Absolute Quantification of Multisite Phosphorylation by Selective Reaction Monitoring Mass Spectrometry

DETERMINATION OF INHIBITORY PHOSPHORYLATION STATUS OF CYCLIN-DEPENDENT KINASES

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Multisite phosphorylation is an important mechanism for achieving intricate regulation of protein function. Here we extended the absolute quantification of abundance (AQUA) methodology and validated its applicability to quantitatively study multisite phosphorylation. As a test case, we chose the conserved inhibitory site of the cyclin-dependent kinases (CDKs), Cdk1, Cdk2, and Cdk3, which are important regulators of cell cycle transitions and apoptosis. Inhibitory phosphorylation at Thr14 and Tyr15 of the CDKs is modulated by complex regulatory mechanisms involving multiple kinases and phosphatases. Yet the resulting quantitative dynamics among the four possible phosphorylated and non-phosphorylated versions of CDKs (T14p-Y15p, T14p-Y15, T14-Y15p, and T14-Y15) has not been investigated to date. Hence we used the heavy isotope-labeled tryptic peptides spanning the inhibitory site as internal standards and quantified all four versions by LC-selected reaction monitoring. Quantification of the phosphorylation status of the inhibitory site in the cell extracts provided novel quantitative insights. 1) The transition to mitotic phase was dominated by the conversion of "T14p-Y15p" to the "T14-Y15" form, whereas the two monophosphorylated forms were considerably lower in abundance. 2) The amount of all four forms decreased during the progression of apoptosis but with differing kinetics. Analysis of immunoprecipitated Cdk1 and Cdk2 revealed that the inhibitory site phosphorylation state of both kinases at different stages of the cell cycle followed the same trend. Quantitative immunoblotting using antibodies to Cdk1 and Cdk2 and to the T14-Y15p form suggested that quantification by AQUA was reliable and accurate. These results highlight the utility of internal standard peptides to achieve accurate quantification of multisite phosphorylation status. Molecular & Cellular Proteomics 5:1146–1157, 2006.

Multisite phosphorylation is an important mechanism for achieving intricate regulation of protein function (1–3). Multisite phosphorylation is known to regulate protein stability/degradation, localization, enzymatic activity, protein-protein interactions, and intracellular signaling pathways. It is a recurrent theme in cell cycle regulation as multisite phosphorylation of multiple regulatory proteins drives the cell cycle (3–11). Most importantly, activity of CDKs, Cdk1, Cdk2, and Cdk3, is modulated by both inhibitory and stimulatory phosphorylation at multiple sites (8, 12). Cdk1 (also known as Cdc2) is important for transition to and progression through M phase, whereas Cdk2 and Cdk3 are important for transition to and progression through S phase (13, 14). Cdk2 is also active during G2 phase (15). In addition, accumulating evidences implicate the CDKs in apoptosis (16–18). These CDKs are inhibited by phosphorylation at conserved Thr14 and Tyr15 residues that disorients the bound ATP and misaligns the substrate-binding pocket (13, 19–22). Tyr15 is phosphorylated by Wee1 and Myt1 kinases, whereas Thr14 is phosphorylated mainly by the Myt1 kinase (8, 23, 24). Both Thr14 and Tyr15 are dephosphorylated by the Cdc25 family of dual specificity phosphatases to relieve the inhibition of the CDKs (8, 25–27). Combined action of the above kinases and phosphatases controls the timely activation of the CDKs to drive the cell cycle transitions (8). As a direct result of the above regulatory enzymes, the CDKs can exist in at least four different versions due to phosphorylation of Thr14 and Tyr15 (T14p-Y15p, T14p-Y15, T14-Y15p, and T14-Y15). The resulting quantitative dynamics among these versions of the CDKs has not been investigated to date.

In 2003, Gerber et al. (28) reported a technique termed AQUA to quantify the abundance of endogenous proteins and peptides using heavy isotope-labeled synthetic peptides as internal standards. Using a phosphorylated, synthetic heavy isotope-labeled peptide, AQUA was also applied to follow the dynamics of phosphorylation at a specific residue of Sepa...
rase, which is important for sister chromatid segregation at anaphase (28). Given the general importance of multisite post-translational modification, we extended the AQUA methodology and validated its applicability to quantitatively study multisite phosphorylation status using the inhibitory site of the CDKs as a test case. We used the tryptic peptide spanning this inhibitory residues is chosen for making internal standards to quantify the multisite phosphorylation status of the CDKs. Octa-deuterated valine is used for isotope labeling the internal standards. Structural roles of the inhibitory and stimulatory phosphorylations are also mentioned. a, a representative LC-SRM ion chromatogram of the standard and native peptides corresponding to the non-phosphorylated form is shown. Quantification is based on the ratio of peak area counts and the initial amount of the spiked standard. XIC, extracted ion chromatogram.

**MATERIALS AND METHODS**

**Preparation of Internal Standard Peptides**—Four heavy isotope-labeled synthetic tryptic peptides spanning the conserved inhibitory site of the CDKs were used as internal standards to quantify all four possible combinations of phosphorylated and non-phosphorylated versions (Fig. 1a). For the purposes of discussion in this report, the peptides have been named Tp-Yp, T-Yp, Tp-Y, and T-Y. The tryptic peptide spanning these inhibitory residues is chosen for making internal standards to quantify the multisite phosphorylation status of the CDKs. Octa-deuterated valine is used for isotope labeling the internal standards. Structural roles of the inhibitory and stimulatory phosphorylations are also mentioned. a, a representative LC-SRM ion chromatogram of the standard and native peptides corresponding to the non-phosphorylated form is shown. Quantification is based on the ratio of peak area counts and the initial amount of the spiked standard. XIC, extracted ion chromatogram.
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lysis buffer with the following constituents: 0.5% n-dodecyl-β-D-maltoside, 50 mM Tris-Cl at pH 8, 120 mM NaCl, 4 mM EDTA, a mixture of phosphatase inhibitors (20 mM NaF, 5 mM Na3VO4, 5 mM β-glycerophosphate, 2 mM Na2VO3, and 2 μM microcystin-LR), and a mixture of protease inhibitors (from Roche Applied Science). For rigorous comparison of AQUA data with quantitative immunoblotting, the lysis buffer was supplemented with additional 80 mM NaF, 10 mM Na3PO4, and 25 mM β-glycerophosphate to further suppress all classes of phosphatase activity. The insoluble fraction was separated by centrifugation at 15,000 × g for 5 min. The protein mixture was denatured using 0.4% SDS and 2% β-mercaptoethanol followed by boiling for 6 min at 95 °C. The denatured samples were then divided into aliquots for AQUA and quantitative immunoblotting.

Immunoprecipitation of Cdk1 and Cdk2—Immunoprecipitation of Cdk1 and Cdk2 was carried out with 500 μg of total cell extracts at 1 μg/μl concentration. Cdk1 (2.5 μg, catalog number SC-54, Santa Cruz Biotechnology, Santa Cruz, CA) or Cdk2 antibodies (2.5 μg, catalog number SC-163, Santa Cruz Biotechnology) were precoupled to 20 μl of a slurry of protein A-conjugated beads (UltraLink ImmunoPure beads, from Pierce). After 4 h of incubation of the extracts with the antibody-bead conjugates at 4 °C, the beads were separated by centrifugation, washed once with the lysis buffer, and denatured as described under “Sample Preparation.” The denatured samples were then divided for AQUA and quantitative immunoblotting. An aliquot of the immunoprecipitate was saved for in vitro kinase assay activity prior to denaturation. To quantitatively compare the inhibitory site phosphorylation status of the combined CDK population and the individually immunoprecipitated Cdk1 and Cdk2, the extracts were left at 4 °C for 4 h before denaturation.

Trypsin Proteolysis of Samples in Solution—A known amount of the four internal standard peptides was added prior to trypsinization. The volume of the protein mixture was increased 10-fold using 50 mM NH4HCO3 (pH 8.0, also containing 7% acetonitrile, 200 μM Na2VO3, and 1 μM microcystin-LR) to make the reaction mixture suitable for enzymatic action by trypsin (from Promega). Trypsin was used at a ratio of 1:30 with respect to the total amount of protein being proteolyzed. After 8 h of proteolysis, the same amount of fresh trypsin was added for the second time, and proteolysis was continued for an additional 10 h. Detergent and salt were removed from the tryptic peptide mixture using a solid-phase strong cation-exchange cartridge (MCX Oasis from Waters Corp.). It was critical to reduce the pH of the solvents used for binding and washing the resin bed below 2.0 to effectively bind and then retain the doubly phosphorylated version of the inhibitory site peptide. The eluted peptide mixture was lyophilized and stored at −20 °C until ready for quantitative mass spectrometry.

Quantification by LC-SRM (AQUA)—The setup used for on-line microcapillary LC and microelectrospray was essentially as reported previously (30). The dimension of the fused silica capillary column (Polymicrotechnologies, Inc.) was increased from 11 cm × 100 μm to 17 cm × 125 μm. Magic C18 beads (200 Å, 5 μm, from Michrom BioResources, Auburn, CA) were used for packing the tapered fused silica tubing. The gradient for reverse-phase chromatography was optimized to resolve the four relevant peptides from the inhibitory site of the CDKs into well separated time windows. A 2-μg tryptic peptide equivalent of the initial total protein amount was loaded on the column for quantifying the endogenous native peptides. A 2-D Ion trap mass spectrometer (LTQ from Thermo Finnigan) was used for quantification. Preliminary MS/MS analyses at typical CID settings were carried out to choose suitable prominent y-ions for SRM analysis. Either two or four SRM transitions, corresponding to one or two standard and native peptide pairs, respectively, were recorded in each time window of acquisition. Two such time windows were used in each chromatographic run to quantify either two or all four phosphorylations of the inhibitory site of the CDKs. The typical effective scan rate during SRM was approximately five acquisitions per second. Parent ion isolation width was set at 2 amu, and product ion scan width was set at 4 amu. The larger window for product ion scanning helped in ruling out spurious peaks based on isotope peak pattern. The base peak chromatograms used for quantification were generated from a 1-amu window around the expected product ion m/z. Representative LC-SRM chromatograms for the four peptide pairs are shown in Supplemental Fig. 3. Peak area of the standard and native peptides was quantified using the Xcalibur software (Thermo Finnigan). The known amount of the standard initially spiked and the ratio of peak areas are used for calculating the absolute quantity (Fig. 1b) (28).

Quantitative Immunoblotting—Quantitative immunoblotting was performed to compare with the quantification from LC-SRM. Specific antibodies to Cdk1 (catalog number SC-54) and Cdk2 (catalog number SC-163) were from Santa Cruz Biotechnology. Polyclonal antibody specific to phosphorylated Tyr15 (catalog number 9111) of the inhibitory site of the CDKs was from Cell Signaling Technology (Beverly, MA). A dilution series of one of the samples was included with every immunoblot experiment. Digital densitometry was carried out using ImageQuant Version 5.0 (Amersham Biosciences) with background correction. Densitometry values were used to generate a standard curve from the dilution series and to obtain relative amounts of β-actin, Cdk1, Cdk2, or phosphorylated Tyr15. The relative quantification data were accepted only when the linear regression coefficient was >0.97 for the standard curve generated from that particular immunoblot. When blotting for phosphorylated Tyr15, the following measures were taken to minimize dephosphorylation during processing and development of immunoblots.(a) Phosphate buffers were used instead of Tris buffers. (b) 5% nonfat milk was heat-treated (80 °C for 15 min) to inactivate phosphatases in the skimmed milk. A phosphatase inhibitor mixture of 80 mM NaF, 10 mM Na3PO4, 25 mM β-glycerophosphate, 2 mM Na2VO3, and 1 μM microcystin-LR was also used at every stage of processing of the blotted nitrocellulose membranes.

In Vitro Kinase Assay for the Activity of Cdk1 and Cdk2—Immunoprecipitated Cdk1 or Cdk2 on the beads was resuspended in 24 μl of in vitro kinase reaction buffer (from New England Biolabs) containing 2.1 μg of Histone H1 (from Calbiochem), 0.3 mM ATP, and 5.3 μCi of [γ-32P]ATP (from Amersham Biosciences). After 30 min at 30 °C, the kinase reaction was stopped by boiling. The extent of phosphorylation of Histone H1 was measured by autoradiography of radiolabeled phospho-Histone H1 followed by digital densitometry (using Image-Quant Version 5.0) with background correction. Specific activity was calculated by normalizing the autoradiography data with the amount of Cdk1 in the reaction mixture.

Peptide ELISA—To assess the specificity of the polyclonal antibody to the phosphorylated Tyr15 of the inhibitory site of Cdk1 and Cdk2, peptide ELISA experiments were carried out. The same stretch of residues as in the standard peptides used in this study was used by the commercial supplier to generate the antibody. Hence the standard peptides were directly used to assess the affinity of the antibody for different combinations of phosphorylations of the inhibitory site of CDKs. Different amounts of the standard peptides were coated onto the polystyrene surface of the 96-well ELISA plate (PolySorp from Nalge Nunc). Carbonate buffer (15 mM NaHCO3 and 35 mM NaHCO3, pH 9.6) was used to facilitate adsorption of the peptides to the polystyrene surface. Extent of the antibody binding was assessed using a peroxidase-conjugated secondary antibody followed by colorimetry for peroxidase activity (o-phenylenediamine dihydrochloride, from Sigma).

RESULTS

We first tested the linearity of response of the 2-D ion trap mass spectrometer. Using standard peptides, linear response
was observed from 100 amol to 5 pmol (Supplemental Fig. 2). Consistent response factor across the dynamic range was also observed; this is a requisite for quantification using ratio of peak area counts of native and isotope-labeled peptide pairs. We were able to detect as low as 500 amol of endogenous T-Yp peptide in 2 μg of tryptic peptide mixture derived from Jurkat T cellular extract (Supplemental Fig. 4). Thus, 2-D (linear) ion traps with superior trapping efficiency, ion capacity, and duty cycle time compared with the conventional three-dimensional ion trap are applicable for sensitive quantification of abundance directly from peak area counts, especially from complex protein mixtures.

Next we explored different modes of data acquisition. We performed multiple reaction monitoring (MRM) experiments to assess the interference of chemical noise and co-eluting peptides as we were quantifying directly from total cell extracts. Less than 15% difference was observed among data acquired from MRM (Supplemental Table 1). Similar observations have been made before (32). This indicates that interference from spurious product ions is negligible in our samples. Multiple MRM experiments validated the specificity of the product ions that we chose for quantification of low abundance peptides from complex mixtures. Tp-Yp and Tp-Y peptides give rise to prominent neutral loss of phosphate peaks due to the presence of phosphothreonine. We observed that quantification using the neutral loss of phosphate fragment ion is within 20% of the quantification obtained from separate MRM (Supplemental Table 2). Thus, neutral loss of phosphate ion can be used as any other product ion for quantification, and neutral loss is not necessarily a technical bane for quantification when internal standards are used. Simultaneous quantification of two peptides (four SRM transitions in every duty cycle) was within 20% of the quantification obtained from separate individual acquisitions (Supplemental Table 3). It is likely that faster scan rates of the linear ion traps used in our study enable multiplexing in the SRM acquisition mode (30, 31).

After the above preliminary experiments, we compared the multisite phosphorylation status at the inhibitory site under different biological states. At first, we quantified the multisite phosphorylation status at different stages of the cell cycle in
total cell extracts. The tryptic peptide spanning the inhibitory site is identical in Cdk1, Cdk2, and Cdk3. All three contribute to the abundance values obtained by AQUA from cell extracts. Henceforth in this report the combined population of Cdk1, Cdk2, and Cdk3 is referred as the “CDK” population. In an asynchronously proliferating Jurkat T leukemia cell population in log phase, around 32% of the CDK population was phosphorylated at both Thr14 and Tyr15 residues (Fig. 2b). In M phase ~11% was phosphorylated at both Thr14 and Tyr15 residues, whereas in early S phase ~70% of the CDK population was phosphorylated at both Thr14 and Tyr15 residues. This trend was reversed for the CDK population that was not phosphorylated at both Thr14 and Tyr15 residues. These quantitative observations are consistent with the known qualitative data on the inhibitory site phosphorylation: active Cdc25, a dual specificity phosphatase, dephosphorylates Thr14 and Tyr15 residues of CDKs in M phase, whereas the action of Wee1 and Myt1 kinases is predominate to that of inactive Cdc25 in other phases of the cell cycle (Fig. 2a) (8, 26, 33–35).

One important quantitative insight that we gained was that most of the dynamics was dominated by interconversion between Tp-Yp and T-Y forms, whereas the monophosphorylated forms (T-Yp and Tp-Y) were considerably lower in abundance (Fig. 2b). To our knowledge, this has not been demonstrated before. The dynamics of the inhibitory site phosphorylation in the CDKs is further elaborated under “Discussion.”

In addition we tested the reliability of the above AQUA measurements. If the quantification from AQUA was accurate, then the sum of the four phosphorylated and non-phosphorylated versions of the inhibitory site should yield the total amount of the CDKs. To test this, first we quantified the relative amounts of Cdk1 and Cdk2 in the asynchronous cells and in cells arrested in M phase and early S phase by immunoblotting. One dilution series that was simultaneously blotted along with the samples to generate standard curves is also shown. Ctrl, control; Noc, nocodazole; Aph, aphidicolin; CAK, CDK-activating kinase.

Fig. 2. Cell cycle-dependent quantitative dynamics in the CDKs. a, schematic diagram outlining the regulatory mechanisms that control the dynamics of Cdk1 phosphorylation at the inhibitory site during cell cycle. The dynamics of the inhibitory site phosphorylation of Cdk2 also follow the same trend (see “Discussion”) (34). Abrupt activation of Cdc25 at the G2-M transition leads to dephosphorylation of Thr14 and Tyr15 and activation of Cdk1 (26). The schematic is based on the Cdk2 crystal structures (22). b, quantification of multisite phosphorylation status of the combined CDK population in asynchronously growing cells, cells at M phase, and early S phase by AQUA. Abundance of each version of the inhibitory site is plotted as a fraction of the total abundance of the inhibitory site peptide. The AQUA results were confirmed by replicate AQUA experiments wherein <15% difference with the first set of results was observed. Note that the transition to M phase is dominated by the conversion of Tp-Yp to T-Y. c, comparative analysis of AQUA data with immunoblotting. Total quantities deduced from AQUA peptides were compared with immunoblotting using Cdk1- and Cdk2-specific antibodies. Good agreement between quantification from AQUA and immunoblotting indicates that the AQUA values are valid. d, immunoblots used for comparison with the AQUA data. A dilution series that was simultaneously blotted along with the samples to generate standard curves is also shown.
the inhibitory site on the same relative scale and compared with the quantitative immunoblot data on Cdk1 and Cdk2 (Fig. 2c). Indeed relative quantities deduced from AQUA agreed well with the immunoblot data suggesting that AQUA of multisite phosphorylation status of the inhibitory site of CDKs was accurate and reliable.

Next we quantified the multisite phosphorylation status at different stages of the cell cycle in immunoprecipitated Cdk1 and Cdk2 and compared it with the CDK population measured directly from the cell extracts (Fig. 3). We ensured that immunoprecipitated Cdk1 did not contain Cdk2 and vice versa (Supplemental Fig. 5). The phosphorylation status of the inhibitory site of immunoprecipitated Cdk1 and Cdk2 and the combined CDK population followed the same trend at different stages of the cell cycle (Fig. 3, a, c, and d). This is consistent with previous studies using in vivo labeling with $^{32}$P orthophosphate in HeLa cells (34). Relative levels of the non-phosphorylated version of the inhibitory site were also consistent with the specific activity of Cdk1 toward Histone H1 (Fig. 3b). More than a 25-fold increase in the specific activity of Cdk1 was observed in cells arrested at M phase by nocodazole.

Relative levels of the non-phosphorylated version of the inhibitory site were also consistent with the specific activity of Cdk2 toward Histone H1. However, only a ~5-fold increase in the specific activity of Cdk2 was observed at M phase.

Using a second preparation of immunoprecipitated Cdk1, we compared the AQUA data with quantitative immunoblotting (Fig. 4). If the quantification from AQUA was accurate, then the sum of the four phosphorylated and non-phosphorylated versions of the inhibitory site should yield the total amount of immunoprecipitated Cdk1. To test this, first we quantified the relative amounts of Cdk1 by immunoblotting. Then we plotted the sum of the four different versions of the inhibitory site on the same relative scale and compared with the quantitative immunoblot data for Cdk1 (Fig. 4b). A similar comparison between AQUA and immunoblotting was carried out for T-Yp (Fig. 4c). We also confirmed the specificity of the Tyr$^{15}$ phosphospecific antibody to T-Yp by peptide ELISA (Fig. 5). Indeed relative quantities deduced from AQUA agreed well with the immunoblot data suggesting that AQUA of multisite phosphorylation status of the inhibitory site of CDKs was accurate and reliable. Similar agreement between AQUA and

![Comparison of the multisite phosphorylation status of immunoprecipitated Cdk1 and Cdk2 with that of the combined CDK population.](Image)

- Cdk1 and Cdk2 were immunoprecipitated from control, nocodazole-, and aphidicolin-treated cells for 4 h. Immunoprecipitates and the cell extracts were then denatured and further processed for AQUA. The phosphorylation status of the inhibitory site of immunoprecipitated Cdk1 (a) and Cdk2 (c) and the combined CDK population (d) follows the same trend at different stages of the cell cycle. The data shown are from four repeated AQUA measurements for each of the inhibitory site versions. Relative levels of the non-phosphorylated version of the inhibitory site are also consistent with the specific activity of Cdk1 toward Histone H1 and the stage of the cell cycle (b). Densitometry readings of autoradiographs were normalized with the relative amounts of Cdk1 in the three samples to obtain specific activity. Ctrl, control; Noc, nocodazole; Aph, aphidicolin.

![Quantification of Multisite Phosphorylation Status](Image)
quantitative immunoblotting was obtained with immunoprecipitated Cdk2 and cell extracts (Figs. 4, e–g, and 2c and Supplemental Fig. 7).

Finally we applied the AQUA method to investigate the dynamics of the CDK population during apoptosis. It has been demonstrated before that activity of both Cdk1 and Cdk2 increases and that the Tyr15 phosphorylation level of both Cdk1 and Cdk2 decreases during apoptosis in Jurkat T leukemia cells (36) (Fig. 6a). AQUA results showed that the amount of all four phosphorylation versions of the inhibitory site decreased as anti-Fas-induced apoptosis progressed but with different kinetics (Fig. 6b). Based on the previous report, we had anticipated that the fraction of T-Y would increase (36). Surprisingly the fraction of T-Y did not increase appreciably, and the fraction of Tp-Yp did not decrease appreciably (Fig. 6c). Although T-Yp steadily decreased to ~20% of that of non-apoptotic cells, it constitutes <10% of total CDK population. The changes in the phosphorylation status of the

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**FIG. 4.** Comparison of AQUA and immunoblot data obtained from immunoprecipitated Cdk1 and Cdk2. Cdk1 and Cdk2 were immunoprecipitated from control, nocodazole-, and aphidicolin-treated cells. Immunoprecipitated Cdk1 and Cdk2 were denatured and divided for AQUA and immunoblotting. a and e, average abundances (in femtomoles) of the four different inhibitory site versions of immunoprecipitated Cdk1 and Cdk2, respectively. The sum of the four different versions reflects the total Cdk2 in the immunoprecipitated samples. b and f, good agreement between quantitative immunoblotting of Cdk1/Cdk2 and the sum of the AQUA abundances of the four different versions in the immunoprecipitated Cdk1 and Cdk2, respectively. c and g, good agreement between quantification of T-Yp by AQUA and immunoblotting in the immunoprecipitated Cdk1 and Cdk2, respectively. The discrepancy between AQUA and immunoblotting for T-Yp in the immunoprecipitated Cdk1 from aphidicolin-treated cells was ~28%, which is the highest discrepancy observed among all the comparative analyses. Comparison with quantitative immunoblotting indicates that the AQUA measurements were accurate in both cases. d and h, immunoblots along with the dilution series used for densitometry and relative quantification. Ctrl, control; Noc, nocodazole; Aph, aphidicolin.

**FIG. 5.** Specificity of the Tyr15 phosphospecific antibody assessed by peptide ELISA. Peptide ELISA confirmed that the antibody for phosphorylated Tyr15 is specific for Tp-Yp and does not cross-react with theTp-Yp and Tp-Y forms of the inhibitory site of the CDKs.

quantitative immunoblotting was obtained with immunoprecipitated Cdk2 and cell extracts (Figs. 4, e–g, and 2c and Supplemental Fig. 7).
two most abundant forms were negligible and hence may not explain more than a 5-fold increase in the activity of Cdk1 and Cdk2 that was observed previously (36). (See “Discussion” for more details.)

Again from AQUA measurements we deduced relative -fold changes of the total CDK population during apoptosis and compared with quantitative immunoblotting data. The Cdk1 level decreased more than that of Cdk2 during apoptosis as revealed by specific antibodies (Fig. 6d). Because the internal standards used for AQUA are measuring the multisite phosphorylation status of the combined population of Cdk1 and Cdk2, one would expect the sum of the four phosphorylated and non-phosphorylated versions of the inhibitory site to follow a trend intermediate to that of Cdk1 and Cdk2. As expected, when plotted on a relative scale the decrease in the combined CDK population revealed by AQUA was intermediate to that of Cdk1 and Cdk2, indicating that the quantification from AQUA was accurate and reliable. Furthermore we also compared the -fold changes in T-Yp during apoptosis as deduced from AQUA and from quantitative immunoblotting (Fig. 6e). Both techniques revealed that there was a steady decrease in the level of T-Yp as reported before (36). These

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**Fig. 6. Quantitative dynamics of the CDKs during apoptosis.**

- **a**, schematic of the proposed regulatory mechanism that controls the dynamics of CDK phosphorylation at the inhibitory site during anti-Fas-induced apoptosis (36). Inactivation of Wee1 kinase due to proteolytic processing by caspase-3 leads to a decrease in Tyr¹⁵ phosphorylation and an increase in CDK activity.

- **b**, quantification of multisite phosphorylation status of the combined population of Cdk1 and Cdk2 during apoptosis by AQUA. Four repeated measurements for each version of the inhibitory site at each of the four time points were used (0, 3, 5, and 8 h). The four phosphorylated and non-phosphorylated versions of the CDK population are present at different starting amounts and decrease with varying kinetics during apoptosis. c, the fraction of the abundant forms, T-Y and Tp-Yp, do not increase or decrease appreciably. The fraction of the T-Yp form shows continuous decrease.

- **d**, comparison of relative quantities deduced from AQUA with those obtained from immunoblots using Cdk1- and Cdk2-specific antibodies.

- **e**, good agreement between relative amounts of T-Yp deduced from AQUA and from immunoblots using the Tyr¹⁵ phosphospecific antibody.

- **f**, immunoblots used for comparison with AQUA data along with the dilution series used to generate standard curves are shown.
results yield further proof that the AQUA standard phosphopeptides enable accurate quantification multisite phosphorylation.

During the initial stages of the study we had also included the stimulatory site of Cdk1 for quantification. The tryptic peptide stretch that includes the stimulatory Thr^{161} residue contains tryptophan. Tryptophan in our standard peptides was oxidized heavily and was leading to erroneous quantification. Hence we did not continue with the analysis of Cdk1 phosphorylation at Thr^{161}. Along with tryptophan, methionine and cysteine can also be oxidized and lead to overestimation of the actual amount of the standard peptide. Like oxidation of residues in the standard peptides, many other issues have to be considered for choosing suitable peptides as internal standards and conducting AQUA experiments. We have detailed them in the form of a table (Supplemental Table 5).

**DISCUSSION**

Given the general importance of multisite post-translational modification, we extended the AQUA methodology and validated its applicability to quantitatively study multisite phosphorylation using the inhibitory site of the CDKs as a test case. We showed that the CV of biological and technical replicates for AQUA measurements was within 24%. Furthermore we demonstrated that quantitative measurements of CDK phosphorylation sites using the phosphospecific standard peptides provide novel quantitative insights. We used immunoblotting as an independent technique to test the accuracy of AQUA results. -Fold changes deduced by AQUA were in good agreement with quantitative immunoblotting for Cdk1, Cdk2, and the T-Yp version of the inhibitory site. From our study, we conclude that AQUA provides accurate measurement of multisite phosphorylation status of specific proteins both from complex mixtures, such as cell extracts, and immunoprecipitates.

From our quantitative measurements using the AQUA methodology, major findings are that transition to M phase of the cell cycle was dominated by conversion of Tp-Yp to T-Y form, and all four phosphorylated forms decreased with different kinetics during apoptosis. In addition, at least three quantitative insights with functional consequences can be gained. 1) We found that cell cycle dynamics was dominated by interconversion between Tp-Yp and T-Y forms, whereas the monophosphorylated forms (T-Yp and Tp-Y) were considerably lower in abundance (Fig. 2b). Mutation analysis suggests that the Tp-Yp form of the CDKs is more inactive than the T-Yp form (37). Many previous studies on the CDKs have considered Tyr^{15} phosphorylation alone by using the Tyr^{15} phosphospecific antibody, which only recognizes the T-Yp form (36, 38-42) (Fig. 5). Thus, these studies have ignored the abundant and more potently inhibited Tp-Yp form of CDKs and only considered the less abundant and less potently inhibited T-Yp form. Our results highlight the need to consider all four phosphorylated forms of CDKs for better understanding of their kinase activities during cell cycle and apoptosis. 2) We found that >70% of the combined population of Cdk1 and Cdk2 in the early S phase-arrested cells existed in the potentially inhibited Tp-Yp form (Fig. 2b). We had anticipated that neither the Tp-Yp nor the T-Y form would dominate in the combined population as Cdk1 is inactive and Cdk2 is active in S phase (15). However, consistent with our observation, multiple evidence indicates that the inhibitory site of Cdk2 is phosphorylated even in S phase. (a) Phosphorylation at the inhibitory site of both Cdk1 and Cdk2 is governed by the same set of enzymes (34). (b) Phosphorylation status of the inhibitory site of both Cdk1 and Cdk2 follows the same trends through the cell cycle (34). (c) Only a small fraction of Cdk2 that is not phosphorylated at the inhibitory sites and phosphorylated at Thr^{160} is present in S phase (34, 43). Finally (d) inhibitory site phosphorylation does not hamper Cdk2 activity as much as that of Cdk1 (44). 3) During apoptosis, we observed that the fraction of T-Y, which corresponds to the active form, did not increase appreciably, and the fraction of Tp-Yp, which corresponds to the potently inhibited form, did not decrease appreciably (Fig. 6c). Although T-Yp steadily decreased to ~20% of that of non-apoptotic cells, it constitutes <10% of total CDK population. Activation of CDKs such as Cdk1 and Cdk2 during apoptosis is well documented (16, 17, 36). Inactivation of inhibitory kinase Wee1, which phosphorylates Tyr^{15} of Cdk1 and Cdk2, by caspase-3-mediated cleavage was suggested to contribute to the activation of these CDKs (36). Based on this report, we had anticipated that the fraction of T-Y would increase. But we observed that the changes in the phosphorylation status of T-Y and Tp-Yp were negligible. Thus, our quantitative results do not favor the previous suggestion that more than a 5-fold increase in the activity of Cdk1 and Cdk2 is due to the inactivation of Wee1 during apoptosis (36). Other mechanisms such as accumulation of cyclin A/B, which are cognate partners necessary for CDK activity and/or cleavage of p21, which is a stoichiometric inhibitor of CDKs, may explain the increase in the activity of Cdk1 and Cdk2 seen during the progression of apoptosis (18, 36, 45).

To quantitatively compare the inhibitory site phosphorylation status of the combined CDK population and the individually immunoprecipitated Cdk1 and Cdk2, even the extracts were left at 4 °C for 4 h before denaturation. This was done to account for any phosphatase action that may change the phosphorylation status of the inhibitory site and lead to quantitative discrepancies. In the extracts left at 4 °C for 4 h, the fraction of the phosphorylated versions was considerably lower than that observed in the extracts denatured immediately for AQUA analysis (Figs. 2b and 3d). This suggests that some phosphatase activity was functioning on the CDKs during incubation and immunoprecipitation even at 4 °C in the presence of the phosphatase inhibitors. Earlier we had expected that the concentrations of multiple phosphatase inhibitors were adequate to suppress the multiple classes of phos-
phatases. Analysis of immunoprecipitated Cdk1 and Cdk2 revealed that the inhibitory site phosphorylation status at different stages of the cell cycle followed the same trend in these two kinases. The immunoprecipitated samples also helped in assessing the accuracy of the AQUA data by comparing with the immunoblot data.

Initially when our lysis buffer contained lower concentrations of phosphatase inhibitors, we observed discrepancies mainly due to dephosphorylation of Tp-Yp to T-Yp. With the use of heat-inactivated skimmed milk and higher levels of phosphatase inhibitors during immunoblotting we were able to obtain quantitative agreement between AQUA and immunoblots for T-Yp. It is possible that some dephosphorylation happened during the preparation of the samples for AQUA. However, the presence of internal standards accounts for it by undergoing dephosphorylation to the same extent and thus maintaining the original ratio of the native peptide to the standard peptide intact.

Although Cdk3 also contains the same inhibitory site peptide, we did not consider Cdk3 for immunoblotting when comparing with the AQUA data from cell extracts. It is likely to be considerably less abundant in Jurkat T leukemia cells. Extensive protein identification in this cell line using multiple subfractions and repeated sequencing resulted in the identification of Cdk1 and Cdk2 with multiple unique peptides but not of Cdk3.\(^2\) Immunoblotting for Cdk1 and Cdk2 was sufficient for good agreement with AQUA data from cell extracts (Figs. 2c and 6d). Thus, abundance values obtained by AQUA from cell extracts most likely reflect the combined population of Cdk1 and Cdk2.

Several methods have been commonly used for the quantitative analysis of protein phosphorylation. In vitro kinase assays with \(^{32}\)P labeling have been used to quantify the extent of phosphorylation at multiple sites (46, 47). Two-dimensional phosphopeptide mapping techniques with \(^{32}\)P labeling have been used to quantify the relative amounts of different phosphopeptides (48, 49). Conventional techniques involving double radioisotope labeling, \(^{32}\)P for the phosphate and \(^{35}\)S (or \(^{14}\)C) for the protein backbone, have been used to quantify the stoichiometry/extent of phosphorylation in vivo (49–51). However, due to incomplete labeling of the double radioisotope methodology, accurate stoichiometric measurement of specific phosphorylation sites in vivo has been questioned (51, 52). Therefore, conventional radioisotope labeling approaches may not be applicable to quantify the stoichiometry of phosphorylation at multiple sites from intact cell preparations. Antibodies corresponding to phosphorylated and non-phosphorylated forms have also been used to quantify the extent of phosphorylation at a specific site (53). However, development of good antibodies that recognize distinct phosphorylation states is a cumbersome, time-consuming exercise. Additionally as multisite phosphorylation leads to combinatorial possibilities, many antibodies will have to be developed. In the case of AQUA, combinatorial possibilities due to multiple phosphorylation sites in a short stretch of sequence can be handled easily by having an internal standard peptide for each distinct phosphorylation state. The sequential nature of peptide synthesis ensures that phosphoamino acids can be incorporated at any desired site(s). There is also a possibility of the antibody cross-reacting with a different version of multiphosphorylated protein/peptide leading to misleading quantitative data. In contrast, the specificity of AQUA obtained by the selection of elution time and parent ion and product ion \(m/z\) should enable unambiguous quantification of the combinatorial possibilities of multisite phosphorylation.

Recently mass spectrometry-based methods combining affinity purification, phosphatase treatment, and chemical (or enzymatic) heavy isotope labeling have been used to quantify the extent of phosphorylation at multiple sites of relevant proteins (54–56). Relatively pure protein preparation is critical for these strategies as quantification is obtained from survey scan data, whereas with AQUA we demonstrated that desirable sensitivity (subfemtomole detection) can be achieved even with highly complex samples. In addition, AQUA is more versatile because the sequential nature of peptide synthesis ensures that, just like O-phosphoamino acids, other modified amino acids can be incorporated in place of regular amino acids. Thus, a diverse set of post-translational modifications in addition to phosphorylation such as acetylation, hydroxylation, methylation, and any likely combination of them become amenable for quantification by the AQUA strategy. Finally AQUA provides abundance information of multisite modification status that then can be used to deduce stoichiometric information of individual modified versions and -fold changes among different biological states.

Multisite post-translational modification can give rise to different versions of the same protein in a combinatorial manner. Thus, multisite post-translational modification provides a combinatorial strategy to modulate protein function as exemplified in the case of histones (3, 57). The literature is replete with examples of multisite post-translational modifications mediating intricate regulation of other proteins as well (1–7). Our analysis of multisite phosphorylation status of CDKs highlights the potential of the AQUA methodology to quantitatively study multisite post-translational modification of specific proteins. We anticipate that AQUA will be an invaluable tool in uncovering the quantitative rules and themes underlying regulation by multisite protein modification.

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\(^2\) L. Wu, K. Rezual, and D. K. Han, unpublished observations.
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REFERENCES

1. Cohen, P. (2000) The regulation of protein function by multisite phosphorylation—a 25 year update. Trends Biochem. Sci. 25, 596–601

2. Holmberg, C. I., Tran, S. E., Eriksson, J. E., and Sistonen, L. (2002) Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem. Sci. 27, 619–627

3. Yang, X. J. (2005) Multisite protein modification and intramolecular signaling. Oncogene 24, 1653–1662

4. Welcker, M., Singer, A. S., Luebke, K., Blocher, A., Guerien-West, M., Clurman, B. E., and Roberts, J. M. (2003) Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. Mol. Cell 12, 381–392

5. Bulavin, D. V., Higashimoto, Y., Demidenko, Z. N., Meek, S., Graves, P., Phillips, C., Zhao, H., Moody, S. A., Appella, E., Piwnica-Worms, H., and Fornace, A. J., Jr. (2003) Dual phosphorylation controls Cdc25 phosphatase and mitotic entry. Nat. Cell Biol. 5, 545–551

6. Nash, P., Tang, X., Orlicky, S., Chen, Q., Gerlert, F. B., Mendenhall, M. D., Sichler, F., Watson, T., and Tyers, M. (2001) Multisite phosphorylation of a Cdk inhibitor sets a threshold for the onset of DNA replication. Nature 414, 514–521

7. Yang, J., Song, H., Walsh, S., Barde, E. S., and Kombluth, S. (2001) Combinatorial control of cyclin B1 nuclear trafficking through multisite phosphorylation at multiple sites. J. Biol. Chem. 276, 3604–3609

8. Lew, D. J., and Kombluth, S. (1996) Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr. Opin. Cell Biol. 8, 795–804

9. Albert, A. L., Lavio, S. B., and Vincent, M. (2004) Multisite phosphorylation of Pin1-associated mitotic phosphoproteins revealed by monoclonal antibodies MMP-2 and CC-3. BMC Cell Biol. 5, 22

10. Larsson, N., Marklund, U., Gradin, H. M., Bratsland, G., and Gullberg, M. (1997) Control of microtubule dynamics by oncoprotein 18: dissection of the regulatory role of multisite phosphorylation during mitosis. Mol. Cell. Biol. 17, 5530–5539

11. Knudsen, E. S., and Wang, J. Y. (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol. Cell. Biol. 17, 5771–5783

12. Berry, L. D., and Gould, K. L. (1996) Regulation of Cdc2 activity by phosphorylation at multiple sites. Curr. Opin. Cell Biol. 8, 107–114

13. Morgan, D. O. (1997) Cyclin-dependent kinases: engines, clocks, and mitosis. Annu. Rev. Cell Dev. Biol. 13, 261–291

14. Hofmann, F., and Livingston, D. M. (1996) Differential effects of cdk2 and GSK3 controls cyclin E degradation. Mol. Cell 12, 381–392

15. Meikrantz, W., and Schlegel, R. (1996) Suppression of apoptosis by dom-
43. Coulonval, K., Bockstaele, L., Paternot, S., and Roger, P. P. (2003) Phosphorylations of cyclin-dependent kinase 2 revisited using two-dimensional gel electrophoresis. J. Biol. Chem. 278, 52052–52060
44. Chow, J. P., Siu, W. Y., Ho, H. T., Ma, K. H., Ho, C. C., and Poon, R. Y. (2003) Differential contribution of inhibitory phosphorylation of CDC2 and CDK2 for unperturbed cell cycle control and DNA integrity checkpoints. J. Biol. Chem. 278, 40815–40828
45. Jin, Y. H., Yoo, K. J., Lee, Y. H., and Lee, S. K. (2000) Caspase 3-mediated cleavage of p21WAF1/CIP1 associated with the cyclin A-cyclin-dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells. J. Biol. Chem. 275, 30256–30263
46. Sun, G., Sharma, A. K., and Budde, R. J. (1998) Autophosphorylation of Src and Yes blocks their inactivation by Csk phosphorylation. Oncogene 17, 1587–1595
47. Craig, K. L., and Harley, C. B. (1996) Phosphorylation of human pleckstrin on Ser-113 and Ser-117 by protein kinase C. Biochem. J. 314, 937–942
48. Marth, J. D., Cooper, J. A., King, C. S., Ziegler, S. F., Tinker, D. A., Overell, R. W., Krebs, E. G., and Perlmutter, R. M. (1988) Neoplastic transformation induced by an activated lymphocyte-specific protein tyrosine kinase (p56lck). Mol. Cell. Biol. 8, 540–550
49. Cooper, J. A. (1991) Estimation of phosphorylation stoichiometry by separation of phosphorylated isoforms. Methods Enzymol. 201, 251–261
50. Cooper, J. A., and Hunter, T. (1983) Identification and characterization of cellular targets for tyrosine protein kinases. J. Biol. Chem. 258, 1108–1115
51. Sefton, B. M. (1991) Measurement of stoichiometry of protein phosphorylation by biosynthetic labeling. Methods Enzymol. 201, 245–251
52. Cohen, P., Gibson, B. W., and Holmes, C. F. (1991) Analysis of the in vivo phosphorylation states of proteins by fast atom bombardment mass spectrometry and other techniques. Methods Enzymol. 201, 153–168
53. Czernik, A. J., Girault, J. A., Nairn, A. C., Chen, J., Snyder, G., Kebabian, J., and Greengard, P. (1991) Production of phosphorylation state-specific antibodies. Methods Enzymol. 201, 264–283
54. Hegeman, A. D., Harms, A. C., Sussman, M. R., Bunner, A. E., and Harper, J. F. (2004) An isotope labeling strategy for quantifying the degree of phosphorylation at multiple sites in proteins. J. Am. Soc. Mass Spectrom. 15, 847–853
55. Bonenfant, D., Schmelzle, T., Jacinto, E., Crespo, J. L., Mini, T., Hall, M. N., and Jenoe, P. (2003) Quantitation of changes in protein phosphorylation: a simple method based on stable isotope labeling and mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. 100, 880–885
56. Zhang, X., Jin, Q. K., Carr, S. A., and Annan, R. S. (2002) N-Terminal peptide labeling strategy for incorporation of isotopic tags: a method for the determination of site-specific absolute phosphorylation stoichiometry. Rapid Commun. Mass Spectrom. 16, 2325–2332
57. Berger, S. L. (2002) Histone modifications in transcriptional regulation. Curr. Opin. Genet. Dev. 12, 142–148