Madison, WI). The translated products were diluted 1/20 in SDS sample buffer and applied onto SDS−PAGE. The amount of [^{35}S]methionine in the translated proteins was quantitated with a PhosphorImager (Molecular Dynamics) by comparison to a dot blot of 2-fold serial dilution of the [^{35}S]methionine used (with known specific activity). Only the linear range of the [^{35}S]methionine serial dilution was used. The actual amount of newly synthesized protein standard in the reticulocyte lysate was then calculated by the knowledge of the number of methionines in the protein sequence. In this paper, the N-terminal methionine residue was included in the calculations.

Serial dilutions of the reticulocyte lysate containing the translated proteins were loaded side by side with synchronized cell extracts (10 μg) onto SDS−PAGE and transferred onto PVDF membranes. The blots were probed with specific antibodies, and the amount of cyclins/CDKs in the cell extracts was estimated by comparison with the reticulocyte lysate standards. Signals on immunoblots were analyzed on a Macintosh computer using the NIH Image program (National Institutes of Health).

Blank reticulocyte lysates (not programmed to synthesize exogenous proteins) were always applied onto the SDS−PAGE for immunoblotting, because endogenous proteins in the rabbit reticulocyte lysate may cross-react with the antibodies, and these background signals had to be subtracted. In particular, we found that reticulocyte lysate contained CDC2-related proteins that cross-reacted with our anti-CDC2 monoclonal antibodies. Since it is possible that there was some residual unlabeled methionine in the reticulocyte lysate system, and hence our data may be underestimated, we have measured the amount of CDK2 in the HeLa cell extracts independently using bacterially expressed and purified CDK2. Comparable values of CDK2 in cell extracts were obtained using bacterially expressed and reticulocyte lysate translated CDK2 as standards; hence no compensation was made for the residual methionine in the reticulocyte lysate.

Histone H1 Kinase Assays. Histone H1 kinase assays were performed as described (*24*).

Gel Filtration Chromatography. Samples (200 μL) were applied onto a Superose 6 HR10/30 column (Pharmacia) and subjected to FPLC chromatography in the buffer 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA with a flow rate of 0.4 mL/min. Fractions (one fraction per minute) were lyophilized and dissolved in 60 μL of SDS sample buffer. Size standards [BSA (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa)] were detected by SDS−PAGE and Coomassie blue staining.

Antibodies and Immunological Methods. Rabbit anti-CDK2 polyclonal antibodies (*20*), rabbit anti-CDC2 polyclonal antibodies (*25*), rabbit anti-CDK4 polyclonal antibodies (*26*), anti-cyclin A2 monoclonal antibody E23 (*22*), rabbit anti-cyclin A2 polyclonal antibodies (*27*), rat monoclonal antibodies YL1/2 against mammalian tubulin (*24*), anti-CDC2 monoclonal antibody A17 (*28*), and anti-cyclin E1 monoclonal antibodies HE12 (*24*) were obtained from sources as previously described. Anti-cyclin B1 monoclonal antibodies GNS1 (sc-245), rabbit anti-cyclin F polyclonal antibodies (sc-952), goat anti-SKP1 antibodies (sc-1568), and

goat anti-SKP2 antibodies (sc-1567) were from Santa Cruz Biotechnology. Rabbit anti-cyclin B1 polyclonal antibodies were gifts from Dr. Katsumi Yamashita (Kanazawa University, Japan). Immunoprecipitation and immunoblotting were performed as described (*26*). Immunodepletions were performed with two rounds of immunoprecipitations as described (*22*). Signals on immunoblots were analyzed on a Macintosh computer using the NIH Image program (National Institutes of Health) with the appropriate calibration.

RESULTS

Obtaining Synchronized HeLa Cells for Quantitation of Proteins in Specific Phases of the Cell Cycle. To investigate the quantitative changes of cyclins in the cell cycle, we first have to obtain highly synchronized cells that provide us with an accurate description of the temporal changes in the total protein level of the cyclins, their complex formation with CDK partners, and their protein kinase activities. Synchronized HeLa cells were obtained using a double thymidine block method. In one continuous experiment, cells were released synchronously from the block and harvested at 3 h time points for up to 33 h. To access the synchrony of the cells, the DNA contents were analyzed by flow cytometry. Figure 1A indicates that the cells were blocked at early S phase at the start of the experiment and completed one round of cell cycle into the next S phase by 21 h.

As a further indication of the synchrony of the cell cycle, cell-free extracts were prepared, and the levels of cyclins and other proteins were detected by immunoblotting. Figure 1B shows the expected waves of the cyclins in the order of cyclin E1, cyclin A2, cyclin F, and cyclin B1. At this resolution, cyclin A2 and cyclin F were synthesized and destroyed slightly earlier than cyclin B1. Immunoblotting for tubulin indicates that a similar amount of extracts was loaded in all the lanes. D-type cyclins were not investigated because they are absent in HeLa cells due to the lack of functional Rb protein. SKP1 and SKP2 are two proteins that associate with the cyclin A2−CDK2 complexes and form part of the SCF^{SKP2} complex. We found that the protein level of SKP2 was highest during the S phase, but the variation was not as significant as its mRNA (*29*) nor as striking as cyclin A2.

The kinase activities associated with the mitotic cyclins (A2 and B1) in the cell cycle were detected by immunoprecipitation followed by histone H1 kinase assay (Figure 1C). Like their protein levels, the kinase activity associated with cyclin A2 declined slightly earlier than that of cyclin B1. The kinetics of the formation of the different cyclin−CDK complexes (cyclin A2−CDK2, cyclin E1−CDK2, cyclin A2−CDC2, and cyclin B1−CDC2) was detected by immunoprecipitation followed by immunoblotting. Panels D and E of Figure 1 show that the formation of cyclin E1−CDK2, cyclin A2−CDC2/CDK2, and cyclin B1−CDC2 complexes followed closely the synthesis of the cyclins. Figure 1D also shows that the kinetics of SKP2 binding to CDK2 closely followed that of cyclin A2 (rather than cyclin E1), suggesting that SKP2 associated mainly with cyclin A2−CDK2.

Direct Measurements of Cyclins and CDKs in HeLa Cells. We next developed the following method to directly measure the amount of cyclins and CDKs in the synchronized cell extracts. Cyclin and CDK standards were first synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]-methionine. The translated products were applied onto SDS−PAGE, and the amount of [^{35}S]methionine in the translated protein was quantitated with a PhosphorImager by comparison to a serial dilution of the stock of [^{35}S]methionine used. The actual amount of protein synthesized in the reticulocyte lysate was calculated from the knowledge of the number of methionine residues in the protein sequence. The amount of cyclins/CDKs in synchronized HeLa cell extracts was then estimated by comparison with the reticulocyte lysate translated standards by immunoblotting with specific antibodies (see Experimental Procedures). Figure 2 shows an example of the quantitation exercise for cyclin F. Human cyclin F was made in reticulocyte lysate, and the amount made was quantitated (Figure 2A). The cyclin F standards were compared side by side with cell extracts from HeLa or AG1523 cells (human normal diploid fibroblasts) (Figure 2B). Reticulocyte lysate programmed to produce CDK2 was included to indicate that the reticulocyte lysate itself did not contain any cyclin F background.

Table 1: Summary of the Amount of CDKs and Cyclins in HeLa Cell Extracts[a]

	concn (pmol/g of extracts)	
	G1	G2
CDC2	912/629	912/629
CDK2	120/340	120/340
CDK5	970/485	970/121
cyclin A2	2.80/5.90	11.8/10.8
cyclin B1	5.80/2.90	11.6/13.3
cyclin E1	30.0/11.0	0.00/5.00
cyclin F	5.40/3.50	21.6/10.8

[a] Quantitation of CDC2, CDK2, CDK5, cyclin A2, cyclin B1, cyclin E1, and cyclin F in HeLa cell extracts derived from cells in the G_1 phase (15 h after double thymidine release) or G_2 phase (9 h after double thymidine release) was as described in Experimental Procedures. The data from two independent experiments are shown. The variations were mainly due to the extracts derived from different synchronization experiments rather than from the quantitation procedures.

Table 1 summarizes the abundance of CDC2, CDK2, and their G_1 and G_2 cyclin partners (cyclin A2, cyclin B1, and cyclin E1) during the G_1 phase and G_2 phase of HeLa cells (cyclin D was absent in HeLa cells). The abundance of CDK5 (which has relative constant expression in the cell cycle and no known cyclin partner) and cyclin F (which varied in the cell cycle but with no known CDK partner) is also shown for comparison.

Levels of Cyclins and CDKs in HeLa Cells Relative to Other Cell Lines. To get an idea of how typical the amount of cyclins and CDKs in HeLa cells was in comparison to other cell types, cell extracts from several commonly used cell types were analyzed (Figure 3). Immunoblotting for tubulin indicated that similar amounts of extracts were loaded. It is apparent that although the concentration of CDK2 was comparable between different cell types, the concentration of cyclins varied immensely. The levels of cyclins in the HeLa cells used in this study were in the midrange in comparison to other transformed and cancer cell lines.

Formal Demonstration That CDC2 and CDK2 Are in Excess over Their Cyclin Partners. From the above quantitations, it is clear that CDC2 was in vast excess over its partners cyclin B1 and cyclin A2 even when these cyclins

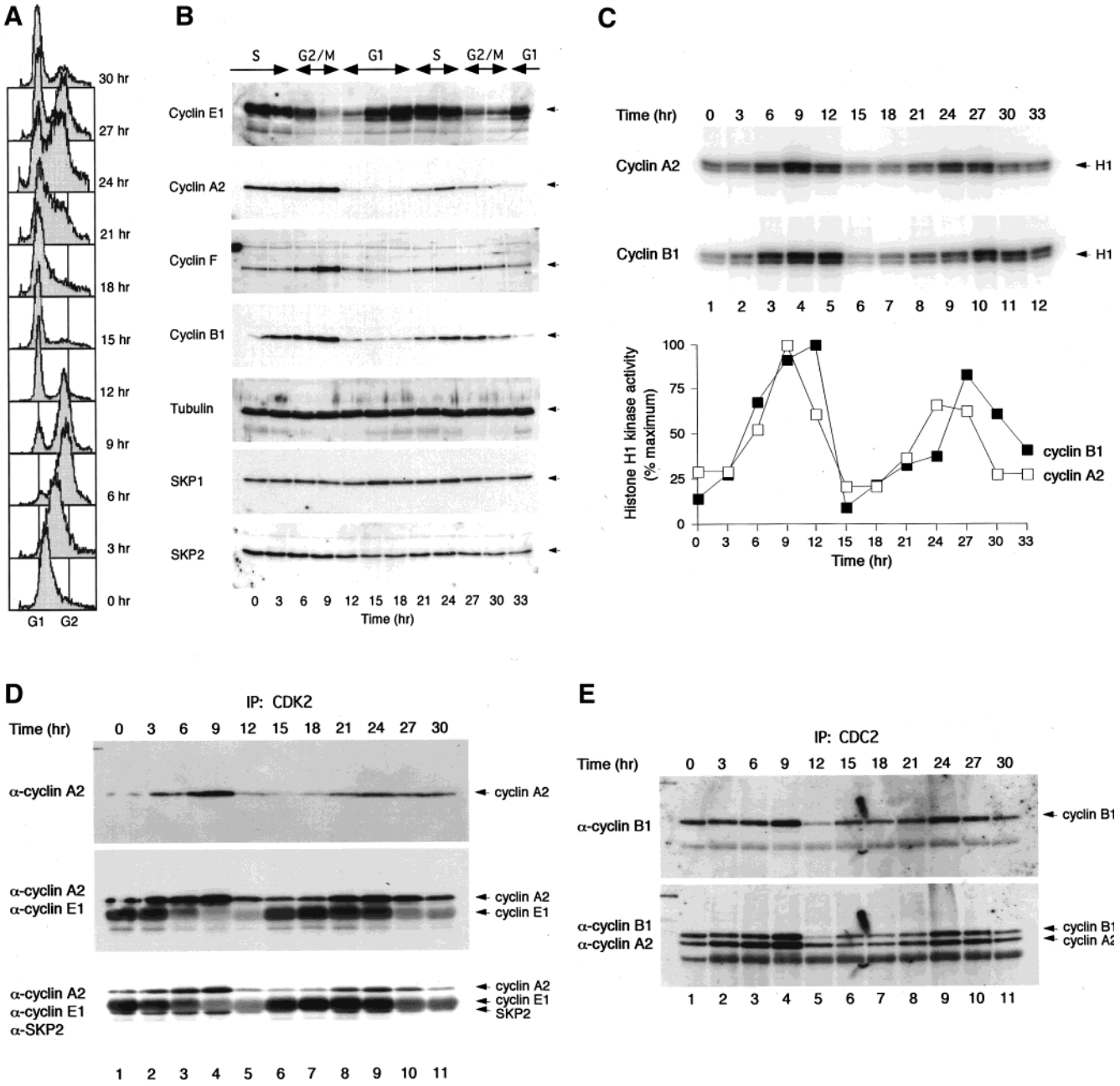

FIGURE 1: Obtaining synchronized HeLa cells by the double thymidine block method. (A) HeLa cells were synchronized by the double thymidine block as described in Experimental Procedures. Cells were harvested at the indicated time points after release from the second thymidine block for flow cytometry analysis. (B) Cell-free extracts were prepared, and the expression of cyclin E1, cyclin A2, cyclin F, cyclin B1, tubulin, SKP1, and SKP2 was determined by SDS−PAGE (10 μg per lane) followed by immunoblotting with the indicated antibodies. The positions of the respective proteins and the estimated cell cycle positions (from flow cytometry) are indicated. (C) Kinase activities of cyclin A2 and cyclin B1. Cell extracts (200 μg) were immunoprecipitated with antibodies against cyclin A2 (white squares) or cyclin B1 (black squares). The histone H1 kinase activities were assayed, and phosphorylations were analyzed with SDS−PAGE and PhosphorImagery (upper panel). The quantitation of the phosphorylated bands is shown in the bottom panel. (D) Association between CDK2 and cyclins. CDK2 immunoprecipitates (from 200 μg extracts) were immunoblotted with the antibodies against cyclin A2, cyclin A2 and cyclin E1 together, or cyclin E1, cyclin A2, and SKP2 together as indicated. (E) Association between CDC2 and cyclins. CDC2 immunoprecipitates (from 200 μg extracts) were immunoblotted with antibodies against cyclin B1 or cyclin B1 and cyclin A2 together as indicated.

were at their peak level during the G_2/M phase (about 30-fold excess). Similarly, but to a lesser extent, CDK2 was in excess over its partners cyclin E1 and cyclin A2 (about 8-fold). At their peak levels, there was more cyclin E1 than cyclin B1 and cyclin A2. The relative concentrations of CDC2, CDK2, and their cyclin partners are summarized in Figure 4A (note that the *y*-axis is in log scale). These data formally confirmed the long-held belief that CDC2 and possibly CDK2 are in excess over their cyclin partners.

To further address the question of whether CDC2 is in a larger excess over its cyclin partners than CDK2 is, we next investigated the proportion of CDKs that was associated with cyclins in the cell extracts. Cell extracts derived from HeLa cells synchronized at the G_1 phase, S phase, or G_2 phase were size fractionated using gel filtration chromatography. The distribution of CDKs in the fractions was detected by immunoblotting. Figure 4B shows that CDK2 was separated into two peaks: a smaller size population (migrated with

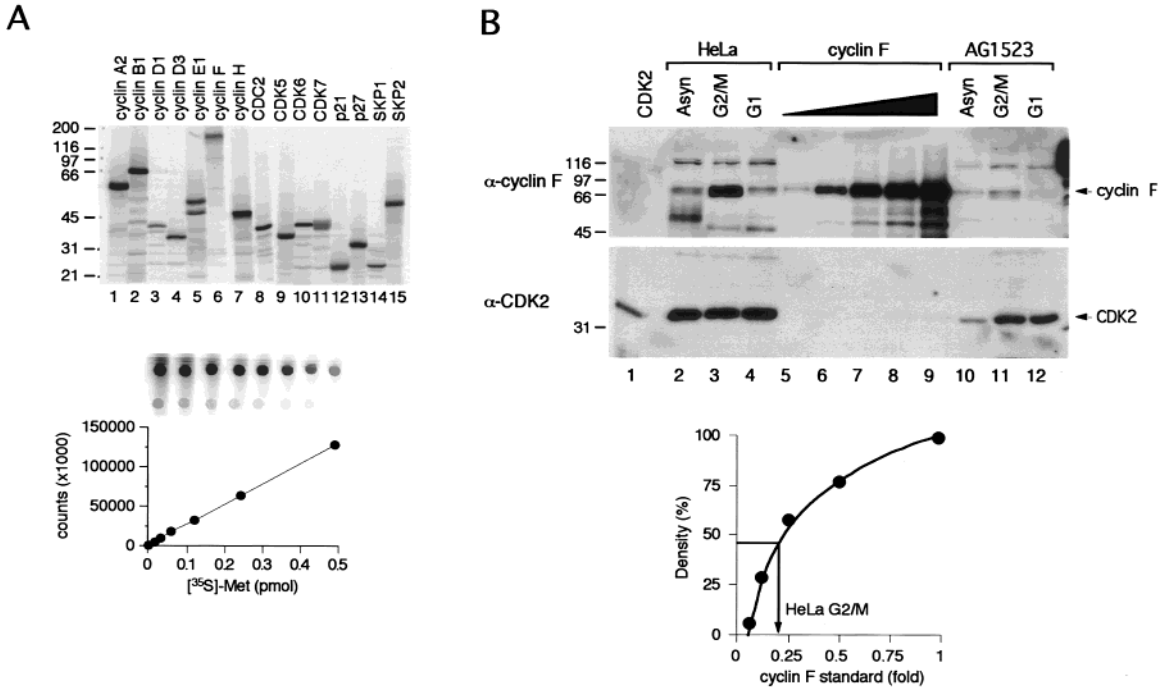

FIGURE 2: Quantitation of cyclins and CDKs in cell-free extracts. (A) Production of protein standards in the reticulocyte lysate. The indicated proteins were translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation products were detected by SDS−PAGE followed by PhosphorImagery (upper panel). The positions of the molecular size standards (in kDa) are indicated on the left. The dot blot of a serial dilution of the [^{35}S]methionine used was analyzed on the same PhosphorImager screen. The linear ranges of the translated proteins and the dot blot (bottom panel) were used to calculate the amount of protein translated. (B) Representational quantitation of protein (cyclin F) in cell extracts. Reticulocyte lysates programmed to express CDK2 (lane 1, 4 μL) or cyclin F (lanes 5−9, 2-fold serial dilution in sample buffer) containing known amounts of cyclin F (see Experimental Procedures), 10 μg of cell-free extracts of HeLa cells (lanes 2−4) or AG1523 (lanes 10−12) that were derived from an asynchronously growing population (lanes 2 and 10), and cells in the G_2/M phase (lanes 3 and 11) or G_1 phase (lanes 4 and 12) were applied onto a 17.5% SDS−PAGE. The samples were transferred onto membranes and immunoblotted with antibodies against cyclin F (upper panel) or CDK2 (lower panel). The positions of the molecular size standards (in kDa) are indicated on the left. The intensity of the cyclin F standards was analyzed with NIH Image software and plotted at the bottom. The corresponding level of cyclin F in the G_2/M HeLa cell extracts is indicated.

the 29 kDa standard) and a larger size population (migrated with the 67 kDa standard). The smaller size population represented the monomeric CDK2, and the larger size population represented CDK2 bound to cyclins and other proteins. The true molecular size of the smaller size population was confirmed to be about 30 kDa by combining the Stokes radius calculated from gel filtration chromatography and the sedimentation coefficient obtained from glycerol gradient centrifugation (data not shown). Figure 4B shows that although the majority of the CDK2 molecules were in the monomeric form during the G_1 phase and S phase, a significant proportion of CDK2 was also found in the complexed form. In contrast to CDK2, only the monomeric form of CDC2 can be detected under similar conditions (Figure 4C). This qualitative analysis is in good agreement with the direct quantitation data that suggest the proportion of CDC2 that was in the cyclin-complexed form was much lower than that of CDK2.

Proportion of Cyclins in Association with CDC2 and CDK2. Given that CDC2 and CDK2 are in excess over their cyclin partners, it is generally assumed that all of the cyclins in the cell are in the CDK-complexed form. To have an idea of the proportion of the cyclins that are associated with CDKs, CDC2 or CDK2 was immunodepleted from cell extracts, and the amount of cyclins remaining in the depleted extracts was measured. The control reaction was performed with normal rabbit serum. Figure 5 shows that CDC2 or CDK2 was completely removed from the cell-free extracts after immunodepletion with the respective antibodies. Furthermore, the antibodies were specific since no CDK2 was lost in the CDC2 immunodepletion and no CDC2 was lost in the CDK2 immunodepletion (lanes 1−6). Significantly, nearly all of the cyclin E1 was depleted with CDK2, suggesting that all of the cyclin E1 was in complex with CDK2 (lanes 13−15). Roughly half of the cyclin A2 was associated with CDC2 and the other half with CDK2 (lanes 7−9). These data indicate that, at least in asynchronous cell extracts, all of the cyclin A2 and cyclin E1 were accounted for by binding to the excess CDC2 and CDK2. About 60% of cyclin B1 could be depleted with CDC2 (lanes 10−12), but it is surprising that a significant portion of cyclin B1 was not depleted. Although cyclin B1 could clearly be co-immunoprecipitated with CDC2 (but not with CDK2; data not shown), we cannot rule out the possibility that some cyclin B1 was not dislodged from CDC2 by the anti-CDC2 antibodies. Hence in agreement with the expectation based on excess levels of CDKs, most of the cyclin A2 and cyclin E1 is in the CDK-complexed form. However, there are some indications that not all of the cyclin B1 is in complex with CDC2.

The Concentration of p21$^{CIP1/WAF1}$ after DNA Damage Is Sufficient To Overcome the Cyclin−CDK2 Complexes. DNA damage and other stresses induce the transcription activator p53, which in turn induces the CDK inhibitor p21$^{CIP1/WAF1}$. We applied the quantitation method to measure the concentration of p21$^{CIP1/WAF1}$ and the cyclin partners of CDK2 after

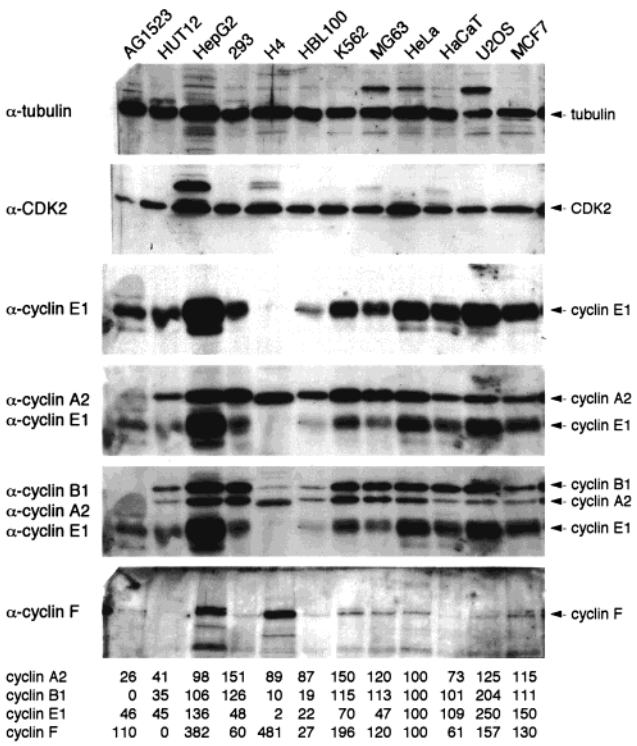

FIGURE 3: Relative levels of cyclins and CDKs in different cell lines. Cell-free extracts (10 μg) derived from an actively growing population of the indicated cell strain and cell lines were applied onto SDS−PAGE and subjected to immunoblotting with antibodies against tubulin, CDK2, cyclin B1, cyclin E1, and cyclin F as indicated. Intensities of the bands on the immunoblots were quantitated. Cyclin levels were normalized with the corresponding tubulin signals, and the values for different cell lines were expressed as a percentage to that of the HeLa cells.

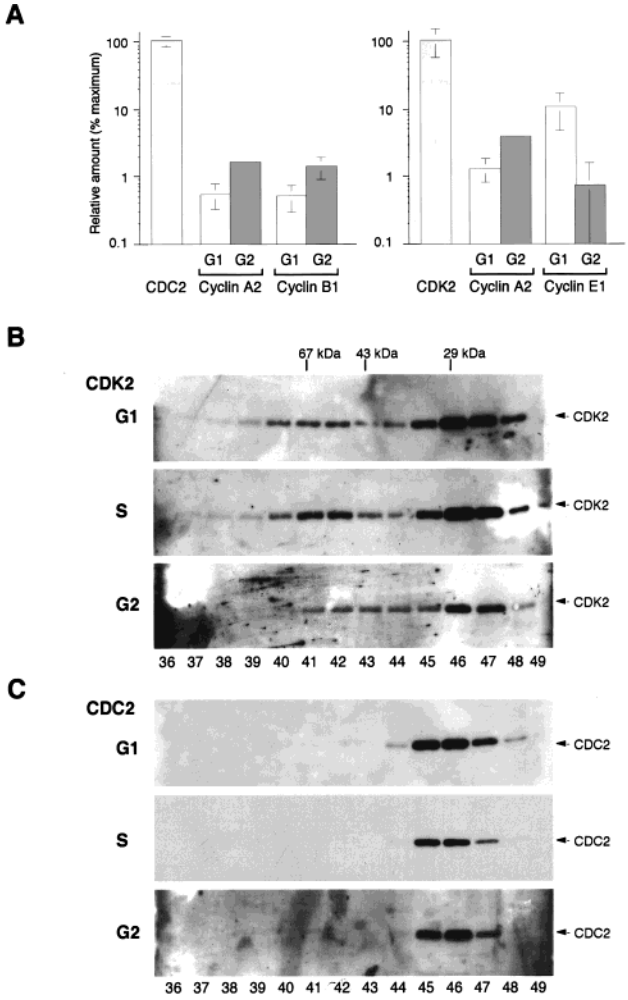

FIGURE 4: Relative abundance of CDC2, CDK2, and their cyclin partners in synchronized HeLa cell extracts. (A) The levels of cyclin A2 and cyclin B1 in the G_1 phase and G_2 phase cell extracts are plotted in relation to that of CDC2 (left-hand panel). The levels of cyclin A2 and cyclin E1 in the G_1 phase and G_2 phase cell extracts are plotted in relation to that of CDK2 (right-hand panel). The average of three independent experiments and their standard deviations are shown. Note that the *y*-axis is in log scale. (B) HeLa cell extracts (200 μg) derived from cells in the G_1 phase (15 h after release from the double thymidine block), S phase (18 h after release), or G_2 phase (9 h after release) were fractionated on a gel filtration column as described in Experimental Procedures. The CDK2 in the factions was detected by SDS−PAGE and immunoblotting. The fraction numbers (bottom) and the positions of the molecular size standards (in kDa) (top) are indicated. (C) Same as panel B except that CDC2 was detected.

DNA damage. The p53-positive human mammary gland adenocarcinoma MCF-7 cells were treated with the DNA damage drug adriamycin. We found that adriamycin induced $p21^{CIP1/WAF1}$ from undetectable levels to ~30 pmol/g in the extracts. The concentration of $p21^{CIP1/WAF1}$ induced by adriamycin was comparable to that of cyclin A2 (~6 pmol/g) and cyclin E1 (~30 pmol/g). Hence the amount of $p21^{CIP1/WAF1}$ induced by DNA damage is theoretically sufficient to inhibit the kinase activity of CDK2.

DISCUSSION

Here we analyzed the concentration of cyclins and CDKs in the cell cycle of HeLa cells. To our knowledge, the amounts of cyclins and CDKs in the cell or cell lysates have never been measured quantitatively. The method described here may be a general approach to measure the concentration of any protein in cell lysates, provided that the expression construct and specific antibodies are available.

The results presented here formally confirmed the long-held presumption that the levels of CDC2 are in excess of its cyclin partners. It is somewhat surprising that CDK2 is not in such a vast excess over its cyclin partners as with CDC2. This is especially true for cyclin E1−CDK2, of which the ratio of the two proteins is less than 10-fold. The relatively small excess of CDK2 over its cyclin partners suggests that some degree of competition between cyclin A2 and cyclin E1 for CDK2 is possible. D-type cyclins and CDK4/CDK6 were not analyzed in HeLa cells because the Rb pathway is inactivated by HPV E7. As another point of reference of the concentration of proteins in the cell extracts, we estimated that about 1 μg of cell lysate was typically derived from 2000 cells.

It is interesting that although CDC2 is in excess over cyclin B1, we found that around 40% of cyclin B1 was not immunodepleted with CDC2. This is in contrast to cyclin A2 and cyclin E1, which appear to be completely complexed with the CDKs. One possibility is that cyclin B1−CDC2 complexes are not very stable, and the two proteins dissociate during extract preparation or experimentation. It is also possible that some cyclin B1 was dislodged from CDC2 by the anti-CDC2 antibodies. Another kind of speculation is that formation of cyclin B1−CDC2 may require an additional step of binding to folding factors or assembly factors. It is

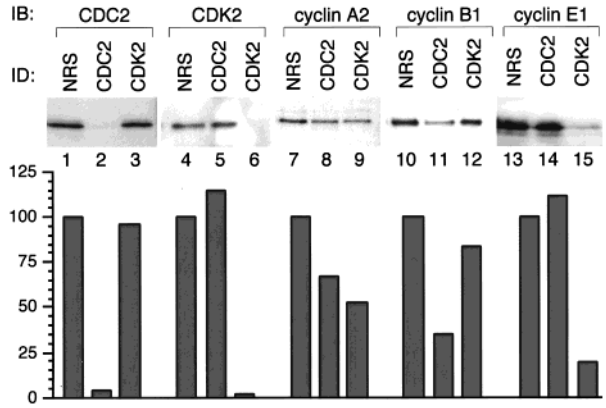

FIGURE 5: Immunodepletion of CDC2 and CDK2 from HeLa cell extracts. HeLa cell extracts were immunodepleted with normal rabbit serum (NRS), anti-CDC2 antibodies, or anti-CDK2 antibodies. The supernatant after the immunodepletion (10 μg) was applied onto SDS−PAGE and immunoblotted with antibodies against CDC2, CDK2, cyclin A2, cyclin B1, or cyclin E1 as indicated. The quantitations of the immunoblot signals were plotted in the bottom panel.

known that CDC37 and HSP90 may act as folding/assembly factors for cyclin D−CDK4/6 (*30*), and the CDK inhibitor $p21^{CIP1/WAF1}$ may act as an assembly factor for cyclin D−CDK4/6 (*31*). In yeast, there is evidence that function of CDK requires interaction with HSP90 (*32*).

The quantitation of cell cycle regulatory proteins is important for a more detailed understanding of the biochemistry of these proteins. When studying the kinetics of CDK phosphorylation of substrates, it would be important to know the concentration of CDK relative to its substrates in the cell. Similar calculations can be made when considering the regulation of CDK itself by phosphorylation and dephosphorylation. In the case of CDK inhibitors, effective inhibition can occur only if the amount of inhibitors present is at least similar to that of the cyclin−CDK complexes. We found that, in MCF-7 cells, the concentration of $p21^{CIP1/WAF1}$ induced by adriamycin was at least comparable to the concentration of cyclin A2 and cyclin E1.

The amount of cyclins and CDKs was relatively high in asynchronously growing HeLa cells in comparison to other cell lines (Figure 3). However, it should be noted that the level of cyclins in the asynchronous population of cells may reflect the relative length of different phases of the cell cycle. The concentration of the cyclins was typically lower in normal fibroblasts than in transformed cell lines, but it is possible that this is due to some degree of contact inhibition and senescence in the normal fibroblasts. It is intriguing that the human neuroglioma cell line H4 contained undetectable amounts of cyclin E1, relatively normal levels of CDK2 and cyclin A, but overexpressed cyclin F. These data only serve to provide a rough reference of the amount of cyclins and CDKs found in different cell lines.

The quantitation method described here that used reticulocyte-expressed standards has several advantages over using bacterially expressed recombinant proteins as standards. Soluble proteins are frequently not obtainable when recombinant proteins are expressed in bacteria. Moreover, quantitation of radioactive signals is more accurate than quantitation of proteins by dye staining. However, cautions are needed in several aspects of this quantitation method. The specificity of the antibodies on immunoblots plays an important part in the method. It is possible that reticulocyte lysate itself contains similar proteins that are also recognized by the antibodies. For instance, both the anti-CDC2 monoclonal antibody A17 and the anti-PSTAIRE monoclonal antibody recognized a CDC2-related protein in rabbit reticulocyte lysate. Hence unprogrammed reticulocyte lysate had to be loaded side by side, and the background was subsequently subtracted from the calculation for CDC2. It is also possible that there was some residual unlabeled methionine in the reticulocyte lysate system, and hence our data may be an underestimation. We have measured the concentration of CDK2 in the HeLa cell extracts independently using bacterially expressed and purified CDK2. Comparable values were obtained using bacterially expressed and reticulocyte lysate translated CDK2 as standards (data not shown); hence no compensation was made for the residual methionine in the reticulocyte lysate. It is possible to add unlabeled methionine to the reticulocyte lysate to adjust the specific activity of [^{35}S]methionine to a specific value. Proteins not in the cell-free extracts but in the particular matters are not included in the quantitation described here. It is possible to lyse the cells directly in SDS sample buffer without making cell-free extracts to avoid the loss of proteins in the pellet. We found that nearly all of the cyclins and CDKs were extracted by the procedures we used (unpublished data); hence we do not think that the values we measured are a gross underestimation in this regard.

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