Biochemical Characterization of p16\(^{INK4}\) - and p18-containing Complexes in Human Cell Lines*  

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The regulation of the D-type cyclin-dependent kinase (CDK4 and CDK6) activity appears to be the key step in the progression of eukaryotic cells through the G1 cell cycle phase. One of the mechanisms involved in this process is the binding of some small proteic inhibitors, with a molecular mass ranging between 14 and 20 kDa, to these CDKs. We have evaluated the amount of two such inhibitors, namely p16\(^{INK4}\) and p18, in normal and transformed cells, as well as the biochemical features of the macromolecular complexes containing these proteins. The results obtained indicated that (i) p18 gene expression, unlike p16\(^{INK4}\) gene, is not regulated by pRB status, (ii) no evident relationship exists between the expression of p16\(^{INK4}\) and p18 genes, (iii) significant amounts of the two proteins are not bound to CDKs but occur as free molecules, (iv) each inhibitor forms a complex with the CDK protein with a 1:1 stoichiometry, and (v) a competition exists between cyclin D and the inhibitor protein toward the CDK protein resulting in the absence of detectable cellular free kinase. Moreover, employing the human native partially purified p16\(^{INK4}\) or the pure recombinant protein, we have been able to demonstrate in vitro the dissociation of CDK4-cyclin D1 complex and the formation of CDK4-p16\(^{INK4}\) bimolecular complex. Our findings suggest that during the cell division cycle the members of the p16\(^{INK4}\) proteic family and cyclin Ds compete for binding to CDK4/CDK6 and that their quantitative ratio is essential for G\(_1\) → S transition.

In eukaryotes the progression through the cell cycle is due to a biochemical cycle in which distinct cyclin-dependent serine-threonine kinases (CDKs) are sequentially activated by different cyclins (1, 2). Then activated CDKs regulate their target molecules by phosphorylation. Finally, these downstream molecules carry out the steps that ultimately allow the ordered development of the cell division cycle (3–7). Thus, the regulation of CDK activity is the key event in the cell cycle progression.

The level of the various CDK proteins generally does not vary remarkably during the cycle, whereas the amount of each cyclin undergoes dramatic changes. During the early G\(_1\) phase, in particular, there occurs the accumulation of three D-type cyclins (D1, D2, and D3) (8–11), which assemble into holoenzymes with either CDK4 (5, 7, 12) or, in a minor number of cell types, CDK6 (13, 14). Interaction between cyclin Ds and these CDKs is not sufficient to activate the kinase activity of the holoenzyme, since functioning CDKs also require phosphorylation at a single threonyl residue (15).

Additional proteins are normal constituents of complexes formed by D-type cyclins and CDK4 (or CDK6), including the proliferating cell nuclear antigen and a small protein named p21. Proliferating cell nuclear antigen, the processivity factor required by eukaryotic DNA polymerases \(\delta\) and \(\epsilon\) (16–21), is involved in the control of the rate of chromosome replication as well as in the repair of damaged DNA (22, 23). p21 is a negative regulator of all CDK multisubunit complexes, including the kinases of this family involved in the progression of the cycle through S, G\(_2\), and M phases (24–27). The protein is a usual constituent of these holoenzymes in their active form (24), but when the level of p21 increases, the stoichiometry of the complexes appears modified with the subsequent inhibition of the kinase activity (26).

A proteic inhibitor structurally and functionally linked to p21, namely p27\(^{kip1}\), was also found to bind to and thus to inhibit various cyclin-CDK enzymatic activities. This protein seems to play a key role in the regulation of G\(_1\) cell cycle arrest by transforming growth factor \(\beta\), cAMP, and cell-cell contact (28–30).

A further key CDK proteic inhibitor, in addition to the above described, is a small peptide named p16\(^{INK4}\). Two main functional and structural differences appear to exist between p21/p27\(^{kip1}\) and p16\(^{INK4}\), namely (i) p21/p27\(^{kip1}\) are expressed mainly as a consequence of external stimuli that inhibit cell growth (25, 31, 32) and/or that might induce cell differentiation (33, 34), whereas p16\(^{INK4}\) seems to belong to an intrinsic regulatory loop mainly related to the control of pRB phosphorylation status (35); and (ii) p21 (and p27\(^{kip1}\)) forms multisubunit complexes containing CDK and other proteins, whereas p16\(^{INK4}\) seems to form a binary complex with CDK4 or CDK6, thus destroying or preventing the formation of the kinase active complexes. It is noteworthy, in this context, that very few direct biochemical analyses have been carried out to investigate the cellular amount and stoichiometry of complexes involving p16\(^{INK4}\) that occur in the G\(_1\) phase (36).

The importance of a detailed characterization of in vivo occurring p16\(^{INK4}\)-containing complexes has greatly increased since the discovery of a strict connection between p16\(^{INK4}\) gene alterations and cancer development. Indeed a tremendous number of studies clearly revealed the inactivation of this gene...
in a very large range of human tumors and transformed cell lines (37–45), thus pointing to this gene as a new important tumor suppressor gene.

It is noteworthy that 9p21 deletions, the locus of the p16NK4 gene, often involve a gene strictly linked to p16NK4, which is named p15NK4B gene or p14NK4B gene (46, 47). Such a gene codifies for a protein that presents a noticeable degree of structural homology with p16NK4 and also a superimposable mechanism of action. However, the few regulatory data available (46) indicate that p15NK4B gene expression is up-regulated by external cellular stimuli (tumor growth factor β treatment), while p16NK4 appears to be an intrinsic and constitutive brake of cell proliferation.

Recently, a third member of the p16NK4 gene family has been identified and cloned (47). This gene codifies for a protein, named p18, which also shows considerable structural and functional homology with p16NK4, being able to bind to and inhibit strongly CDK6 and, perhaps in a weaker way, CDK4. Interestingly, the p18 gene is localized at the 1p32 chromosomal region, which is an area well known in molecular oncology, being altered in several tumors (47).

Due to the strict linkage between CDK inhibitors, including p16NK4 and p18, and cancer development we have begun research aimed at investigating the amount and distribution of these putative tumor suppressor proteins in normal and transformed cells. In order to get information on these aspects we have analyzed the stoichiometry and the levels of the p16NK4, and p18-containing complexes in some established cell lines by means of gel filtration chromatography followed by immunoblotting analysis of the single fractions. We have also attempted to reproduce in vitro the effect of p16NK4 protein on the structure of cellular CDK4-containing complexes.

Our results allow (i) a direct evaluation of the type of complexes involving p16NK4 and p18 and (ii) the determination of the stoichiometry of complexes involving these CDK inhibitors. Moreover, in this paper we propose a new methodology to investigate the interactions existing between the molecules involved in the cell division cycle. The findings are discussed in light of the possible meaning of p16NK4 and its homologue p18 in cell division cycle progression and in cancer development.

**EXPERIMENTAL PROCEDURES**

Materials—Alkaline phosphatase-conjugated goat anti-rabbit IgG, 3-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, phenylmethylsulfonyl fluoride, tosylphenylalanin chloromethyl ketone, reduced dithiothreitol, Nonidet P-40, chymostatin, isopropyl-ß-D-thiogalactoside, ovalbumin (43 kDa, fractions 42–43), carbonic anhydrase (30 kDa, fractions 49–50) and cytochrome c (12.4 kDa, fractions 61–63). The standards were run before and after three sample analyses to verify the efficiency of separation. Less than 5% variation in the elution times was noticed among all the chromatographic analyses carried out. The fractions were added with protease inhibitor mixture (final concentrations as indicated above for the lysis buffer) and stored at 4°C when immediately analyzed or at −80°C when stored for prolonged time periods.

Effect of Partially Purified p16NK4 or Recombinant p16NK4 on U-118 Cell Extracts—Three fractions (53, 54, and 55) obtained from three different FPLC chromatographic analyses of HBL-100 extract were pooled (total volume, 1.8 ml). 200 μl of such partially purified free p16NK4 protein (about 3–6 μg of protein) were added to 300 μl of U-118 cell extract (about 2 mg of protein) and incubated for 30 min at 30°C. The assay mixture was then applied onto a Superdex-75 HR column and chromatographed as described above. Each fraction was then analyzed by immunoblot for cyclin D1, CDK4, and p16NK4 proteins. Moreover an additional 200-μl aliquot of the p16NK4 pool was analyzed by FPLC, and the fractions were studied by immunoblotting employing anti-p16NK4 antibodies.

An identical experimental scheme was employed to investigate the effect of human recombinant pure p16NK4 on naturally occurring cyclin D1-CDK4 complex. In this case, different amounts of protein (namely 1, 10, or 100 ng of p16NK4) were added to 2 mg of U-118 extracts, and the mixture was processed as described above.

In the investigations employing partially purified native p16NK4, we also performed a negative control experiment using 200 μl of the 16NK4 pool that was depleted by this polypeptide as follows. 15 μl of antiserum anti-p16NK4 were incubated with 3 h with 100 μl of protein A-agarose was incubated with 300 μl of such partially purified free p16NK4 protein (about 3–6 μg of protein) and incubated for 30 min at 30°C. The assay mixture was then applied onto a Superdex-75 HR column and chromatographed as described above. Each fraction was then analyzed by immunoblot for cyclin D1, CDK4, and p16NK4 proteins. Moreover an additional 200-μl aliquot of the p16NK4 pool was analyzed by FPLC, and the fractions were studied by immunoblotting employing anti-p16NK4 antibodies.

Immunoblotting and Immunoprecipitation—About 40 μl of each FPLC fraction or 50–200 μg of cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis employing a 15% polyacrylamide resolving gel, transferred to a nitrocellulose membrane, and incubated with different antiserum (48). The immunocomplexes were detected by the alkaline phosphatase method or by the ECL techniques as described in Ref. 51. Immunoprecipitation experiments on cell extracts were carried out essentially as described in Ref. 51, employing various antibodies. The immunoprecipitates were then analyzed by immunoblotting as described above.
Characterization of p16INK4- and p18-containing Complexes

p16INK4 and p18 Protein Level in Human Cells—Although both p16INK4 and p18 proteins have been demonstrated to be capable of binding to and in inhibiting CDK4 and CDK6 activities, few data on the amount of these two proteins in a specific cell type are available. To clarify this point, we have examined few data on the amount of these two proteins in a specific cell capable of binding to and inhibiting CDK4 and CDK6 activities, NA, not available.

Table I

| Cell type     | Normal | Transformed |
|---------------|--------|-------------|
| Normal Cell   |        |             |
| Osteoblasts   | Bone   | HBL-100     |
| Fibroblasts   | Skin   | Breast      |
| Condrocytes   | Cartilage | U-118  |
| Transformed   |        |             |
| ZR-75         | Breast | HBL-100     |
| HBL-100       | Breast | Breast      |
| MCF-7         | Breast | Breast      |
| MDA-231       | Breast | Breast      |
| Saos-2        | Osteosarcoma | + + + + |
| U-118         | Gloma  | + + + + +   |
| MG-P          | Gloma  | + + + + +   |
| Caco-2        | Colon  | + + + + +   |
| Saos-2        | Colon  | + + + + +   |
| K562          | Leukemia | + + + +   |
| CCRF-CEM      | Leukemia | + + + + + |
| OCC-A         | Ovary  | + + + + +   |
| Hep-LT        | Liver  | + + + + +   |

RESULTS

p16INK4 and p18 Protein Level in Human Cells—Although both p16INK4 and p18 proteins have been demonstrated to be capable of binding to and in inhibiting CDK4 and CDK6 activities, few data on the amount of these two proteins in a specific cell type are available. To clarify this point, we have examined their levels in three primary cultures obtained from normal mesenchymal tissues and in a number of transformed cell lines by means of immunoblotting technique. Some of them, like many established cell lines, do not express p16INK4 as a consequence of the homozygous deletion of the codifying gene.

As reported in Table I, all the primary cell cultures express clearly evident and quite similar levels of both p16INK4 and p18 proteins (Fig. 1); in particular, the osteoblastic cells appear to produce remarkably high levels of p16INK4 protein. On the other hand, due to either the malignant condition of the cell or their different histological origin, signals of different intensity were obtained by analyzing the established cell lines (Fig. 1).

Moreover, and more importantly, no relationship seems to exist between the level of p16INK4 and p18, thus suggesting the absence of common regulatory loop(s) that could control the expression of the two genes. Table I also reports the information available on the status of RB1 gene in some of the cells analyzed. These data confirm that the functional inactivation of pRB is a strong signal for p16INK4 gene expression and, moreover, interestingly, suggest that this mechanism does not control the p18 gene.

The probable independence of p16INK4 and p18 gene expression along with the notion that both of these proteins are known to form complexes with CDK4 and CDK6 prompted us to investigate the biochemical features of the complexes involving these CDK inhibitors by using cell extracts prepared from some of the cell lines reported in Table I. Indeed, as stated in the Introduction, while several studies have been carried out by following the inhibition of the kinase activity and by using recombinant proteins, few biochemical investigations have been performed by employing cellular extracts.

We used the data obtained (Table I and Fig. 1) to select three cell lines (namely U-118, HBL-100, and Saos-2) in order to characterize the composition and stoichiometry of the complexes involving p16INK4 and p18 proteins. The rationale of this choice was the following. U-118, like a number of glioma cell lines, does not express p16INK4 protein; thus, it seems an excellent source to analyze only complexes involving p18 protein. Saos-2, an osteosarcoma-derived cell line, contains a significant amount of both p16INK4 and p18 proteins. Although this cell line has an inactivated RB1 gene, the levels of both p16INK4 and p18 are similar to those of its normal counterpart, namely human osteoblasts (Table I). Thus, Saos-2 could represent an appropriate model to investigate the type and amounts of complexes containing these two CDK inhibitors. Finally, the HBL-100 cell line showed a high level of p16INK4 and scarce amounts of p18; thus, it might be an excellent source of human native p16INK4 protein.

The cellular contents of CDK4, cyclin D1, p15INK4B, p21, and p27kip1 were then analyzed in the selected lines by using direct immunoblotting with specific antibodies. The levels of these proteins were evaluated since they represent either proteins potentially interacting with p16INK4 and p18 or additional CDK inhibitors. The results of direct immunoblotting analyses of extracts from these cell lines were reported in Fig. 2. CDK4 was clearly expressed in U-118, Saos-2, and HBL-100, although the amount of the kinase was higher in Saos-2 and U-118 than in HBL-100. Cyclin D1 was distinctly detectable only in U-118, whereas it appeared lacking in HBL-100 and Saos-2. p21 (data not reported) and p15INK4B signals were not evident in the cell extracts while faint bands (probably a doublet) of p27kip1 protein were observable in all three cell lines.

The levels of cyclin D2 and D3 and CDK6 were not investigated, since these proteins were not expressed in these cell lines with the only exception of small amounts of CDK6 in U-118 (14, 51).

On the basis of these results, we hypothesized that U-118 cells should contain complexes formed by CDK4-p18 and/or CDK4-cyclin D1, while both CDK4-p16INK4 and CDK4-p18 might be present in Saos-2 and only CDK4-p16INK4 in HBL-100. Moreover, the high level of p16INK4 protein demonstrated in HBL-100 allowed us to choose this cell line as an optimal source of this protein in order to prepare adequate amounts of native p16INK4 protein, possibly free from other components of the cell cycle.

Characterization of p16INK4, and p18-containing Complexes in HBL-100, Saos-2 and U-118 Cell Lines—Although we could not rule out the possibility that the entire amount of p16INK4 was bound to CDK4, the high level of this protein in HBL-100 suggested to us the possible presence of free protein. To investigate this possibility, we decided to analyze the molecular mass of the complexes involving the CDK inhibitors in this cell line by separating an HBL-100 cellular extract on a gel filtration column and analyzing the amount of p16INK4 and CDK4 occurring in each fraction by direct immunoblotting. The cellular extracts were separated by means of a Superdex-75 HR
column, which allows a very high resolution of native proteins with a molecular mass lower than 80 kDa. Fig. 3A shows a typical result of these experiments; each fraction obtained after the chromatographic separation was analyzed by immunoblotting for the p16\(_{\text{INK4}}\) and CDK4 content. Cyclin D1 was not investigated, since such a cell line lacks this protein (see Fig. 2).

Two clear peaks of p16\(_{\text{INK4}}\) were detectable: one occurring at a molecular mass of about 45–55 kDa and the other one at about 15–20 kDa. The two peaks are completely resolved, and no additional signals were evident in other areas of the chromatographic analysis, even when high amounts of sample (10 mg of total proteins) were applied to the column (data not reported). When CDK4 occurrence was analyzed in the same fractions, only a peak of about 45–55 kDa could be detected. Totally superimposable results were obtained when Saos-2 cell extracts were analyzed by means of this methodology (data not shown). Indeed, in this cell line, two distinct pools of p16\(_{\text{INK4}}\) protein occurred, one probably bound to CDK4 and a second free of bound protein(s). Moreover, when Saos-2 was analyzed for p18, only a peak corresponding to the unbound p18 form was observed (data not shown).

The fractions containing free p16\(_{\text{INK4}}\) protein from HBL-100 cell line were rechromatographed under the same conditions, and the protein was again eluted with an estimated molecular mass between 15 and 20 kDa (data not shown).

From a rough estimation of the p16\(_{\text{INK4}}\) content of each elution area by scanner analysis we could calculate that the free protein represented more than 80\% of its total content in HBL-100, whereas in Saos-2 it was about 60\%. These findings indicate that in these two cell lines a significant amount of p16\(_{\text{INK4}}\) protein occurs in a free form, which largely exceeds that bound to CDK4. Moreover, in order to confirm the interaction between CDK4 and p16\(_{\text{INK4}}\) protein in HBL-100, the cellular extract and the relevant fractions were immunoprecipitated with anti-p16\(_{\text{INK4}}\) or anti-CDK4 antisera, and then the immunocomplexes were analyzed using antibodies against CDK4 and p16\(_{\text{INK4}}\). The results showed the occurrence of both p16\(_{\text{INK4}}\) and CDK4 in the immunoprecipitates, thus confirming the presence of the interaction between these two proteins (Fig. 3B).

Successively, we analyzed the complexes occurring in U-118 cells employing the same methodology described above. In this case, we evaluated in each fraction the occurrence of p18, CDK4, and cyclin D1, since this cell line lacks p16\(_{\text{INK4}}\) protein while presenting a detectable amount of the D-type cyclin (Fig. 2). As showed in Fig. 4, like for p16\(_{\text{INK4}}\) in HBL-100, two peaks of p18 were identified corresponding to the free form and to that bound to CDK4. This cyclin-dependent kinase was clearly detectable in the flow-through of the column, occurring in complexes with a molecular mass higher than 80 kDa. Moreover, a faint but distinct CDK4 signal, representing about 5–10\% of the total, was observable at a molecular mass around 45–55 kDa. Cyclin D1 was detectable exclusively in the flow-through fractions, and no additional cyclin signals could be evidenced (Fig. 4).

Moreover, to gain additional information on the distribution of the two CDK inhibitors, we investigated the cellular distribution of p16\(_{\text{INK4}}\) and p18 in Saos-2, since this cell line contains a level of these two proteins similar to that of the normal counterpart. The results obtained (data not shown) suggest that both the inhibitors are localized at the nuclear and cytoplasmic compartment, and when the localization of p16\(_{\text{INK4}}\) protein was investigated in HBL-100, we observed a detectable amount at the cytoplasmic compartment probably due to the unphysiological overexpression.

Direct in Vitro Effect of p16\(_{\text{INK4}}\) Protein on CDK4 Com-
In order to demonstrate that the observed free $p16^{INK4}$ protein is functionally active and does not represent an inactive form of the inhibitor, we studied the effect of fractions containing free $p16^{INK4}$ protein on the CDK4 complexes occurring in U-118 extracts. Indeed, U-118 cells do not express $p16^{INK4}$ protein (see Fig. 1) and show the great majority of CDK4 (more than 90%) occurring in multisubunit complexes with a molecular mass higher than 80 kDa (Fig. 4). Since this cell line contains remarkable levels of cyclin D1 (and not cyclin D2 and D3), it is highly probable that such complexes also include cyclin D1 and/or additional proteins. The addition of $p16^{INK4}$ to preformed cyclin D1-CDK4 complexes should also provide information on the possibility of forming ternary complexes.

Prior to carrying out these experiments, the fractions containing unbound $p16^{INK4}$ protein (prepared from HBL-100 extracts) were analyzed by immunoblotting by using sera directed against $p15^{INK4}$, $p18$, $p21$, and $p27^{INK4}$ protein. Such analysis was performed to rule out possible interference due to additional known small CDK inhibitors. As shown in Fig. 5, the pool of fractions containing free $p16^{INK4}$ did not show any of the above-mentioned inhibitors.

The sample containing partially purified HBL-100 $p16^{INK4}$ was then added to U-118 cell extracts and incubated at 30 °C for 30 min. The mixture was then applied to a Superdex-75 HR column and separated as described above. Finally, each fraction was analyzed by immunoblotting for CDK4, cyclin D1, and $p16^{INK4}$ content. Similar experiments were also carried out by employing various amounts of human recombinant pure $p16^{INK4}$. As seen in Fig. 6A, a clear shift of CDK4 molecules from fractions at high molecular mass to fractions around 45-55 kDa was observable by using both partially purified native $p16^{INK4}$ or known amounts of recombinant protein. Moreover, the amount of CDK4-$p16^{INK4}$ complex formed appeared to be strictly dependent on the quantity of the added recombinant protein (Fig. 6C). The displacement of CDK4 from the complex with cyclin D1 and the formation of the CDK4-$p16^{INK4}$ complex was also confirmed by the shift of $p16^{INK4}$ from the molecular free form to that bound to CDK4 (Fig. 6B).

Several negative control experiments were carried out to verify that the effect on CDK4-cyclin D1 containing complexes of the partially purified preparation from HBL-100 was due to $p16^{INK4}$. In particular, we observed that incubation of U-118 extracts with (i) fractions from FPLC chromatography lacking $p16^{INK4}$ protein or (ii) fractions depleted of $p16^{INK4}$ by treatment with specific antibodies (see "Experimental Procedures" for details) did not modify the elution pattern of the U-118 CDK4-complexes or other known small CDK inhibitors that were eluted. B. 200 μL of partially purified $p16^{INK4}$ protein were separated by gel filtration FPLC and 40-μL aliquots of the reported fractions were analyzed by immunoblot and probed with antibodies against CDK4. At the top of the panel, the arrows show fractions in which the indicated molecular mass protein standards were eluted. C. Effect of different quantities of recombinant $p16^{INK4}$ protein on the preformed CDK4-cyclin D1 complex. The amount of CDK4-$p16^{INK4}$ complex was determined by scanning the immunoblotting analyses (carried out in triplicate) and reported as percentage of total CDK4. The amount of recombinant $p16^{INK4}$ protein employed was reported in nanograms.

**DISCUSSION**

The data reported in the present paper allowed a direct biochemical evaluation of the amount and composition of the...
cellular complexes containing p16<sub>INK4</sub> and p18 proteins, two important CDK inhibitors and putative tumor suppressor proteins. These results have been obtained by analyzing total cell extracts and thus should correspond to the native condition. Moreover, the methodology we developed allowed an accurate estimation of the molecular mass of the complexes evidenced and consequently a clear definition of the stoichiometric ratio of their components.

Some main conclusions could be drawn by our findings. First of all, p18 expression is largely independent of pRB status, and no relationship seems to exist between the expression on p16<sub>INK4</sub> and p18 genes. Second, the occurrence (as demonstrated in U-118 cells) of CDK4-cyclin D1 complexes in the presence of high levels of free p18 indicates that the ratio of CDK inhibitors to cyclins plays a key role in establishing the very delicate equilibrium among the various CDK-containing complexes. Third, significant amounts of cellular p16<sub>INK4</sub> and p18 proteins might occur in a free active form. This finding has been demonstrated in all three cell lines analyzed and, in particular, in one (namely Saos-2) that contains levels of inhibitors superimposable to those of normal osteoblasts, its untransformed counterpart.

Finally, this paper reports, for the first time, direct biochemical experiments demonstrating the in vitro disassembling of a CDK4-cyclin D1 complex after the addition of human p16<sub>INK4</sub> protein with the contemporaneous formation of a CDK4-p16<sub>INK4</sub> complex.

The interest in p16<sub>INK4</sub> protein has enormously increased since the almost definitive demonstration that the p16<sub>INK4</sub> gene is inactivated in a tremendous number of different human cancers (37, 38, 40, 42–45). This finding along with p16<sub>INK4</sub> gene has been demonstrated in all three cell lines analyzed and, in particular, in one (namely Saos-2) that contains levels of inhibitors superimposable to those of normal osteoblasts, its untransformed counterpart.

We were also able to reproduce the formation of the CDK4-p16<sub>INK4</sub> complex in vitro by adding p16<sub>INK4</sub> protein (both native and recombinant) to cell extract that did not contain p16<sub>INK4</sub> protein. Interestingly, no cyclin D1 was observed in the fractions containing CDK4 and p16<sub>INK4</sub> thus allowing the exclusion of the formation of a ternary complex. It has been previously reported that when recombinant p16<sub>INK4</sub> is added to a preassembled cyclin D-CDK complex, it forms a stable ternary complex and inhibits the kinase activity without displac-
ing the cyclin (53). Conversely, our data rule out the formation of ternary complexes and argue in favor of a competition between p16INK4 and cyclin D toward CDK. It is of note that we used partially purified p16INK4 protein and total cellular extracts in an attempt to carry out experiments under conditions similar to the in vivo situation.

From a regulatory point of view, the results shown in Fig. 1 and Table I further support the idea of an inverse relationship between the level of functioning pRb1 and the level of p16INK4 protein (47), which suggests that the p16INK4 gene is under the control of pRb1 status (35, 36, 47). The same regulatory loop does not appear to involve the p18 gene, since cell lines that lack functioning pRb1 protein do not express a p18 level higher than that of cell lines containing a wild-type Rb1 gene (Table I). In the U-118 cell line, which lacks active p16INK4 gene, we observed a strong p18 signal (Fig. 1), and a similar result was obtained in three additional glioma cell lines with a homozygous deletion at the p16INK4 gene level (Fig. 1 and data not shown). These findings initially suggested to us the possibility that p18 overexpression might compensate for the absence of p16INK4 protein, allowing the hypothesis of a regulatory loop involving the two CDK inhibitor genes. However, our successive screening on a panel of cell lines did not confirm this view, since we did not observe any clear up-regulation of p18 gene expression in p16INK4 gene-deleted cell lines (Table I and Fig. 1). Possibly, the high levels of p18 in glioma cells are related to the pattern of p18 tissular expression (47, 53).

An additional consideration is that the occurrence of high levels of p18 might cast some doubts on the role of p16INK4 gene as a tumor suppressor gene since the two proteins have apparently similar functions. However, the very great amount of data on the inactivation of p16INK4 gene in human cancers seems to be an excellent indication for its role in cancerogenesis. In addition, the p18 gene, given its chromosomal localization on 1p32 chromosome, has also been proposed as a further potential tumor suppressor gene. However, experiments carried out in our laboratory on primary tumors (acute lymphoblastic leukemias, neuroblastomas, and rhabdomyosarcomas)2 and on malignant cell lines of various origin (Table I) seem to rule out the p18 gene inactivation in human cancer. Such a conclusion also suggests that p18 protein, unlike p16INK4, might have a different function in the control of the cell division cycle (or other processes) irrespective of the apparent structural and functional similarities between these two CDK inhibitors.

Overall the data obtained in normal and transformed cells suggest that the cellular content of the members of the p16INK4 family is redundant, especially taking into consideration that apparently all of them seem to have the same function. Thus, it is totally conceivable that an important aliquot of cellular CDK4 and CDK6 is present in inactive binary complexes in all the phases of cell cycle, whereas only a fraction of this kinase pool is activated in G1 phase by the build-up of cyclins. Our findings indirectly support this hypothesis, since we were unable to detect free CDK4. We can therefore hypothesize that the amount of CDK4/CDK6/p16INK4 (and CDK4/CDK6/p15INK4/p18/p19) complexes varies during the cell cycle as a consequence of cyclin D1 level changes.

In conclusion, we strongly believe that in the future major efforts should be devoted to characterizing the molecular structure and the precise quantity of each complex involved in the cell cycle progression and that the methodology we have proposed might be particularly useful to this aim. Moreover, these analyses should be carried out mainly by employing primary cell cultures from adult mammalian tissues and by holding in due account the specific cell lineage origin.

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