Multiplexing Fluorescent and Chemiluminescent Live Cell Reporters to Dissect NFκB Signal Transduction Requirements

Cell Biology Group, Clontech Laboratories, Inc., in collaboration with Molecular Devices, now a part of MDS Analytical Technologies

We have employed the chemiluminescent Ready-To-Glow™ Secreted Metridia *Luciferase reporter system to monitor NFκB response element activation by TNF-α, and combined it with the fluorescence-based ZsGreen Proteasome Sensor. We used this combined assay to directly prove the requirement for active proteasomes in TNF-α-induced, NFκB-dependent signaling. We took advantage of the multidetection capacity of the SpectraMax® M5 multimode microplate reader from Molecular Devices to measure both reporters in a completely homogeneous manner, in the same well of a 96-well plate, without cell lysis.*

NFκB Translocation is Proteasome-Dependent

The proteasome is a large protein complex that degrades proteins into short peptides in a ubiquitin-dependent manner. It plays a vital role in processes such as gene transcription, cell-cycle progression, DNA repair, cellular differentiation, viral infection, and oncogenesis (1). Proteasome activity has been implicated in the regulation of NFκB-based transcription: in order to activate NFκB upon TNF-α stimulus, IκB must be phosphorylated and then degraded by the proteasome.

If proteasome degradation is blocked, NFκB activation is compromised (2–3). However, until now it has not been possible to monitor proteasome activity and NFκB signaling simultaneously, in the same sample.

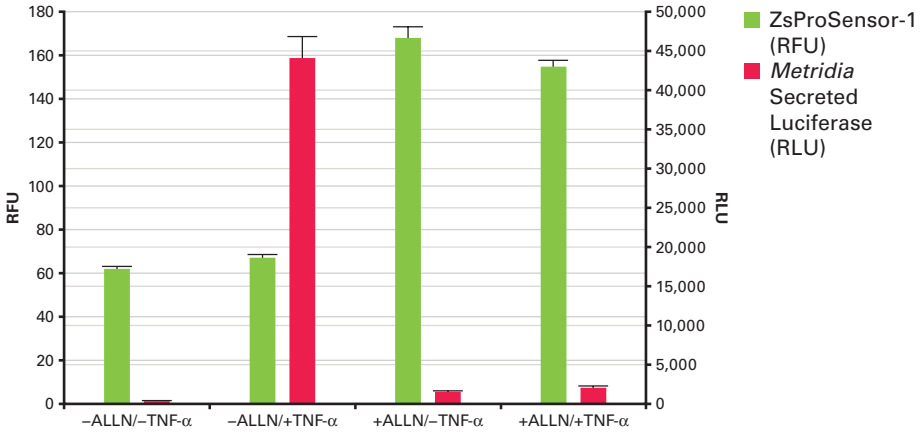

Figure 1. NFκB activation by TNF-α requires proteasomal activity. High levels of *Metridia* luciferase signal were only observed in the absence of ALLN and the presence of TNF-α. When the proteasome is inactivated by ALLN (monitored by increasing levels of ZsProSensor-1 fluorescence), the NFκB signaling pathway cannot respond effectively to TNF-α stimulation. ALLN = N-acetyl-leucyl-leucyl-norleucinal.

The ZsGreen Proteasome Sensor

The **Living Colors® HEK 293 ZsGreen Proteasome Sensor Cell Line** (Cat. No. 631535) provides a simple and noninvasive way to monitor proteasome activity (4). This stably transfected cell line is based on our **Proteasome Sensor Vector** (Cat. No. 632425), which encodes a fusion of the wild-type ZsGreen fluorescent protein (5) with a proteasome targeting sequence (6). Normally, the resulting fusion protein, ZsProSensor-1, is rapidly degraded. However, when proteasome function is compromised, ZsProSensor-1 accumulates quickly, and can be easily monitored in a fluorescence plate reader or by flow cytometry (4, 7–8). The ZsGreen Proteasome Sensor has previously been used to successfully monitor proteasome activity in a quantitative fashion on the SpectraMax® M5 plate reader from Molecular Devices (8).

Secreted *Metridia* Luciferase

Clontech's **Ready-To-Glow Secreted Luciferase** is a chemiluminescent, enzyme-based reporter which utilizes our sequence- and human codon-optimized secreted *Metridia longa* luciferase (9). Secreted *Metridia* luciferase is ideal for characterizing signal transduction pathways in a homogeneous, live cell format. It exhibits high fold-induction; extended signal stability and intensity; and possesses the high sensitivity of an enzyme-based system (9–10). The assay's high sensitivity and broad dynamic range generally eliminate the need for sample dilution (11).

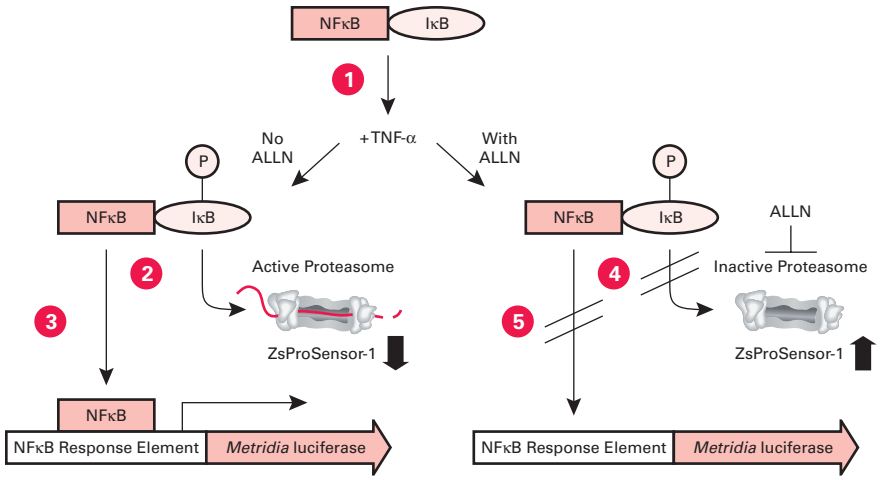

Figure 2. Requirement for active proteasomes in TNF-α-induced, NFκB-dependent signaling. Inactive NFκB is sequestered in the cytoplasm by IκB. IκB must be phosphorylated upon TNF-α induction (1) and degraded by the proteasome (2) in order for NFκB to translocate to the nucleus and initiate signaling (3). Alternatively, when the proteasome is inhibited by the peptide ALLN (4), IκB is not degraded and NFκB cannot translocate (5). The status of the proteasome (active or inactive) can be monitored based on ZsProSensor-1 levels.

The SpectraMax® M5 Multimode Microplate Reader

The SpectraMax® M5 microplate reader is a benchtop, dual-monochromator, multidetection instrument with assay performance similar to dedicated single-mode readers. It has five detection modes: UV-visible absorbance, fluorescence intensity, time-resolved fluorescence, fluorescence polarization, and luminescence (12).

Data collection, analysis, and management were performed with SoftMax® Pro software, which provides these functions for all Molecular Devices microplate readers, allowing cross-plate analysis and custom calculations (13). Protocols for use with Ready-To-Glow are available directly from the pulldown menus in the SoftMax® Pro software.

Observing Proteasome Status & NFκB Signal Transduction

In order to make the connection between proteasomal activity and NFκB-dependent signal transduction, we transiently transfected the Proteasome Sensor Cell Line with a reporter vector encoding Ready-To-Glow secreted *Metridia* luciferase driven by the NFκB response element. ZsGreen fluorescence was used to monitor the activity of the proteasomes, while *Metridia* luciferase chemiluminescence was used to measure the transcriptional activation of the NFκB response element via NFκB, upon addition of TNF-α. Since the Proteasome Sensor and Ready-To-Glow assays are both live-cell assays and the SpectraMax® M5 microplate reader functions in both fluorescence and chemiluminescence modes, the measurements were performed in a multiplex format on the same cells, in the same well.

Transfected cells were induced with TNF-α (25 ng/ml) in the presence or absence of the proteasome inhibitor ALLN (50 µM). The ZsGreen fluorescent signal was used to monitor the proteasomal activity of the cells; then the secreted *Metridia* luciferase substrate was added to the same well, and the chemiluminescent signal was measured.

In the absence of ALLN, only low levels of ZsGreen were observed, indicating that the proteasomes were active and degrading the ZsProSensor-1 protein. Upon TNF-α induction of cells that were not treated with the proteasome inhibitor ALLN, a high level of the *Metridia* luciferase reporter was detected in the media supernatant of transfected cells, indicating that the NFκB response element was active and driving the expression of secreted *Metridia* luciferase (Figure 1).

However, if cells were treated with TNF-α in the presence of ALLN, high levels of ZsGreen fluorescence were measured, indicating that the proteasomes were inactivated. Despite TNF-α induction, only low levels of the *Metridia* luciferase transcription reporter were detected, indicating that NFκB was not effectively activated (Figure 1). These combined results demonstrate the essential need for proteasomal activity in TNF-α-induced, NFκB-dependent signaling (Figure 2).

Conclusions

By combining the Proteasome Sensor with the Ready-To-Glow System, we were able to observe, in single cultures of live cells, that active proteasomes are required for NFκB to initiate transcription upon induction by TNF-α (Figure 2). Thus, the classical view that phosphorylation/dissociation of IκB is sufficient for NFκB signaling is incomplete.

Multiplexing Fluorescent and Chemiluminescent Live Cell Reporters to Dissect NFκB Signal Transduction Requirements...continued

The SpectraMax® M5 microplate reader and accompanying SoftMax® Pro software enabled us to unify the experiment as follows: immediately following the ZsGreen fluorescence measurement, we were able to add the *Metridia* luciferase substrate to the same well and measure the resulting chemiluminescence. Both readings were captured from the same cells, on the same instrument, and with the same software. Thus, proteasomal activity and NFκB dependent signaling induced by TNF-α were monitored simultaneously (Figure 1).

In the past, multiplex assay design was limited to (a) multiplexing different fluorescent proteins to create a live-cell assay, or (b) multiplexing different luciferases to create a lysis-based assay. However, since *Metridia* luciferase is a secreted reporter that does not rely on cell lysis, we were able to multiplex fluorescence- and chemiluminescence-based reporter systems for the first time, combining the advantages of both into one homogeneous, live-cell assay. Multiplexing fluorescent and chemiluminescent live-cell assays within the same well opens new avenues for intelligent assay development.

Acknowledgements

We would like to thank Cathy Olsen of Molecular Devices for her technical assistance.

References

1. Adams, J. (2002) *Trends Mol. Med.* **8**(4 Suppl):S49–S54.
2. Haas, M. *et al.* (1998) *J. Leukocyte Biol.* **63**(3):395–404.
3. Sun, L. and Carpenter, G. (1998) *Oncogene.* **16**(16):2095–2102.
4. Proteasome Sensor Vector. (April 2003) *Clontechniques* **XVII**(2):14.
5. Matz, M. V. *et al.* (1999) *Nature Biotech.* **17**(10):969–973. Erratum in *Nature Biotech.* **17**(12):1227.
6. Li, X. *et al.* (1998) *J. Biol. Chem.* **273**(52): 34970–34975.
7. Living Colors® Cell Lines. (April 2004) *Clontechniques* **XVII**(2):14.
8. Measurement of proteasome inhibition in live cells in Molecular Devices microplate fluorometers. (2006) *SpectraMax Application Note.* Molecular Devices.
9. Ready-To-Glow™ Secreted Luciferase System. (July 2006) *Clontechniques* **XXI**(2):12–13.
10. Ready-To-Glow™ Dual Secreted Reporter System. (October 2006) *Clontechniques* **XXI**(3):1.
11. Detection Limit and Linear Range of Ready-To-Glow Secreted *Metridia* Luciferase Determined on the Molecular Devices SpectraMax® L. (July 2007) *Clontechniques* **XXII**(3):20–21.
12. SpectraMax® M5/M5e multi-detection reader: A five-mode microplate reader with three-mode cuvette port. *Molecular Devices Data Sheet* (2006).
13. SoftMax® Pro software: The industry standard in microplate data analysis. *Molecular Devices Data Sheet* (2006).

Product	Size	Cat. No.
Proteasome Sensor Vector	20 µg	632425
Living Colors HEK 293 ZsGreen Proteasome Sensor Cell Line	1 vial	631535
Ready-To-Glow Secreted Luciferase pMetLuc Vector Kit	each	631729
Ready-To-Glow Secreted Luciferase Reporter Assay	100 rxns	631726
	500 rxns	631727
	1, 000 rxns	631728
Ready-To-Glow Secreted Luciferase Reporter System[1]	100 rxns	631730
	500 rxns	631731
	1,000 rxns	631732
Ready-To-Glow Dual Secreted Reporter Vector Kit	4 x 20 µg	631735
Ready-To-Glow Dual Secreted Reporter Assay	500 rxns	631734

1 The Reporter System combines the Reporter Assay and the pMetLuc Vector Kit components.