Phosphorylations of cyclin-dependent kinase 2 revisited using two-dimensional gel electrophoresis

Katia Coulonval§, Laurence Bockstaele, Sabine Paternot, and Pierre P. Roger§

Institute of Interdisciplinary Research (K.C., L.B., S.P., P.P.R.) and Protein Chemistry Department (K.C.), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, B-1070 Brussels, Belgium.

§Address all correspondence and requests for reprints to : Pierre P. Roger or Katia Coulonval, I.R.I.B.H.M., ULB, Campus Erasme, Building C, 808 Route de Lennik, B-1070 Brussels, Belgium.
Tel. : 322 555 4153
Fax. : 322 555 4655
E-mail : proger@ulb.ac.be, koulonv@ulb.ac.be

Running title : 2D-gel electrophoresis pattern of CDK2 phosphorylations
SUMMARY

To control the G1/S transition and the progression through the S phase, the activation of the cyclin-dependent kinase (CDK) 2 involves the binding of cyclin E then cyclin A, the activating Thr160-phosphorylation within the T-loop by CDK-activating kinase (CAK), inhibitory phosphorylations within the ATP-binding region at Tyr15 and Thr14, dephosphorylation of these sites by cdc25A, and release from Cip/Kip family (p27kip1 and p21cip1) CDK inhibitors. In order to re-assess the precise relationship between the different phosphorylations of CDK2, and the influence of cyclins and CDK inhibitors upon them, we introduce here the use of the high resolution power of two-dimensional gel electrophoresis, combined to Tyr15- or Thr160- phosphospecific antibodies. The relative proportions of the potentially active forms of CDK2 (phosphorylated at Thr160 but not Tyr15) and inactive forms (non phosphorylated, phosphorylated only at Tyr15, or at both Tyr15 and Thr160), and their respective association with cyclin E, cyclin A, p21 and p27, were demonstrated during the mitogenic stimulation of normal human fibroblasts. Novel observations modify the current model of the sequential CDK2 activation process: (i) Tyr15-phosphorylation induced by serum was not restricted to cyclin-bound CDK2; (ii) Thr160-phosphorylation engaged the entirety of Tyr15-phosphorylated CDK2 associated not only with a cyclin but also with p27 and p21, suggesting that Cip/Kip proteins do not prevent CDK2 activity by impairing its phosphorylation by CAK; (iii) the potentially active CDK2 phosphorylated at Thr160 but not Tyr15 represented a tiny fraction of total CDK2 and a minor fraction of cyclin A-bound CDK2, underscoring the rate limiting role of Tyr15-dephosphorylation by cdc25A.
INTRODUCTION

The major events of the eukaryotic cell cycle depend on the sequential and ordered formation, activation (by phosphorylation and/or dephosphorylation) and then inactivation of different complexes of cyclin-dependent kinases (CDKs). CDK2 plays an essential role in controlling the G1/S transition and the progression through the S phase: it participates in the inactivating phosphorylations of pRb and related p130 (1-4) and it phosphorylates other key substrates whose activities are necessary to trigger and organize the DNA synthesis phase while preventing DNA re-replication (5-9). Since the critical roles of CDK2, both in positive and negative mitogenic controls of cell cycle and as the endpoint target of DNA damage checkpoint mechanisms, have been well established (10-13), understanding the details of CDK2 regulation is of fundamental importance.

Based on the cdc2/CDK1 activation model (14-16) and on structural (17) and enzymatic studies (18-24), the consensual framework of CDK2 activation is the following. The binding of a cyclin partner (cyclin E at the G1/S transition, cyclin A during the S phase) confers a low basal activity to the cyclin-CDK2 complex and enables subsequent phosphorylation of CDK2 by the CDK-activating kinase (CAK / cyclin H-CDK7) on a conserved threonine residue in the activation loop (Thr160, T160), which is essential for proper alignment of the kinase domain. Further phosphorylation within the ATP-binding region of CDK2, at Tyr15 (Y15) by the Wee1 kinase and, to a lesser extent, at Thr14 (T14) inhibits the kinase activity, which can be restored through dephosphorylation by the dual-specificity cdc25A phosphatase (25,26). The activity of cyclin-CDK2 complexes is also negatively regulated by the binding of inhibitory proteins belonging to the Cip/Kip family (10) (p27kip1, rapidly degraded consecutively to Thr187 phosphorylation by the cyclin E-CDK2 complexes (27), and p21cip1). In vitro studies have indicated that p27 and p21 interfere with CAK-induced phosphorylation of CDK2 (21,28,29), which is supported by cristal
structure analysis of the p27-cyclin A-CDK2 complex (30). p27 also inhibits the activity of cyclin A-CDK2 by other mechanisms, including insertion in the catalytic cleft of CDK2 (30). In human fibroblasts, low stoichiometry p21-binding was nevertheless found to support rather than prevent the catalytic activity of cyclin A-CDK2 complexes (31,32). This was not observed by others (33,34) and questioned by an in vitro investigation (35).

The precise relationship between the different inhibitory and activating phosphorylations of CDK2, and the influence of cyclins and CDK inhibitors on these phosphorylations, have never been investigated in the context of endogenous proteins in a normal human cell. Here, we introduce the utilization of the high resolution power of the two-dimensional gel electrophoresis, combined to Tyr15- or Thr160- phosphospecific antibodies, to demonstrate the relative proportions of the different CDK2 phosphoforms and their respective association with cyclin E, cyclin A, p21 and p27, during the mitogenic stimulation of normal human diploid fibroblasts. Some unexpected observations prompt a reevaluation of the accepted sequence of regulatory events involved in the complex activation process of CDK2.
EXPERIMENTAL PROCEDURES

Cell culture - Human diploid fibroblasts (IMR-90) (American Type Culture Collection, Manassas, VA) were obtained at population doubling 24 and grown for not more than 40 total population doublings in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO) supplemented with antibiotics and 10% fetal calf serum (FCS) as described (36). After reaching semiconfluency, they were synchronized by starvation in 0.2% FCS for 3 days. Quiescent cells were then growth stimulated by addition of FCS (20%). Bromodeoxyuridine (BrdU) labeling of DNA-replicating cells was obtained at the end of the mitogenic stimulation by a 30-min incubation in the presence of 10\(^{-4}\) M BrdU, and detected as described (37).

Immunoprecipitation - For the analysis of protein complexes, IMR-90 cells in 10-cm Petri dishes were washed with calcium/magnesium-free PBS and lysed on ice in 1 ml NP-40 lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM \(\beta\)-glycerophosphate, 10 mM DTT and protease inhibitors (pefablock, leupeptin). The homogenized (glass/glass) cellular lysate was sonicated twice, precleared with protein A sepharose (Amersham Biosciences, Uppsala, Sweden) and then incubated at 4°C for 3 hours with protein A-sepharose which had been preincubated overnight with 2 \(\mu\)g of antibody (monoclonal antibody against cyclin A (E72, NeoMarkers, Fremont, CA), rabbit polyclonal antibody against cyclin E (06-459, Upstate Inc, Charlottesville, VA), a mixture of the K25020 anti-p27 monoclonal antibody from Transduction Laboratories and the C-15 p27 polyclonal antibody (Santa Cruz Biotechnology, CA), the C-19 p21 polyclonal antibody from Santa-Cruz, or the DCS-11 cyclin D1 monoclonal antibody kindly provided by J. Bartek).

Alternatively, for the analysis of the 2D-gel electrophoresis pattern of the whole population of CDK2, cells in 10-cm Petri dishes were washed with PBS and scraped in 200 \(\mu\)l
of denaturing lysis buffer (50 mM Tris-HCl (pH 7.5), 0.6% SDS, 10 mM DTT, 50 mM NaF, 100 µM vanadate and protease inhibitors), boiled for 5 min and frozen. Cell lysates containing 300-500 µg of protein were cleared by centrifugation and diluted by adding five volumes of RIPA buffer without SDS (10 mM Tris-HCl (pH 7.5), 1% Na deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM NaF, 100 µM vanadate and protease inhibitors). They were then subjected to immunoprecipitation as above, except that the M2 polyclonal antibody (Santa-Cruz) was used for CDK2.

**Gel electrophoresis and western blotting** – For two-dimensional gel electrophoresis separations, immunoprecipitated proteins were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated by isoelectric focusing using the IPGphor apparatus from Amersham Biosciences and active in-gel rehydration as described (38) on immobilized linear pH gradient (pH 3 to 10) (IPG) strips (Amersham Biosciences). Isoelectric focusing was performed for a total of 30000 Vh, starting at 200 V and gradually raising the voltage to 8000 V. The IPG strip was then equilibrated for 15 minutes in 50 mM Tris (pH 8.5), 6 M urea, 30% glycerol, 1% SDS and 1% DTT, and for 15 minutes in 50 mM Tris, 6 M urea, 30% glycerol, 1% SDS and 5% iodoacetamide before loading onto SDS-polyacrylamide slab gels (12.5%) for separation according to molecular mass. After transfer on PVDF membranes, CDK2 was immunodetected using the phosphospecific-CDK2/CDK1 (Thr160 or Tyr15) antibodies from Cell Signalling Technology (Beverly, MA) or the M2 polyclonal CDK2 antibody (Santa-Cruz); membranes detected with the phosphospecific antibodies were reprobed with the M2 polyclonal antibody for detection of total CDK2. In some cases, long exposures were required to detect minor phosphorylated forms of CDK2 from low abundance complexes, which markedly enhanced the appearance of diffuse « background » spots. These very characteristic spots were clearly non-specific as exactly the same spots were obtained
when other polyclonal antibodies were used both for the immunoprecipitation and the immunodetection (e.g. CDK4 or p27 antibodies). In that cases, to improve the visibility of the specific CDK2 spots, the Adobe Photoshop program was used to decrease the grey levels of the background spots in the respective Figure panels (as indicated in Figure legends).

Whole cell extract proteins were separated according to molecular mass on SDS-PAGE (7, 10 or 12%) and immunodetected after western blotting. Equal amounts of protein were loaded for each condition. The phosphospecific and total CDK2 antibodies were as above. The JG39 cyclin A polyclonal antibody and the HE-12 cyclin E and MO.1.1 CDK7 monoclonal antibodies were kind gifts of Julian Gannon and Tim Hunt, and Jiri Bartek, respectively. The p21, p27 and p38 MAP kinase polyclonal antibodies were from Santa-Cruz and the cdc25A DCS-121 monoclonal antibody was from NeoMarkers (Fremont, CA).

All the experiments were reproduced at least twice with identical results.
RESULTS

CDK2 phosphorylations and CDK2 regulators during mitogenic stimulation of human fibroblasts – A maximum of DNA-synthesizing cells was observed 20 h after serum stimulation of quiescent IMR-90 cells (Fig. 1A). CDK2 and CDK2 regulatory proteins were analysed from whole cell extracts from quiescent unstimulated (control, Cont) cells or from cells that were stimulated by serum for 16 h or 24 h, i.e. when a maximum of cells were in early S-phase or late S-phase/G2 phase, respectively (Fig. 1B). CDK2 was present in quiescent cells and gradually accumulated after stimulation. CDK2 activating (T160) or inhibitory (Y15) phosphorylations were detected using phosphospecific antibodies, which also recognize the corresponding T161- or Y15-phosphorylated forms of cdc2/CDK1 (Fig. 1B). The T160-phosphorylation of CDK2 was already detected in quiescent cells and similarly increased after 16 h and 24 h of stimulation, preceding the T161-phosphorylation of cdc2 only observed at 24 h. By contrast, the Y15-phosphorylation was almost undectable in quiescent cells and appeared after cell stimulation on both CDK2 and cdc2, being maximum at 24 h. Cyclin E was detected in quiescent cells and was maximally expressed 16 h after serum stimulation. It thus preceded the accumulation of cyclin A, which was absent from quiescent cells and maximally expressed at 24 h. Like cyclin E, p21\textsuperscript{cip1} was present in quiescent cells and transiently enhanced 16 h after serum stimulation. By contrast, the presence of p27\textsuperscript{kip1} progressively decreased after cell stimulation. Cdc25A displayed several forms, which were increased or induced after serum stimulation. CDK7 was present in quiescent cells and slightly enhanced by serum stimulation. These different observations are in perfect agreement with previous reports from IMR-90 cells and other normal human fibroblasts (5,20,33,36,39-41).

In serum (16 h)-stimulated IMR-90 cells, a very large fraction of CDK2 was associated with p21 (Fig. 1C). A much smaller fraction of CDK2 was bound to p27. Serum
increased not only the presence of cyclin E-CDK2 complexes, but also the association of CDK2 with p21 and p27 (Fig. 1C). Both the inhibitory Y15- and activating T160-phosphorylations of CDK2 were found not only in cyclin E-CDK2 complexes, but also in CDK2 bound to p21 or p27 (Fig. 1C).

2D-gel electrophoresis analysis of the phosphorylation pattern of total CDK2 – CDK2 was immunoprecipitated (using the M2 CDK2 antibody) from quiescent unstimulated cells (Cont) or cells stimulated by serum for 16 or 24 h, and its different forms were resolved by isoelectric focusing (pH 3–10 linear gradient) followed by SDS-PAGE, and then detected by Western blotting using either the M2 CDK2 antibody, or the T160 or Y15 phosphospecific antibodies (Fig. 2). After immunodetection with the phosphospecific antibodies, membranes were systematically reprobed with the total CDK2 M2 antibody to ascertain the identity of the different forms and the homogeneity of the blotting transfer. In stimulated cells, multiple forms of CDK2 were distributed within an interval of 2.3 pH units (assuming the linearity of the supplied pH gradient gels) (Fig. 2). The most basic, and most abundant, form of CDK2 (form 0) focused at approximatively pH 9, which is close to the calculated isoelectric point of human CDK2 (8.80 ; as calculated according to (42) using the Compute Mw/pI tool provided by the ExPASy website). Besides CDK2 form 0, all the other forms of CDK2 were phosphorylated at Y15 and/or T160, as detected by the phosphospecific antibodies² (Fig. 2). These different CDK2 phosphoforms focused at positions that roughly correspond to those predicted for the addition to CDK2 of entire numbers of negative charges (computed position scale at the bottom of Fig. 2, calculated according to (42,43)). They were thus numbered according to their position relative to this scale (Fig. 2) and further identified by their demonstrated phosphorylation(s) (T, T160 ; Y, Y15 ; T,Y , T160+Y15). Considering that phosphate groups produce a two-charge isoelectric point shift above pH 7 (PO₄³⁻) or a one-
charge shift below pH 6 (HPO$_4^{2-}$) (43,44), CDK2 phosphoforms 2, 4 and 5/6 were expected to contain one, two or three phosphorylations, respectively. The minor intermediate spots could be explained by a small proportion of phosphate group in the HPO$_4^{2-}$ form or by other enzymatic or non-enzymatic covalent modifications (44). Together, informations on the isoelectric points and the detection by the phosphospecific antibodies allowed us to identify CDK2 forms 2T and 2Y as mono-phosphorylated at T160 and Y15, respectively; form 4T,Y as phosphorylated at both T160 and Y15; form 4Y as phosphorylated at Y15 and presumably T14; and minor forms 5T,Y and 6T,Y as three-fold phosphorylated at T160, Y15 and presumably T14. This determination is in perfect agreement with a previous investigation of CDK2 phosphorylations by $^{32}$Pi incorporation, tryptic peptide and mutagenesis analyses (18), which did not detect any other important phosphorylations, and demonstrated that the major phosphorylated sites are Y15 and T160 while the T14 phosphorylation is restricted to a fraction of Y15-phosphorylated CDK2. An identical 2D-gel electrophoresis pattern of CDK2 was obtained from primary cultures of canine thyroid epithelial cells (not shown), indicating that the present identification of CDK2 phosphoforms might apply to a wide variety of systems.

In control quiescent IMR-90 cells, CDK2 was essentially not phosphorylated$^2$. Only a small proportion of mono-T160-phosphorylated CDK2 was detected (Fig. 2). The inhibitory Y15-phosphorylation was induced by serum and was mostly represented by the mono-phosphorylated 2Y form. Smaller proportions of twice phosphorylated CDK2 forms containing either both the Y15- and T160-phosphorylations (form 4T,Y), or the Y15-phosphorylation but not the T160-phosphorylation (form 4Y, putatively containing the T14-phosphorylation), were also observed 16 h and even more 24 h after serum stimulation (Fig. 2). In control quiescent cells, the detection of a trace of the twice phosphorylated 4T,Y form required long exposures of the blots (Fig. 2). The activating T160-phosphorylation was also
strongly increased by serum stimulation. Surprisingly, it was essentially associated with Y15-phosphorylation (4Y,T and other minor Y,T forms). The potentially active mono- T160-phosphorylated form (2T) thus represented only a small fraction of T160-phosphorylated CDK2 and an extremely minor fraction of total CDK2 (Fig. 2).

Phosphorylations of cyclin E-bound CDK2 – Phosphospecific and total CDK2 antibodies were applied to 2D-gel separations from cyclin E co-immunoprecipitations (Fig. 3). In control quiescent cells, the twice T160+Y15-phosphorylated forms were the only CDK2 forms associated with cyclin E, as detected after long exposures of the blots. This complex should be present at a very low abundance, because the 4T,Y CDK2 form was faintly detectable only after long blot exposures from total CDK2 immunoprecipitations (Fig. 2). 16 Hours after serum stimulation, when cyclin E was maximally expressed (Fig. 1B), cyclin E-bound CDK2 was represented by non-phosphorylated CDK2 (form 0), mono- T160-phosphorylated CDK2 (form 2T) and T160+Y15-phosphorylated CDK2 (form 4T,Y) (Fig. 3). In sharp contrast with the situation observed in total CDK2 (Fig. 2), the most abundant cyclin E-bound CDK2 form was the potentially active mono- T160-phosphorylated one, and the Y15-phosphorylation was only observed in association with the T160-phosphorylation (Fig. 3). The CDK2 forms phosphorylated at Y15 but not T160 observed in total CDK2 (the abundant 2Y and minor 4Y forms in Fig. 2) were undetectable from cyclin E complexes (Fig. 3).

Phosphorylations of cyclin A-bound CDK2 – In the present experiments, cyclin A was absent from control quiescent cells and maximally expressed 24 h after serum stimulation (Fig. 1B). The phosphorylations of cyclin A-bound CDK2 were thus only analyzed at this time point (Fig. 4). At variance with total CDK2 and cyclin E-CDK2 complexes, almost no
unphosphorylated CDK2 was associated with cyclin A (Fig. 4). Though a small proportion of mono- Y15-phosphorylated CDK2 (2Y) was detected, the immense majority of Y15-phosphorylation was associated with the T160-phosphorylation in cyclin A complexes (Fig. 4). The potentially active mono- T160-phosphorylated form (2T, 1T) represented only a minor fraction of cyclin A-bound CDK2 (Fig. 4), in contrast to cyclin E-CDK2 complexes where this form was the most abundant one (Fig. 3).

Phosphorylations of p21-bound CDK2 – A large fraction of CDK2 was associated with p21 as judged from comparisons of CDK2 amounts (co)precipitated by p21 and CDK2 antibodies (Fig. 1C). At variance with cyclin E/A-CDK2 complexes, p21-bound CDK2 was clearly observed both in quiescent control and serum stimulated IMR-90 cells (Fig. 5A, B). Moreover, it was predominantly represented by the unphosphorylated 0 form, even in serum-stimulated cells (Fig. 5B). In control cells, the small proportion of T160-phosphorylation seen in p21 complexes (Fig. 5A, B) was similar to what was observed in total CDK2 (Fig. 2). Some p21-CDK2 complexes should thus exist without being associated with cyclin E or cyclin A. They could be associated with cyclin D1 (45), as cyclin D1-bound CDK2 only consisted of the unphosphorylated form 0 and of a very small proportion of the mono- T160-phosphorylated form 2T, both in unstimulated cells and 16 h after serum stimulation (data not shown). Aside from the association of unphosphorylated CDK2 with p21, in serum stimulated cells, p21 complexes contained cyclin E or cyclin A (Fig. 5A) and the pattern of p21-bound CDK2 phosphorylations (Fig. 5B) much resembled the CDK2 phosphorylations that are enriched in cyclins E/A complexes (Fig. 3, 4). The activating T160-phosphorylation was unexpectedly present in p21 containing complexes, associated or not with the Y15-phosphorylation, and no mono- Y15-phosphorylated CDK2 was detected (Fig. 5A, B). As compared with the T160+Y15-phosphorylated form 4T,Y, the mono- T160-phosphorylated
form (2T) was more abundant at 16 h than at 24 h (Fig. 5B). The distribution of p21-bound CDK2 phosphoforms was thus more similar to the CDK2 pattern associated with cyclin E at 16 h (Fig. 3), and to the pattern associated with cyclin A at 24 h (Fig. 4), consistently with the relative abundance of both cyclins at these time points (Fig. 1B).

**Phosphorylations of p27-bound CDK2** – We repeatedly failed to detect CDK2 in p27-immunoprecipitates from control unstimulated IMR-90 cells, as also mentioned by others (5). In serum-stimulated cells, p27-CDK2 complexes were also less abundant than p21-CDK2 complexes (Fig. 1C and (5)). At least part of p27 complexes contained cyclin E, as shown by reprobing with a p27 antibody the 2D gel blots of cyclin E immunoprecipitates (data not shown). The phosphorylation profile of p27-bound CDK2 16 h after serum stimulation (Fig. 6) was quite similar to the phosphorylation pattern of cyclin E-bound CDK2 (Fig. 3), with a predominant presence of the mono- T160-phosphorylated form (2T). As in the case of p21-bound CDK2, this surprising observation contrasted with the concept that Cip/Kip proteins inhibit the activity of CDK2 in part by preventing its phosphorylation by CAK. A small proportion of mono- Y15 phosphorylated form was also detected (Fig. 6), as in cyclin A complexes (Fig. 4).

**Comparison of total and complexed CDK2 from serum stimulated fibroblasts** – As illustrated in Fig. 7 and summarized in Table 1, the main novel observations from this study are: (i) the enrichment of T160-phosphorylated CDK2 forms, not only in cyclin E complexes and even more in cyclin A complexes (where unphosphorylated CDK2 was almost undetectable), but also in p21 and p27 complexes; (ii) the predominant presence in total CDK2 of the Y15-phosphorylation in forms devoid of the T160-phosphorylation (2Y, 3Y, 4Y), whereas in the different complexes the Y15-phosphorylation was restricted totally or almost
totally to T160-phosphorylated forms (3T,Y, 4T,Y, 6T,Y). This last intriguing observation implies that the Y15-phosphorylation induced by serum was not restricted to cyclin-CDK2 complexes and thus did not depend on cyclin binding, unlike the T160-phosphorylation. It might also imply that in the different complexes the Y15-phosphorylated forms could be preferentially recruited for T160-phosphorylation.
DISCUSSION

This study constitutes the first analysis of CDK2 phosphorylations using 2D-gel electrophoresis. In one-dimensional SDS-PAGE, the activating T160-phosphorylation generates a downward electrophoretic shift (18) and thus it has been easily investigated in numerous studies, which recently identify it as a direct target of mitogenic controls (46-48). By contrast, the inhibitory Y15-phosphorylation does not affect the SDS-PAGE migration of CDK2 (18), which explains in part the paucity of published information concerning the relationship of this phosphorylation with the other regulatory mechanisms of CDK2, despite the strong evidence of the crucial role of the Y15-phosphorylation emerging from the investigation of the cell cycle function of cdc25A (25) and the elucidation of DNA damage checkpoint mechanisms (49). The present characterization of the 2D-gel electrophoresis pattern of CDK2 using phosphospecific antibodies is fully consistent with the previous identification of major phosphorylation sites of human CDK2 (18). It allows to distinguish the different T160- and/or Y15-phosphorylated forms of CDK2 and thus to separate for the first time the potentially active CDK2 phosphorylated at T160 but not Y15 (nor T14), which was demonstrated here as a very minor fraction of CDK2 in human fibroblasts. As discussed below, the visualization of the relative stoichiometry of the different (un)phosphorylated forms of CDK2 associated or not with cyclins and CDK inhibitors alters the current concept of the sequence of regulatory events leading to CDK2 activation.

**T160-phosphorylated CDK2 is associated with cyclins and CDK inhibitors** – CAK (cyclin H-CDK7) has been initially reported to phosphorylate monomeric as well as cyclin-bound CDK2 *in vitro* (50). Furthermore, monomeric Cak1p-type CAKs from yeasts and plants exhibit a preference for cyclin-free CDKs (51). Nevertheless, the crystal structure study suggests that cyclin A-binding exposes the T-loop of CDK2 making it a better substrate for phosphorylation by CAK (52), in agreement with a more recent study clearly showing that
mammalian CAK preferentially phosphorylates cyclin-CDK2 complexes (21), as previously demonstrated for cdc2 (14). The nuclear translocation of CDK2 (40,53) associated with cyclin-CDK2 complex formation should also favor CDK2 phosphorylation by permitting the colocalization with nuclear CAK (54). Moreover, cyclin-binding protects T160-phosphorylated CDK2 from dephosphorylation by the KAP phosphatase (55). The strong enrichment of T160-phosphorylated forms of CDK2 in cyclin E/A complexes observed in the present study (Fig. 7; Table 1) thus fully confirms in vivo the dependence of T160-phosphorylation upon prior cyclin-binding. Interestingly, cyclin-binding might be the sole rate-limiting factor for phosphorylation by CAK in cyclin A complexes (as they contained almost only CDK2 phosphorylated at T160; Fig. 4), but not in cyclin E complexes from stimulated IMR-90 fibroblasts (which contained a significant proportion of unphosphorylated CDK2; Fig. 3), which is consistent with previous observations from these cells (33).

As CAK is reported to be constitutively active (54,56), T160-phosphorylation within cyclin-CDK2 complexes is generally believed to be limited by their association with p21\textsuperscript{cip1} or p27\textsuperscript{kip1}, which interfere with phosphorylation by CAK in vitro (21,28,29), possibly by hindering T-loop access (30). The present observations from intact cells challenge this concept essentially derived from in vitro experiments performed using bacterially produced p21 and p27, which are presumably unphosphorylated and possibly misfolded. Unexpectedly, T160-phosphorylated CDK2 was largely associated with the CDK inhibitors p21 and p27 (Table 1). Moreover, while a large proportion of cyclin A-CDK2 complexes contain p21 in proliferating human fibroblasts (32,39), almost all the CDK2 was phosphorylated at T160 in cyclin A complexes (Fig. 4). Similarly, the inactive cyclin E-CDK2 complexes from unstimulated IMR-90 cells contain p21 (5) and/or p27 (40) (though we repeatedly failed to detect p27-bound CDK2 from quiescent cells, in agreement with (5)), but the entirety of CDK2 from these cyclin E complexes was phosphorylated at T160 (Fig. 3). Our results are
thus more consistent with the paradoxical observation by some authors that the vast majority of active CDK2 is associated with p21 in normal human fibroblasts (31,32), implying that, at least at low stoichiometry binding, p21 should permit CDK2 phosphorylation by CAK. The abundant association of T160-phosphorylated CDK2 with p27 (Fig. 6) was even more intriguing. This is not to say that p27 is not a strong CDK2 inhibitor (10), since p27 also inhibits the activity of T160-phosphorylated CDK2 bound to cyclin E (57), in agreement with the multiplicity of p27 inhibitory mechanisms suggested from the structural study of cyclin A-CDK2-p27 complexes (30). At this stage, we cannot formally exclude the possibility that p27 might have joined the cyclin E-CDK2 complex after CDK2 phosphorylation by CAK, since p27 associates with only a fraction of CDK2 complexes. Nevertheless, at variance with the in vitro evidence obtained with bacterially produced p21 and p27, our results clearly indicate that, at least in intact human fibroblasts, p21 and possibly p27 do not prevent the activity of CDK2 complexes by impairing the phosphorylation of CDK2 by CAK.

Y15-Phosphorylation is not restricted to cyclin-bound CDK2 - By analogy with the situation demonstrated for cdc2/CDK1 (58), the Y15-phosphorylation of CDK2 has been inferred to depend on cyclin-binding, like the T160-phosphorylation by CAK (18). Nevertheless, to our knowledge, this question has not been investigated directly. Here, the Y15-phosphorylations of CDK2 and cdc2 were strongly induced by serum, but, unlike the T160-phosphorylation of CDK2, the Y15-phosphorylation of CDK2 was clearly independent of prior cyclin-binding. Indeed, Y15-phosphorylation was mostly represented in total CDK2 by the mono-Y15-phosphorylated form 2Y (Fig. 2), which was faint or undetectable in CDK2 complexed to cyclins E/A or Cip/Kip proteins (Table 1). The marked increase of Y15-phosphorylation of CDK2 thus likely depends on the regulation of the Wee1 kinase (59), which is induced during cell cycle progression (20,60).
**Y15-Phosphorylated CDK2 is preferentially recruited for T160-phosphorylation** – In HeLa cells, almost all of the phosphorylation of CDK2 on Y15 occurs on CDK2 molecules that are also phosphorylated on T160 (18). We have observed a different situation in IMR-90 fibroblasts. While mono- Y15-phosphorylated forms were abundantly detected in total CDK2 from stimulated cells, almost all of the Y15-phosphorylation was associated with the T160-phosphorylation in CDK2 complexed to cyclins or CDK inhibitors (Fig. 7; Table 1). In CDK2 complexes associated with cyclin E and/or p21 or p27, it is thus apparent that the phosphorylation by CAK recruited the entirety of Y15-phosphorylated CDK2. This contrasts with the persistence in these complexes of a significant proportion of unphosphorylated CDK2, which thus was less efficiently engaged by T160-phosphorylation (Fig. 7; Table 1). Moreover, the mono- T160-phosphorylated CDK2 form might be generated, not through phosphorylation by CAK of unphosphorylated CDK2, but most likely through dephosphorylation of the T160+Y15-phosphorylated forms by a cdc25 phosphatase. Indeed, the addition of cdc25A to co-immunoprecipitated cyclin E/A-CDK2 complexes considerably increases their catalytic activity (41), and the rapid disappearance of cdc25A following cell irradiation prevents most of CDK2 activity without affecting cyclin E-CDK2 complex formation (26), which demonstrates that CDK2 activity critically depends on its Y15-dephosphorylation. The mechanism restricting phosphorylation by CAK to Y15-phosphorylated CDK2 is unknown. Its physiological importance would be clearly to prevent a premature activation of cyclin-CDK2 complexes by CAK from bypassing the tight control by cdc25-dependent checkpoint pathways, such as those activated by DNA damage (12).

**Activation of CDK2 during mitogenic stimulation** – As designed, the present experiments only provide snapshots of the complex dynamic process leading to the full activation of cyclin-CDK2 complexes in response to proliferation stimulation. Nevertheless, new insights about this process can be incidentally deduced from the comparison of the
profile of CDK2 forms associated or not with cyclin E, cyclin A and/or p21 in serum-stimulated versus quiescent IMR-90 cells, within the context of previous observations by others.

In quiescent unstimulated cells, CDK2 was mainly present as an unphosphorylated, uncomplexed form. A proportion of unphosphorylated CDK2 and the minor fraction of mono-T160-phosphorylated CDK2 were also associated with p21 (Fig. 5) and cyclin D1 (not shown), but not with cyclins E/A. An even tinier fraction of CDK2, consisting only of the T160+Y15-phosphorylated forms, was complexed with cyclin E and possibly p21. It is likely to be totally inactive (5,40). At variance with cyclin E-bound CDK2, CDK2 associated with cyclin D1 and p21 was only faintly phosphorylated at T160, even in serum-stimulated cells (not shown), in agreement with previous reports indicating that cyclin D1 is an inhibitor for CDK2 that prevents its phosphorylation by CAK (45,61,62).

Upon serum stimulation, the abundance of cyclin E and CDK2 increases (Fig. 1B) and new cyclin E-CDK2 complexes are formed, perhaps depending in part on CDK2 nuclear import (40). The most impressive effect of serum was the appearance of the inhibitory Y15-phosphorylation in both uncomplexed CDK2 and CDK2 associated with cyclins E/A and p21 or p27. The activating T160-phosphorylation also increased. Unlike the Y15-phosphorylation, it likely depended on the formation of cyclin E/A-CDK2 complexes, which may comprise p21 or p27. Within these complexes, CAK phosphorylates the entirety of Y15-phosphorylated CDK2, but possibly not unphosphorylated CDK2 (as discussed above) (Fig. 8), thus constituting the important reservoir of pre-activated T160+Y15-phosphorylated forms. Depending on serum stimulation, likely through activation of cdc25A (as possibly reflected by the appearance of an undetermined up-shifted form of this phosphatase (Fig. 1B)), T160+Y15-phosphorylated CDK2 was then dephosphorylated in cyclin E complexes to generate the potentially active mono- T160-phosphorylated CDK2 (Fig. 8), which became the
predominant form associated with this complex at the G1/S transition (but still represents only a very minor fraction of total CDK2).

In contrast, paradoxically when the activity of cyclin A-CDK2 is maximum in cells in S- or G2 phases (36), cyclin A-bound CDK2 and p21-bound CDK2 at 24 h remained largely phosphorylated at both T160 and Y15 with a surprizingly small proportion of mono- T160-phosphorylated form (Figs. 4,5). This intriguing observation is consistent with the abrupt dephosphorylation at mitosis of most Y15 residues in both cdc2 and CDK2 described by Gu and collaborators (18), which might be explained by the strong stabilization of cdc25A in mitotic cells (63). Though cdc25A can equally activate CDK2 bound to cyclin E or cyclin A in vitro (41), this might suggest that cyclin A-CDK2 complexes could be more resistant to dephosphorylation by cdc25A, perhaps due to an increased association with p21 which competes with cdc25A for binding to the same cyclin site (64). On the other hand, at S/G2 phases (24 h after stimulation), cyclin A-CDK2 complexes are likely to be more abundant than the cyclin E-CDK2 complexes observed at the G1/S transition (16 h after serum stimulation), relative to unchanged levels of cdc25A (Fig. 1B). Even during unperturbed S phase progression (65,66), cdc25A presence and thereby activity are critically restricted by the checkpoint kinase Chk1, which is induced at the S to M phase and maximally activated 24 h after serum stimulation in human fibroblasts (67). Cdc25A might thus be especially rate-limiting for the dephosphorylation of cyclin A-CDK2. A fraction of cyclin A-CDK2 associates with DNA replication sites (68-70) to phosphorylate various pre-replication complex proteins (9,71) such as cdc6 (6,72), and thus to monitor ongoing DNA replication and control re-replication (8,73). We surmise that to tune the dynamic processivity of chromosome replication, the modulation of cyclin A-CDK2 activity by the S-phase checkpoint Chk1-cdc25A cascade (65,66) and/or through p21 release (13) might well need to be tightly restricted spatiotemporally, thus engaging at a given time only a minor fraction (as
observed in Fig. 4) of the reservoir of pre-activated cyclin A-CDK2 complexes that would accumulate in a pan-nuclear manner through Y15-phosphorylation, cyclin A-binding and T160-phosphorylation.

To conclude, the identification of the various CDK2 phosphoforms by 2D-gel electrophoresis has allowed for the first time to distinguish the potentially active T160-phosphorylated CDK2 from its T160+Y15-phosphorylated precursor forms. While T160-phosphorylation appears to depend on both cyclin-binding and prior Y15-phosphorylation, the Y15-dephosphorylation is underscored as the rate-limiting step of the complex activation process of cyclin-CDK2 complexes. This methodology should prove a valuable experimental tool in any future investigation of CDK2 regulation.

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Footnotes

1. The abbreviations used are: CDK, cyclin-dependent kinase; pRb, retinoblastoma susceptibility protein; CAK, CDK-activating kinase; IPG, immobilized pH gradient; IEF, isoelectric focusing; 2D, two-dimensional.

2. The most basic and most abundant CDK2 form \( \theta \) was therefore assumed to be unphosphorylated in agreement with (18), though this was not directly assessed in the present study.

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FIGURE LEGENDS

Figure 1. **CDK2 and CDK2 regulatory proteins in human IMR-90 cells stimulated to re-enter the cell cycle.** G0 cells (Cont) were reactivated by addition of 20% serum and sampled at the indicated time points. A, DNA synthesis was monitored by counting the fraction (mean + range of duplicate dishes) of nuclei having incorporated BrdU during the last 30 min of the stimulation. B, Western blotting analyses of CDK2, P-T160/P-T161-phosphorylated forms of CDK2/cdc2, P-Y15-phosphorylated forms of CDK2 and cdc2, cyclin E, cyclin A, p21, p27, cdc25A and CDK7 from whole cell extracts. p38 MAP kinase was detected as a loading control. C, The presence of p21, CDK2 and its P-T160- and P-Y15- phosphorylations was detected by Western blotting in (co) immunoprecipitates (IP) of p21, p27, cyclin E or CDK2.

Figure 2. **The potentially active mono-T160-phosphorylated CDK2 form represents an extremely minor fraction of total CDK2.** Western blotting analysis of the 2D-gel electrophoresis pattern of total CDK2 (immunoprecipitated with an anti-CDK2 antibody) from quiescent unstimulated IMR-90 cells (Cont) or cells stimulated by 20% serum for 16 or 24 h. The immunodetection was performed using a CDK2 antibody (CDK2), or the T160 or Y15 phosphospecific antibodies (P-T160 or P-Y15, respectively). In the two 24 h panels, the same membranes were first detected using either the P-T160 or P-Y15 phosphospecific antibodies and then reprobed using the total CDK2 antibody. Two different exposures (1 min and 5 min) of the Cont panel are shown. The 1 min exposures of Cont, 16 h and 24 h panels can be compared. To improve the visibility of the specific CDK2 spots, the Adobe Photoshop program was used to decrease the grey levels of the background spots in the P-T160 and P-Y15 panels (see Experimental Procedures). The different CDK2 forms were numbered according to their position relative to a computed scale (bottom) of isoelectric point shifts.
generated by the addition to CDK2 aminoacid sequence of entire numbers \((n)\) of negative charges (see Results) and labeled according to their demonstrated phosphorylation(s) (T, T160-phosphorylation; Y, Y15-phosphorylation).

Figure 3. Phosphorylations of cyclin E-bound CDK2. IMR-90 cells remained quiescent \((Cont)\) or were stimulated by 20% serum for 16 h. Lysates were subjected to co-immunoprecipitation with an anti-cyclin E antibody, separated by 2D-gel electrophoresis, and the Western blotting membranes were immunodetected first with the T160 or Y15 phosphospecific antibody \((P-T160\) or \(P-Y15\), respectively), and then reprobed with the total CDK2 antibody \((CDK2)\). Different exposure times were used for the \(Cont\) and 16 h panels. To improve the visibility of the specific CDK2 spots, the Adobe Photoshop program was used to decrease the grey levels of the background spots (see Experimental Procedures). Notice the enrichment of the potentially active mono-T160-phosphorylated CDK2 form \(2T\) at 16 h.

Figure 4. Phosphorylations of cyclin A-bound CDK2. 2D-gel separation of a co-immunoprecipitation with a cyclin A antibody from IMR-90 cells stimulated with serum for 24 h. Western blotting membranes were immunodetected first with the T160 or Y15 phosphospecific antibodies \((P-T160\) or \(P-Y15\), respectively), and then reprobed with the total CDK2 antibody \((CDK2)\).

Figure 5. Phosphorylations of p21-bound CDK2. IMR-90 cells remained quiescent \((Cont)\) or were stimulated by 20% serum for 16 or 24 h. Lysates were subjected to co-immunoprecipitation with an anti-p21 antibody and separated by one-dimensional \((A)\) or 2D-gel electrophoresis \((B)\). In \(B\), the Western blotting membranes were immunodetected first with the T160 or Y15 phosphospecific antibody \((P-T160\) or \(P-Y15\), respectively), and then
reprobed with the total CDK2 antibody (CDK2). To improve the visibility of the specific CDK2 spots in the Cont panels, the Adobe Photoshop program was used to decrease the grey levels of the background spots (see Experimental Procedures).

Figure 6. **Phosphorylations of p27-bound CDK2.** Lysates from 16 h-serum stimulated IMR-90 cells were subjected to co-immunoprecipitation with anti-p27 antibodies, separated by 2D-gel electrophoresis, and Western blotting membranes were immunodetected first with the T160 or Y15 phosphospecific antibody (P-T160 or P-Y15, respectively), and then reprobed with the total CDK2 antibody (CDK2). To improve the visibility of the specific CDK2 spots in the phosphospecific antibody detections, the Adobe Photoshop program was used to decrease the grey levels of the background spots (see Experimental Procedures).

Figure 7. **Comparison of total and complexed CDK2 from serum stimulated fibroblasts.** Same lysates from 16 h- or 24 h- serum stimulated IMR-90 cells were subjected to (co-) immunoprecipitation with anti-CDK2, -cyclin E, -cyclin A, -p27 or -p21 antibodies, separated by 2D-gel electrophoresis, and the resulting Western blots were immunodetected using the total CDK2 antibody.

Figure 8. **Suggested relationship of cyclin E/A binding and phosphorylations of CDK2.** The inhibitory Y15-phosphorylation of CDK2 induced by serum is clearly independent of prior cyclin-binding. It likely precedes the activating T160-phosphorylation by CAK, which depends on cyclin E/A binding and recruits the entirety of Y15-phosphorylated CDK2 within cyclin complexes, irrespective of their association with p21 or p27. The potentially active CDK2 phosphorylated at T160 but not Y15 is subsequently generated by dephosphorylation by cdc25A, which appears as the rate-limiting step of the CDK2 activation process.
**Summary of the main observations.** The different CDK2 phosphoforms detected in the different (co-) immunoprecipitations (first column) from IMR-90 cells serum-stimulated for 16 and/or 24h or not (Cont), are divided into four categories: 0 (not phosphorylated at T160 or Y15), PY15 (phosphorylated at Y15 but not T160), PT160 (phosphorylated at T160 but not Y15), PT160 + PY15 (phosphorylated at both T160 and Y15). For each CDK2 complex, the relative abundance of the main representative form (as indicated in bold) of each category was scored from - (undetectable) to +++ (most abundant form).
Figure 1

A

BrdU-labeled nuclei (%) vs. time (hour)

B

CDK2
P-T161 cdc2
P-T160 CDK2
P-Y15 cdc2
P-Y15 CDK2
cyclin E
cyclin A
p21
p27
{cdc25A
CDK7
p38 MAPK

C

IP: p21 p27 cyclin E CDK2

cont 16h cont 16h cont 16h cont 16h
Figure 2

CDK2
1 min
0 2T

CDK2
5 min
0 2T 4T,Y

CDK2
1 min
0 1Y 2T,2Y 3Y 4T,Y 4Y

CDK2
1 min
0 1T 2T 3T,Y 4T,Y 5T,Y 6T,Y

P-T160

P-Y15

n negative charges (computed position)
Figure 3

cyclin E-bound CDK2

CDK2

Cont

P-Y15

4T,Y

CDK2

0 2T 4T,Y

P-Y15

16h

2T 4T,Y

P-T160
Figure 4

cyclin A-bound CDK2 (24h)

CDK2

1T  2T  2Y  3T,Y  4T,Y  6T,Y

P-Y15

P-T160

1T  2T  2Y  3T,Y  4T,Y  6T,Y

CDK2
Figure 5

A

p21-bound proteins

- cyclin E
- cyclin A
- CDK2
- P-T161 cdc2
- P-T160 CDK2
- P-Y15 cdc2
- P-Y15 CDK2

Cont 24h

B

p21-bound CDK2

CDK2

0 2T 4T,Y

P-T160

0 2T 4T,Y

P-Y15

0 2T 4T,Y 6T,Y

Cont

16h

CDK2

0 2T 4T,Y 6T,Y

P-T160

0 2T 4T,Y 6T,Y

P-Y15

0 2T 4T,Y 6T,Y

24h

CDK2

0 2T 4T,Y 6T,Y

P-T160

0 2T 4T,Y 6T,Y

P-Y15

0 2T 4T,Y 6T,Y
Figure 6

p27-bound CDK2 (16h)

CDK2

0 2T 2Y 4T,Y

P-T160

2Y 4T,Y

P-Y15
Figure 8
Phosphorylations of cyclin-dependent kinase 2 revisited using two-dimensional gel electrophoresis
Katia Coulonval, Laurence Bockstaele, Sabine Paternot and Pierre P. Roger
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