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Mini review

Optimising methods for the preservation, capture and identification of ubiquitin chains and ubiquitylated proteins by immunoblotting

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ABSTRACT

Immunoblotting is a powerful technique for the semi-quantitative analysis of ubiquitylation events, and remains the most commonly used method to study this process due to its high specificity, speed, sensitivity and relatively low cost. However, the ubiquitylation of proteins is complex and, when the analysis is performed in an inappropriate manner, it can lead to the misinterpretation of results and to erroneous conclusions being reached. Here we discuss the advantages and disadvantages of the methods currently in use to analyse ubiquitin chains and protein ubiquitylation, and describe the procedures that we have found to be most useful for optimising the quality and reliability of the data that we have generated. We also highlight commonly encountered problems and the pitfalls inherent in some of these methods. Finally, we introduce a set of recommendations to help researchers obtain high quality data, especially those new to the field of ubiquitin signalling. The specific topics addressed in this article include sample preparation, the separation, detection and identification of particular ubiquitin chains by immunoblotting, and the analysis of ubiquitin chain topology through the combined use of ubiquitin-binding proteins and ubiquitin linkage-specific deubiquitylases.

1. Introduction

The discovery of ubiquitin-mediated proteolysis [1] was one of the most seminal papers published in *Biochemical and Biophysical Research Communications* (BBRC), which won Aaron Ciechanover and Avram Herschko the Nobel Prize for Chemistry 26 years later. Subsequently, ubiquitylation was found to control many other cellular processes and, to date, eight different types of ubiquitin chain linkage have been identified in cells [2,3]. These linkages are formed by the covalent attachment of the C-terminus of ubiquitin to the ε-amino groups of any of the seven lysine (K) residues in ubiquitin (K6, K11, K27, K29, K33, K48 and K63) or the α-amino group of its N-terminal methionine (M1) residue. In addition, some proteins become mono-ubiquitylated or multi-monoubiquitylated, in which the first ubiquitin attached to a protein does not undergo polyubiquitylation. Finally, hybrid (also called branched or mixed) ubiquitin chains containing more than one type of ubiquitin linkage have also been identified in cells [4–6], introducing further complexity into the system (Fig. 1). Protein ubiquitylation is a versatile and reversible protein modification with regulatory roles that extend far beyond the proteasome-dependent degradation of substrate proteins, and include cellular signalling and trafficking, as well as the control of the cell division cycle and DNA repair.

In recent years there has been an explosion of interest in ubiquitylation and the number of publications in this area is increasing exponentially (Fig. S1). It is self evident that the experiments aimed at enhancing our understanding of this process are conducted to the highest standards of quality control. However, to our knowledge, no simple, clear guidelines or standardised

Abbreviations: DMP, dimethyl pimelidinate; DUB, deubiquitylase; GST, glutathione S-transferase; HEK, human embryonic kidney; IAA, iodoacetamide; IL-1R, IL-1 receptor; IMAC, immobilised metal ion affinity chromatography; IP, immunoprecipitation; IRAK, interleukin receptor associated kinase; LUBAC, Linear UBiquitin Assembly Complex; MES, 2-(N-morpholino) ethane sulfonic acid; MOPS, 3-(N-morpholino) propane sulfonic acid; NC, nitrocellulose; NEM, N-ethylmaleimide; NEMO, NF-κB essential modulator; NZF, Npl4 zinc finger; POI, protein of interest; PPase, phage λ-phosphatase; pUb, polyubiquitin; PVDF, polyvinyl difluoride; RIP, receptor interacting protein; SDS, sodium dodecyl sulphate; TA, tris-acetate; TG, tris-glycine; TNF, tumour necrosis factor; TNF-R, TNF receptor; TUBEs, tandem-repeated ubiquitin-binding entities; UBA, ubiquitin-associated; UBAN, ubiquitin binding in ABIN and NEMO domain; UBD, ubiquitin-binding domain; UIM, ubiquitin-interacting motif.

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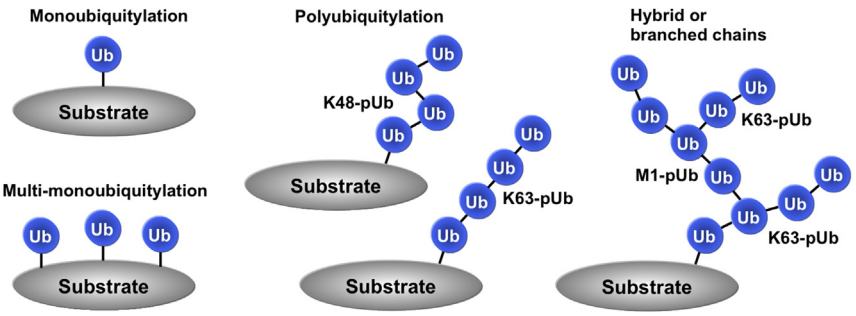

Fig. 1. Different types of ubiquitylation. Ubiquitin modifications can be classified into three general types, termed monoubiquitylation, multi-monoubiquitylation and polyubiquitylation. Polyubiquitylation can be further subdivided into homotypic ubiquitylation (each ubiquitin chain comprising just one type of ubiquitin linkage) or heterotypic ubiquitylation (containing more than one type of ubiquitin chain). The latter are usually termed hybrid, branched or mixed ubiquitin chains.

methodologies for the preservation, detection and analysis of ubiquitylation events by immunoblotting are available. In this article, we therefore introduce a number of recommendations about how to optimise the quality of the information that can be obtained from such experiments, based on our own experiences and other published papers in the literature.

2. Preserving the ubiquitylation state of proteins

2.1. Inhibition of deubiquitylases

Protein ubiquitylation is reversible and this modification can therefore easily be lost through the hydrolysis of ubiquitin chain linkages, which is catalysed by protein ubiquitin hydrolases, termed deubiquitylases (DUBs). For this reason it is essential to include DUB inhibitors in the buffers used for cell lysis, to preserve proteins in the state of ubiquitylation at which they were present in the intact cell. The inclusion of DUB inhibitors is particularly critical during immunoprecipitation (IP) or other "pull-down" experiments, where cell extracts may be incubated for several hours in non-denaturing conditions. There are five different families of DUBs, one of which encodes metallo-proteinases, the other four being cysteine proteinases. Therefore, to block DUB activity, EDTA or EGTA must be included in the lysis buffer to remove traces of heavy metal ions, and Iodoacetamide (IAA) or N-ethylmaleimide (NEM) must also be added to alkylate the active site cysteine residues of DUBs. Although IAA or NEM have typically been included at concentrations of 5–10 mM in many publications, we find that up to 10-fold higher concentrations are needed to preserve the ubiquitylation status of some proteins (e.g. Interleukin receptor associated kinase-1 (IRAK1) (Fig. 2A) and ubiquitin chains (Fig. 2B). High concentrations of NEM are better at preserving K63-Ub chains and M1-Ub chains than high concentrations of IAA, probably due to the instability of the latter compound.

An advantage of IAA over NEM is that it is destroyed by light within minutes, preventing the continued alkylation of cysteine residues on many proteins. However, the covalent 2-acetamidoacetamide adduct ($C_4H_6N_2O_2$) formed by the reaction of IAA with cysteine residues has a molecular mass of 114 Da [7], which is identical to that of the Gly–Gly dipeptide that remains attached to the ε-amino group of lysine residues of proteins after ubiquitylated proteins have been digested with trypsin. This modification may therefore interfere with the identification of ubiquitylation sites by mass spectrometry. It is therefore recommended to use NEM instead of IAA when such mass spectrometry experiments are to be performed. NEM and IAA are equally compatible for experiments where immunoblotting is the final readout.

If the ubiquitylation of proteins is to be studied in cell extracts only, then DUBs can be inactivated by extracting the cells directly into boiling lysis buffer containing 1% sodium dodecyl sulphate (SDS). Ubiquitin with a C-terminal cysteine-reactive probe is reported to inactivate some DUBs [8] and, provided that this compound inhibits every DUB, it may also be useful for preventing deubiquitylation after cell lysis. Other broad-spectrum chemical inhibitors of DUBs may be identified in the future.

2.2. Proteasome inhibition

Proteins modified by all types of ubiquitin linkage, except for K63-linked and M1-linked chains, can be targeted to the 26S Proteasome for rapid degradation. For example, in yeast, proteins modified by K6-, K11-, K27-, K29- and K33-linked polyubiquitin (pUb) chains, as well as by K48-linked ubiquitin chains, accumulated in cells when the proteasome was inhibited [9]. Many inhibitors of the chymotryptic like protease of the proteasome have been described [10], of which the most widely used is Z-leucyl-leucyl-leucyl-CHO, termed MG132. Treatment with MG132 blocks protein degradation and preserves the ubiquitylated form of the protein of interest (POI), thereby facilitating its detection. As an example, pUb-IκBα was only detectable by immunoblotting if the cells were incubated with MG132 prior to cell lysis and enriched from the cell extracts using immobilised Halo-TUBEs (Tandem-repeated Ubiquitin-Binding Entities) (Fig. 2B, left panel), which capture every type of ubiquitin chain (Section 5). However, prolonged (12–24 h) treatment with MG132 can have cytotoxic effects [11] and ubiquitylation observed after these long incubations might be a consequence of one or more stress responses.

3. Resolution and identification of ubiquitin chains and ubiquitylated proteins by SDS-PAGE

3.1. Choice of gel and running buffer

Different gels and running buffers for resolving ubiquitylated proteins by SDS-PAGE are available. Proteins can be modified by 20 or more ubiquitin molecules that can add >200 kDa to their molecular mass, resulting in a smear of pUb chains that typically stretch upwards towards the top of the gel, so that selection of the most appropriate separation system is important (see Section 10). The action of E1, E2 and E3 ligases can generate pUb chains *in vitro* that differ greatly in length (Fig. 3A). When using pre-poured gradient gels, a MES (2-(N-morpholino) ethane sulfonic acid) buffer gives improved resolution of relatively small ubiquitin