Benchmarks

Using dot blot with immunochemical detection to evaluate global changes in SUMO-2/3 conjugation

Markéta Častorálová, Tomáš Ruml, Zdeněk Knejzlík
Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, Czech Republic

Small ubiquitin-related modifier-2/3 (SUMO-2/3) is a member of the ubiquitin-like (Ubl) protein family. Conjugation of SUMO-2/3 to target proteins is influenced by various stress conditions and chemical inhibitors. SUMO-2/3 conjugation may serve as a neuroprotective mechanism and may play a role in protein quality control. A method for screening global changes in SUMO-2/3 conjugation would facilitate further research of SUMO-2/3 cellular function. Here we show that dot blot with immunochemical detection allows evaluation of changes in global cellular SUMO-2/3 conjugation and offers an alternative to more laborious Western blot analysis. The method is based on a change of SUMO-2/3 signal intensity upon its conjugation. The dot blot analysis presented here is a time-saving method that enables screening of large numbers of samples and easy statistical evaluation of the results.

Keywords: SUMO-2/3; SUMO-2/3 conjugation; dot blot

Method summary:
We show that dot blot analysis is an efficient and valuable method for quantifying changes in SUMO-2/3 conjugation in cell cultures and animal tissues. The method readily distinguishes differences in total protein concentration between individual samples, requires less time than Western blot analysis, enables researchers to screen large numbers of samples, and allows for easy evaluation of the results.
since large sets of proteins may be modified by SUMO-2/3 (18,19), a smear is often observed in Western blot analysis (5,10, Figure 1). The signal intensity of the smear corresponding to conjugated SUMO-2/3 is dramatically increased compared with the original signal from unconjugated SUMO-2/3. Although the higher signal intensity of conjugated SUMO-2/3 has been observed many times (5,10), the nature of this phenomenon has not been discussed. One possible explanation is that immunoreactive epitopes in SUMO-2/3 become easier for antibodies to recognize following conjugation because of immobilization of SUMO-2/3 with its protein targets on the membrane. Other possibilities are that SUMO-2/3 conjugates move transfer more readily from the polyacrylamide gel than free SUMO-2/3 or that free SUMO-2/3 has extremely low-binding affinity for the membrane. Here we used heat shock and treatment with proteasomal inhibitor MG132 as model conditions to induce accumulation of SUMO-2/3 conjugates in HEK 293T cells and analyzed SUMOylation by Western blot analysis on nitrocellulose (0.45 µm) or PVDF (0.2 µm) membranes (Figure 1, lanes 2 and 3); PVDF membranes proved to be more suitable for simultaneous detection of free SUMO-2/3 and its conjugates under the same exposure time. Parallel analysis of lysates where SUMO-2/3 was deconjugated by the SUMO-specific isopeptidase Ulp1 showed that the increased signal intensity of SUMO-2/3 is caused exclusively by SUMO-2/3 conjugation and not by increased expression because similar signal levels from deconjugated SUMO-2/3 were detected under all tested conditions (Figure 1, compare lanes 4–6). Surprisingly, similar qualitative findings for SUMO-2/3 conjugate intensities were observed when lysates were directly dot-blotted onto nitrocellulose or PVDF membranes and probed with antibodies against SUMO-2/3 (Figure 1). The signal change caused by conjugation did not depend on membrane or antibody type because N-terminally HA fused SUMO-3 protein expressed in HEK 293T cells showed identical behavior using mouse monoclonal anti-HA antibody (data not shown). The samples used for Western blot analysis contained intentionally high total protein concentrations to obtain a visible increase of free SUMO-2/3 bands as an indicator of increased conjugation. Although unconjugated SUMO-2/3 was detectable by Western blot, almost no signal was observed by dot-blot analysis on either nitrocellulose or PVDF membranes (Figure 1, lanes 4–6). This observation was surprising considering that in addition to free SUMO-2/3, there were three additional cross reactive bands detected with Western blot (Figure 1, lanes 4–6; 20). Independent of the type of membrane, these results indicate that the SUMO-2/3 signal on the dot blot is a function of the level of SUMO-2/3 conjugates. Free SUMO-2/3 is far less detectable by dot blot than by Western blot, possibly because of lower affinity to the membrane, competition with other cellular proteins for membrane binding sites, or shielding of epitopes by other cellular proteins or the membrane. Dot blot previously has been used to detect global cellular levels of SUMO-1 (21). However, because of the increase of SUMO-2/3 signal intensity upon its conjugation, it is not possible to use this method to assess global cellular levels of SUMO-2/3 as it was used for SUMO-1 (21).

Next, we examined the dependence of the Western blot and dot blot signal intensity on the percentage of conjugated SUMO-2/3 (Figure 2). To obtain defined levels of conjugated SUMO-2/3 in total cell lysates, the lysate from heat-shocked cells was mixed at various ratios with cell lysate of the same origin but treated with Ulp1 for global SUMO-2/3 deconjugation. The signal intensity of free SUMO-2/3 and SUMO-2/3 conjugates in the individual samples with comparable total protein content was verified by Western blot analysis (Figure 2A). The decrease of free SUMO-2/3 signal correlated with the theoretical increase of the level of SUMO-2/3 conjugates. However,
for the increase of SUMO-2/3 conjugate, the signal was visible in the interval of 0% (only unconjugated SUMO-2/3) to approximately 60% (SUMO-2/3 conjugated on nitrocellulose), and up to 90% on PVDF (Figure 2). Dot blot analysis showed an approximately linear correlation between the signal and the level of conjugated SUMO-2/3 in the interval from 0% to 60% of SUMO-2/3 conjugated on nitrocellulose and 0 to 90% conjugated on PVDF, which was in agreement with results obtained by Western blot (Figure 2). Even with reduced protein quantities near the detection limit of dot blot, we observed a constant signal above 60%–70% of SUMO-2/3 conjugated on nitrocellulose membrane. Based on these results, we recommend using PVDF membranes for evaluation of global changes in SUMO-2/3 conjugation by dot blot.

Finally, we used dot blot to screen for changes in global SUMO-2/3 conjugation in HEK 293T cells grown under different conditions (Figure 3). Each sample was tested in four replicates using dot blot and the signal intensities were evaluated using Total Lab TL100 software and statistically processed (Figure 3). The results obtained from these dot blot analyses on PVDF membranes reflect the fact that treatment by MG132-induced SUMO-2/3 conjugate accumulation in a time dependent manner (Figure 3, MG132–1h and 2h), while treatment with the translational inhibitor cycloheximide strongly decreased the MG132-induced SUMO-2/3 conjugation. This negative effect of cycloheximide on MG132-induced conjugation of SUMO-2/3 in our assay agrees with previous observations (10, 17), where SUMO-2/3 deconjugation assays showed that cycloheximide treatment did not influence global cellular SUMO-2/3 levels, but instead affected SUMO-2/3 conjugation by blocking production of novel SUMOylation targets (17).

As shown above, our approach to evaluate global changes in cellular SUMO-2/3 conjugation is based on increased signal intensity of SUMO-2/3 upon its conjugation. We used this phenomenon to screen for changes in cellular SUMO-2/3 conjugation under different conditions by dot blot analysis on nitrocellulose and PVDF membrane, and showed that the PVDF membrane was more suitable for this appli-
cation. When compared with Western blot, this technique offers the advantages of shorter time requirements, potential to screen large numbers of samples, and ease in evaluating the results using quantification software and statistical analysis. It should be noted that dot blot analysis can be used only to screen for changes in global cellular SUMO-2/3 conjugation; this method is not intended for evaluation of changes in SUMOylation of individual proteins. In those cases, Western blot analysis is still required. Dot blot is particularly useful for comparing SUMO conjugates contained in tissues (in individuals or under different pathological conditions), and screening for activators or inhibitors of SUMO-2/3 conjugation in cell cultures for potential development of drugs modulating SUMO-2/3 conjugation.

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Competing interests
The authors declare no competing interests.

References
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2000. Functional