An assay for dual-translocation of MAPKAP-k2 and ATF-2 using an automated cellular imaging system – IN Cell Analyzer 1000

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Introduction
Image-based analysis of the movement of cell signalling molecules can be used as an indicator of cellular activation. Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-k2) and activating transcription factor-2 (ATF-2) are members of p38 MAPK and SAPK/JNK stress-activated signalling pathways respectively (1). Measurement of the cellular translocations of these proteins can be used to monitor the activation of their upstream signalling cascades. An assay for simultaneous anisomycin-stimulated cellular translocation of MAPKAP-k2 and ATF-2 has been developed (Fig 1). The p38 MAPK signalling pathway has been widely studied using the p38-specific inhibitor SB 203580 (2). The effect of this compound on the SAPK/JNK pathway has been examined. The assay has been modified to use the IN Cell Analyzer 1000 (Amersham Biosciences Corp.), an automated lamp-based fluorescence imaging system combined with high-throughput image analysis.

Methods
Dual-translocation assay
A BHK-derived cell line stably expressing GFP-MAPKAP-k2 (Amersham Biosciences Corp.) was used to directly monitor translocation of MAPKAP-k2. Fluorescence immunostaining for ATF-2 was employed to demonstrate the translocation of ATF-2 in the same cells. The cells (6000 per well) were incubated for 24 hrs in a Greiner 96-well plate and treated with either anisomycin or SB203580 (Sigma) for 1 hr. The cells were then fixed (4% paraformaldehyde) and immunofluorescently stained with the phospho-ATF-2 specific mouse antibody (Cellomics) and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes). Nuclei were labeled with Hoechst (Molecular Probes). The cells were imaged with a 10x objective using a 10x numerical field and 3 different channels [(1) red: Ex-620/60, Em-700/75, (2) green: Ex-475/20, Em-535/50, and (3) blue: Ex-360/40, Em-535/50]. The analysis software quantitatively measures the fluorescent intensity in both nucleus and cytoplasm.

Results
Translocation of GFP-MAPKAP-k2 and ATF-2
Upon stimulation with the agonist anisomycin, GFP-MAPKAP-k2 translocates to the cytoplasm from the nucleus (compare upper Figs 2A and 2B). This translocation event was inhibited by the p38 specific inhibitor SB203580 (upper Fig 2C). Red fluorescence (Alexa 594) indicates the presence of ATF-2 in the untreated cells, with weaker staining in the nuclei (lower Fig 2A). After anisomycin treatment, ATF-2 moved from the cytoplasm to the nucleus (lower Fig 2B), but the p38 specific inhibitor SB203580 failed to inhibit this translocation (lower Fig 2C).

Quantification of GFP-MAPKAP-k2 and ATF-2
Images were analysed automatically for nuclear and cytoplasmic intensity ratios (nu/cy) using a Nuclear Trafficking Algorithm. The anisomycin stimulation resulted in increased GFP-MAPKAP-k2 translocation to cytoplasm from nucleus and a resultant decrease in nu/cy ratio (Fig 3A). Blocking the translocation with the p38-specific inhibitor SB203580 increased the nu/cy ratio. In contrast anisomycin increased the nuclear localization of ATF-2, resulting in a higher nu/cy ratio compared to control cells (Fig 3B). The nu/cy ratio was also higher in cells treated in combination with anisomycin and SB 203580, indicating a lack of inhibitory effect for this p38-specific inhibitor on ATF-2 translocation.

Dose response of anisomycin induced dual-translocation of MAPKAP-k2 and ATF-2
Increasing the anisomycin concentration resulted in a decreasing (nu/cy) ratio of ATF-2, and an increasing (nu/cy) ratio of ATF-2 in a dose-responsive manner (Fig 4). The EC50’s for anisomycin stimulation were 93nM for MAPKAP-k2 and 90nM for ATF-2, indicating that this agonist was equally potent for both translocations.

Figure 1. Overview of translocation assays

Figure 2. Upper panel: cell images of GFP-MAPKAP-k2. Lower panel: cell images of ATF-2. (A) Untreated cells; (B) cells treated with 300nM anisomycin; (C) cells treated with 300nM anisomycin and 150µM SB203580. The ratios are means (±SD) of 8 wells (1 image per well).

SB203580, a specific inhibitor of p38MAPK, inhibited the anisomycin-induced MAPKAP-k2 translocation with an IC50 of 0.9µM, but had no effect on anisomycin–induced ATF-2 translocation (Fig 5).

Figure 3(A). GFP-MAPKAP-k2 translocation and (B) ATF-2 translocation in: (●) control cells, (●) cells treated with 300nM anisomycin and (●) cells treated with 300nM anisomycin and 150µM SB203580. The ratios are means (±SD) of 8 wells, one image/well, and approximately 300 cells/image.

Figure 4. Effect of increasing concentration of anisomycin on MAPKAP-k2 and ATF-2 translocation. The cells were treated with 10-1000nM anisomycin for 1 hr. The nuclear to cytoplasmic intensity ratio is plotted, each data point representing the mean (±SD) of 8 wells (1 image per well). SB 203580 inhibition of MAPKAP-k2 translocation

CONCLUSIONS

• An image-based dual-translocation assay for simultaneous measurement of MAPKAP-k2 and ATF-2 has been successfully developed.
• The assay provides an information-rich measurement of both p38 MAPK and SAPK/JNK signalling pathways in the same cells.
• Study of pathway-specific drug interactions is possible for both drug discovery and lead profiling.
• IN Cell Analyzer 1000, a multiple wavelength fluorescence detector with high-resolution image acquisition and automated image analysis, is an enabling platform for analysis of multiplexed cellular events.

References

Figure 5. SB203580 inhibition of MAPKAP-k2 and ATF-2 translocations. Cells were treated with 0.15µM to 150µM SB203580 in the presence of 300nM anisomycin for 1 hr. The nu/cy intensity ratio is plotted, each data point representing the mean (±SD) of 8 wells (1 image per well).

The results show that the known p38 MAPK-specific inhibitor SB203580 acted solely on the MAPK pathway, but not on the SAPK/JNK pathway, consistent with published results (1). Small molecule inhibitors of MAPK pathways are being actively studied as potential therapeutics for a number of disease states (3). This multiplexed assay allows study of pathway-specific interactions for drug discovery and lead profiling.