Cisplatin-Induced Inhibition of $p34^{cdc2}$ is Abolished by 5-Fluorouracil

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Clinical (Dimery and Hong, J Nat Cancer Inst 1993; 85: 95–111) and experimental studies (Scanlon et al., Proc Natl Acad Sci USA 1986; 83: 8923–5; Lewin et al., In Vivo 1990; 4: 277–82) have indicated an increased cytotoxic effect, when cisplatin (CDDP) is combined with 5-fluorouracil (5-FU). Addition of 5-FU abolishes the G_2-arrest induced by CDDP (Lewin et al., In Vivo 1990; 4: 277–82; Nylén et al., Acta Oncol 1996; 35: 229–35). The mechanism for the synergy is unclear. Activation of $p34^{cdc2}$ is necessary for progression from G_2 to mitosis (Lewin et al., Anti-Cancer Drugs 1995; 6: 465–70). The aim was to study $p34^{cdc2}$, cdc25C and wee1 after treatment of mammalian tumour cells in vivo with CDDP as single agent or in combination with 5-FU. CDDP prevented activation of $p34^{cdc2}$ by keeping cdc25C inactive and wee1 active. Addition of 5-FU to CDDP decreased the expression of wee1 and promoted cdc25C-activation. $p34^{cdc2}$ was dephosphorylated by cdc25C and activated. Alterations in activity of cdc25C and wee1 after drug combination were due to changes in the protein amount, rather than to changes in the phosphorylation degree.

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Treatment with a combination of CDDP and 5-fluorouracil (5-FU) in patients with squamous cell carcinoma of the head–neck region has resulted in improved tumour response compared to the situation with single drug treatment (1). Experimental studies have supported the presence of a synergistic effect when the drugs are combined (2, 3). The combination of CDDP and 5-FU to in vivo-growing mouse ascites sarcoma cells (Bp8) causes imbalance in the deoxyribonucleotide pools and elevated rate of replicative DNA synthesis (3, 4). Despite these findings there was no evidence of misincorporation of deoxyribonucleotides in DNA (4). No change was observed in induction or repair of CDDP-induced DNA cross links (5). However, the cell cycle arrest in G_2, seen after CDDP as single drug, could not be seen when CDDP was combined with 5-FU (3, 4).

In most cases an activated $p34^{cdc2}$-cyclin B complex is required for progression from G_2 into mitosis (6, 7). The $p34^{cdc2}$ kinase activity is dependent on availability of cyclin B, which accumulates with progression through S and G_2 (8). The $p34^{cdc2}$ kinase activity is regulated by a balance of stimulatory and inhibitory factors acting on the degree of phosphorylation (9). The $p34^{cdc2}$ kinase activity is inhibited, when the threonine-14 and tyrosine-15 residues in the enzyme are phosphorylated. Phosphorylation of the tyrosine-15-site is a key function and in eucaryotes is promoted by the tyrosine kinase wee1 (6), in fission yeast wee1/mik1 phosphorylate tyrosine-15 of $p34^{cdc2}$ (10), in Xenopus wee1/mik1 phosphorylate both threonine-14 and tyrosine-15 (11). In mammalian cells there is also a capacity for phosphorylation of threonine-14, but the responsible enzyme has not yet been described. The presence of more than one signal transduction pathway regulating the activity of $p34^{cdc2}$ is likely.

While the $p34^{cdc2}$ activity is negatively regulated by kinases, such as wee1, $p34^{cdc2}$ is positively regulated by cdc25 phosphatases. In human cells there are at least three cdc25 homologues (A, B and C). In mammalian cells, cdc25C has the ability to dephosphorylate both threonine-14 and tyrosine-15 of $p34^{cdc2}$ prior to onset of mitosis (12). Cdc25C is activated at the end of G_2 by phosphorylation (12). This can be achieved by inactivation of the phosphatases PP1 and PP2A (13). There is a positive feedback loop between cdc25C and $p34^{cdc2}$, which enhances the activities of both enzymes (14). There might also be a coordinated inhibition of wee1 and activation of cdc25C by PP1 and PP2A, which promotes activation of $p34^{cdc2}$ by a rapid, synchronous dephosphorylation of tyrosine-15 (15).

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Any damage to DNA, such as strand breaks and cross-links induced by agents such as etoposide (16) and nitrogen mustard (17), can inhibit the activity in $p34^{cdc2}$ and arrest the cell cycle progression in G_2. The mechanism for propagation of signals from damaged DNA to G_2 checkpoint enzymes is, as yet, not fully understood but DNA-PK and p53 protein may be involved. There is evidence to suggest that a prolonged G_2 phase is of importance for repair of damage and that abrogation of the cell cycle delay, for example by the addition of caffeine, does increase the sensitivity of mammalian cells to DNA damage, caused by CDDP and other agents (18, 19).

CDDP has a cytotoxic effect, mainly due to induction of inter- and intra-strand DNA cross links, but effects on cell membrane function and expression of oncogenes have also been reported (20). CDDP does inhibit the cell cycle progression with accumulation of cells in S and arrest in the G_2 phase (3, 4, 21). CDDP can inactivate the $p34^{cdc2}$-kinase by suppression of the dephosphorylation of $p34^{cdc2}$ necessary for onset of mitosis (19–21), but CDDP did not influence the amount of wee1 or cyclin B (21).

5-fluorouracil is an antimetabolite, which exerts its cytotoxic effect through inhibition of thymidylate synthase (22) incorporation in DNA and RNA (22, 23) and influences the maturation process and metabolism of RNA (24). Treated cells accumulate in early S-phase (3, 4).

Our hypothesis is that the synergistic effect of the combination of CDDP and 5-FU is caused by induction of damage to DNA in parallel with disturbed regulation of cell traverse from G_2 to M phase. The mechanism could thus resemble what has been registered when caffeine is added to DNA-damaging agents with increase in toxicity by abolishment of the G_2 arrest (18, 19). The aim of the present study was to analyse the relationships among wee1, cdc25C, cyclin B1 and $p34^{cdc2}$ at the G_2/M-transition check point.

MATERIAL AND METHODS

Experimental animals

Mouse sarcoma cells (Bp8) were grown intraperitoneally in male NMRI-mice 3 to 5 months old (25–30 g). The mice were kept on a 12 h light and 12 h dark cycle at a constant temperature of 23°C and relative humidity of 50 to 55%. Water and standard food were given ad libitum.

Cytostatic treatment

CDDP (25 μg/mouse corresponding to 1 mg/kg body weight) was diluted in normal saline to a total volume of 0.2 ml/mouse. The drug was injected intraperitoneally. The control mice were at corresponding times injected with the same volume of normal saline.

5-FU (5-fluorouracil, Roche, 2.7 mg/mouse corresponding to 108 mg/kg body weight) was diluted in normal saline to a total volume of 0.2 ml/mouse. The drug was injected intraperitoneally. When 5-FU was given in combination with CDDP, 5-FU was injected 30 min after CDDP. The control mice were at corresponding times injected with the same volume of normal saline.

After 6 h the mice were killed by cervical dislocation and the ascites cell suspension collected and quickly put on ice.

Centrifugal elutriation

Cell separation was carried out using a Beckman JE-G elutriation rotor in a Beckman J21 B centrifuge as described (25). The rotor speed was maintained at 2800 rpm at 4°C. Fractions 2, 3, 4 and 5 were collected at buffer flow rates of 24, 38, 52 and 64 ml/min, respectively. Fraction one, containing dead cells, was discarded.

Cell cycle composition

Aliquots of cells were fixed in ice-cold ethanol and the DNA was stained with ethidiumbromide. The distribution of cells in the cell cycle was determined by flow-cytofluorometry as described earlier (26). The relative number of cells in G1, S and G2 + M phases were calculated using a computer program (Ahrens, Germany).

Cell preparation

Cells were lysed in a lysing buffer (10 mM Tris–HCl, 25 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 1 μg/ml leupeptin and aprotinin, 10 μg/ml soybean trypsin inhibitor and tosylphenylanine chloromethyl ketone) for 30 min at 4°C. After centrifugation for 30 min at 45000 g, the supernatant was removed and cleaned by filtration (Millex GV 0.22 μm, Millipore) and then incubated with 30 μl Protein Sepharose A CL-4B (Pharmacia Biotech, 1 × 1 with lysing buffer) for 30 min at 4°C. The Sepharose was removed by centrifugation at 11000 g for 10 min at 4°C. The protein concentration was determined (Lowry) and the samples were diluted with lysing buffer to a concentration of 4 mg/ml.

Immunoprecipitation of cyclin B1-$p34^{cdc2}$-complex

Cell extracts (200 μg) were incubated with 2 μg anti-cyclin B1 mouse monoclonal antibody (Santa Cruz Biotechnology) for 1 h at 4°C, then 25 μl Protein A Sepharose CL-4B (Pharmacia Biotech) was added. After a further hour, the precipitate was washed three times with lysing buffer containing 0.5 M NaCl by centrifugation at 11000 g for 10 min. The pellet was finally resuspended in Laemmli sample buffer (0.0625 M Tris, 10% β-mercapto-ethanol, 20% glycerol, 4% SDS, pH 6.8), kept in 95°C for 3 min and run on a SDS gel electrophoresis (Bio-Rad, Miniprotean) or used for $p34^{cdc2}$ activity assay.

Western blot

The immunoprecipitate was diluted in 50 μl 1 × Laemmli sample buffer. When the unprecipitated cell extracts were

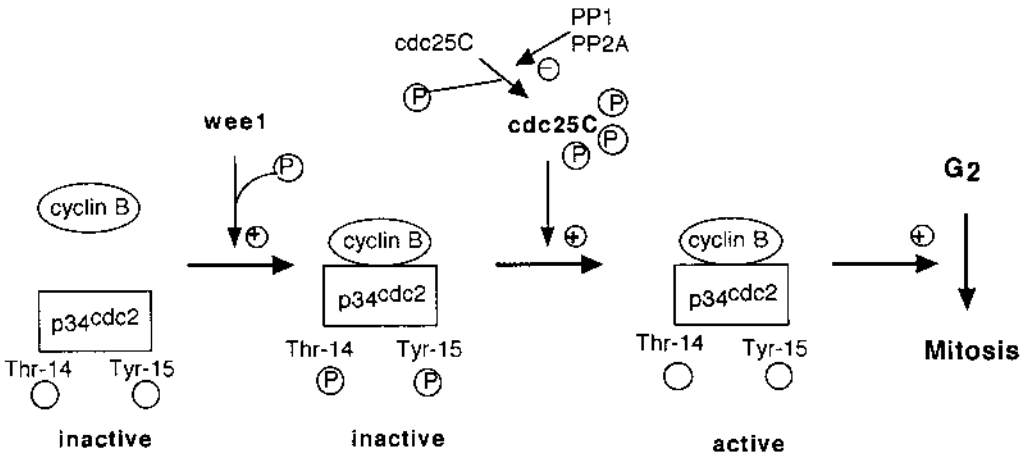

Fig. 1. Schematic presentation of interacting factors regulating the G_2-M-check point described in the text. During the G_2 phase, cyclin B1 associates with p34^{cdc2} and a functional enzyme complex is formed. As long as Tyr-15 of p34^{cdc2} is phosphorylated by wee1 kinase, the p34^{cdc2} enzyme activity is inhibited. In late G_2 the cdc25C phosphatase is activated by phosphorylation. Thr-14 and Tyr-15 in p34^{cdc2} are then dephosphorylated and the mitosis initiated.

used, they were diluted 1:1 with 2 × Laemmli sample buffer. Ten μl (20 μg) of the sample was put on SDS polyacrylamide gel and the electrophoresis was run as described by Bio-Rad. After electrophoretic transfer to HybondTM-ECL membrane, the immunostaining was performed as described by Amersham LIFE Science (1993, ECL Western blotting protocols, Amersham Int. plc, Bucks/UK 1993), slightly modified. The membrane was blocked in TBS-T buffer (0.02 M Tris, 0.137 M NaCl, 0.01% Tween-20, pH 7.6) containing 5% non-fat milk and 0.02% NaN_3 for 4 h at room temperature. As primary antibody one of anti-cyclin B1 monoclonal antibody, anti-p34^{cdc2} polyclonal antibody, anti-wee1 polyclonal antibody, anti-c25C polyclonal antibody, all from Santa Cruz Biotech, or anti-phosphotyrosine monoclonal antibody (4G10) from Upstate Biotechnology was added and incubation proceeded overnight at 4°C. The membranes were incubated for 1 h at room temperature with a second biotinylated antibody (1:5000) followed by 1 h incubation with avidin HRP conjugate (1:5000). The membrane was washed several times with TBS-T between each step. Cyclin B1, p34^{cdc2}, wee1 and cdc25C were detected by ECL (Amersham). A laser densitometer (LKB, Ultrascan XL) was used for quantification of the results. The values were expressed as area units/mg protein.

p34^{cdc2} kinase activity

The cyclin B1 immunoprecipitate was washed three times with lysing buffer containing 0.5 M NaCl and once with assay buffer (50 mM Tris–HCl, pH 74, 10 mM $MgCl_2$, 1 mM DTT). For the kinase reaction 25 μl assay buffer with Histon 1 (50 μg/ml Boehinger, Mannheim) and ATP (5 μCi ^{33}P, Amersham/50 mM ATP) was added and incubated at 37°C for 30 min. The reaction was stopped by addition of 25 μl 2 × Laemmli sample buffer. The samples were kept at 95°C for 3 min and run on a 15% SDS gel electrophoresis, as described above. After autoradiography the Histon 1-kinase activity was determined by using a laser densitometer (LKB, Ultrascan XL).

Cdc25C activity

Cdc25C phosphatase activity was measured by a spectrophotometric colour reaction from the dephosphorylation of pNPP (p-nitrophenyl phosphate disodium 6 H_2O). Cell extract (200 μg) was incubated with 2 μg anti-cdc25C polyclonal antibody (Santa Cruz Biotech), for 1 h at 4°C. Twenty-five μl Protein-A Sepharose was added and the incubation proceeded for one more hour at 4°C and constant rotation. The immuno-precipitate was washed four times with lysing buffer containing 0.5 M NaCl and centrifuged at 11000 g at 4°C. After the last wash, 100 μl assay buffer (1 mM EDTA, 20 mM dNPP, Sigma and 0.1% 2-mercaptoethanol) was added to the immuno-precipitate. After incubation at 37°C for 1 h, the kinase reaction was stopped by addition of 50 μl 0.5 M NaOH. The degree of dephosphorylation was measured spectrophotometrically at 400 nm.

RESULTS

CDDP causes a transient, dose-dependent arrest in G_2 (3, 4). When the regulation of G_2-M check-point traverse is to be investigated after CDDP treatment, it is of interest to analyse cells when the mechanism causing arrest is still active. Therefore, the investigations were made 6 h after treatment. Fig. 1

Synchronized cells are required for investigations of cell cycle-regulating factors at specific check points in the cell cycle. For that purpose we have used centrifugal elutriation, which allows cell separation without impairment of cellular functions. We separated the cells in four cell cycle

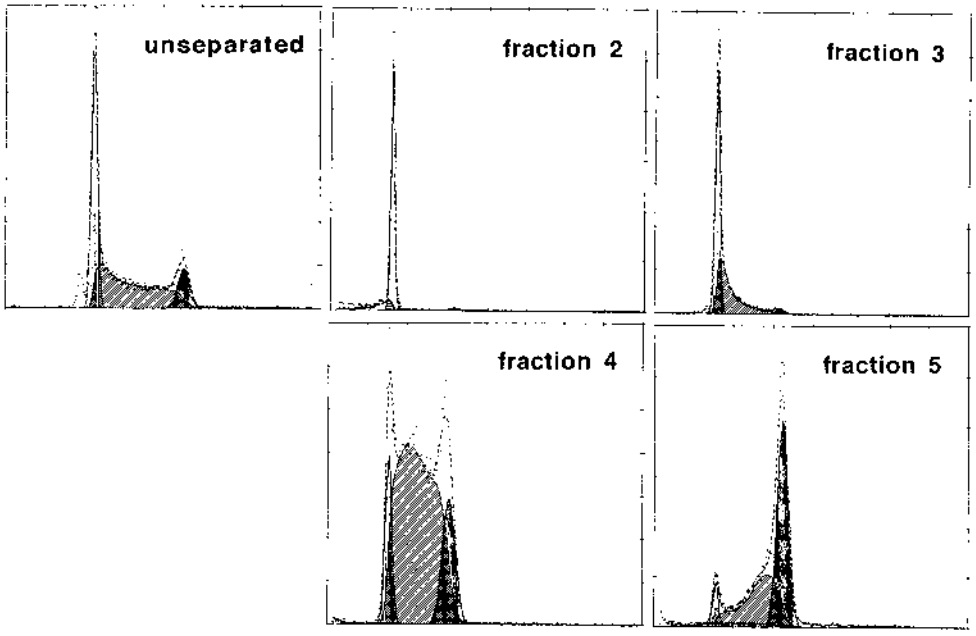

Fig. 2. Representative histograms of untreated Bp8 tumour cells showing the cellular content of DNA in fractions collected in centrifugal elutriation. In the histogram of unseparated cells the very small peak to the left represents normal mouse cells. The largest peak, i.e. the second peak from the left, corresponds to the G_1 and the peak to the right corresponds to the G_2 cells of the tumour. The S phase is shown as the area between G_1 and G_2 peaks. Fraction 2 represents G_1-enriched cells. Fraction 3 represents G_1-S cells. Fraction 4 represents mainly S cells. In fraction 5 about 50% of the cells are in G_2.

fractions, numbered 2 to 5. Fraction No. 5 contained 50–60% of cells in the G_2 phase (Figs. 2 and 3). The various cell cycle regulating factors were studied by means of Western blots. The amounts of wee1, cdc25C and cyclin B1 were determined in cell extract, while the amounts of phosphorylated tyrosine-15 of $p34^{cdc2}$ as well as the activity of $p34^{cdc2}$ were measured in the immunocomplex cyclin B1/$p34^{cdc2}$. The cdc25C activity was determined after immunoprecipitation with anti-cdc25C polyclonal antibody. The degree of phosphorylation of cdc25C was determined from the shift in molecular weight in the Western blot.

In the G_2-enriched fraction (No. 5) of untreated tumour cells a reduced phosphorylation of $p34^{cdc2}$ (Tyr-15-site) was found, as compared to cell fractions from the earlier cell cycle phases. This was consistent with an increased phosphorylating activity by $p34^{cdc2}$. The amount of cdc25C was constant in all fractions (Nos. 2–5) but the proportion of the phosphorylated form of cdc25C increased from about 50% in G_1 cells to about 80% in the G_2-enriched cell fraction. The phosphatase activity of cdc25C increased markedly in the G_2-enriched fraction (No. 5) of the elutriated cells. The amount of wee1 was decreased in G_2, as compared with G_1- and S-phase cells. The amount of cyclin B1 was slightly increased in G_2. The results from untreated tumour cells were thus in line with what could be expected (Figs. 4 and 5).

CDDP as well as 5-FU does affect the cell cycle progression; CDDP gives rise to G_2 arrest, while 5-FU mainly arrests cells in early S phase (4). In this investigation we studied the effect of CDDP and 5-FU 6 h after treatment. At that time the redistribution of the cell cycle is not yet pronounced (Fig. 3).

CDDP treatment

Six hours after treatment with CDDP, the activity of $p34^{cdc2}$ in the G_2-enriched fraction (No. 5) was reduced, while the amount of cyclin B1 was increased, as compared with the untreated control (Figs. 4 and 5). The reduction of $p34^{cdc2}$ activity was further supported by an elevated phosphorylation of the $p34^{cdc2}$ on Tyr-15 (Figs. 4 and 5). In parallel, the amount and activity of cdc25C were reduced (Figs. 4 and 5). However, there was no shift in phosphorylation degree of the enzyme, as determined from the Western blots (Fig. 4). The amount of wee1 was increased (Figs. 4 and 5).

5-FU treatment

The effect of 5-FU on the cell cycle regulation factors was partly similar to the effect of CDDP. Thus, 6 h after treatment, the activity of $p34^{cdc2}$ in the G_2-enriched fraction was diminished, when compared with untreated cells (Figs. 4 and 5). This was accompanied by an elevated phosphorylation of Tyr-15 (Figs. 4 and 5). The amount of wee1 was increased (Figs. 4 and 5). The effect of 5-FU on cdc25C did, however, differ from that of CDDP treatment. The amount of cdc25C was increased, which is likely to indicate an increased activity. However, we were not able to confirm that, since the enzyme activity of cdc25C was

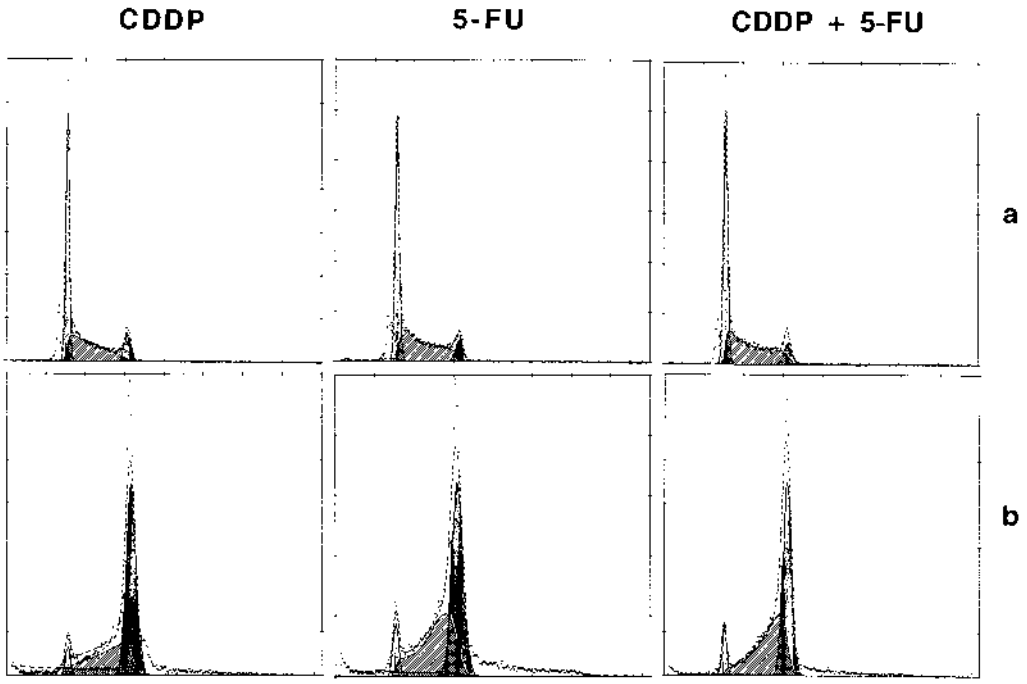

Fig. 3. Representative DNA histograms of Bp8 tumour cells treated with CDDP, 5-FU or a combination, as described in Material and Methods. a) Unseparated cells, b) fraction 5 after centrifugal elutriation (G_2-enriched). The different peaks are described in Fig. 2.

not determined. No shift in the phosphorylation degree was found, however. The amount of cyclin B1 was increased (Figs. 4 and 5).

Treatment with a combination of CDDP and 5-FU

When 5-FU was added 30 min after CDDP treatment, the activity of p34^{cdc2} was not reduced in the G_2-enriched fraction of cells 6 h after treatment and the p34^{cdc2} activity was almost equal to that in untreated cells (Figs. 4 and 5). This contrasted with the result when CDDP or 5-FU were used as single agents. After treatment with the drug combination the amount of phosphorylated p34^{cdc2} was reduced when compared to untreated cells (Figs. 4 and 5). The amount of wee1 was reduced, while the amount and activity of cdc25C were elevated as compared to untreated cells (Figs. 4 and 5). As was the case when cells were treated with CDDP or 5-FU alone, no shift in the phosphorylation degree of cdc25C was found. The amount of cyclin B1 was increased, as after single drug treatment (Figs. 4 and 5).

DISCUSSION

The present results show that CDDP-induced G_2 arrest is caused by alterations in the activities of the p34^{cdc2}, wee1 and cdc25C, which regulate the G_2-M-transition check point. CDDP treatment tends to inhibit dephosphorylation of the p34^{cdc2} on Tyr-15 with persistence of inactivity in p34^{cdc2}. The abolished dephosphorylation is caused by failure in activation of cdc25C and probably also in failed deactivation of wee1. Moreover, the available amount of wee1 is increased. Addition of 5-FU to CDDP results in normal activation of p34^{cdc2} at the G_2-M-transition check point. Changes in activation of p34^{cdc2} in cells treated with CDDP, 5-FU or a combination of the drugs is paralleled by alterations in the expression of both wee1 and cdc25C (Fig. 6).

The modification by 5-FU of the CDDP effects on the enzymatic regulation of mitotic onset has not been reported earlier. How the 5-FU effect is exerted on the molecular level in this context is still unanswered. However, we can conclude, that the change in activity of the mitotic regulating p34^{cdc2} kinase, when CDDP or a combination of CDDP and 5-FU is given, is well in accordance with our earlier findings. In these experiments we registered an abolished G_2 arrest with ensuing increase in cytotoxicity after the drug combination (3, 4).

To improve the specificity in the cell cycle-related analyses, a synchronized cell population is necessary. We used the elutriation technique and could sort living cells in different fractions of the cell cycle. For analysis of the cell cycle-regulating factors at the G_2-M check point a G_2-enriched fraction of about 50% was used (Fig. 2). For the analysis of p34^{cdc2} activity, the immunocomplex of cyclin B1/p34^{cdc2} was used, which eliminated interference by the p34^{cdc2} kinase active in G_1 and early-/mid-S phase on the measurements of p34^{cdc2} activity at the G_2-M-transition check point. For cdc25C the activity is confined to the final part of G_2. Wee1 accumulates and exerts activity in S and early/mid G_2, when it is of vital importance for cells to

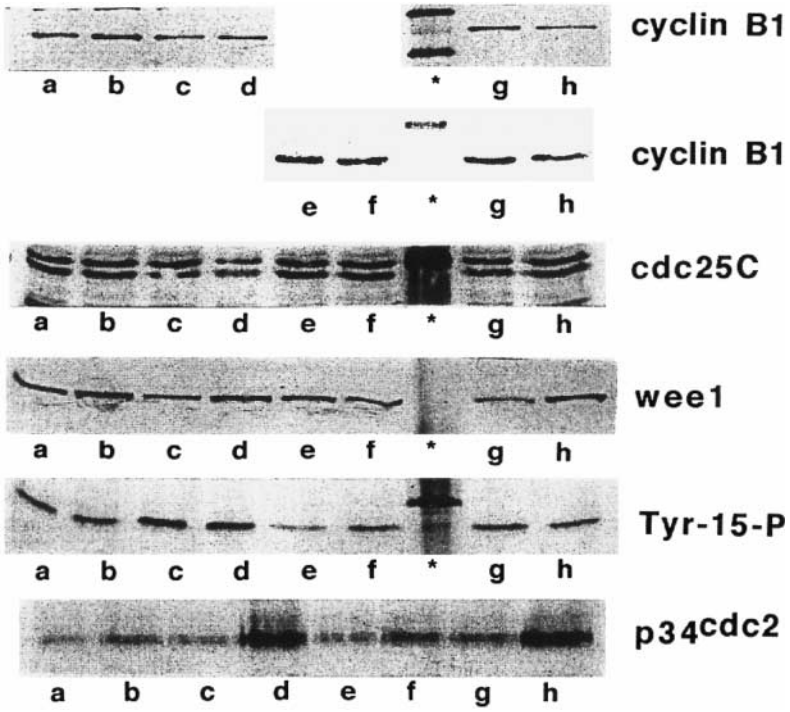

Fig. 4. Representative examples of Western blot results. The concentration of SDS in the gel was 7% (wee1), 10% (cyclinB1, cdc25C) or 15% ($p34^{cdc2}$, PTy). The intensities of the bands were scanned, as described in Material and Methods, and the values adjusted for the content of protein. a) Fraction 4, CDDP; b) fraction 5, CDDP; c) faction 4, 5-FU; d) fraction 5, 5-FU; e) fraction 4, CDDP + 5-FU; f) fraction 5, CDDP + 5-FU; g) fraction 4, untreated cells; h) fraction 5, untreated cells, *lane of mw-marker.

avoid a premature mitosis (6). The G_2-enriched cell fraction (No. 5) in our study (Figs. 3 and 4) contained up to 50% of cells in S phase. The wee1 that we detected in fraction No. 5 thus originates from S as well as from G_2. A decrease in wee1 amount/activity at the G_2-M border may be too small to be detectable over the activity present in S and early-/mid-G_2 cells.

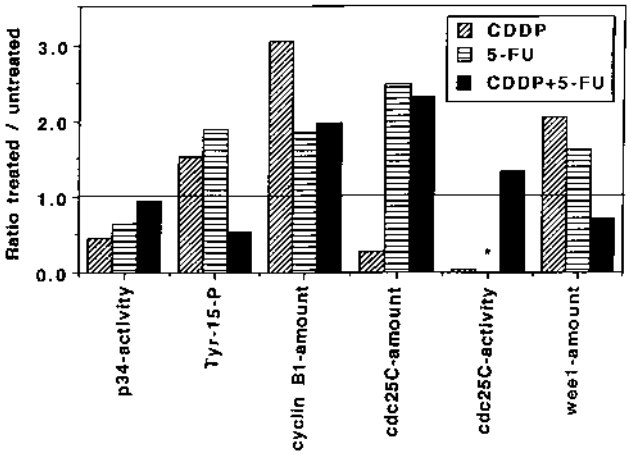

Fig. 5. Diagram showing the results from Western blots of cells from the G_2-enriched fraction. For $p34^{cdc2}$ the diagram shows the result of enzyme activity measurement by the histone 1 phosphorylation assay. 'Tyr-15-P' shows the degree of phosphorylation of Tyr-15 in $p34^{cdc2}$. The cdc25C amount refers to the total amount of hypophosphorylated and phosphorylated enzyme. Cdc25C activity refers to measurement of the kinase activity. All results are shown as ratios of values from treated cells to untreated cells at each experiment. The results are representative examples from 3–6 experiments. (*The cdc25C activity was not measured in the 5-FU treated cells.)

The active wee1 present in murine cells is composed of 646 amino acids (38) and constitutes a protein of about 66 kD. In the Western blot of Bp8 cell extract we found, that wee1 corresponded to 98 kD, which represents a phosphorylated form of the native, murine 66 kD protein. However, different molecular weights of wee1 have been reported even though the antibody has been identical. Thus, in extract of human lung cancer cells the molecular weight was 110 kDa (21), whereas the molecular weight was 49 kDa in human malignant B-cells after anti-wee1 antibody immunoprecipitation before the Western blot (27). We found results in parallel, as Western blot of the wee1 kinase in cell extract indicated a molecular weight of about 98 kDa, whereas anti-wee1 antibody immunoprecipitation before the Western blot gave a molecular weight of about 50 kDa. The antibody we used thus enabled detection of both the dephosphorylated, active form and the phosphorylated, inactive form of wee1. The 98 kD band, presented in 'Results', corresponded to the dephosphorylated, active form of the kinase.

Cdc25C is cytoplasmic but translocates, together with cyclin B1, to the nucleus at the G_2-M transition (28). According to literature and our data from untreated cells (not shown), the protein level of cdc25C is fairly constant

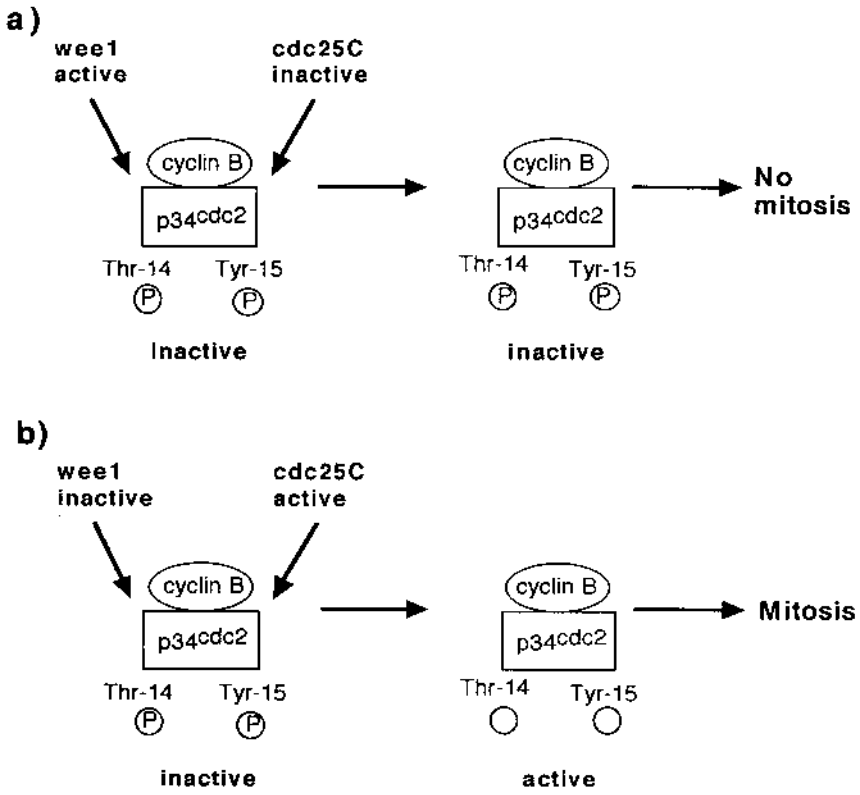

Fig. 6. Schematic presentation of how CDDP and CDDP + 5-FU influence the G_2-M-transition. a) CDDP. During the G_2 phase, cyclin B1 associates with p34^{cdc2} and a functional enzyme complex is formed. By lack of degradation of wee1 the Tyr-15 phosphorylation of p34^{cdc2} is maintained. The amount of cdc25C is decreased and the cdc25C-phosphatase activity low. The kinase activity of p34^{cdc2} is inhibited and the cells remain in G_2. b) CDDP + 5-FU. The amount of wee1 kinase is decreased and the expression and activity of cdc25C is increased as compared to untreated cells. The Tyr-15 phosphorylation of p34^{cdc2} is removed and p34^{cdc2} kinase activated. The mitosis is initiated.

during the cell cycle. The enzyme undergoes a shift to a phosphorylated, active form prior to mitosis (14, 15), which we also found in the untreated cells. Active cdc25C dephosphorylates Tyr-15 and Thr-14 of p34^{cdc2} and initiates the mitosis. Furthermore, activation of cdc25C is dependent on presence of active p34^{cdc2}-cyclin B complexes. Thus, a positive feed-back loop is created, which may be responsible for the rapid activation of p34^{cdc2}-cyclinB complexes at the onset of mitosis (14, 15) (Fig. 1).

The p34^{cdc2}cyclin B complex is reported to activate mitosis only when located in the nucleus. This implies that changes in subcellular localization of the complex can regulate the enzyme activity (28). It also seems that the wee1 activity in the nucleus is of prime importance in the regulation of the mitotic onset. There is a report claiming that the nuclear fraction of wee1 enzyme phosphorylates and inactivates incoming p34^{cdc2}-cyclin B complexes during G_2 (28). The initiation of mitosis thus can be regulated by different precise mechanisms.

In untreated as well as in treated cells we found a close correlation between the activity of p34^{cdc2} and its degree of phosphorylation. This confirms that p34^{cdc2} activity is regulated by phosphorylation. For cdc25C, however, such a correlation was found only in the untreated cells, where a shift from an underphosphorylated to a hyperphosphorylated form was seen, when cdc25C was activated in G_2. When cells were treated with CDDP and 5-FU in combination, cdc25C was activated as in untreated cells. However, the elevated activity of cdc25C was paralleled with an increase in amount of the enzyme rather than by a shift in degree of phosphorylation. Furthermore, when the cells were treated with CDDP only, the amount of cdc25C was decreased in G_2 cells. These results may indicate regulation of the cdc25C activity on the transcriptional or translational level. The results were unexpected, since the cdc25C activity in untreated, normally growing cells seems to be regulated by phosphorylation, rather than by a change in expression of the protein. When compared with untreated cells, the amount of wee1 was maintained after single-agent treatment with CDDP or 5-FU. This may indicate influence on the degradation process. The conclusion is, that treatment with CDDP alone or in combination with 5-FU seems to exert its effect on cdc25C and wee1 activities by influencing the expression of the proteins rather than by influencing the regulation of phosphorylation.

Cyclin B1 accumulates during G_2 and forms a complex with p34^{cdc2} before mitosis. In the present experiments,

after both single-agent treatment and the combination treatments, the amount of cyclin B1 was increased in comparison to the untreated cells. This eliminates a deficit of cyclin B1 as explanation for G_2 arrest after single-agent treatment.

The principal way of action for CDDP is induction of DNA cross-links. Our results of down-regulation of the $p34^{cdc2}$ activity and G_2 arrest after CDDP treatment are in good accordance with earlier reports. Cell cycle targets for the check-point regulation to bring about G_2 delay in response to DNA damage, however, remain to be established. When cell division is prevented in response to damaged DNA, there may be a converging signal to the same target in the cell as when cell division is withheld due to the presence of unreplicated DNA (29). Tyrosine-15 phosphorylation of $p34^{cdc2}$ could be a target for the negative regulation. This can be achieved by upregulation of wee1 kinase and/or downregulation of cdc25C phosphatase. Formation of the $p34^{cdc2}$/cdc25C autocatalytic feedback loop, which normally brings about rapid activation of $p34^{cdc2}$, can also be suppressed (15, 17). In yeast the wee1 function is needed for induction of G_2 arrest by CDDP. When the wee1 function was defective the sensitivity to CDDP was increased (30). Thus, there is support for the conclusion, that wee1 is important for the inhibition of $p34^{cdc2}$ after CDDP.

The abolished G_2 arrest, which can be explained by the present results, is likely to be central for the increased toxicity of the combination. At present, the question remains to be answered whether the action of 5-FU, when it abolishes the CDDP-induced G_2 arrest, is the result of interaction on one step in the cell cycle regulatory mechanism or whether 5-FU is acting on different targets in parallel. However, the effects on cdc25C and wee1 can explain the abolished G_2 arrest, when 5-FU is added to CDDP.

Further studies of the combination of agents that damage DNA with agents that influence cell cycle regulation will show whether this concept can improve clinical tumour treatment.

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