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Research paper

A native antibody-based mobility-shift technique (NAMOS-assay) to determine the stoichiometry of multiprotein complexes

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Abstract

Characterization of multiprotein complexes (MPCs) is an important step toward an integrative view of protein interaction networks and prerequisite for a molecular understanding of how a certain MPC functions. Here, we present a technique utilizing monoclonal subunit-specific antibodies for an electrophoretic immunoshift assay in Blue Native-gels (NAMOS-assay), which allows the determination of the stoichiometry of MPCs. First, we use the B cell antigen receptor as a model MPC whose stoichiometry is known, confirming the $HC_2LC_2Ig\alpha/\beta_1$ stoichiometry. Second, we demonstrate that the digitonin-extracted T cell antigen receptor (TCR) extracted from T cells has a stoichiometry of $\alpha\beta\varepsilon_2\gamma\delta\zeta_2$. We then show that the NAMOS-assay does not require purified MPCs, since it can determine the stoichiometry of an MPC in cell lysates. The NAMOS-assay is also compatible with use of epitope tags appended to the protein of interest, as e.g. the widely used HA-tag, and anti-epitope antibodies for the assay. Given its general applicability, this method has a wide potential for MPC research.

Keywords: Blue Native; BN-PAGE; Proteomics; Stoichiometry; Composition; TCR

1. Introduction

Most proteins fulfill their functions in multi-protein complexes (MPCs). In fact, the human proteome is estimated to contain around 10^4 different MPCs (Sali et al., 2003). In the post-genomic era there is an increasing need to analyze MPCs in order to obtain an integrative view of the protein–protein interaction networks that define protein function and cell behavior. Furthermore, at the level of individual MPCs, it is necessary to study the complex in terms of its composition and stoichiometry, in order to comprehend its function in molecular terms.

MPC stoichiometry can be determined if the atomic structure of an MPC has been solved by X-ray crystallography or by NMR spectroscopy (Sali et al., 2003). Obviously, these approaches require large amounts of highly purified MPC, and very often require the use of

Abbreviations: BN; Blue Native; BCR; B cell antigen receptor; Fab; antigen-binding fragment; HA-tag; hemagglutinin epitope; HC; heavy chain; LC; light chain; MPC; multiprotein complex; NAMOS; native antibody-based mobility shift; sc; single chain; TCR; T cell antigen receptor.

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expression systems that do not fully incorporate the post-translational modifications existing *in vivo*. Metabolic labeling of the MPC and estimation of the ratio between subunits according to the radioactivity that each subunit incorporates, has been described (Schamel and Reth, 2000; Call et al., 2004). If the copy number of one subunit is known, then the complete stoichiometry of the complex can be calculated. But this method poses difficulties due to the fact that the MPC has to be present in pure form and the possibility of different subunits having distinct half-lives. Quantitative flow cytometry (Portoles et al., 1989; Thibault and Bardos, 1995) and also immunoblotting (Li and Hazelbauer, 2004) have been used to determine stoichiometries. These methods might not always provide reproducible results (Portoles et al., 1989; Thibault and Bardos, 1995) and measurements of the individual subunits are difficult to normalize. Furthermore, co-purification of two distinct versions of the same subunit (Schamel and Reth, 2000; Siegel et al., 2000; Alarcon et al., 2003) or fluorescence resonance energy transfer (FRET) between two subunits (Fernandez-Miguel et al., 1999; Siegel et al., 2000) has been applied to determine whether a subunit is present more than once in an MPC. Thus, no rapid and reliable methods to determine stoichiometries exist.

We reasoned that Blue Native (BN)-PAGE (Schägger and von Jagow, 1991; Schamel and Reth, 2000; Swamy et al., 2006a) would be an appropriate method to develop a technique to determine exact stoichiometries. BN-PAGE is a charge shift method for the native separation of proteins in a polyacrylamide gradient gel. After solubilization of the proteins, the anionic dye Coomassie blue G-250 is added to the sample. The dye binds unspecifically to all proteins and imparts a net negative charge to the proteins, allowing them to migrate to the anode during electrophoresis. Thus, the electrophoretic mobility of an MPC is determined by the charge of the bound Coomassie dye and the size and shape of the complex (Schägger and von Jagow, 1991; Schägger et al., 1994). Coomassie does not act as a detergent and preserves the MPC structure. The resolution of BN-PAGE is higher than that of gel filtration or sucrose gradient ultracentrifugation (Schägger and von Jagow, 1991; Schägger et al., 1994) and therefore of potential use in our assay. To develop and validate our new technique in Blue Native gels, we used the B cell antigen receptor (BCR) and the T cell antigen receptor (TCR) as model systems.

The BCR consists of two heavy chains (HCs) covalently bound to two light chains (LCs) and one Ig-α/β heterodimer with a stoichiometry of HC_2LC_2-Igα/β_1 (Fig. 1A). This stoichiometry was determined by co-purification of two distinct versions of the same subunit (Schamel and Reth, 2000), by calculations based on the amount of radioactivity incorporated into each subunit upon metabolic labelling (Schamel and Reth, 2000) and by comparison of the sizes of the BCR and a variant thereof in which Ig-α/β had been removed (Schamel, 2001). Recently, these biochemical data were confirmed by FRET studies in living B cells (Tolar et al., 2005).

The TCR, which represents one of the most complex transmembrane receptors, is composed of the ligand-binding TCRα/β heterodimer and the signal-transducing CD3ε/γ, CD3ε/δ, and ζζ dimers (Alarcon et al., 2003). To date it remains an enigma as to how the TCR transmits the signal from ligand binding across the membrane, thus initiating the activation of the T cell. Key to answering this question is an understanding of the stoichiometry of the TCR. The latter is still a major debate among immunologists, since different studies led to TCR stoichiometries with disparate results. Suggested stoichiometries include $\alpha\beta\varepsilon_2\gamma\delta\zeta_2$ (Blumberg et al., 1990; Punt et al., 1994; Call et al., 2004), $(\alpha\beta)_2\varepsilon_2\gamma\delta\zeta_2$ (Exley et al., 1995; San Jose et al., 1998; Fernandez-Miguel et al., 1999) and $\alpha\beta(\varepsilon\gamma)_2\varepsilon\delta\zeta_2$ (Rubin et al., 2002). We have recently shown that the TCR is not one defined complex, but rather exists in various forms on the cell surface (Schamel et al., 2005; Alarcon et al., 2006). This was shown on intact fixed T cells by immuno-gold labeling of the TCR followed by electron microscopy. In addition, we could show that the basic TCR unit can be extracted by the detergent digitonin and contains one TCRβ and two CD3ε subunits. Thus, to date the full stoichiometry of this complex receptor has still not been demonstrated.

We present here a general method to determine the stoichiometries of MPCs based on Blue Native gel electrophoresis (BN-PAGE) (Schägger and von Jagow, 1991; Schamel and Reth, 2000). The MPC of interest is separated by BN-PAGE and then detected by Western Blotting. Antibodies against the different subunits are added separately to the complex before electrophoresis, so that antibody-MPC conjugates can form. These conjugates migrate slower in the native gel, so that the size shift directly reflects the numbers of antibodies that are bound to the complex. Thus, the number of copies of each subunit can directly be determined using this method.

2. Materials and methods

2.1. Cell lines and antibodies

The murine T cell line 2B4, the transfectant 31-13. scTCRβ and the J558 Lμm/Ig-αflN B cell line were grown as described (Siegers et al., 2006; Minguet et al.,