An Important Role for the Retinoblastoma Protein in Staurosporine-induced G₁ Arrest in Murine Embryonic Fibroblasts*

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In this study, we investigated the molecular basis of the ability of staurosporine to induce G₁ arrest in murine embryonic fibroblasts (MEFs). We used MEFs from transgenic mice lacking several negative regulators of the G₁/S phase transition including cells from mice lacking p53, p21, retinoblastoma (Rb), or p16 genes. We found that p53 function was not essential for staurosporine-induced G₁ arrest. In contrast, MEFs from mice lacking Rb genes showed approximately a 70% reduced capacity to arrest in the G₁ phase following staurosporine treatment. In support of a role for Rb in staurosporine-induced G₁ arrest, ret embryonic fibroblasts stably overexpressing cyclin D1/Cdk4R24C exhibited approximately a 50% reduced G₁ arrest response to staurosporine. The role of Rb in determining the degree of staurosporine-induced G₁ arrest did not depend on the function of the cyclin-dependent kinase inhibitors p16 or p21 because MEFs lacking either of these genes were still capable of undergoing G₁ arrest following staurosporine exposure. Our studies provide evidence of an important role for the Rb protein in determining the degree of staurosporine-induced G₁ arrest in the first cell cycle.

Staurosporine, a broad spectrum protein kinase inhibitor, has been shown to induce a G₁ and G₂ phase arrest in normal cells, and importantly the G₁ arrest response has been shown to be selectively lost in a variety of tumor cell lines (1, 2). The mechanistic basis underlying the loss of the G₁ arrest response in tumor cells is presently unresolved. In this study we focused our attention on the importance of the p53 and/or retinoblastoma (Rb) tumor suppressor proteins in the mechanism of staurosporine-induced G₁ arrest. These studies were prompted by the findings that the p53 and/or Rb pathways are commonly altered in human tumor cells (3–6), raising the possibility that such disruptions might account for the lack of staurosporine-induced G₁ arrest in tumor cells.

Interestingly, the effects of staurosporine on the cell cycle are reminiscent of DNA damage in that both induce a G₁ and G₂ arrest in normal cells, but the G₁ arrest is lost in many tumor cell lines (1, 2). The p53 tumor suppressor is essential for G₁ arrest induced by ionizing radiation and other DNA damaging agents, as well as G₁ arrest following suppression of ribonucleoside triphosphate pools (3, 7). In fact, loss of p53 activity by gene mutation, gene deletion, or overexpression of dominant-negative acting factors such as mutant-p53, Mdm2, or the human papillomavirus E6 protein blocks G₁ arrest following DNA damage (8–10). Although the lack of DNA damage-induced G₁ arrest in tumor cells has been linked to p53 dysfunction, a role for p53 in staurosporine-induced G₁ arrest still remains to be established. An important mediator of p53-induced G₁ arrest is the p21Waf1/Cip1 gene product (p21) (11, 12). The p21 protein binds to and inhibits a variety of G₁/S phase cyclin-dependent kinases (Cdk), which in turn are prevented from phosphorylating and inactivating the Rb protein (13).

Previous observations have indicated that staurosporine promotes the hypophosphorylation of Rb in cell types susceptible to G₁ arrest (2, 14, 15). Hypophosphorylated Rb would retain its ability to repress genes that are regulated through E2F-dependent consensus elements in their promoters (16, 17). When Rb function is inactivated through Rb gene mutation, gene deletion, expression of certain viral, or cellular proteins, the cells lose the capacity to G₁ arrest in response to some negative regulators of the G₁/S phase transition, including the Cdk inhibitor protein p16 (5, 9, 18–20). Previous studies in bladder carcinoma cell lines have indicated that p21 may participate in staurosporine-induced G₁ arrest (14). Definitive evidence of this possibility was, however, not obtained in these earlier studies.

In this study we investigated the mechanistic basis of staurosporine-induced G₁ arrest by using murine embryonic fibroblasts (MEFs) as a model system. Our choice of MEFs in this respect was fueled by the availability of cells from transgenic mice lacking important negative regulators of the G₁/S phase transition. Such a model system enabled us to determine the importance of p53, p21, Rb, and p16 in the staurosporine-induced G₁ arrest mechanism in this cell type. Here we report that p53 function was not essential for staurosporine-induced G₁ arrest in MEFs. In contrast, the Rb protein played an important role in determining the degree of G₁ arrest observed in the first cell cycle following exposure to staurosporine. Supporting evidence of a role for Rb in staurosporine-induced G₁

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‡ The abbreviations used are: Rb, retinoblastoma; STP, staurosporine; MEF, murine embryonic fibroblasts; Cdk, cyclin-dependent kinase; NOC, nocodazole; p21, p21Waf1/Cip1.
arrest was provided by the observation that rat embryonic fibroblasts stably overexpressing cyclin D1/Cdk4R24C exhibited a reduced G1 arrest response to staurosporine. Staurosporine did not induce a Rb-dependent G1 arrest in the first cell cycle by solely acting through the Cdk inhibitor proteins p16 or p21, because MEFs lacking either of these genes were still capable of undergoing G1 arrest following staurosporine treatment. Our studies provide evidence of an important role for the Rb protein in determining the degree of staurosporine-induced G1 arrest in the first cell cycle.

**MATERIALS AND METHODS**

**Chemical Treatments**—Staurosporine was obtained from Sigma and prepared as a 500 μm stock solution in Me2SO, and aliquots were stored at −20 °C until needed. Nocodazole (NOC) was purchased from Sigma.

**Cell Culture**—Early passage MEFs and rat embryonic fibroblasts were grown at 37 °C in 95% air/5% CO2 in Dulbecco’s modified Eagle medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 50 units/ml penicillin G, 50 μg/ml streptomycin (Life Technologies, Inc.). The wild type/wild type and p16−/− MEFS were graciously supplied by Dr. Ronald A. DePinho (Albert Einstein College of Medicine, New York, NY), whereas the Rb−/− and p53−/− MEFs were generously provided by Drs. Allan Bradley and Larry Donehower, respectively (Baylor College of Medicine, Houston, TX). The p21−/− MEFs were a kind gift from Dr. Chuxia Deng (NIDDK, National Institutes of Health, Bethesda, MD).

Rat embryonic fibroblasts (REF52) overexpressing either the vector or cyclin D1/Cdk4R24C were generously provided by Dr. Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Cell counts were determined using a Coulter counter with Channelizer attachment to monitor cell size (Coulter Electronics, Hialeah, FL).

**Flow Cytometry**—Samples were prepared for flow cytometry essentially as described previously (21). Briefly, cells were washed with 1× phosphate-buffered saline, pH 7.4, and then fixed with ice-cold 70% ethanol. Samples were then washed with 1× phosphate-buffered saline and stained with propidium iodide 60 μg/ml (Sigma) containing RNase 100 μg/ml (Sigma) for 30 min at 37 °C. Cell cycle analysis was performed using a Becton Dickinson florescence-activated cell analyzer and Cell Quest version 1.2 software (Becton Dickinson Immunocytometry Systems, Mansfield, MA). For each sample at least 10,000 cells were analyzed, and quantification of the cell cycle distribution was performed using the ModFit LT version 1.01 software (Verity Software House Inc., Topsham, ME).

**RESULTS**

**Staurosporine-induced G1 Arrest Does Not Operate through a p53 or p21-dependent Pathway**—We initially investigated if staurosporine-induced G1 arrest required the function of the p53 tumor suppressor. For this purpose we investigated the effects of p53 gene disruption on the ability of MEFs to arrest in G1 and G2 phases of the cell cycle following staurosporine treatment. Dose-response curves (5–50 nM STP) were performed to establish the dose of staurosporine that completely inhibited cell division for each sample, and each point in C represents either the mean and S.D. from three independent experiments or data from two individual experiments.

**The Rb Protein Is an Important Mediator of Staurosporine-induced G1 Arrest in the First Cell Cycle**—Having established that the p53 and/or p21 gene products were not essential for staurosporine-induced G1 arrest, we next turned our attention to the role of the Rb pathway in the staurosporine-induced G1 arrest mechanism. For this purpose we conducted studies in MEFs with intact versus disrupted Rb genes. Again, dose-response curves (5–50 nM STP) were performed to establish the dose of staurosporine that completely inhibited cell division within 24 h of addition. The maximal growth inhibitory dose (50 nM) was then chosen to investigate the cell cycle responses following addition of staurosporine (Fig. 2). In the case of MEFs from normal littermates, we found that staurosporine induced both a sustained G1 and a G2 arrest, and this was evident within 24 h of staurosporine addition (Fig. 1, A and C). Comparable cell cycle responses were observed in MEFs from mice lacking p53 genes (Fig. 1, A and C), suggesting that p53 function was not essential for staurosporine-induced G1 or G2 arrest. The role of the cyclin-dependent kinase inhibitor p21, an important mediator of p53-induced G1 arrest (11, 22–24) that can also be activated through p53-independent mechanisms (25), was also investigated. When MEFs from mice lacking p21 genes were exposed to staurosporine (50 nM) for 24 h, they underwent both a G1 and a G2 arrest (Fig. 1, A and C). These results suggested that p21 was similar to p53 in the respect that it also was not essential for staurosporine-induced G1 or G2 arrest. Thus, although it has previously been suggested that p21 participates in the mechanism by which staurosporine induces G1 arrest (14), our studies in MEFs show that p21 is not essential for G1 arrest in the first cell cycle of this cell type.
population was 14 ± 3% (n = 3) compared with 41 ± 5% (n = 3) for the vehicle-treated control cells. This amounted to approximately a 70% reduction in the degree of G1 arrest seen in the presence of staurosporine (p < 0.01, Student’s t test).

To delineate whether staurosporine-induced G1 arrest was a transient or sustained event, we chronically exposed either wild-type or Rb−/− MEFs to staurosporine for up to 72 h and performed flow cytometric analysis at 24-h intervals over this time course. As indicated in Fig. 3, staurosporine induced a sustained G1 arrest in wild-type MEFs, whereas a substantial proportion of Rb−/− cells were able to escape the G1 arrest in the first cell cycle. These cells went on to arrest at the G2/M junction, a response that did not require Rb function. Interestingly, although Rb disruption enabled approximately 70% of the original G1 population to escape the G1 phase over this time course. This result suggested that a subpopulation of G1 cells, presumably in early G1 phase, might still be capable of G1 arrest despite the absence of Rb genes (see “Discussion”).

We also investigated the effects of staurosporine in REF52 cells infected with viruses expressing the Rb-inactivating kinase complex, cyclin D1-Cdk4. A Cdk4 mutant (Cdk4R24C) that is resistant to the inhibitory influence of p16 was used in these studies (26, 27). Our view here was that if we could inactivate Rb through cyclin D1-Cdk4-mediated hyperphosphorylation, these cells would show a reduced capacity to G1 arrest in response to staurosporine. In support of a role for Rb in the staurosporine-induced G1 arrest mechanism, we found that REF52 cells expressing cyclin D1-Cdk4R24C were less susceptible to staurosporine-induced G1 arrest as compared with the empty vector control cells (Fig. 4). Cell cycle analysis of REF52 cells 24 h after a chronic exposure to staurosporine showed that approximately 49 ± 11% (n = 3) of the original G1 population of cyclin D1-Cdk4R24C overexpressing cells remained in G1 phase compared with 96 ± 5% (n = 3) for the empty vector control cells. This amounted to approximately a 50% reduction in the degree of staurosporine-induced G1 arrest in cyclin D1-Cdk4R24C overexpressing cells as compared with empty vector control cells (p < 0.01, Student’s t test).

Staurosporine-induced G1 Arrest in the First Cell Cycle Does Not Require the Function of the p16 Tumor Suppressor Gene—Because the Cdk inhibitor p16 has previously been shown to induce an Rb-dependent G1 arrest in mammalian cells (18–20), we investigated whether staurosporine might act through p16 to induce G1 arrest in MEFs. Our investigations showed, however, that MEFs from mice lacking p16 genes underwent a combined G1 and G2 arrest comparable with that observed in wild-type MEFs (Fig. 2, A and C). These results suggested that p16, a well recognized regulator of Rb function, was not essential for staurosporine-induced G1 arrest in MEFs.

DISCUSSION

In this study we investigated the mechanistic basis of staurosporine-induced G1 arrest in murine cells derived from transgenic mice lacking important negative regulators of the G1/S phase transition. Such a model system enabled us to investigate the importance of the p53, p21, Rb, and p16 genes in the mechanism through which staurosporine induces G1 arrest in otherwise normal cells. We found that in contrast to the p53 tumor suppressor, the Rb protein was an important mediator of...
Rb remained arrested in the G1 phase (sporine, whereas in contrast virtually all of the cells with intact analyzed in the empty vector control and cyclin D1-Cdk4 R24C-overexpressing cells by flow cytometry as described under “Material and Methods.” Samples shown are from representative experiments that were repeated three times with similar results.

the degree of staurosporine-induced G1 arrest observed in the first cell cycle. Further evidence implicating Rb in the mechanism of staurosporine-induced G1 arrest was evidenced in rat embryonic fibroblasts stably overexpressing cyclin D1-Cdk4 R24C. Such cells exhibited a reduced G1 arrest response to staurosporine compared with empty vector control cells. Staurosporine did not, however, induce an Rb-dependent G1 arrest by acting through the Cdk inhibitor proteins p16 or p21, because MEFs lacking these genes were still capable of G1 arrest following staurosporine treatment. Our studies provide evidence of an important role for Rb in determining the degree of staurosporine-induced G1 arrest.

Our finding that MEFs lacking p53 still underwent a combined G1 and G2 arrest in response to staurosporine provided evidence that in this cell type, p53 was not essential for staurosporine-induced G1 arrest. Because the Cdk inhibitor, p21, can be induced by p53-independent mechanisms (25) and because p21 has previously been implicated in the mechanism by which staurosporine induces G1 arrest (14), we examined the importance of p21 in staurosporine-induced G1 arrest. However, we again found that MEFs lacking p21 still underwent G1 arrest in response to staurosporine, indicating that in this cell type, p21 was not essential for the G1 arrest response.

Studies indicating the common occurrence of Rb disruptions in tumor cells and studies indicating that a tumor cell line overexpressing a Rb transgene underwent G1 arrest following staurosporine exposure (14) prompted us to also test the role of Rb in the majority of G1 arrest seen in the first cell cycle and that investigations aimed at determining the mechanism through which staurosporine maintains Rb in an active form could ultimately provide the target of staurosporine as it relates to Rb.

We speculate on some potential targets through which staurosporine might act to induce a Rb-dependent G1 arrest. In Fig. 5. We represent Rb in an equilibrium between its hyperphosphorylated (relatively inactive) state and its hypophosphorylated (active) state. This equilibrium is governed by cyclin-dependent kinases such as cyclin D1-Cdk4, cyclin D1-Cdk6, cyclin E-Cdk2, and cyclin A-Cdk2 (17, 31, 32). The state of Rb phosphorylation by an as yet poorly defined phosphatase (29, 30). Although DNA damage-induced G1 arrest has been linked to a pathway involving the p53 tumor suppressor and the p21 Cdk inhibitor, staurosporine does not elicit G1 arrest through a pathway involving p53 or p21 function. Another Cdk inhibitor, p16, which induces a G1 arrest in a Rb-dependent fashion (18–20) was also found not to be required for staurosporine-induced G1 arrest. Staurosporine might induce a Rb-dependent G1 arrest through direct inhibition of G1/S phase cyclin-dependent kinase activity. Important here would be cyclin D1-Cdk4 or cyclin D1-Cdk6 complexes whose inactivation causes a Rb-dependent G1 arrest (4), and in these studies we found that overexpression of cyclin D1-Cdk4 R24C could reduce the ability of staurosporine to induce G1 arrest. Alternatively, staurosporine could conceivably inhibit a kinase that normally negatively regulates a phosphatase that maintains Rb in an active, hypophosphorylated state. A potential Rb-independent arrest point in early G1 phase is represented by X. Whether this arrest point is actually present or not will require further studies outside the scope of this study.
tion of approximately 20 nM, data not shown), and we found that overexpression of cyclin D1-Cdk4R24C in REF52 cells reduced the ability of staurosporine to induce G1 arrest compared with the empty vector control cells. Such results, although suggestive of cyclin D1-Cdk4 as the direct target of the actions of staurosporine, are not by themselves conclusive. Indeed, staurosporine in G1 phase might induce a Rb-dependent G1 arrest through the activation of a phosphatase that dephosphorylates and activates Rb (Fig. 5). This could occur if staurosporine inhibited a kinase that normally negatively regulated this Rb phosphorylation (29, 30). It will be interesting to determine if the phosphorylation status and activity of this type I phosphatase is affected by staurosporine.

In the different MEFs we studied, we did not observe any obvious differences in the ability of cells to arrest in the G2 phase 24 h after staurosporine treatment. This indicated that p53, p21, Rb, or p16 were not essential components of the induction of this G2 arrest response. A possible target of the action of staurosporine in G2 has been presented before as the Cdc2 kinase (36).

In summary, our results highlight the importance of the Rb protein in the mechanism through which staurosporine induces G1 arrest in the first cell cycle. Continued investigation of the actions of staurosporine on Rb phosphorylation status could uncover the route and eventual target of the actions of staurosporine as it relates to Rb.

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