Formulated Glyphosate Activates the DNA-Response Checkpoint of the Cell Cycle Leading to the Prevention of G2/M Transition

Julie Marc, Robert Bellé[1], Julia Morales, Patrick Cormier, and Odile Mulner-Lorillon

Station Biologique de Roscoff, Cycle Cellulaire et Développement, Unité Mer & Santé (FRE 2775), Centre National de la Recherche Scientifique (CNRS) and Université Pierre et Marie Curie (UPMC), BP 74, 29682 Roscoff, France

A glyphosate containing pesticide impedes at 10 mM glyphosate the G2/M transition as judged from analysis of the first cell cycle of sea urchin development. We show that formulated glyphosate prevented dephosphorylation of Tyr 15 of the cell cycle regulator CDK1/cyclin B *in vivo*, the end point target of the G2/M cell cycle checkpoint. Formulated glyphosate had no direct effect on the dual specific cdc25 phosphatase activity responsible for Tyr 15 dephosphorylation. At a concentration that efficiently impeded the cell cycle, formulated glyphosate inhibited the synthesis of DNA occurring in S phase of the cell cycle. The extent of the inhibition of DNA synthesis by formulated glyphosate was correlated with the effect on the cell cycle. We conclude that formulated glyphosate's effect on the cell cycle is exerted at the level of the DNA-response checkpoint of S phase. The resulting inhibition of CDK1/cyclin B Tyr 15 dephosphorylation leads to prevention of the G2/M transition and cell cycle progression.

Key Words: CDK1-cyclin B; formulated glyphosate; cell cycle checkpoint; DNA synthesis.

INTRODUCTION

The cell cycle is the universal process by which cells reproduce and which underlies the growth and development of all living organisms. The most important events of the cell cycle are those concerned with the copying and partitioning of the hereditary material that is replicating chromosomal DNA during the S phase and separating the replicated chromosomes during mitosis. Timely progression through cell division requires phosphorylation events carried out by cyclin-dependent protein kinases (CDKs), the universal cell cycle regulators (Murray, 2004; Nurse, 2002). Cells have developed a number of surveillance mechanisms to ensure that the genome is correctly replicated and partitioned between the two daughter cells during the process of cell division. These genome-surveillance mechanisms, known as checkpoints (Hartwell and Weinert, 1989), act by delaying cell cycle progression until errors have been corrected. Usually, the parallel activation of a program of cell death (apoptosis) ensures that the abnormal cells are eliminated if no repair occurs (review in Norbury and Zhivotovsky, 2004). Failure to activate these checkpoints has consequences both for intrinsic and environmentally induced genomic instability. The essential nature of checkpoint responses in human health and genomic stability is most clearly indicated by the observation that defects in nearly all identified component proteins are associated with severe hereditary human genetic disorders or predisposition to cancer (reviews in Hoeijmakers, 2001; Molinari, 2000; Schneider and Kulesz-Martin, 2004).

In all eukaryotic cells, CDKs are initially activated by their association with cyclin subunits, by phosphorylation on a threonine residue located at position 161 (based on the cdc2 *Schizosaccharomyces pombe* sequence) and by dephosphorylation of two crucial residues Tyr 15 and Thr 14, located within the ATP-binding loop of the CDKs (reviewed in Nurse, 2002). The dephosphorylation of Thr 14 and Tyr 15, mediated by dual specificity phosphatases of the cdc25 family, is the rate-limiting step for CDK activation (Donzelli and Draetta, 2003; Margolis and Kornbluth, 2004; Wolfe and Gould, 2004). Cyclin-dependent protein kinases function as key targets of the checkpoints response. Thus, upon DNA damage or replication errors, activation of the checkpoint pathways leads to cell cycle arrest in G1, S, or G2 (reviewed in Motoyama and Naka, 2004). It has been demonstrated that G2 arrest is triggered by the maintenance of the inhibitory phosphorylations of CDK1, the CDK responsible for the G2/M transition (reviews in Norbury and Zhivotovsky, 2004; Nurse, 2002). The checkpoint operates for the most part through the regulation of cdc25 activity (Donzelli and Draetta, 2003; Margolis and Kornbluth, 2004; Wolfe and Gould, 2004).

Among the most obvious models used to study cell cycle regulation, yeasts, mammalian cultured cells or oocytes and embryos from amphibians and invertebrates, sea urchin embryos won their *lettres de noblesse* by being at the origin of the discovery of cyclins (Evans *et al.*, 1983). The molecular basis of cell cycle control is highly conserved from single unicellular eukaryotes to complex metazoans such as humans and Echinoderm

[1] To whom correspondence should be addressed at Station Biologique, BP 74, 29682 Roscoff Cedex, France. Fax: 33 (0) 2 98 29 23 06. E-mail address: belle@sb-roscoff.fr.

gametes, and embryos constitute a recognized powerful model for the study of cell cycle regulation (Nurse, 2002). Fertilization of ovulated sea urchin eggs arrested in a G1-like state of the cell cycle triggers entry into S phase followed by the G2 and M phases. Fertilized eggs then divide and subsequent cell cycles proceed as rapid rounds of highly synchronous S and M phases without gap (G1 or G2) phases. A number of reports suggested that cells from rapidly developing embryos (from fertilization to the mid-blastula transition) were devoid of mitotic checkpoints and apoptotic response (Clute and Masui, 1997; Epel 2003; Finkielstein *et al*., 2001; Newport and Dasso, 1989). Nevertheless, the presence and activation of checkpoint signaling mechanism have been thoroughly demonstrated during the first mitotic embryonic cell cycle (Greenwood *et al*., 2001; Ikegami *et al*., 1978; Kawahara *et al*., 2000; Kim *et al*., 1999; Lesser *et al*., 2003; Meijer *et al*., 1991; Sluder *et al*., 1995; Stokes and Michael, 2003; Voronina and Wessel, 2001; You *et al*., 2002).

Widely spread pesticides containing glyphosate as the active herbicide (Malik *et al*., 1989) are currently of no health or environmental concern (Williams *et al*., 2000). However, formulated glyphosate was recently shown to provoke cell cycle dysfunction when applied to sea urchin embryos (Marc *et al*., 2002, 2003, 2004). Formulated glyphosate affected CDK1/cyclin B activation, thus impeding entry into the M phase of the cell cycle (Marc *et al*., 2002, 2003, 2004). The mechanism of action of the pesticide was shown to be independent of the formation of the CDK1/cyclin B complex (Marc *et al*., 2003), which normally occurs for the G2/M transition as a result of cyclin B synthesis (Murray, 2004; Nurse, 2002). Therefore, formulated glyphosate may affect CDK1/cyclin B activation through inhibition of the dephosphorylations of Thr 14/Tyr 15. In this work, this hypothesis was tested and we present evidence that formulated glyphosate acts on the G2/M transition through mobilization of the DNA-response checkpoint as a consequence of interference with DNA replication during S phase.

MATERIALS AND METHODS

Chemicals. The formulated glyphosate used was Roundup 3plus, a trademarked product from Monsanto (170 g/L glyphosate as isopropylamine salt) and was from a commercial source. *N*-(Phosphonomethyl) glycine (glyphosate) was purchased from Cluzeau Info Labo (France). The polyclonal antibody directed against sea urchin cyclin B (Lozano *et al*., 1998) was a generous gift from Gérard Peaucellier (Banyuls, France). Glutathione S-transferase (GST)-fusion protein containing full-lengh human cdc25C (83 units/mg; 1 unit = 1 nanomol of phosphate hydrolyzed from 3-*O*-methylfluorescein phosphate (OMFP) per min) was from Upstate (Euromedex). The monoclonal anti-PSTAIR antibody, 3-*O*-methylfluorescein phosphate (OMFP), sodium orthovanadate, and aphidicolin were purchased from Sigma. The anti-PSTAIR antibody is directed against a 16 amino acid oligopeptide containing the PSTAIR (aminoacids in one letter coding) sequence of CDK1. The peroxidase-coupled secondary antibodies (goat anti-mouse Ig G (H + L) and goat anti-rabbit Ig G (H + L)) were from Biorad. The monoclonal anti-phospho-tyrosine HRP conjugate antibody was obtained from Santa Cruz Biotechnology. The ECL detection reagents and [methyl-^{3}H] thymidine (86 Ci/mmol) were obtained from Amersham Pharmacia Biotech. Okadaic acid was purchased from Calbiochem and ethylene diamine tetra-acetic

acid (EDTA) from Amresco. The BCA protein assay reagents were obtained from Pierce.

Handling of eggs and embryos and cytological observations. The *Sphaerechinus granularis* sea urchins were collected in the Brest area (France), 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 µm Millipore-filtered seawater and rinsed twice by centrifugation at 2,000 rpm for 2 min. For DNA synthesis determination experiments, eggs were dejellied by swirling twenty seconds in 3.5 mM citric acid pH 5 and rinsed three times in filtered seawater prior to fertilization. For fertilization, eggs were suspended in Millipore-filtered seawater (5% suspension) containing 0.1% glycine. Diluted sperm was added to the eggs and withdrawn after fertilization envelope elevation. Experiments were only performed on batches exhibiting greater than 90% fertilization, and each experiment used gametes from a single female. Cultures were performed at 16°C with constant agitation. Thousands of embryos were incubated for each experimental determination. Pesticide solutions in Millipore-filtered seawater were adjusted to pH 7.5 and added to the embryo suspension 10 min after fertilization. Approximately 100 embryos were scored at short time intervals by phase contrast microscopy for cytokinesis. For some experiments, 0.2 ml aliquots of the embryo suspension at various times after fertilization were fixed for at least 2 h in 0.5 ml methanol/glycerol (3:1) in the presence of the DNA dye bisbenzimide (0.1 µg/mL) and were mounted in 50% glycerol. Chromatin state observation under fluorescence microscopy was used to determine metaphase stage timing.

Affinity purification of CDK1/cyclin B from embryos. The CDK1 protein was affinity purified at different times after fertilization using p13^{suc1}-Sepharose beads (Dunphy *et al*., 1988). Every 10 min after fertilization, embryos from 2 ml samples of the 5% 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. Embryos were homogenized through a 25-gauge needle in 400 µL ice-cold buffer (60 mM β-glycerophosphate pH 7.2, 15 mM *p*-nitrophenyl phosphate, 25 mM 4-morpholinepropanesulfonic acid (MOPS), 15 mM ethylene glycol tetraacetic acid (EGTA), 15 mM MgCl2, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenylphosphate, 10 µg/ml soybean trypsin inhibitor (SBTI), 100 µM benzamidine, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.1% Triton). The homogenates were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. The CDK1/cyclin B complex was isolated by incubating the 14,000 rpm supernatant (400 µL) at 4°C for 45 min under constant rotation in the presence of 50 µL p13^{suc1}-Sepharose beads (1/5 dilution) prepared using bacterially produced p13^{suc1} protein coupled to Sepharose beads as described elsewhere (Arion and Meijer, 1989). After incubation, the beads were collected by centrifugation, washed twice in ice-cold bead buffer and once in 1 mL ice-cold 50 mM Tris-HCl pH 7.5. Proteins bound to the beads were eluted by boiling for 3 min in 35 µL electrophoresis sample buffer (Laemmli, 1970).

Western blot analysis. After clearing by centrifugation, 15 µl of the affinity-purified proteins were resolved by one-dimensional electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Laemmli, 1970). Proteins were transferred to nitrocellulose (Towbin *et al*., 1979), stained with Ponceau red and processed for Western blotting. The anti-cyclin B antibody and the anti-PSTAIR were used, respectively, at a dilution of 1:1000 and 1:3000 in 5% skimmed milk, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20. The anti p-Tyr was used at a dilution of 1:1000 in 5% bovine serum albumin (BSA), 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20. Membranes were then incubated with peroxidase-coupled secondary antibodies (Biorad): goat anti-mouse IgG (H + L)/HRP conjugate for PSTAIR detection (1:5000) and goat anti-rabbit IgG (H + L)/HRP conjugate for cyclin B detection (1:10 000). The antigen–antibody complexes were revealed by the chemiluminescence system according to the manufacturer's instructions (Amersham Biosciences). When needed, membranes were stripped in dehybridization buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 40 min at 60°C under constant rocking and processed as above with a new antibody.

Phosphatase assays. The specific 3-*O*-methylfluorescein phosphate (OMFP) artificial substrate was used for the estimation of cdc25 activity (Gottlin *et al.*, 1996). Pure human cdc25C was assayed at 50 m units (1 unit = nanomole of 3-*O*-methylfluorescein phosphate hydrolyzed per minute). Embryo extracts were prepared as described for the CDK1/cyclin B purification procedure, except that the homogenization buffer was 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 μg/mL soybean trypsin inhibitor (SBTI), 10 μg/ml pepstatin A. Supernatants used for phosphatase assays were from metaphase-stage embryos as judged by the chromatin state.

Phosphatase assays were performed in 1 mL reactions at 30°C for at least 1 h. The mixture contained either 50 m units of commercial recombinant cdc25C or embryo extracts (100 μg protein) in substrate buffer (500 μM 3-*O*-methylfluorescein phosphate (OMFP), 100 mM Tris-HCl pH 8.2, 40 mM NaCl, 1 mM DTT, 10 mM glutathione, 20% glycerol; Gottlin *et al.*, 1996). Absorbance was measured at 477 nm in a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Activity of pure cdc25 C was 0.12 OD units per hour.

Determination of DNA synthesis **in vivo.** Ten minutes post-fertilization, methyl-^{3}H-thymidine (10 μCi/ml final concentration), was added to the embryo suspension as described by Arion and Meijer (1989). When used, formulated glyphosate at the indicated concentration or aphidicolin (10 μg/mL final) were added 10 min post-fertilization. At different times thereafter, embryos from 1 mL samples of the suspension were rapidly packed by centrifugation for 5 s in an Eppendorf centrifuge, rinsed in 1 mL Millipore-filtered seawater, and suspended in 1 mL ice-cold 20% trichloroacetic acid (TCA). Aliquots were taken for the determination of total thymidine uptake. After precipitation for one night at 4°C, the pellets were collected by centrifugation, then washed twice with 20% TCA. The pellets were dissolved in 1 ml 0.125 M NaOH. Radioactivity was measured on duplicate 500 μL aliquots of the dissolved samples in the presence of Optiphase Supermix scintillation liquid in a 1450 Wallac MicroBeta-Counter (Wallac, EG&G Instruments). The protein content was measured on the remaining sample using the BCA protein assay kit.

RESULTS

Formulated Glyphosate Prevents Dephosphorylation of CDK1 at Tyr 15

The effect of formulated glyphosate on cyclin B accumulation, CDK1 abundance, and phosphorylation states at Tyr 15 (based on the cdc2 *S. pombe* sequence) was analyzed during the first cell cycle of sea urchin early development. The three parameters were examined after CDK1/cyclin B affinity purification from the same post-fertilization embryo extracts (Fig. 1). In the reported experiment, formulated glyphosate at 10 mM glyphosate delayed the occurrence of the first cell cycle by 30 min in concordance with previous results (Marc *et al.*, 2002, 2003, 2004) and related to the prevention of the activation of the protein kinase activity of CDK1/ cyclin B (Marc *et al.*, 2002, 2004). The abundance of CDK1 was verified by immunoblotting the affinity-purified proteins using the anti-PSTAIR antibody. As expected, the abundance of CDK1 remained constant during the first cell cycles and was not affected by formulated glyphosate treatment (Fig. 1 α-PSTAIR). The anti-PSTAIR detection was therefore subsequently used as a loading control. Cyclin B levels and phosphorylation changes were analyzed using an anti-cyclin B antibody. The main features of the cyclin B changes

observed were these: (1) the pattern of cyclin B accumulation and association with CDK1; (2) the resolution of cyclin B as a doublet at 46/49 kDa at the M phase of the cell cycle and its abrupt degradation at anaphase, both events resulting from CDK1 activity; and (3) further changes related to the second cell division (Fig. 1; α-cyclin B). As already reported (Marc *et al.*, 2002, 2003), formulated glyphosate does not affect the time course of cyclin B accumulation nor the association of cyclin B with CDK1 during the first 80 min post-fertilization, whereas formulated glyphosate delays the cyclin B pattern changes associated with the activity of CDK1 (Fig. 1; compare the kinetic of cyclin B electrophoretic pattern in control and formulated glyphosate-treated embryos).

The phosphorylation of CDK1 tyrosine was monitored by immunoblotting the affinity-purified proteins with the anti-phospho-tyrosine antibody. In control embryos, phospho-tyrosine content of CDK1 at 32 kDa increased progressively from 10 min to 70 min post-fertilization, abruptly disappearing at 80 min and reappearing again during the second cell cycle (Fig. 1 control, α-pTyr). As already reported, it was observed that the appearance of cyclin B as a doublet, corresponding to activation of CDK1/cyclin B, preceded the complete dephosphorylation of CDK1 (Fig. 1 control, compare the profiles at 60 and 80 min; Meijer *et al.*, 1991). The observed changes in tyrosine phosphorylation level are associated with the metaphase and anaphase stages of the cell cycle, occurring within the first and second cell cycles (Murray, 2004; Nurse, 2002). When the embryos were incubated in the presence of formulated glyphosate, dephosphorylation of CDK1 tyrosine was significantly delayed by 30 min compared to the control (Fig. 1 Formulated glyphosate, α-pTyr). The delay in tyrosine dephosphorylation provoked by formulated glyphosate corresponded to the delay in the appearance of the cyclin B doublet, the indicator of CDK1/cyclin B activation (Fig. 1 Formulated glyphosate, α-cyclin B). When observed in different independent experiments, the delay in tyrosine dephosphorylation was comparable to the delay in cyclin B resolution as a doublet and to the delay in cytokinesis (Table 1). Therefore, formulated glyphosate prevents the activating dephosphorylation of CDK1 on tyrosine.

A possible direct inhibition of cdc 25 activity by formulated glyphosate or glyphosate alone was investigated. Glyphosate at 10 mM or formulated glyphosate containing 10 mM glyphosate had no inhibitory effect on the hydrolysis of 3-*O*-methylfluorescein phosphate (OMFP) by the pure recombinant cdc25C protein. Hydrolysis of 3-*O*-methylfluorescein phosphate (OMFP) by embryo extract was 0.7 OD units per hour, it was orthovanadate sensible (ID 50 of 260 μM) and insensible to okadaic acid or EDTA, corresponding to the characteristics of cdc25 activity (Millar *et al.*, 1991). Glyphosate at 10 mM or formulated glyphosate containing 10 mM glyphosate had no effect on the embryos' activity.

Together, these results demonstrate that the delay in cytokinesis due to the prevention of CDK1/cyclinB complex activation induced by formulated glyphosate is exerted through the
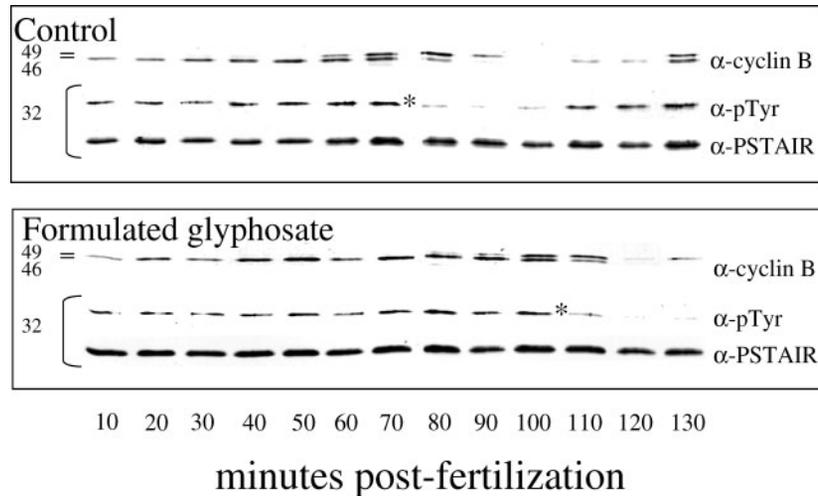
FIG. 1. Formulated glyphosate effects on cyclin B and CDK1 levels and on CDK1 phosphorylations during the first cell cycles of early development. Sea urchin gametes were fertilized and the embryos transferred 10 min post-fertilization into fresh seawater (Control) or into seawater containing 1 % formulated glyphosate i.e. 10 mM equivalent glyphosate (Formulated glyphosate). At the indicated times post-fertilization, 100 µl of pelleted embryos were homogenized and processed for CDK1 affinity purification as indicated in Material and Methods. Affinity-purified proteins were resolved by electrophoresis and processed for immunoblotting using anti-cyclin B antibody (α-cyclin B), anti-phospho-tyrosine antibody (α-pTyr) and anti-PSTAIR antibody (α-PSTAIR). Molecular weight markers were run on the same electrophoretic gel and used to indicate the molecular weights of proteins in kDa (left). Tyrosine dephosphorylations on CDK1 for control and formulated glyphosate-treated embryos are indicated by *. In the illustrated experiment, control embryos underwent 50% cytokinesis at 115 min and formulated glyphosate-treated embryos at 150 min. The figure is representative of three independent experiments.

TABLE 1
Formulated Glyphosate Effects on the Timing of Cell Cycle Events

| Experiment | Event | Time of event (minutes) | |
		Control	Formulated glyphosate
1	Tyrosine dephosphorylation	80	110
	Maximal cyclin B phosphorylation	80	110
	50% cytokinesis	115	150
2	Tyrosine dephosphorylation	100	120
	Maximal cyclin B phosphorylation	100	120
	50% cytokinesis	150	175
3	Tyrosine dephosphorylation	70	80
	Maximal cyclin B phosphorylation	80	90
	50% cytokinesis	105	115

The timing of CDK1 tyrosine dephosphorylation, cyclin B phosphorylation and cytokinesis throughout the first cell cycle was determined in three independent experiments. At 10 min time interval post-fertilization, embryos were homogenized and processed for CDK1 affinity purification, electrophoresis and immunoblotting using anti-cyclin B antibody and anti-phospho-tyrosine antibody as indicated in Material and Methods. The times of tyrosine dephosphorylation and maximal cyclin B phosphorylation were determined by quantification of the western blots. The criterion for determining the cyclin B phosphorylation state was the appearance of the electrophoretic doublet. The time of 50% cytokinesis was established by phase contrast microscopy observation of the embryos. Formulated glyphosate was used at a 1% dilution (10 mM equivalent glyphosate) for female 1 and at a 0.8% dilution (8 mM equivalent glyphosate) for females 2 and 3.

inhibition of CDK1 dephosphorylation on tyrosine *in vivo*, which occurs as the result of an indirect effect of formulated glyphosate. Since the prevention of CDK1/ cyclin B dephosphorylation is reminiscent of the DNA damage or unreplicated-DNA checkpoint of the cell cycle (review in Motoyama and Naka, 2004; Nurse, 2002), the effect of formulated glyphosate on DNA synthesis was investigated.

Formulated Glyphosate Inhibits DNA Synthesis In Vivo

Incorporation of radiolabeled thymidine into DNA was measured as a function of time post-fertilization in control and formulated glyphosate-treated embryos. In control embryos, incorporation was first detected at 20 min post-fertilization, increased rapidly, reaching a plateau at 50 min (Fig. 2). Incorporation of thymidine into DNA was totally inhibited by the presence in the incubation medium of aphidicolin, a DNA polymerase 1 inhibitor, indicating that the thymidine incorporation measurement reflected the DNA synthesis activity of the embryos (Fig. 2). In a series of independent experiments, 10 µg/mL aphidicolin delayed cytokinesis by a mean of 105 min (S.D. = 30; n = 7). This result is in accordance with existence of mitotic checkpoints as already reported in *Sphaerechinus granularis* embryos (Meijer *et al.*, 1991) and in other sea urchin species (Patel *et al.*, 1997). When the embryos were treated with a dose of formulated glyphosate that prevented entry into M phase, DNA synthesis was reproducibly inhibited by around 75% (Fig. 2). A comparison between the inhibitory effects of formulated glyphosate on DNA synthesis and on the first cell
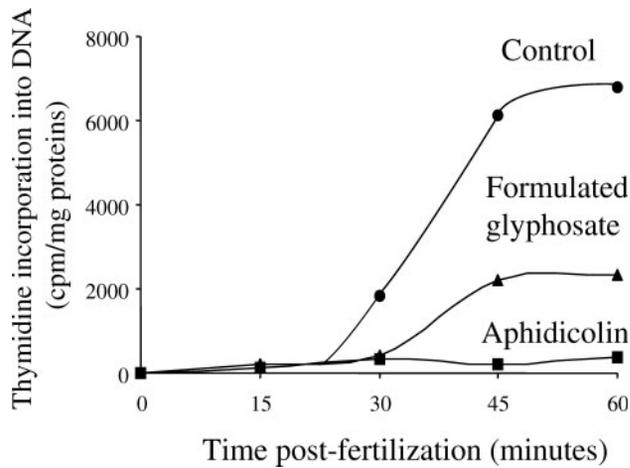
FIG. 2. Effect of formulated glyphosate on of DNA synthesis kinetics *in vivo*. Sea urchin gametes were fertilized and the embryos transferred 10 min post-fertilization into fresh seawater (Control), into seawater containing 1% formulated glyphosate (i.e., 10 mM equivalent glyphosate) or into seawater containing 10 µg/ml aphidicolin. Embryos were processed for DNA synthesis determination as indicated in the Material and Methods. The figure is representative of four independent determinations. In this experiment, the first cleavage began at 90 min for control embryos, at 125 min for aphidicolin-treated embryos and at 165 min for formulated glyphosate-treated embryos.
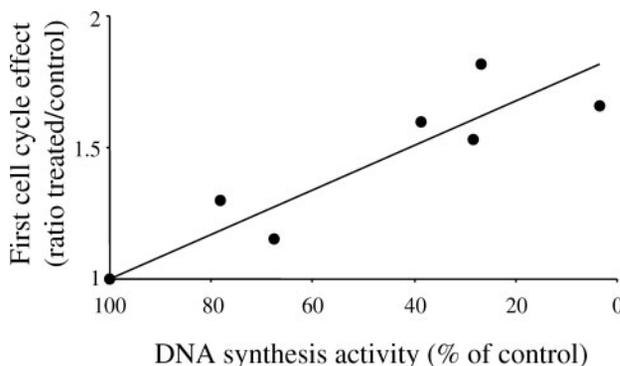

FIG. 3. Correlation between the first division delay and the inhibition of DNA synthesis in formulated glyphosate-treated embryos. DNA synthesis was determined from control and formulated glyphosate-treated embryos as indicated in *Materials and Methods*. DNA synthesis activity is expressed in percentage of thymidine incorporation in control embryos. Cell cycle inhibition was determined from the time to reach 50% cytokinesis and expressed as the ratio control versus treated. The figure is a compilation of experiments performed with formulated glyphosate ranging from 0.6 to 1.6%, i.e. containing 6 to 16 mM glyphosate.

cycle demonstrated a strict correlation between the two events (Fig. 3).

Therefore, formulated glyphosate at concentrations affecting cell cycle regulation exerts an adverse effect on DNA synthesis in embryos during the S phase period of the first cell cycle.

DISCUSSION

Sea urchin early development was proved to be a excellent model both for the study of cell cycle regulation (Nurse, 2002) and for the sensitive analysis of cell cycle deregulation by anthropogenic substances (Marc *et al.*, 2002, 2003, 2004). Formulated glyphosate, a increasingly widely used pesticide, was shown to impede cell cycle progression at the G2/M transition by preventing CDK1/cyclin B activation, but without affecting the formation of the CDK1/cyclin B complex (Marc *et al.*, 2002, 2003, 2004). The effect of formulated glyphosate was shown to be due to its active herbicide component glyphosate, acting at millimolar range concentration in synergy with permeabilizing agents and surfactants present in the formulation commercialized products (Marc *et al.*, 2002 and 2004). The formulation products are required, as for herbicide usage (Williams *et al.*, 2000), for the penetration of glyphosate into the cells to reach its intracellular targets. We demonstrate here that the inhibition of CDK1/cyclin B activation by formulated glyphosate involves preventing dephosphorylation of the critical Tyr 15 residue of CDK1 *in vivo*, and, furthermore, that formulated glyphosate inhibits DNA synthesis occurring at the S phase of the first cell cycle.

It is well established that the transition from the G2 to the M phase of the cell cycle requires activation of the mitotic kinase CDK1/cyclin B, and that activation of this complex is restrained by Wee1/Myt1-mediated phosphorylation of CDK1 (Murray, 2004; Nurse 2002). Moreover, reversal of these phosphorylations (on Thr 14 and Tyr 15) is catalyzed by a dual-specific phosphatase of the cdc25 phosphatase family (Donzelli and Draetta, 2003; Margolis and Kornbluth, 2004; Wolfe and Gould, 2004). Implication of cdc25 protein phosphatases in the activation of CDK1/cyclin B during the cell cycle is clearly established. On the other hand, the mechanism of cdc25 activation and regulation during the normal cell cycle is not fully understood. In mammals, cdc25 phosphatases are translated from three different genes leading to three proteins (cdc25 A, B, and C). The three cdc25 protein phosphatases share highly similar structural and mechanistic properties, and it is likely that their specific biological roles are determined by the timing of their activation, their subcellular localization and substrate recognition. Regulation of cdc25 activity has been reported to be mediated by both the abundance of the protein resulting from modulation of expression and degradation (for cdc25A), and the modulation of the activity of the enzyme through the phosphorylation of a number of alternative sites, which affects its localization (through 14–3–3 binding) and possibly also its catalytic properties (Donzelli and Draetta, 2003; Margolis and Kornbluth, 2004; Wolfe and Gould, 2004). Formulated glyphosate had no direct effect of purified cdc25 activity or on cdc25 extractable activity from the metaphase embryos judged as 3-*O*-methylfluorescein phosphate (OMFP) hydrolysis.

Formulated glyphosate may have maintained the Tyr 15 phosphorylation of CDK1/cyclin B through an indirect pathway reminiscent of the cell cycle checkpoint, which inhibits the

activation of the CDK1/cyclin B kinase as a consequence of damaged or unreplicated DNA (review in Motoyama and Naka, 2004; Nurse, 2002). An effect of formulated glyphosate at the level of DNA synthesis was investigated and demonstrated during the S phase of the cell cycle. DNA synthesis inhibition correlated well among experiments with the timing of the inhibition of cell cycle progression, strongly suggesting a link between the two effects on formulated glyphosate acting at S phase and resulting in an inhibition of G2/M progression. The fact that formulated glyphosate acts during the first hour of the first cell cycle (Marc *et al.*, 2002) is compatible with an initial effect of the pesticide at the level of S phase.

Altogether, the effect of formulated glyphosate presents two essential features of G2/M cell cycle checkpoint activation: sensing damaged or unreplicated DNA in S phase and preventing cell cycle progression at M phase through inhibition of CDK1/cyclin B activation (review in Motoyama and Naka, 2004; Nurse, 2002). Obviously, we cannot formally exclude the possibility of multiple formulated glyphosate targets mimicking a checkpoint mechanism.

The adverse effect of formulated glyphosate toward the cell cycle is in the millimolar range concentration when applied for short time exposure to sea urchin embryos. At soil or water residual concentrations of glyphosate in the nanomolar range, there is no indication that glyphosate exerts any genotoxic effect (Williams *et al.*, 2000). However, mobilization of the DNA response checkpoint may reflect interference of the product at the DNA level, potentially leading to genetic instability, which is recognized as one the main forces driving the onset and progression of carcinogenesis (Hoeijmakers, 2001; Molinari, 2000; Schneider and Kulesz-Martin, 2004). Because formulated glyphosate is sprayed for herbicide usage at a recommended concentration of 40 mM, it would be of interest to analyze the consequence for humans of the inhalation of the micro-droplets in the vicinity of the pesticide usage.

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