TECHNICAL NOTE

Cell-based ATM/ATR signaling

Optimization of ATM/ATR signaling conditions in various human cells

Introduction

Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) are serine/threonine kinases that regulate cell cycle checkpoints in response to DNA damage. These proteins phosphorylate a conserved S-Q/T-Q motif, which is present in many cellular proteins. ATR is activated by a variety of agents that can induce DNA damage, including UV light, and particular chemicals that inhibit DNA replication or directly modify DNA, where ATM is induced primarily by conditions that induce double strand breaks in the DNA, such as ionizing radiation.

Upon induction, ATM/ATR regulate the activity of several diverse classes of protein, many of which have been directly implicated in cellular checkpoint signalling pathways. These include scaffolding proteins, regulatory proteins, and other kinases. Among the better understood ATM/ATR substrates are p53, Chk1 and Chk2. Located immediately downstream of ATM/ATR, parallel analysis of phosphorylation of a combination of Chk1, Chk2 and p53 provides an ideal platform for interrogating the induction of ATM/ATR-mediated signalling.

Materials used

A431, HEK 293, HeLa, U2OS, NIH 3T3 and MCF7 cells are available from ATCC. AlphaScreen SureFire phospho-Chk1, phospho-Chk2, phospho-p53 and total p53 assay kits are available from PerkinElmer. Western blotting detection reagents and secondary antibody-HRP conjugates are available from Perkin Elmer. Tissue culture plasticware is from Nunc, and AlphaScreen® *SureFire*® assay plates are available from PerkinElmer.

Results

Cell line selection

Several cell lines were analyzed for the expression of Chk1, Chk2 and p53 to determine a cell lines that could be used for the analysis of all 3 targets of interest. Of these cells, Chk1 phosphorylation was readily detectable in NIH 3T3, MCF7, HeLa and HEK 293 cells, but signal was strongest in HEK 293 cells (Figure 1). Chk2 phosphorylation was analysed in A431, HEK293, HeLa and MCF7 cells, and signal was strongest in A431 cells, but was also readily detectable in HEK 293 cells. Finally, total and phospho-p53 was analyzed in A431, Hek 293 and U2OS cells, and was readily detected in all cell types.

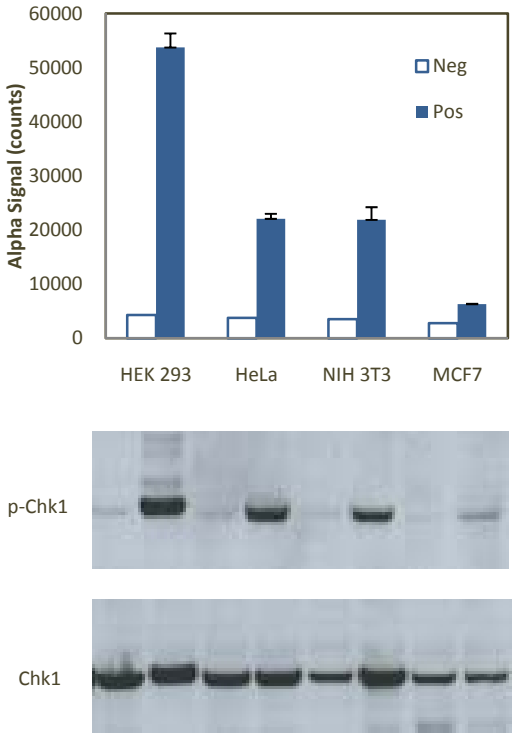

Figure 1. Detection of Chk1 phosphorylation in several cells. Cells were seeded in 96-well plates, and grown until confluent. The cells were treated with either nocodazole overnight (neg), or calyculin A (pos) for 1 hour, then lysed. The lysates were separated by SDS-PAGE, and analysed for phospho-Chk1 by AlphaScreen® *SureFire*®, or Western blot.

Stimulation of ATM/ATR activity in HEK cells

Several chemical agents known to cause DNA damage were assessed for their ability to induce ATM/ATR activity in HEK 293 cells and U2OS cells, as measured by induction of phospho-Chk1, phospho-Chk2 and phospho-p53 (Figure 2).

In HEK 293 cells, phosphorylation of Chk2 was induced for all treatments except hydroxyurea, which was only slightly induced after 1 hour. In contrast, phosphorylation of Chk1 was induced strongly by hydroxyurea at this timepoint, and comparatively weakly by doxorubicin and neocarzinostatin. Etoposide strongly induced both Chk1 and Chk2 phosphorylation in the HEK cells. Changes to levels of phospho-p53 or total p53 were poorly detected in HEK 293 cells after an 18 hour treatment period, probably due to the already high basal levels of phospho-p53 that were present in the cells.

In U2OS cells, Chk1 and Chk2 phosphorylation was more difficult to detect, but showed a similar pattern of induction as seen in HEK 293 cells. Chk1 was most strongly induced by etoposide and hydroxyurea, whereas Chk2 was induced by all agents except hydroxyurea after 1 hour. In contrast to HEK 293 cells, however, changes in p53 phosphoryation and accumulation were much more readily detected, and was induced strongly by doxorubicin and etoposide after 18 hours, but comparatively poorly by either hydroxyurea or neocarzinostatin at this timepoint.

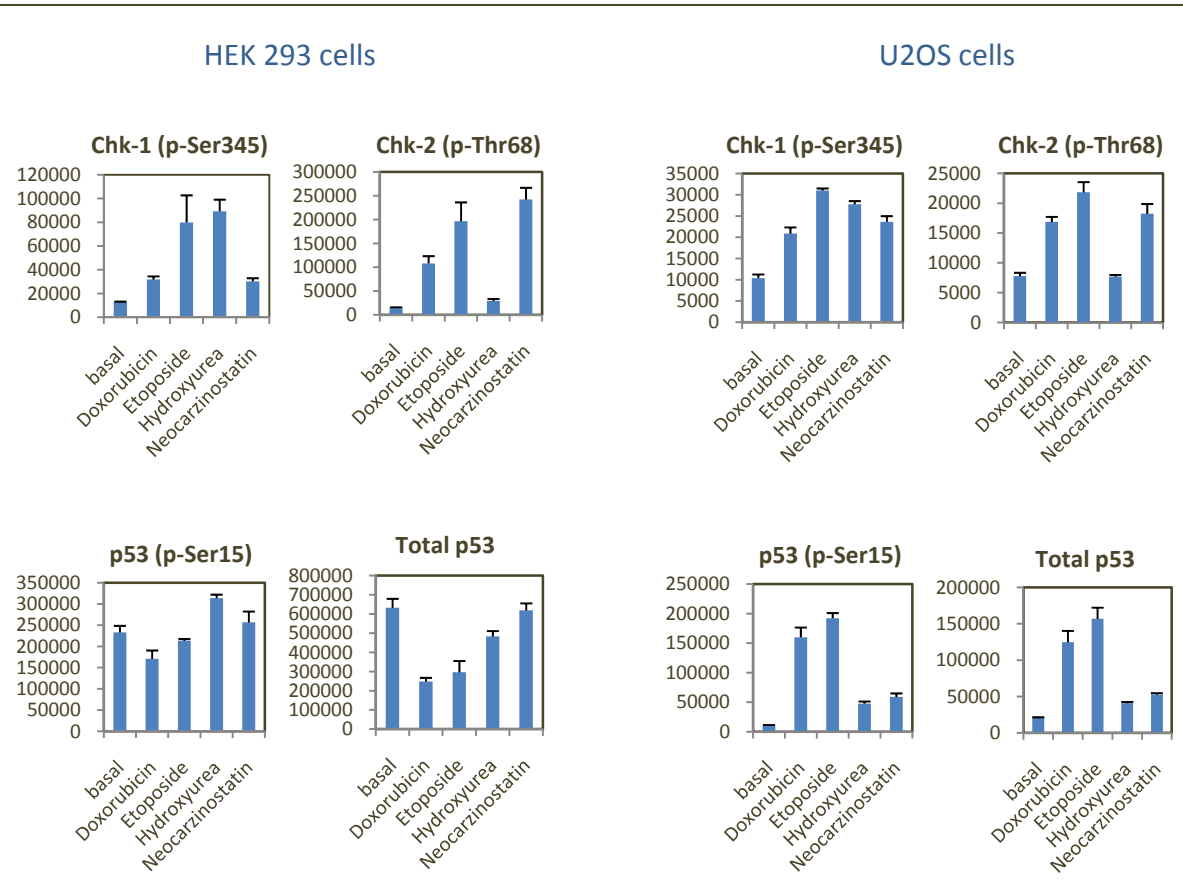

Figure 2. Detection of ATM/ATR-mediated phosphorylation in HEK 293 and U2OS cells. Cells were seeded in 96-well plates, and grown until confluent. The cells were treated as indicated for 1 hour for Chk1 or Chk2 measurements, or 18 hours for p53 measurements, then lysed. The lysates were transferred to 4 replicate low-volume 384-well Proxiplates (PerkinElmer) and analyzed for either phospho-Chk1, phospho-Chk2, phospho-p53 or total p53, using standard AlphaScreen® *SureFire*® assay protocols. The units are Alpha counts.

Stimulation of Chk1-specific phosphorylation in HEK cells

Hydroxyurea, which strongly induces Chk1 phosphorylation, but only weak Chk2 phosphorylation in HEK 293 afer 1 hour, was used to examine ATR-specific activity. HEK cells were either pre-treated with nocodazole, which causes cell cycle arrest in G2 or M phase, or left untreated. The cells were subsequently stimulated with hydroxyurea for varying times, and lysed. The lysates were examined for phospho-Chk1 (Figure 3). The stimulation of Chk1 was much stronger in the untreated cells, in contrast to nocodazole cells, where accumulation of Chk1 phosphorylation was much slower and also weaker.

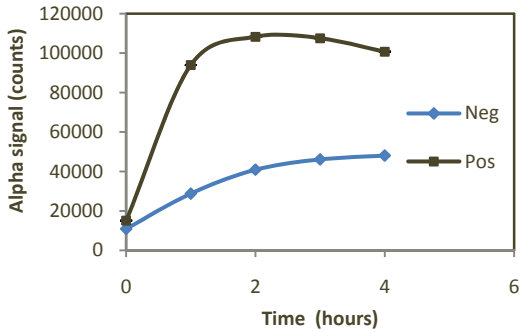

Figure 3. Detection of ATR-mediated phosphorylation in HEK 293 cells. Cells were seeded in 96-well plates, and grown until confluent. The cells were either pre-treated with nocodazole overnight (neg), or left untreated. The cells were subsequently stimulated with 5 mM hydroxyurea for varying periods of time, then lysed. The lysates were transferred to 4 replicate low-volume 384-well Proxiplates (PerkinElmer) and analyzed for phospho-Chk1, using standard AlphaScreen® *SureFire®* assay protocols.

Inhibition of ATM activity in HEK cells

Inhibition of ATM in HEK cells was analyzed using the specific ATM inhibitor KU-55933. HEK cells were pre-treated with inhibitor for 60 minutes, and subsequently stimulated with doxorubicin 90 minutes.

Cellular lysates were examined for phospho-Chk2, and a clear dose-dependant inhibition of Chk2 phosphorylation was observed (Figure 4).

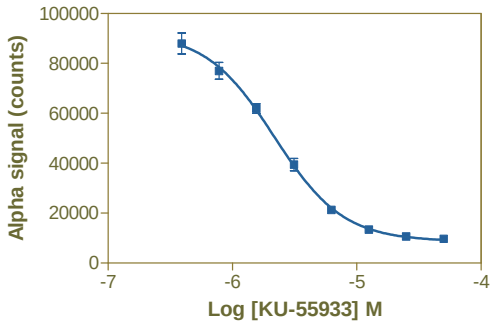

Figure 4. Inhibition of ATM-mediated phosphorylation in HEK 293 cells. Cells were seeded in 96-well plates, and grown until confluent. The cells were treated with the ATM inhibitor KU-55933 for 60 minutes, and subsequently stimulated with doxorubicin for 90 minutes. The cells were lysed, and a portion of lysate was transferred to 4 replicate low-volume 384-well Proxiplates (PerkinElmer) and analyzed for phospho-Chk2 using a standard AlphaScreen® *SureFire®* assay protocol.

Conclusions

We have analyzed several cell lines for ATM/ATR signaling, using phosphorylation of Chk1, Chk2, and p53 as markers. The targets are generally detectable in most cells that were examined, but were most readily detected in HEK 293 cells, although changes in p53 phosphorylation and accumulation were more readily detected in U2OS cells, most likely because high basal levels were present in HEK 293 cells. Thus, these cellular resources used in conjunction with these assay systems are readily amenable for analysis of the ATM/ATR signaling pathway.

Ordering Information

All cell lines are available from ATCC (www.atcc.org).

AlphaScreen® SureFire® cellular assay kits, available in 500pt, 10,000pt and 50,000pt pack sizes, are from PerkinElmer.

Catalog#

AlphaScreen SureFire Phospho-Chk1 (p-Ser345)
TGRCHK1S500, TGRCHK1S10K and TGRCHK1S50K

AlphaScreen SureFire Phospho-Chk2 (p-Thr68)
TGRCHK2S500, TGRCHK2S10K and TGRCHK2S50K

AlphaScreen SureFire Phospho-p53 (p-Ser15)
TGRP53S500, TGRP53S10K, TGRP53S50K

AlphaScreen SureFire total p53
TGRT53S500, TGRT53S10K, TGRT53S50K

Note: These kits must be used in conjunction with the AlphaScreen protein A IgG detection kits.