

## Pesticide Roundup Provokes Cell Division Dysfunction at the Level of CDK1/Cyclin B Activation

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To assess human health risk from environmental chemicals, we have studied the effect on cell cycle regulation of the widely used glyphosate-containing pesticide Roundup. As a model system we have used sea urchin embryonic first divisions following fertilization, which are appropriate for the study of universal cell cycle regulation without interference with transcription. We show that 0.8% Roundup (containing 8 mM glyphosate) induces a delay in the kinetic of the first cell cleavage of sea urchin embryos. The delay is dependent on the concentration of Roundup. The delay in the cell cycle could be induced using increasing glyphosate concentrations (1–10 mM) in the presence of a subthreshold concentration of Roundup 0.2%, while glyphosate alone was ineffective, thus indicating synergy between glyphosate and Roundup formulation products. The effect of Roundup was not lethal and involved a delay in entry into M-phase of the cell cycle, as judged cytologically. Since CDK1/cyclin B regulates universally the M-phase of the cell cycle, we analyzed CDK1/cyclin B activation during the first division of early development. Roundup delayed the activation of CDK1/cyclin B *in vivo*. Roundup inhibited also the global protein synthetic rate without preventing the accumulation of cyclin B. In summary, Roundup affects cell cycle regulation by delaying activation of the CDK1/cyclin B complex, by synergic effect of glyphosate and formulation products. Considering the universality among species of the CDK1/cyclin B regulator, our results question the safety of glyphosate and Roundup on human health.

### Introduction

Widespread use of pesticides leads to chronic exposure to a combination of various products. Despite the obvious benefits of pesticides, their extensive use has posed problems for both environment and human health. Among the human health concerns, exposure to several groups of pesticides has been associated with various cancers (1, 2) or reproductive and developmental disorders (3). Therefore, searches for mechanisms by which pesticides could interfere with cell cycle or developmental processes are of great interest.

The cell cycle is the universal process by which cells reproduce and underlies the growth and development of all living organisms. Controls operate during the cell cycle to regulate the onset of DNA replication and segregation of the replicated chromosomes. Surveillance mechanisms, called checkpoint (4) pathways, ensure the proper order and correct execution of cell cycle events. The molecular basis of these controls is highly conserved from single unicellular eucaryotes to complex metazoans such as humans (for review, see refs 5–7). Human cancers are associated with defects in the control of cell cycle transitions (8). Errors in the choreography of the process of mitosis and cytokinesis can lead to genetic instability fostering cell death or disease. Failures of these checkpoints allow cells to divide when DNA is incorrectly replicated, or when chromosomes are incor-

rectly segregated to daughter cells, resulting in increased genetic damages that is crucial in the generation of cancer (for review, see refs 5–7).

Marine invertebrates, including sea urchin gametes and embryos (9) have proven to be powerful models for conceptual advances in the cell cycle and its regulation (5). Early development offers the opportunity to analyze synchronous embryonic cell divisions, during first cleavages after fertilization in the absence of transcriptional control (9).

We have studied early development in sea urchin in order to provide a molecular basis for assessing the risk of pesticide usage for altering cell cycle controls.

We have investigated effects of the pesticide Roundup. Roundup contains glyphosate as the active ingredient; it has been used since 1974 and is currently spread worldwide (for review, see ref 10). Glyphosate is a nonselective herbicide that inhibits plant growth through interference with the production of essential aromatic amino acids (11). The adverse effects of glyphosate and Roundup are periodically reevaluated and toxicity of the pesticide has been described for severe exposure conditions (for review, see ref 10). It has been suggested that the toxicity of glyphosate-containing products was related to the surfactant components of the formulation products (12). In normal usage and at chronic exposure, several regulatory agencies and scientific institutions worldwide have concluded that there is no indication of any human health concern with glyphosate and Roundup (10).

We report here that Roundup causes changes in cell cycle regulation that may raise questions about the effect of this pesticide on human health.

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## Materials and Methods

**Chemicals.** *N*-(Phosphonomethyl) glycine (glyphosate) was purchased from Cluzeau Info Labo (France) and the pesticide Roundup (containing 170 g/L isopropylamine glyphosate salt) was from a commercial source. [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol) and [ $^{35}$ S]-L-methionine (1000 Ci/mmol) were obtained from Amersham (France). The purified protein kinase CDK1/cyclin B from Starfish was gift from Dr. L. Meijer (Roscoff, France). Emetine and Histone H1 (type III-SS) were purchased from Sigma. CNBR-activated Sepharose was from Pharmacia France. A polyclonal antibody directed against cyclin B (13) was a generous gift from Gérard Peaucellier (Banyuls, France).

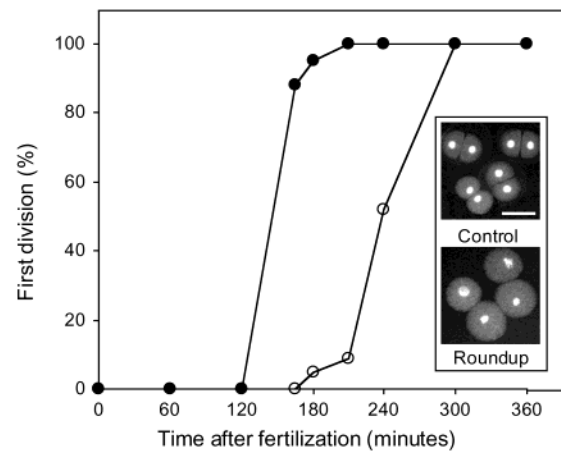
**Handling of Eggs and Embryos.** The sea urchins *Sphaerechinus granularis* were collected in the Brest area, kept in seawater and used within 5 days. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22  $\mu$ m-Millipore-filtered seawater and rinsed twice by centrifugation at 2000 rpm for 2 min. For fertilization, eggs were suspended in Millipore-filtered seawater (5% suspension) containing 0.1% glycine. Dilute sperm was added to the eggs and withdrawn after fertilization membrane elevation. Experiments were only performed on batches exhibiting greater than 90% fertilization and each experiment used gametes from a single female. Pesticide solutions were adjusted to pH 7.5 before addition to the embryos suspended in Millipore-filtered seawater. Thousands of embryos were incubated for each experimental determination from which around one hundred were scored for the developmental stage.

**Sea Urchin Development and Cytological Observations.** Embryos were cultured at 16 °C with constant stirring and observed at short time intervals by phase contrast microscopy for developmental progression. At various times after fertilization, 0.2 mL aliquots of the egg suspension was fixed for at least 2 h in 0.5 mL methanol/glycerol (3:1) in the presence of the DNA dye bisbenzimidazole (0.1  $\mu$ g/mL), mounted in 50% glycerol and observed under fluorescence microscopy.

**Preparation of Egg and Embryo Extracts.** Every 15 min after fertilization, 1 mL samples of an embryo suspension were rapidly packed by centrifugation for 5 s at full speed in an Eppendorf centrifuge, immediately frozen in liquid nitrogen and kept at -80 °C until further processing. For homogenization through a 25 gauge needle, embryos were suspended in 400  $\mu$ L ice-cold buffer (60 mM  $\beta$ -glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.2, 15 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenyl phosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10  $\mu$ g/mL soybean trypsin inhibitor, 100  $\mu$ M benzamide, 1% Triton). The homogenates were clarified by centrifugation at 14 000 rpm for 10 min at 4 °C.

**Measurement of CDK1/Cyclin B Activity in Vitro.** CDK1/cyclin B activity was assayed under standard conditions using histone H1 as a substrate (14). The reaction mixture (50  $\mu$ L) contained 1  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP (20  $\mu$ Ci/mL final), 10  $\mu$ M unlabeled ATP, 2.5  $\mu$ g of histone III-SS (0.05 mg/mL final) in buffer containing 60 mM  $\beta$ -glycerophosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM MOPS, pH 7.2, 5 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM sodium orthovanadate. After 10 min at 30 °C, the reaction was stopped by chilling the tubes on ice. Duplicate aliquots of 10  $\mu$ L were spotted onto Whatman P81 phosphocellulose paper, which were washed five times in 1% phosphoric acid and counted in water in a 1450 MicroBeta-counter (Wallac, EG&G Instruments).

**Analysis of CDK1/Cyclin B Activation in Vivo.** The activation state of CDK1/cyclin B was determined from activity measurements of the protein kinase purified from the embryos at various times post-fertilization. At each time point, the CDK1/cyclin B complex was isolated from the embryo extracts by affinity chromatography on p13<sup>suc1</sup>-Sepharose beads (15), prepared using bacterially produced p13<sup>suc1</sup> protein coupled to Sepharose beads (16). The embryo extracts (300  $\mu$ L) were incubated for 45 min under constant rotation at 4 °C in the



**Figure 1.** Kinetics of the first division of early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater (full circles) or into seawater containing 0.8% Roundup (open circles). One hundred embryos were scored for the first cleavage by phase contrast microscopy as a function of time after fertilization. Insert: nuclear division shown at 150 min following fertilization scored by fluorescence microscopy observation of control- and Roundup-treated embryos stained with Hoechst dye. Bar: 90  $\mu$ M.

presence of 50  $\mu$ L p13<sup>suc1</sup>-Sepharose beads. After three washes, the activity of the bound kinase was determined under the standard conditions reported above.

**Determination of Protein Synthesis Rate in Vivo.** Batches of embryos (1 mL) were incubated in the presence of 10  $\mu$ Ci/mL [ $^{35}$ S]-L-methionine. After 5 min pulse incubations, embryos were rapidly pelleted and frozen. [ $^{35}$ S]Methionine incorporation into proteins was measured on duplicate aliquots of the embryo extracts after 10% TCA precipitation on Whatman 3M filters and counting in the presence of Optiphase Supermix scintillation liquid.

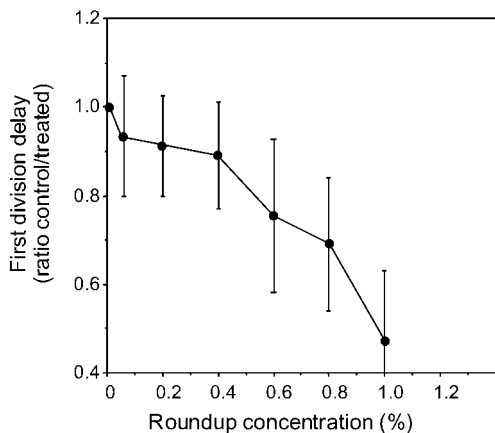
**Analysis of Cyclin B by Immunoblot.** Cyclin B detection was performed on whole extracts from embryos taken every 15 min after fertilization. Pellets from 200  $\mu$ L of a 5% egg suspension were boiled for 3 min in 200  $\mu$ L of electrophoresis sample buffer (17). After clearing by centrifugation, 10  $\mu$ L of the embryo extract were resolved on 12% acrylamide gel electrophoresis (17), transferred on nitrocellulose (18), stained by Ponceau Red and processed for western blotting using anti-cyclin B antibody (13) detected with goat anti-rabbit Ig G (H+L)-AP conjugate (Bio-Rad) using the NBT-BCIP reagent (Fulka).

## Results

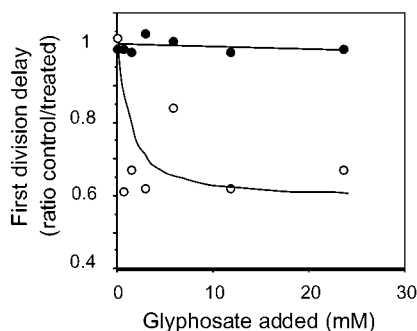
**Effect of Roundup on the First Cell Cycle.** Sea urchin early development was used to monitor cellular dysfunction induced by Roundup. Several steps of early development were affected by the presence of Roundup in the incubation medium. Abnormality in the first cell cycle of early development was observed and further analyzed.

The presence of Roundup at 0.8% (containing 8 mM glyphosate) applied at 10 min postfertilization affected the first cell cycle as judged by a delay in the occurrence of the first cleavage. In a typical experiment (Figure 1), the cleavage occurred 120 min postfertilization in the earliest embryos and had occurred in 100% of the embryos at 210 min, whereas cleavage started at 165 min postfertilization in the earliest pesticide-treated embryos and had occurred in all pesticide-treated embryos at 300 min.

Pesticide-treated embryos were undistinguishable from the control foster embryos at the cytological level after Hoechst staining (see Figure 4). The insert of Figure 1



**Figure 2.** Dose-response effect of Roundup on the first division of early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater or into seawater containing Roundup at various indicated concentrations. The time at which 50% of the embryos cleaved was determined by observation of the embryos by phase contrast microscopy and compared for untreated versus treated embryos in nine different experiments. Vertical bars represent the standard deviation for the nine independent experiments.



**Figure 3.** Synergistic effects of Roundup and glyphosate on the first division of early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater containing increasing concentrations of glyphosate, together with 0.2% Roundup which already contains 2 mM glyphosate (open circles) or without Roundup (full circles). The time at which 50% of the embryos cleaved was determined by phase contrast microscopy and compared in control-versus treated-embryos. The figure is representative of three independent experiments.

illustrates embryos at 150 min postfertilization. Embryos remained healthy as judged by further development of almost all treated embryos through all the cleavage stage. When the pesticide-treated embryos were incubated up to 6 h and then transferred in pesticide-free seawater, their development proceeded as for the control embryos reaching the prisme stage 2 days postfertilization, thus indicating that 6 h incubation in 0.8% Roundup was not lethal and did not provoke early developmental irreversible damage.

The effect of various concentrations of Roundup on the first cell cycle delay was analyzed as the time at which 50% of the embryos had cleaved. Kinetics of first cleavage among different batches of embryos show inherent biological variability. Comparison could be performed using the ratio between the times necessary to reach 50% of first mitotic division for the control embryos versus that for the foster treated-embryos. The delay in the first cleavage kinetic was reproducibly observed and was proportional to the concentration of Roundup (Figure 2). In all experiments, the dose-response effect of Roundup occurred in a narrow concentration window (see Figure

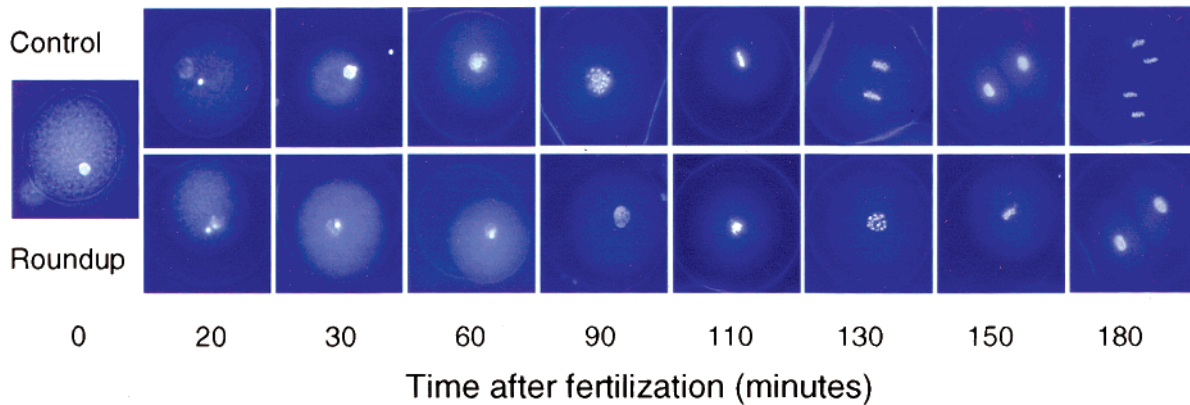
2). A concentration of Roundup of 0.68% (containing 6.8 mM glyphosate) was estimated to produce half the maximal delay effect. When Roundup concentration exceeded 1% (containing 10 mM glyphosate), development was generally arrested at the first cell cycle stage.

The minimal exposure time required for cell cycle dysfunction was examined by scoring first cleavage under various exposure conditions. In a set of incubations, Roundup was added to seawater at different times after fertilization and maintained continuously. A significant delay in first cleavage was observed when the pesticide was added at 10, 30, or 60 min postfertilization. Added after 90 min, Roundup was no longer able to delay the first mitotic division. In a second set of incubations, Roundup was transiently added to the medium for different time intervals. A 30 min pulse exposure, applied from 10, 30, or 60 min postfertilization, did not delay the first cleavage. On the other hand, a 60 min exposure to Roundup, applied from 10 or 30 min postfertilization, consistently delayed the first mitotic division. Therefore, dysfunctions in the first cell cycle occur if 0.8% Roundup applied to embryos for at least 60 min is starting within the first hour following fertilization.

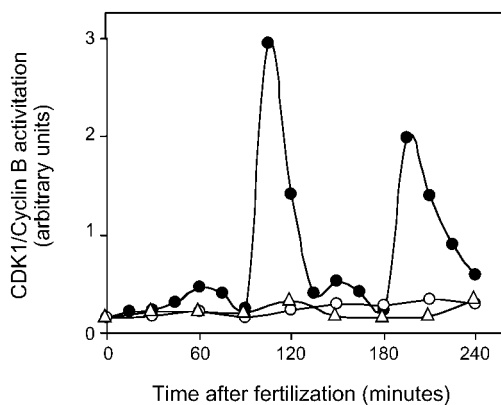
**Effect of Glyphosate on the First Cell Cycle.** The commercial Roundup contains glyphosate, the active product as pesticide, associated with a combination of formulation products. The effect of pure glyphosate on the first cleavage was investigated. At concentrations of glyphosate ranging from 1 mM up to 20 mM (corresponding to the amount present in Roundup 0.1–2%), applied under the same experimental conditions as Roundup, pure glyphosate had no effect on the first mitotic cell division. Therefore, we conclude that either the formulation products of Roundup are directly responsible for the cell cycle dysfunction or glyphosate and formulation products act in synergy.

Possible synergy between the formulation products and glyphosate was assayed by analyzing the effects of various concentrations of glyphosate on the first cell cycle, in the absence or in combination with subthreshold doses of Roundup. Increasing concentrations of glyphosate were effective in dysregulating the first cell cycle in the presence of 0.2% Roundup, whereas 0.2% Roundup alone was inefficient (Figure 3). As observed for the effect of Roundup alone, the combined effect of glyphosate and Roundup occurred in a narrow concentration range. We conclude that glyphosate and formulation products present in Roundup exert synergistic effects that delay the first cell cycle of early development.

**Cellular Analysis of Roundup-Induced Cell Cycle Dysfunction.** The effects of Roundup on first cell cycle were analyzed at a cellular level. Fertilized embryos were treated with 0.8% Roundup (containing 8 mM glyphosate) at 10 min postfertilization. At various times, batches of embryos were stained with Hoechst dye and observed by fluorescence microscopy. Chromatin morphology during all phases of the first cell cycle was comparable in the Roundup-treated and control embryos (Figure 4). However, stages of chromatin evolution during the cell cycle were delayed in the treated embryos compared to control embryos (Figure 4). Careful time course observations from fertilization to cytokinesis were performed. No delay was observed in the Roundup-treated embryos during the first 30 min, from fertilization to the close association of the two pronuclei. The period between association of the pronuclei up to their fusion, in which the male pronucleus



**Figure 4.** Changes in chromatin morphology during early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater (control: upper panel) or into seawater containing 0.8% Roundup (Roundup: lower panel). Chromatin state was observed by fluorescence microscopy of Hoechst stained embryos as a function of time after fertilization. Left, an unfertilized egg. Bar: 20  $\mu$ M.

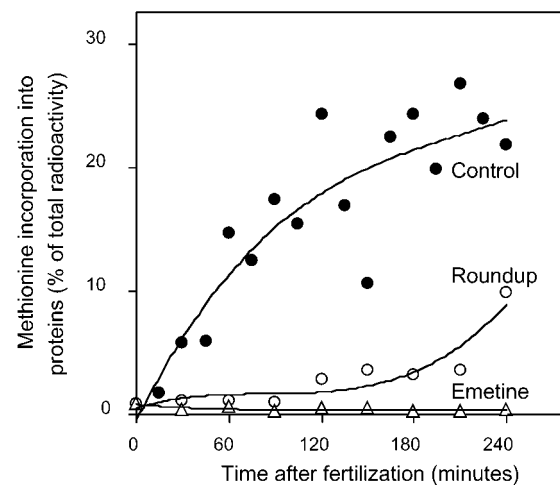


**Figure 5.** Kinetics of CDK1/cyclin B activation during early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater (filled circles), into seawater containing 0.8% Roundup (open circles) or into seawater containing 0.2 mM emetine (open triangles). At the indicated times postfertilization, CDK1/cyclin B activation state was determined from the measurement of its H1 kinase activity as indicated under material and methods. In this experiment, first cleavage occurs around 170 min for control embryos and around 240 min for 50% of the Roundup-treated embryos. The figure is representative of four independent experiments.

undergoes decondensation, went on for 20 min in control embryos and for 50 min in the Roundup-treated embryos. The phase of zygotic chromatic condensation lasted around 50 min in both Roundup-treated and control embryos. The characteristic mitotic stages, metaphase to cytokinesis, occurred with comparable time lags for all embryos.

Thus, Roundup induces a delay in the onset of M-phase of the cell cycle without affecting significantly the kinetic of mitotic progression itself or the period of cytokinesis.

**Effects of Roundup on H1 Kinase Activation *In Vivo*.** The complex CDK1/cyclin B controls entry into M-phase (for review, see refs 5-7). We first tested for a direct effect of Roundup on the activity of the protein kinase. Neither Roundup nor glyphosate inhibited the activity of purified CDK1/cyclin B *in vitro*. The effect of Roundup was therefore tested on the activation of CDK1/cyclin B *in vivo*, determined from H1 kinase activity of extracts prepared at time intervals after fertilization. Figure 5 shows that control embryos presented characteristic peaks of H1 kinase activity at 105 and 195 min postfertilization corresponding respectively to the first

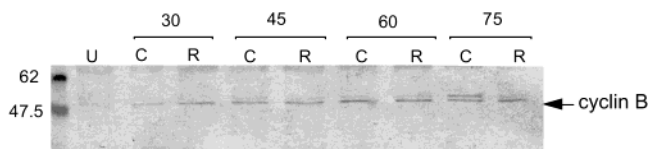


**Figure 6.** Kinetics of the increase in protein synthesis during early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater (filled circles), into seawater containing 0.8% Roundup (open circles) or into seawater containing 0.2 mM emetine (open triangles). At the indicated times after fertilization, batches of embryos were processed for protein synthetic rate determination, by 5 min pulse incubations in the presence of [ $^{35}$ S]-methionine and measurement of the labeled proteins as indicated under material and methods. In this experiment, first cleavage occurs around 170 min for control embryos and around 240 min for 50% of the Roundup-treated embryos. The figure is representative of four independent experiments.

and second cell cycle. The presence of Roundup 0.8% in the incubation medium totally abolished the peaks of H1 kinase activity. Therefore, Roundup acts on the mechanism of activation of the complex.

Inhibition of protein synthesis has been reported to delay or block first cell cycle in sea urchin (16, 19, 20). In our experimental conditions, the protein synthesis inhibitor, emetine, did inhibit the CDK1/cyclin B activation *in vivo* (Figure 5). The effect of Roundup on protein synthesis was therefore investigated. In the control embryos, protein synthesis rate readily increased after sperm addition as well documented (9) and was prevented by emetine treatment (Figure 6). Roundup 0.8% was found to strongly inhibit the increase in protein synthesis associated with fertilization (Figure 6).

**Analysis of Cyclin B during Early Development.** The synthesis of cyclin B is a prerequisite for the activation of CDK1 (5-7). Roundup might have hindered



**Figure 7.** Analysis of cyclin B during early development. Sea urchin gametes were fertilized and the embryos transferred 10 min postfertilization into fresh seawater or into seawater containing 0.8% Roundup. Batches of embryos were processed for immunoblotting using anti-cyclin B antibody as indicated under material and methods, at the indicated time in minutes postfertilization. Roundup-treated embryos (R) were compared to control (C) and to unfertilized (U) eggs processed in parallel. Molecular weight markers were run on the same electrophoretic gel and are indicated (kDa) on the left side. Position of Cyclin B is arrowed on the right side. Control embryos underwent the first division at 90 min postfertilization, cell division of the Roundup-treated embryos was delayed by 60 min in this experiment.

the activation of CDK1/cyclin B through the deficiency in the amount of cyclin B as a result of the general inhibition of the protein synthesis machinery. We analyzed the level of cyclin B during early development using a polyclonal antibody directed against sea urchin cyclin B. The antibody recognized a major single band produced postfertilization at 46 kDa (Figure 7). The protein amount increased and progressively resolved as a doublet 46–49 kDa at M-phase (Figure 7). The upper band of the doublet then strongly decreases at anaphase (data not shown). The appearance of the doublet reflected the phosphorylated form of the protein that takes place after the activation of the CDK1/cyclin B complex (5–7). In the Roundup-treated embryos, cyclin was synthesized postfertilization (Figure 7). Resolution of the protein as a doublet was delayed compared to control (Figure 7), reflecting the delay in CDK1 activation. The synthetic rate of cyclin synthesis was estimated from densitometry of immunoblots. No significant difference was found between the Roundup-treated and control embryos indicating comparable rates in the accumulation of cyclin B postfertilization.

## Discussion

Our results demonstrate that Roundup affects the first cell cycle of early development by impeding activation of the cell cycle regulator CDK1/cyclin B. Cell cycle is regulated through an all-or-nothing switch of CDK/cyclin activity after progressive synthesis of the cyclin component (7). The narrow concentration range of Roundup efficiency (see Figure 2 and 3) may be related to this “all-or-nothing” mechanism. The switch is under posttranslational control by protein dephosphorylation/phosphorylation modifications. The timing of the entry into M-Phase is dependent on the activation of the mitotic switch that is under the control of the checkpoint pathways (for review, see refs 5–7). Since CDKs and cell cycle checkpoints are universal from unicellular eucaryotes to human, the molecular target of Roundup is likely to be of universal meaning. Deregulations of cell cycle checkpoints are directly linked to genomic instability, which can generate diseases and cancer (see introduction). Our results therefore question whether human health could be affected by Roundup (10).

The concentration of Roundup that provokes cell cycle dysfunction appears to largely exceed the recommended usage concentration as an herbicide. The residual con-

centration of Roundup and/or glyphosate in soil or water is lower, glyphosate being in the nanomolar range present almost constantly. Cellular dysfunction in the cell cycle occurred at the millimolar range concentration of glyphosate for minute range exposure. Nevertheless, the discrepancy between the residual doses of glyphosate or Roundup and the doses inducing dysfunction should not lead one to underestimate the risk. First, the large difference in glyphosate concentrations could be partially compensated by the great difference in the time exposure. Second, the initial target of the pesticide in the embryos is not yet identified and might be affected by much lower concentrations of Roundup. Third, in our experiments, the pesticide affects 100% of the cells whereas cancers and tumors develop from few cells under the same environmental exposure (21), suggesting potential efficiency of much lower concentrations of the pesticide.

Roundup contains surfactants, which promote wetting of plant surface and rapid herbicide penetration. In previous reports, the toxicity of Roundup was ascribed to the surfactant component (12). We show that glyphosate and formulation products act in synergy on the cell cycle indicating an effect of glyphosate by itself. Our experiments indicate that glyphosate requires the presence of the formulation products to be effective on the embryo. It is likely that the formulation products favor the penetration of glyphosate in the embryos that were already reported to be impermeable to some compounds (9).

The synthesis of cyclin B is required during the first cell cycle in sea urchin (16, 19, 20). Roundup inhibits the rise in protein synthesis associated with early development, without affecting significantly the accumulation of cyclin B as judged from immunoblots performed with anti-cyclin B antibody. The discrepancy between the inhibition of global protein synthesis together with the maintenance of specific synthesis of cyclin has already been described using sea urchin (20). Global protein synthesis is very low in gametes and strongly increases after fertilization (9) due to the de-repression of the translation machinery at the level of initiation control (22, 23) and to unmasking of maternal mRNAs (24). Since the synthesis of cyclin B is not affected significantly, Roundup may hinder, through global protein synthesis inhibition, the synthesis of a yet unidentified protein that would be necessary to turn on the mitosis switch and activate the CDK1/cyclin B complex (7). Existence of such protein has been postulated since threshold doses of emetine can delay cell cycle by inhibiting global protein synthesis without affecting accumulation of cyclin (20). This hypothetical protein would be involved in the pathway leading to the switch activation CDK1/cyclin B. Roundup could affect the activation switch by hindering the putative protein action or more directly by impeding with the mechanism of the switch itself such as dephosphorylation/phosphorylation activities (7).

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