Subject: Utilization of Signal-Seeker™ kits and reagents to characterize the PTM profile of the nuclear transcription factor, cJUN

Clone:

Catalog #: BK160, BK161, BK162

Description: Tyrosine phosphorylatin, ubiquitination, acetylation, and SUMOylation 2/3 Signal-Seeker™ kits

Identification of novel post-translational modifications (PTM) for a target protein can be challenging and often requires extensive optimization of the antibody, buffer compatibility, and enrichment of the PTM of interest. Here we describe a newly developed signal-seeker PTM identification kit that rapidly identifies PTM modifications for tyrosine phosphorylation, SUMOylation 2/3, ubiquitination, and acetylation for target proteins of interest. The affinity beads for these PTMs have been optimized to detect very low abundance PTMs and have been validated against several known targets. Importantly, the kit was developed to perform the immunoprecipitation of all four PTMs in the same lysis system to identify potential PTM cross-talk while also streamlining the assay in order to enhance usability. To highlight the utility of the signal-seeker kit, the transcription factor cJUN was examined and both established and novel PTMs of this target protein was identified. cJUN ubiquitination was further defined using a simple method whereby the deubiquitinase inhibitor was removed from the lysis buffer. Endogenous changes in cJUN ubiquitination in response to the physiologic stimulus, EGF was monitored using the Signal-Seeker kit. Collectively, the signal-seeker kit provides a user-friendly tool to rapidly identify novel mechanisms of regulation for a protein of interest through identification of novel PTMs as well as providing users with the capability to study endogenous changes in their target-protein's PTM profiles.

Introduction

Post-translational modifications (PTMs) are highly regulated alterations to a protein, whereby the modification is added or removed in a specific manner as part of an overall cellular response to a stimulus. PTMs are often dynamic, transient changes that significantly alter the protein's structure, interacting partner proteins, and spatial localization; ultimately, enabling the protein to perform distinct functions. Identifying Post-translational modifications and defining their effect on a target protein is critical towards understanding the protein's physiologic function; moreover, disruption of PTM regulation is a common mechanism to disrupt proper signaling mechanisms, cellular function, and promotion of pathogenesis for an array of diseases. Because of the nature of PTMs, such as their low abundance and transient nature they can be challenging to study. PTM proteomics has significantly improved scientist's ability to rapidly identify new PTM modifications (1). Signal-Seeker kits offer the non-proteomics specialist the chance to quickly and simply examine a PTM profile for any target protein. Signal-seeker kits are comprised of affinity beads that target tyrosine phosphorylation (pY), ubiquitination (ub), SUMOylation 2/3 (SUMO 2/3), and acetylation (Ac) modifications, as well as a specialized lysis buffer system, and an optimized detection system.

cJUN is a well-characterized transcription factor that binds to AP-1 DNA recognition elements as a homodimer or heterodimer with c-Fos family members. It was one of the first transcription factors identified as an oncoprotein in the mid-1980s; thus, significant work was performed to better understand how cJUN was regulated; such as, defining its binding partners, its leucine zipper binding domain, and its PTM regulation (2, 3). cJUN serine/threonine phosphorylation by JNK kinases regulate its transcriptional activity and is the best characterized modification of cJUN. Recent studies showed that cJUN can also be tyrosine phosphorylated (4), ubiquitinated (5-7), and acetylated (8). While there is compelling evidence that cJUN is SUMO-1 (9, 10) modified, whether or not it is SUMO-2/3 modified is unknown. In this study the signal-seeker kits were utilized to study the PTM profile of the essential transcription factor cJUN.

Results & Discussion

PTM modification of cJUN

To examine cJUN's PTM profile untreated and EGF-treated A431 cells were lysed using BlastR™ lysis buffer to isolate total protein from all cellular compartments (Figure 1). Complete cellular lysis is essential as cJUN is a nuclear localized transcription factor. Importantly, standard buffers such as RIPA, or other commercial buffers were much less effective at isolating proteins from both the membrane and nuclear fractions (Figure 1). pY, ub, Ac, and SUMO 2/3 modified proteins were isolated from the untreated and EGF-treated cell lysates using signal-seeker affinity beads, and western blots of cJUN was performed. Figure 2 shows that the signal-seeker kit effectively identified cJUN pY, ub, and Ac; furthermore, these three PTMs of cJUN were induced in response to EGF treatment. These data validated previously published findings that cJUN can be modified by pY, ub, and Ac and also suggest that these

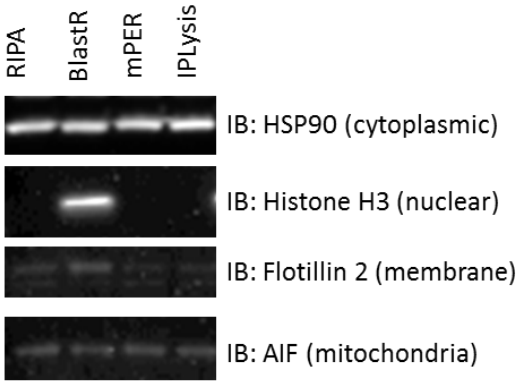

Figure 1 Legend: A431 cells were lysed with RIPA, BlastR (Cytoskeleton Inc.), mPER (Thermofisher), or IP Lysis (Pierce). 10ug of lysate was separated by SDS-PAGE and cellular organelle markers for the cytoplasm (HSP90), nucleus (Histone H3), membrane (Flotillin 2), and mitochondria (AIF) were utilized to determine efficacy of protein isolation.

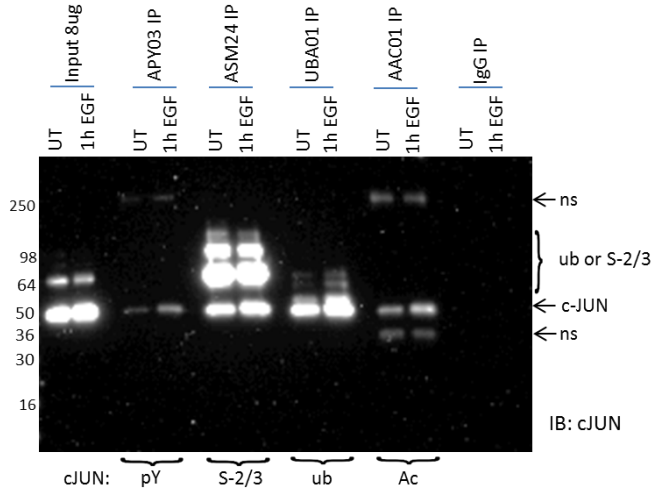

Figure 2 Legend: A431 cells, untreated or treated with EGF (33ng/mL for 60 minutes), were isolated using BlastR buffer. IP was performed for tyrosine phosphorylation (APY03), SUMOylation 2/3 (ASM24), ubiquitination (UBA01), and acetylation (AAC01). Total cell lysate (Input) and immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blot with a cJUN antibody (Thermofisher).

modifications may play a role in EGF induced cJUN regulation; however, further studies to define a causal relationship as well as the potential crosstalk between these PTMs are warranted. Interestingly, cJUN also appeared to be SUMO 2/3 modified which has not been reported previously; but, the SUMOylated 2/3 form of cJUN was not regulated by EGF stimulation at 1 hour of treatment. The data shows that the signal-seeker kit can effectively identify both established and novel PTMs of a nuclear protein like cJUN. Ultimately, this study produced three novel research avenues to pursue such as further characterizing cJUN SUMO 2/3 modification, EGF regulation of cJUN Ac and pY, and the potential crosstalk between these PTMs and how they regulate cJUN activity.

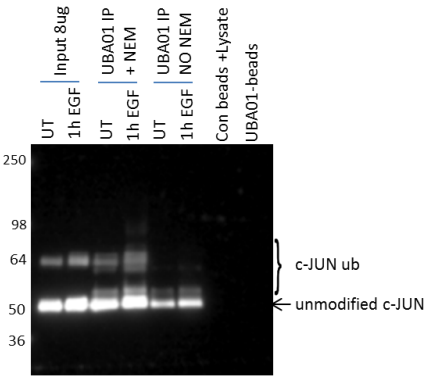

Figure 3 Legend: A431 cells were harvested with BlastR lysis buffer with or without NEM. Lysates were incubated with ubiquitin binding beads (UBA01) or control beads (CUB01). Total cell lysate (Input) and immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blot with a cJUN antibody (Thermofisher).

Validating cJUN ubiquitination

A simple confirmatory experiment to determine if a target protein of interest is modified by a specific PTM can be performed by removing the specific de-PTM inhibitor from the BlastR™ lysis buffer prior to lysing the cells. For example, to validate that cJUN was ubiquitinated as shown in figure 2, untreated and EGF treated A431 cells were lysed with BlastR lysis buffer supplemented with or without NEM. NEM is a pan deubiquitinase inhibitor; thus, lysing cells without NEM will significantly decrease the ubiquitinated protein profile (data not shown). The ubiquitinated proteins from each sample were captured with UBA01 signal-seeker ubiquitin affinity beads and ubiquitinated cJUN species were identified by performing western blots with cJUN antibody. The data confirms that the profile that was observed in figure 2 was ubiquitinated cJUN as the ubiquitinated cJUN bands in either the untreated or EGF-treated samples disappeared without NEM present (Figure 3); presumably due to the loss of ubiquitinated cJUN in response to active de-ubiquitinases in the lysate. This approach was utilized for several other target proteins for all four PTMs (data not shown) and specific depletion of the PTM modified bands was observed. While other methods should be utilized to further validate a novel modification for a protein of interest this method provides an additional level of confidence that the novel PTM identified is valid and worthy of further investigation.

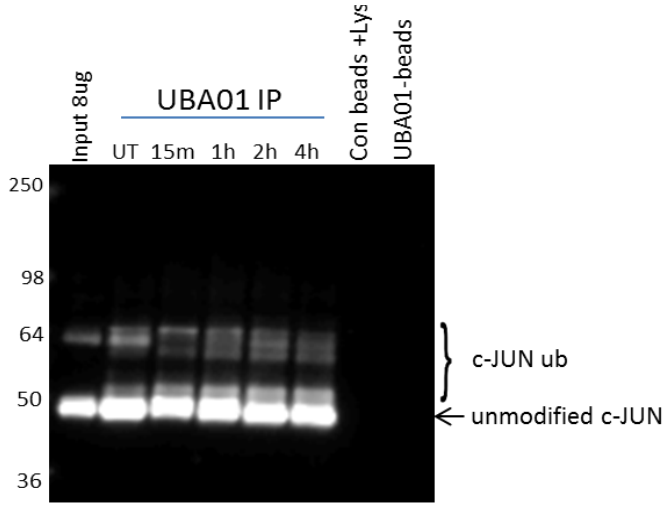

Figure 4 Legend: Serum-restricted A431 cells were stimulated with EGF for the given time period. Lysates were incubated with ubiquitin binding beads (UBA01) or control breads (CUB01). Total cell lysate (Input) and immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blot with a cJUN antibody (Thermofisher).

Endogenous cJUN ubiquitination is dynamically regulated by EGF

PTMs are relatively low in abundance and often transient by nature; thus, it can be difficult albeit necessary to study their dynamic and endogenous changes. A benefit of the signal-seeker kit is its ability to identify dynamic and endogenous changes of a PTM for any protein of interest. Evidence in the literature suggests that cJUN is ubiquitinated in response to EGF stimulation, and data from figure 2 support these findings. However, looking at two data points can often miss critical changes; therefore, the signal-seeker kit was utilized to monitor cJUN ubiquitination in response to EGF over a given time course. Figure 4 provides evidence that cJUN ubiquitination occurs rapidly in response to EGF and is maintained for several hours before declining around 4 hours. Tools utilized to enhance a PTM signal for a target protein, such as plasmid overexpression can often mask important endogenous physiologic changes. Other tools like mass spectrometry may be too cumbersome to study PTM changes over multiple time points, and antibodies that target a specific PTM for a protein of interest is very limited to that single target. The signal-seeker kit circumvents these aforementioned limitations; as a result, it is an extremely useful tool to investigate dynamic, endogenous changes of these four PTMs for any protein of interest over a given time course or in response to physiologic stimulants and inhibitors.

Identification of novel PTMs for a protein of interest from a complex lysate usually has several optimizations steps such as: 1) identifying and optimizing a protein-specific, IP-compatible antibody, 2) performing the IP while learning and optimizing buffer conditions for a specific PTM of interest, and 3) further validation with overexpression and proteomic studies. This is a huge undertaking with significant risk and a high failure rate, which is not surprising considering the transient nature of PTMs. Signal-seeker kits aims to revolutionize this process by providing users with a highly optimized system to quickly and easily determine if their protein is modified by pY, ub, Ac, or SUMO 2/3 modification with minimal optimization or investment in reagent development. This approach will save valuable time and resources; ultimately, allowing scientist to focus their efforts on promising PTM targets that lead to novel findings , which may have otherwise been too "risky" to perform. Importantly, the signal-seeker kit is very complementary to the current available PTM identification tools; for example, it could be used to validate proteomic studies.

These studies provided clear evidence of the utility of the signal-seeker kit using cJUN as a model protein and have yielded several novel avenues for investigating cJUN regulation.

Materials and Methods

Cell Culture and Reagents
A431 cells were grown in DMEM media (ATCC, VA) supplemented with 10% FBS (Atlas Biologicals, CO) and penicillin/streptomycin (ThermoFisher, MA). Trypsin/EDTA was obtained from Gibco (ThermoFisher, MA). Unless otherwise noted, chemicals were obtained from Sigma Chemical Co. (Sigma, MO). Human EGF was obtained from Cytoskeleton, Inc. (Cytoskeleton, CO). For EGF stimulation experiments A431 cells were serum restricted for 24 hours with serum free DMEM in order to synchronize the cells. The cells were then treated with 33ng/ml EGF for 15 minutes, or 1,2, and 4 hours in individual 15cm dishes (Corning, NY) followed by subsequent lysis with BlastR lysis buffer (Cytoskeleton, CO).

Western blotting
A431 cells were lysed with ice-cold BlastR lysis (Cytoskeleton, CO), RIPA, mPER (Thermofisher, MA), or IP Lysis (Thermofisher, MA) buffer containing a cocktail of NEM, TSA, Na_3VO_4, and protease inhibitors (PIC02) (Cytoskeleton, CO). DNA was removed by passing the lysate through the BlastR filter system (Cytoskeleton, CO). After dilution with BlastR dilution buffer, protein concentrations were determined with protein reagent, ADV02 (Cytoskeleton, CO), and measured at 600nm OD. Protein lysate samples were separated using Tris-glycine SDS-polyacrylamide gel electrophoresis (ThermoFisher, MA) and transferred to Immobilon- P membranes (Millipore, MA). Membranes were blocked for 1 hr at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween-20 (TTBS) and 5% milk (Thrive Life, UT), and then incubated with 2.5% milk in TTBS solution containing primary antibodies for 2 hrs at room temperature (RT). Membranes were washed in TTBS 3x10minutes, prior to secondary antibody for 1hr at RT. Bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies and chemiluminescent reagent (Cytoskeleton, CO) according to the manufacturer's directions. Antibodies used: cJUN (Thermofisher, MA), tubulin (Cytoskeleton, CO), HRP-anti- sheep secondary (Cytoskeleton, CO), and HRP-anti - Rabbit secondary (Jackson ImmunoResearch, PA).

Co-immunoprecipitation assay
A431 cells were lysed with ice-cold BlastR lysis buffer containing a cocktail of NEM, TSA, Na_3VO_4, and protease inhibitors (PIC02). DNA was removed by passing the lysate through the BlastR™ filter system (Cytoskeleton, CO). After dilution with BlastR dilution buffer, protein concentrations were determined with ADV02 and measured at 600nm OD. Samples were immunoprecipitated, using signal-seeker-kits, with equal protein concentration and IP volumes according to the manufacturer's protocol (Cytoskeleton, CO). The appropriate amount of pY (APY03), ub (UBA01), SUMO 2/3 (ASM24), Ac (AAC01), IgG beads (CIG01), or con beads (CUB01) were added to the respective samples for 1-2 hr at 4°C on an end-over- end tumbler. After incubation, the affinity beads from each sample were pelleted, and washed 3X with BlastR wash buffer. Bound proteins were eluted using bead elution buffer (Cytoskeleton, CO) and detected by western immunoblotting. For reciprocal EGFR IP experiment samples were incubated with 8mg of EGFR antibody (Millipore, MA) for 1-2 hr at 4°C on an end-over- end tumbler. 50 µl of a 50% slurry of Protein G beads (Biovision,) was added to each sample and incubated for 2 hours at 4 °C on an end-over- end tumbler. After incubation the resin from each sample was pelleted, and washed 3X with BlastR wash buffer. Bound proteins were eluted using bead elution buffer and detected by western immunoblotting.

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