A GFP-based assay for rapid screening of compounds affecting ARE-dependent mRNA turnover

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ABSTRACT

A reporter transcript containing the green fluorescent protein (GFP) gene upstream of the destabilizing 3′-untranslated region (3′-UTR) of the murine IL-3 gene was inserted in mouse PB-3c-15 mast cells. The GFP–IL-3 transcript was inherently unstable due to the presence of an adenosine–uridine (AU)-rich element (ARE) in the 3′-UTR and was subject to rapid decay giving a low baseline of GFP fluorescence. Transcript stabilization with ionomycin resulted in an increase of fluorescence that is quantitated by FACS analysis of responding cells. Using this system we have identified okadaic acid as a novel stabilizing compound, and investigated the upstream signaling pathways leading to stabilization. This reporter system has the advantage of speed and simplicity over standard methods currently in use and in addition to serving as a research tool it can be easily automated to increase throughput for drug discovery.

INTRODUCTION

Regulating gene expression by modulating the turnover of mRNA is an important post-transcriptional mechanism to ensure a rapid cellular response to appropriate stimuli (1). This is achieved by the generation of short-lived transcripts with high turnover rates in the cytoplasm. The lability of these transcripts is largely due to the presence of *cis*-elements present in the transcript and a corresponding binding protein that targets the transcript for rapid decay. Several such destabilizing elements, located both in the coding and non-coding region, have been described: the CRD (coding region determinant) of the c-myc and c-fos oncogenes, the CDE (constitutive decay element) in the tumour necrosis factor α (TNFα) 3′-UTR and adenosine–uridine (AU)-rich elements (ARE). The ARE is the major *cis*-determinant and up to 8% of genes in the mammalian genome contain putative ARE sequences in their 3′-UTR (2). Based on sequence and functional criteria, AREs are divided into three main groups. Class I AREs contain multiple scattered AUUUA pentamer motifs in a U rich region, class II AREs contains the AUUUAUUUA motif which is composed of multiple overlapping AUUUA pentamers, while class III AREs which do not contain the AUUUA motif nevertheless are

uridine-rich and serve to promote decay. Most transcripts encoding cytokines, growth factors and proto-oncogenes contain AREs, examples of clinical significance being the c-fos, c-myc and c-jun oncogenes, the cytokines IL-2, IL-3, IL-4, IL-6 and Granulocyte–macrophage colony-stimulating factor (GM–CSF), the VEGF growth factor and the inflammatory cytokine TNFα.

While such transcripts are present at low steady-state levels in the cytoplasm due to their inherent lability, transient stabilization under appropriate conditions leads to their rapid accumulation and expression (3). Conventional methods of detecting transcript stabilization are based first on blocking transcription with a transcriptional inhibitor such as actinomycin D, and harvesting the RNA at appropriate intervals. The RNA is resolved by gel electrophoresis, transferred to membranes and probed for the transcript of interest via northern hybridizations. Alternatively, the levels of a particular transcript can also be detected via quantitative PCR. Stability is then assessed by the decay or persistence of the transcript over the time-course following actinomycin D addition. Such methods are labor intensive and time consuming making them ill-suited for the rapid screening of large numbers of compounds for their ability to influence mRNA turnover rates.

We describe here the application of a green fluorescent protein (GFP)-based cellular assay where the GFP coding transcript is rendered unstable by fusion to the IL-3 3′-UTR which contains a canonical class II ARE that is responsible for destabilizing the native IL-3 transcript. Changes in transcript stability are mirrored by altered GFP levels which are directly measured by FACS analysis. Using this reporter system we have identified okadaic acid as a potent stabilizer of the IL-3 ARE-containing mRNA. In addition, drugs with proven clinical relevance score strongly and specifically in this assay. The research potential of this system is also demonstrated by the identification of upstream signaling pathways involved in stabilization.

MATERIALS AND METHODS

Reagents

Chemicals were purchased from the following suppliers: ionomycin, cyclosporin A, wortmannin, SB202190, FK506 and actinomycin D (Calbiochem); okadaic acid, rapamycin, butyric acid, MG132 (Sigma); and SP600125, PD169316, PD98059, U0126, Akt inhibitor (Alexis). Antibodies against phospho-p38, p38, phospho-JNK, JNK were obtained from

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Cell Signaling Technologies. Goat anti-mouse and goat anti-rabbit secondary antibodies were from Promega.

Cell culture

PB-3c-15 is a pre-malignant subclone of murine PB-3c mast cells that is known to rapidly degrade endogenous IL-3 mRNA. PB-3c-15 cells that have been transformed with the v-H-ras oncogene were inoculated and oncogenically transformed in syngeneic mice and the V2D1 cell line was derived from the resulting tumors (4). A V2D1 subclone, VG59, was used in this study. All cells were maintained in Iscove's medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 1% conditioned medium from X63-mIL3 cells as a source of exogenous IL-3. Stable transfectants were obtained by electroporation with a GenePulser (BioRad) and selection with hygromycin (1 mg/ml).

Plasmid constructs

The GFP–IL3wt reporter construct has been previously described (5). Briefly, it consists of the mouse genomic 1.9 kb HindIII–ApaI fragment containing the IL-3 promoter inserted upstream of the EGFP promoter in pEGFP-N1 (Clontech). The entire IL-3 promoter–GFP construct was cleaved out as a HindIII–XbaI fragment and inserted into the IL3MXh-wt vector. This step fused the GFP coding sequence upstream of a portion of the terminal exon of IL-3 containing the entire 3′-UTR including the poly(A) signal (Figure 1A). The GFP–IL3ΔAU construct is similar except for the deletion of a 216 bp NcoI–StyI fragment from the 3′-UTR thereby removing the entire ARE.

Flow cytometry

Around 10^5 cells were grown in 12-well dishes and stimulated with the respective compounds for 4 h. After harvesting by centrifugation, the cells were resuspended in a small volume (200–400 µl) of phosphate-buffered saline (PBS) containing propidium iodide (5 µg/ml) and kept on ice in the dark for 30 min. A total of 10^4 cells were analyzed using a FACScan (Becton Dickinson) and the data processed with the CellQuest program. GFP fluorescence was excited at 488 nm and emission was measured with a 510 nm filter. Propidium iodide staining was detected with a 580 nm filter which allowed exclusion of dead cells and cellular debris during data analysis.

Actinomycin chase mRNA decay assay

Control and treated cells were transcriptionally blocked with actinomycin D (5 µg/ml) and cytoplasmic RNA was extracted at the following time points: 0, 30, 60, 90 and 120 min. Twenty micrograms of cytoplasmic RNA was resolved on a 1.1% agarose–formaldehyde gel and blotted onto HyBondN+ nylon membranes (Amersham) overnight by capillary transfer with 20× SSC. Membranes were probed for GFP-IL3 mRNA using an SP6 radiolabeled RNA probe from the XbaI–EcoRI fragment of murine IL-3 cDNA containing the IL-3 3′-UTR. For loading controls, cells were stripped and re-probed against G3PDH, a stable, constitutively expressed transcript. Quantification of hybridizing signals was performed on a PhosphorImager (Molecular Dynamics) using ImageQuant software. The reporter GFP–IL3 mRNA signal was normalized to the respective G3PDH reference signal after subtraction of the filter background.

Western blotting

Cells were grown in 12-well plates with about 10^5 cells per well. After pelleting, the cells were extracted with lysis buffers containing Phosphatase Inhibitor Cocktail 1 and 2 (Sigma) and protease inhibitors (Complete, Roche). Forty micrograms of cell lysate protein was resolved on 12% SDS–PAGE gels and electrophoretically transferred onto Immobilon membranes (Millipore). Membranes were blocked in 5% skimmed milk in TBS–0.1% Tween-20 (TBS-T) and shaken at 4°C overnight with the respective antibody. The membrane was washed four times for 10 min in TBS-T and incubated with the secondary alkaline phosphatase conjugated antibody for 1 h at room temperature. The membrane was washed as before and the signal developed with CDP-Star (Roche) for autoradiography.

RESULTS

Construction of GFP–IL3 reporter cell lines

PB-3c-15 cells were transfected with the GFP–IL3 plasmid and selected with hygromycin to obtain a pool of stable transfectants. The GFP–IL3 construct consists of the GFP coding sequence upstream of the entire IL-3 3′-UTR. The 59 nt long ARE region has been previously shown to confer instability on the IL-3 and heterologous transcripts (6). As shown schematically in Figure 1A, the inherent instability of the reporter transcript results in correspondingly low steady-state mRNA levels of the transcript. This is mirrored by weak GFP fluorescence in cells expressing the reporter. However, upon stabilization, levels of the transcript rise rapidly which results in a rightward shift of the GFP peak as measured by FACS analysis. The reverse situation holds true in the case of further destabilization of a transcript. GFP fluorescence of the transfected cells was assessed by eye to select for GFP expressing cells and was confirmed by FACS (data not shown). Individual clones were obtained by dilution cloning for further testing.

To confirm that the GFP–IL3 transcript was indeed subject to ARE-dependent destabilization we treated the subclones with ionomycin, a calcium ionophore that is known to be a potent stabilizer of IL-3 mRNA in these cells (7,8). FACS profiles identified subclones with a low GFP baseline that responded upon ionomycin treatment with significant increases in GFP fluorescence. Of these subclones, one was selected for further study and designated 15-GFP–IL3. A similar procedure was followed to obtain the control cell line 15-GFP-ΔAU lacking the ARE.

Screening of mRNA stabilizing compounds

Signaling pathways have been shown to regulate mRNA stability (9–12). Therefore we tested the effects of various kinase and phosphatase inhibitors to promote or inhibit stabilization, as well as previously described stabilizing agents. Compounds screened were U0126 (MEK1/2 inhibitor), PD98059 (MEK1 inhibitor), SB202190 (p38MAPK inhibitor), PD169316 (p38MAPK inhibitor), wortmannin (PI3K inhibitor), rapamycin (mTOR inhibitor), ionomycin (calcium ionophore), butyric acid (HDAC inhibitor, pleiotropic effects), okadaic acid
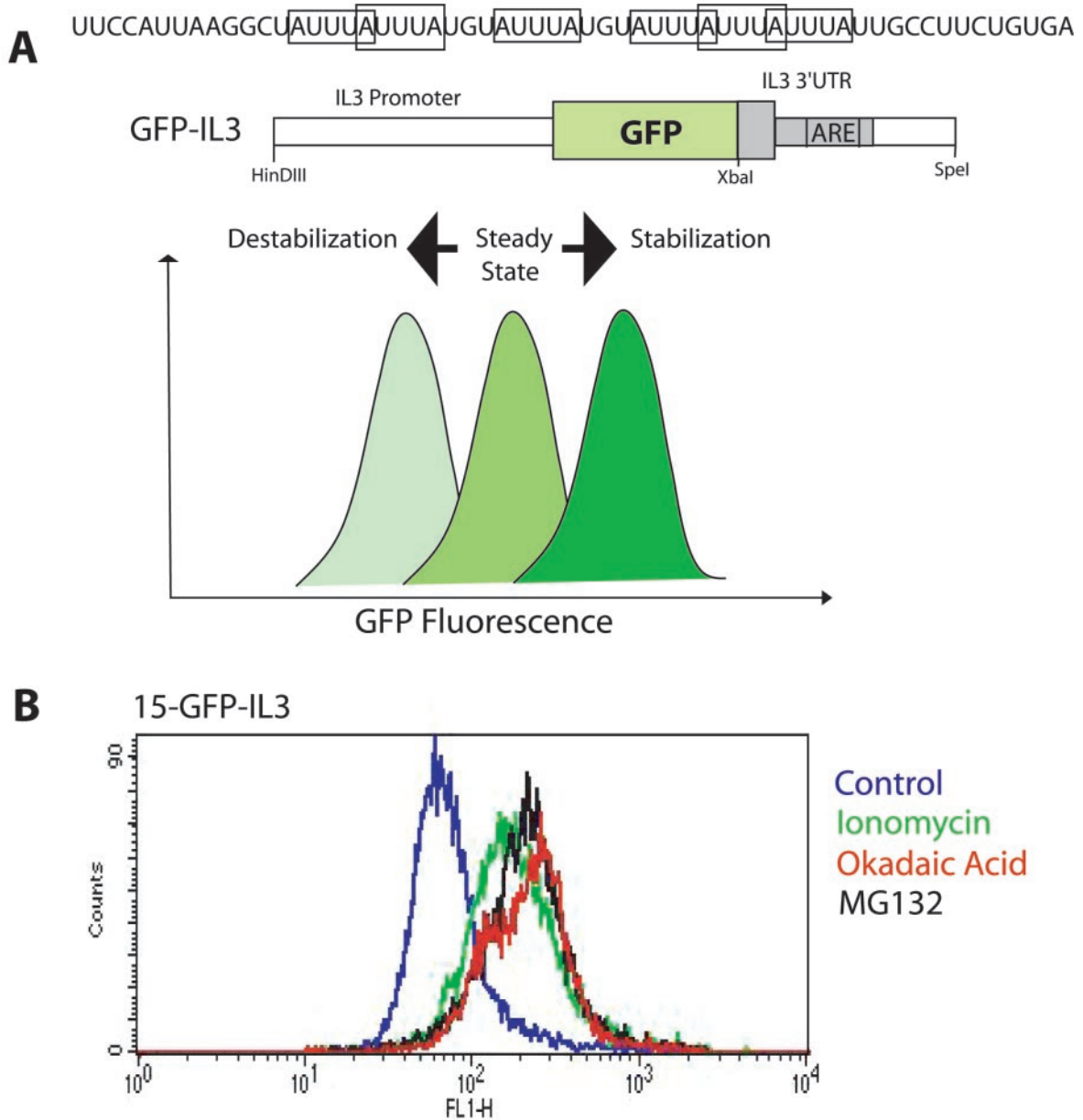
Figure 1. (**A**) The 59 nt long ARE region of the mouse IL-3 3′-UTR with AUUUA pentamers shown in boxes. A schematic representation of the GFP–IL3 reporter construct and the expected FACS profiles under conditions of mRNA stabilization and destabilization. (**B**) A representative FACS profile of 15-GFP–IL3 cells after treatment with ionomycin (1 µM), okadaic acid (400 nM) and MG132 (500 nM) for 4 h showing a substantial increase in GFP fluorescence.

(PP2A inhibitor), Akt inhibitor, MG132 (proteasome inhibitor), SP600125 (JNK inhibitor), cyclosporin A and FK506. Cells were treated for 4 h and harvested for FACS analysis. This was done in order to exclude non-specific effects and to shorten the time for analysis as mRNA stabilization is in any case a rapid process. Of all the compounds tested, only okadaic acid (an inhibitor of the cellular phosphatase PP2A) and MG132 (a proteasome inhibitor) gave a significant shift in GFP fluorescence (Figure 1B) that was comparable in magnitude to that induced by the known stabilizer ionomycin.

The effects of both compounds were further investigated in the control cell line 15-GFP-ΔAU. This contains a similar GFP–IL3 reporter construct except for the deletion of the entire ARE thereby resulting in the production of a stable transcript. Any increase of GFP fluorescence in this cell line would hence be due to a non-ARE-dependent mechanism, most probably transcriptional upregulation or even a translational effect. As seen in Figure 2A, neither okadaic acid nor MG132 cause a major shift in the GFP peak of 15-GFP-ΔAU.

To definitely confirm the RNA stabilizing properties of these compounds, we performed an actinomycin D chase mRNA decay assay. This confirmed okadaic acid as a genuine stabilizing agent (Figure 2B). However, MG132 was shown to be unable to stabilize the reporter transcript in this assay despite inducing a shift in GFP fluorescence (Figure 1B).
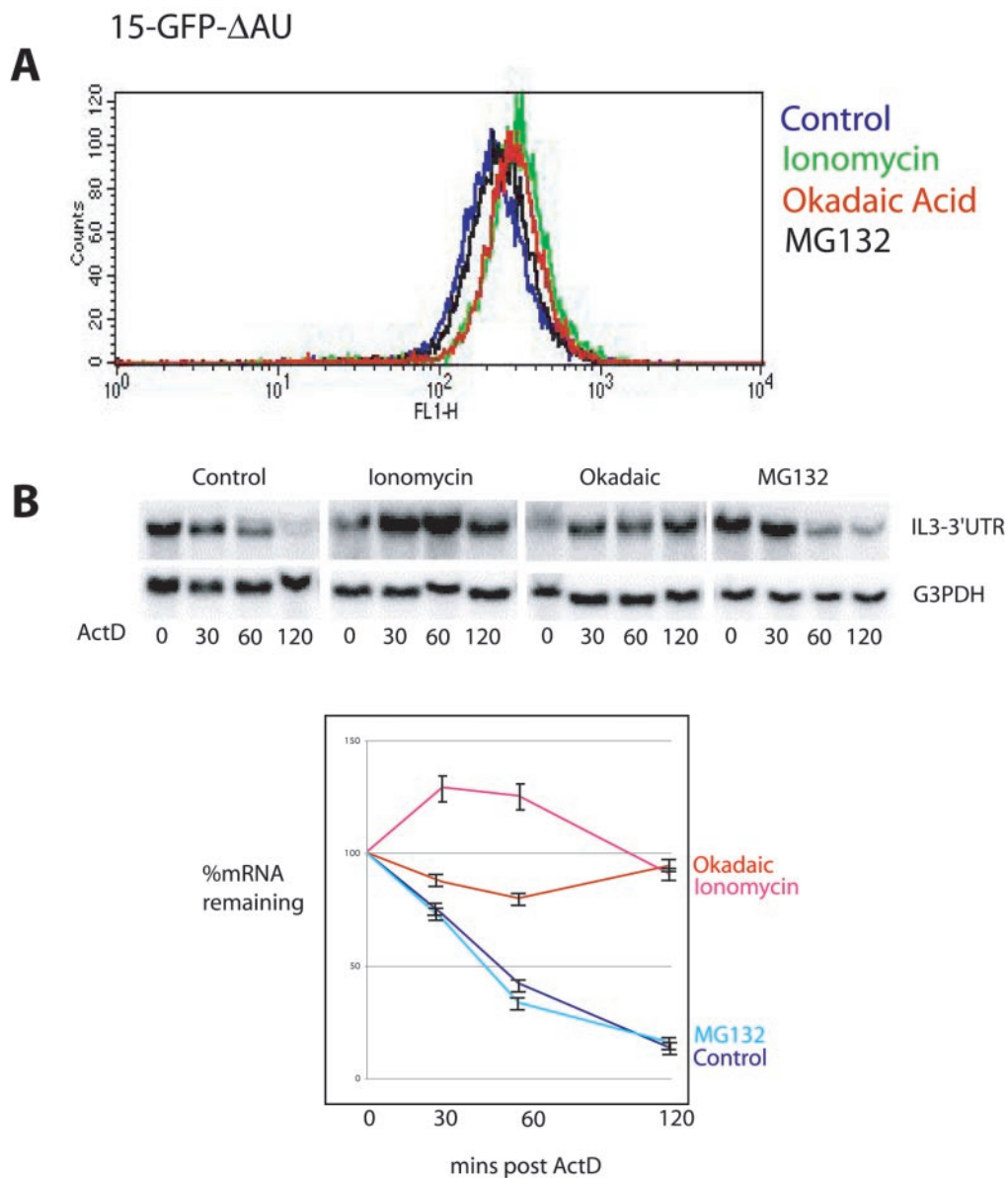
Figure 2. (**A**) A representative FACS profile of the control non-ARE cell line, 15-GFP-ΔAU, showing no significant increase in GFP fluorescence after similar treatments with ionomycin, okadaic acid and MG132 as for the reporter cell line 15-GFP–IL3. (**B**) Actinomycin D chase mRNA decay assay of 15-GFP–IL3 cells over a 2 h time course. The blots were probed for the GFP–IL3 3′-UTR reporter to determine the rate of decay, then stripped and re-probed for the stable G3PD transcript for loading controls and to normalize the GFP–IL3 hybridization signals for quantification. Quantified results (±SE) for four independent experiments are shown on the graph. As shown, ionomycin and okadaic acid stabilize the reporter mRNA while MG132 is ineffective.

Regulation of okadaic acid- and ionomycin-induced mRNA stabilization

Previous work from our laboratory has shown that ionomycin-induced IL-3 mRNA stabilization in PB-3c cells operates via activation of calcineurin (PP2B), a calcium-dependent phosphatase, as it could be antagonized by the calcineurin inhibitors cyclosporin A and FK506 (13,14). Among the downstream signaling pathways triggered by calcineurin activation, the JNK pathway was shown to be required but not sufficient for stabilization (10). We therefore sought to confirm these earlier findings with our reporter system and, in addition, to identify kinase/phosphatase inhibitors that were able to antagonize pharmacologically induced stabilization. As expected, ionomycin-induced stabilization was clearly antagonized in cells pre-incubated with cyclosporin A and SP600125 [a JNK kinase inhibitor, (15)] (Figure 3A and C). In contrast both cyclosporin A and SP600125 were unable to inhibit okadaic acid-induced stabilization (Figure 3B and D) indicating that it acts either by a distinct mechanism or downstream of calcineurin. Interestingly, a p38 MAPK inhibitor, PD169316, could block stabilization in both cases (Figure 3E and F) pointing to a central role of the p38 pathway in IL-3 mRNA stabilization in mast cells. To confirm the activation of both pathways, western blotting on control and treated PB-3c-15 cell lysates was performed with antibodies directed against
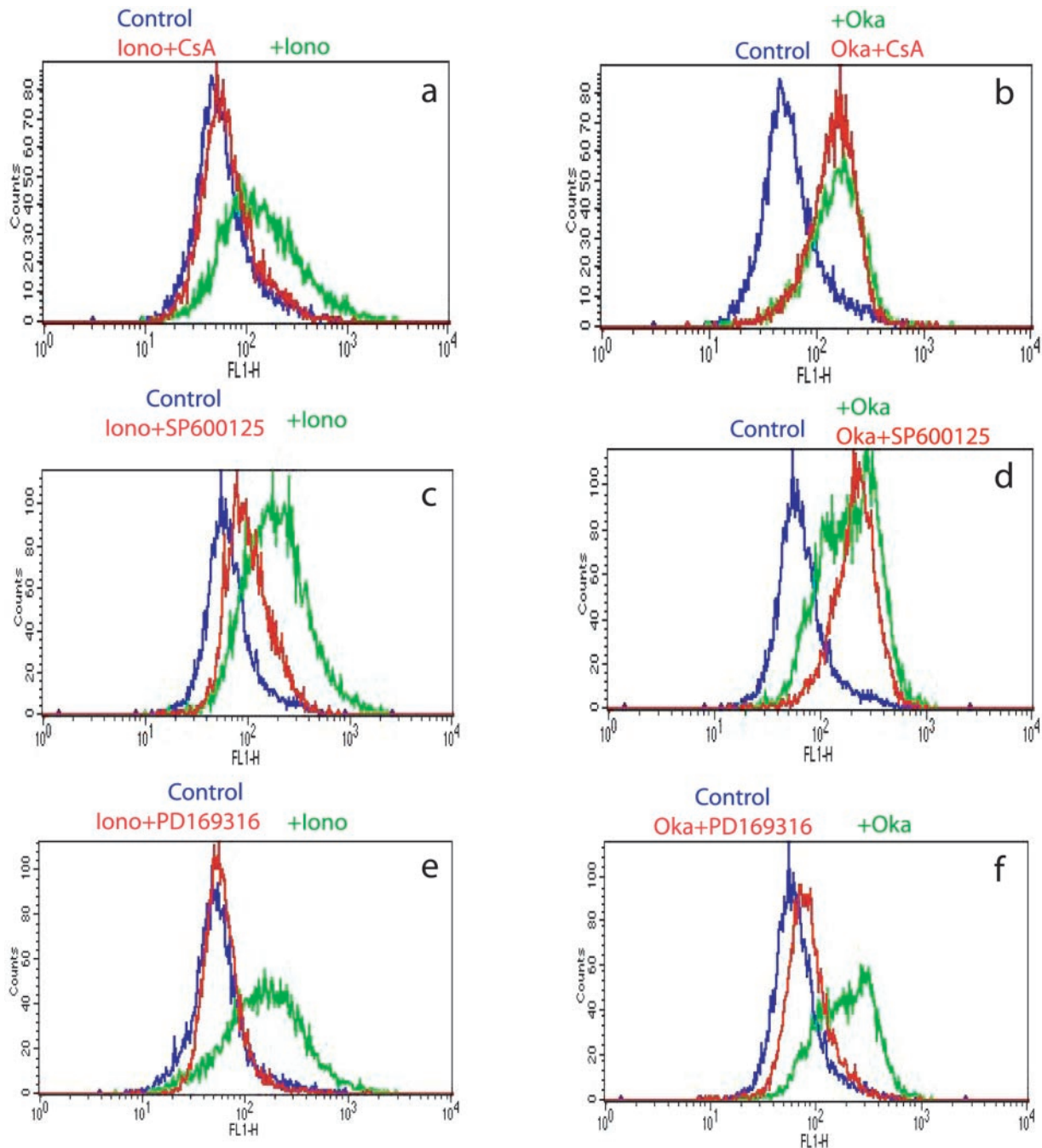
Figure 3. Antagonization of ionomycin-induced mRNA stabilization by cyclosporin A (0.5 µg/ml), PD169316 (10 µM) and SP600125 (10 µM). In contrast, okadaic acid-induced stabilization was uninhibited by cyclosporin A and SP600125 but was also sensitive to PD169316.

the active, phosphorylated forms of the p38 and JNK kinases (Figure 4). Both pathways were shown to be activated by ionomycin in line with our previous data; interestingly, the JNK pathway is even more strongly activated by okadaic acid but is nevertheless inessential for okadaic acid-induced stabilization (Figure 3).

Destabilization of GFP–IL3 mRNA

V2D1 is an autocrine tumor line derived from PB-3c-15 cells expressing the v-H-ras oncogene after inoculation into syngeneic

mice. The key feature of V2D1 is the loss of IL-3 dependence which is achieved by the establishment of an autocrine loop for IL-3 production through constitutive stabilization of the IL-3 mRNA (16,17). A V2D1 subclone stably expressing the GFP–IL3 reporter was created and designated VG59. Our previous work has shown that stable IL-3 transcripts in these cells can be destabilized with cyclophilins such as cyclosporin A and FK506 (14). To test if the reporter system is also capable of detecting mRNA destabilization, we treated VG59 cells with FK506 for 72 h followed by FACS analysis. The longer time required for treatment is due to the stability of pre-existing GFP

protein which needs to be cleared before it can accurately reflect the steady-state levels of the encoding transcript. In Figure 5 a distinct back shift of the GFP peak is visible after FK506 treatment in VG59 cells in line with expectations that this method can, in principle, be used to detect mRNA destabilization.

DISCUSSION

While the number of genes known to be post-transcriptionally regulated has grown, this has not been accompanied by similar progress in identifying compounds capable of modulating mRNA stability. This is largely due to the time-consuming and labor-intensive nature of standard mRNA decay assays which do not permit rapid, large-scale screening of potential stabilizing agents.
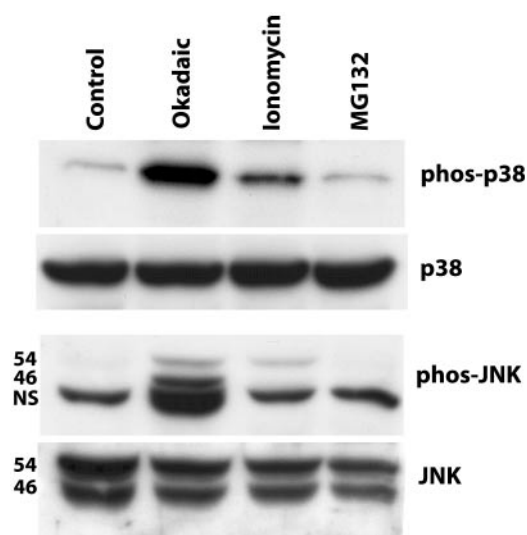

Figure 4. Western blots of 15-GFP–IL3 lysates treated with ionomycin, okadaic acid and MG132 for 30 min. Activation of the p38 MAPK pathway was determined with an antibody directed against the phosphorylated, active form of p38. JNK pathway activation was determined with a similar phospho-specific antibody that detects two phosphorylated isoforms of JNK (46 and 54 kDa), and a non-specific (NS) band. Blots were stripped and re-probed with antibodies against the unphosphorylated forms of the proteins as loading controls.

The reporter system presented here has the advantage of providing quick (<5 h), reliable and quantitative results. As only a small number of cells are required for FACS analysis (10^4 cells), the size of the experiment can be scaled up or down as required in an appropriate multi-well format to screen any number of compounds with the relevant time and dilution series. In addition, the small number of steps required suggests that it will lend itself easily to automation. Using this system we have identified okadaic acid and MG132 as potential stabilizers of IL-3 mRNA (Figure 1B). Okadaic acid, but not MG132, was confirmed as a genuine mRNA stabilizer via standard methods (Figure 2B). MG132 has previously been shown to stabilize the class II ARE present in the GM–CSF mRNA in HeLa cells (18). The apparent GFP shift observed with MG132 treatment is most likely due to reduced turnover of GFP at the protein level due to its anti-proteosomal properties (19) rather than actual stabilization of the reporter transcript. This illustrates that this reporter system would function best as a large-scale primary screen for potential stabilizing compounds that would then require further confirmation.

We also obtained evidence for the involvement of several signaling pathways in inducing stabilization (Figure 3). The central role of the p38 MAPK pathway in ionomycin- and okadaic acid-induced stabilization is consistent with other reports (20,21) indicating common regulatory mechanisms controlling stability. While this demonstrates the research potential of the technique, it may also have practical application as the regulation of mRNA stabilization is of potential clinical significance as in, e.g. modulating the levels of cytokines.

Although we have constructed the ARE and the non-ARE reporters in separate cell lines, it is technically feasible to insert both constructs in the same cell line with the proviso that they are linked to reporter molecules with different emission wavelengths (e.g. a GFP–ARE reporter versus an RFP–ΔARE reporter). As a large-scale screen will presumably uncover effects unrelated to ARE-dependent decay but capable of affecting GFP protein levels (i.e. stimulation of transcription/translation, reduced turnover of GFP protein, etc.), it is critical that such a double-reporter cell line is used for actual screening. This will, in addition, provide simultaneous data collection while serving as an internal control to distinguish between ARE-dependent and non-ARE-dependent stabilization.
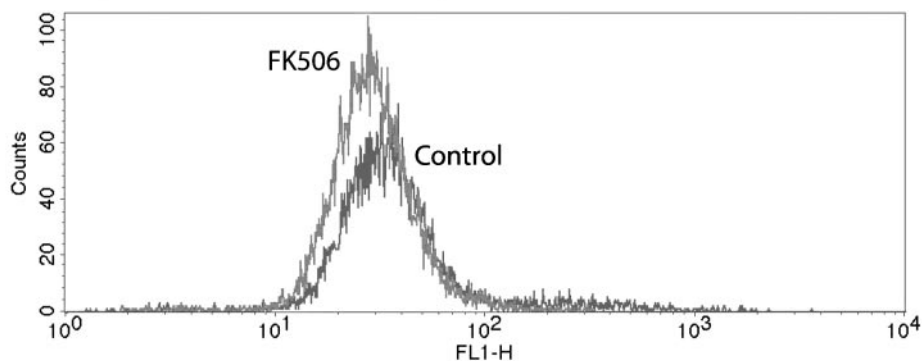

Figure 5. FACS profile of VG59 cells treated with FK506 (1 µg/ml) for 72 h.

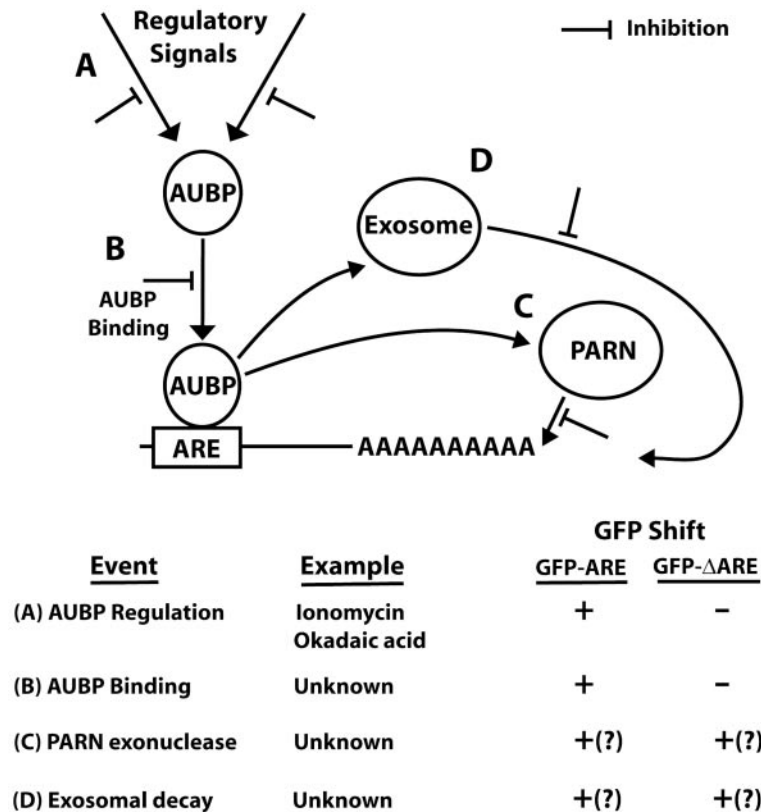| Event	Example	GFP Shift	
		GFP-ARE	GFP-ΔARE
(A) AUBP Regulation	Ionomycin Okadaic acid	+	–
(B) AUBP Binding	Unknown	+	–
(C) PARN exonuclease	Unknown	+(?)	+(?)
(D) Exosomal decay	Unknown	+(?)	+(?)

Figure 6. Overview of the steps in mRNA decay. (**A**) Regulatory signals act on the AUBP to determine binding ability and hence stability/decay of the target transcript; (**B**) Binding of the AUBP to the ARE; (**C**) Deadenylation of the poly(A) tail by poly(A) ribonuclease (PARN); (**D**) $3'$–$5'$ degradation of the deadenylated transcript by the cytoplasmic exosome. Decay can be potentially blocked by inhibition at any specific step. The table shows the expected results for different classes of inhibitors when screened using a GFP–ARE and the complementary ARE-deleted GFP–ΔARE cell lines. Question marks in parenthesis denotes where a particular interaction is hypothetical.

A further technical refinement would be the use of a more labile reporter molecule to facilitate investigations into mRNA destabilization. Our results with a destabilizing agent, FK506, while establishing a proof in principle of the applicability of this method in detecting mRNA destabilization (Figure 5), may not represent an ideal situation as the long treatment times required (72 h) can conceivably give rise to non-specific secondary effects. The availability of more labile forms of GFP (22) provides a ready solution that should feature in future developments of this method.

Regulated mRNA decay is a multi-step process that can be divided into: regulation of AUBP activity, AUBP binding to the ARE, deadenylation of the poly(A) tail by PARN and $3'$–$5'$ degradation of the deadenylated transcript by the cytoplasmic exosome (Figure 6). The AUBP can promote decay either by accelerating the degradation of the poly(A) tail or by direct recruitment of the exosome. Due to the sequential nature of events involved in effecting decay, it is possible in theory to screen for inhibitors at several steps of the process. Our results with phosphatase/kinase inhibitors indicate that ionomycin- and okadaic acid-induced stabilization is effected at the level of AUBP regulation. It is possible with a larger screen, to perhaps identify inhibitors of PARN or the exosome.

While we have limited our studies to the IL-3 ARE, which is a class II ARE, this approach can obviously be applied to any ARE or *cis*-determinant of interest and similar flexibility applies to the choice of cell line to be studied. Indeed we have constructed similar reporter systems for the CDE of the TNFα $3'$-UTR (23), and in various cell lines, namely RAW264.7 and HT1080 (24).

ACKNOWLEDGEMENTS

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