Subunit stoichiometry determination by chemical cross-linking

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Method Article

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Abstract

Introduction

This procedure adapted from Kedei, N. _et al._ \(2001) allows the oligomerization status of a protein to be determined using a chemical cross-linking approach. Chemical cross-linkering reagents create covalent links between adjacent subunits of a multiprotein complex that will therefore migrate as one single complex during denaturing polyacrylamide gel electrophoresis \(PAGE). The number of subunits is deduced from the molecular mass of each monomer compared to the molecular mass of the cross-linked homo-multimeric complex. In the present study, we applied this approach to determine the subunit stoichiometry of the Orai proteins that constitute the CRAC channel, expressed in _Drosophila_ S2 cells. Two alternative protocols are described: one on total cell lysates; the other on intact cells. We used the following homobifunctional reagents \(Pierce): the lysine-reactive _N_-hydroxysuccinimide esters bis\[sulfosuccinimidyl]suberate \(BS3, water-soluble, membrane-impermeant, spacer arm length 11.4 Å) and dithiobis\[succinimidylpropionate] \(DSP, water-insoluble, membrane-permeant, spacer arm length 12 Å), the lysine-reactive aryl halide 1,5-difluoro-2,4-dinitrobenzene \(DFDNB, water-insoluble, membrane-permeant, spacer arm length 3 Å) and the cysteine-reactive maleimide 1,6-bismaleimidohexane \(BMH, water-insoluble, membrane-permeant, spacer arm length 11.4 Å). Useful additional information can be found on the "pierce website":http://www.piercenet.com/products/browse.cfm?fldID=0203&WT.mc_id=go_Crosslinkers_CL_brj&gclid=CJb6n8OeopUCFQv7agodpQVEkQ

Procedure

Chemical cross-linking of total cell lysates 1- Rinse the cells twice with ice-cold phosphate-buffered saline \(PBS). 2- Harvest the cell pellet in ice-cold cross-linking buffer \(PBS pH 8 for lysine-reactive cross-linkers or PBS pH 7.4, 2.5 mM EDTA for cysteine-reactive reagents) containing protease inhibitors \(complete-mini EDTA-free, Roche Molecular Biochemicals) and sonicate at 4°C. 3- Measure the protein content by a small-volume micromethod using the Bio-Rad D_C protein assay. 4- Distribute 15 µg of protein in an equal volume of cross-linking buffer in one 1.5 ml tube per condition, pre-cooled to 4°C. 5- Weigh and dissolve the chemical cross-linkers either in PBS for water-soluble reagents or DMSO for the water-insoluble ones. 6- Mix the protein samples with an equal volume for each condition of stock cross-linker solutions \(or vehicle only as a control) to reach the desired final concentrations. 7- Incubate for the time and at the temperature that you have previously optimized for your own protein. In the present study on Orai expressed in _Drosophila_ S2 cells, incubations were performed for 10 min at 37°C for the lysine-reactive cross-linkers and for 20 min at room temperature for the cysteine-reactive reagents. 8- Stop the reaction by the addition of the quenching solution at a final concentration of 20 mM Tris pH 7.5 for the lysine-reactive cross-linkers or 25 mM DTT for the cysteine-reactive cross-linkers and incubate 10 min at room-temperature. 9- Mix the stopped reaction with denaturing gel loading buffer and analyze the size of the cross-linked products by a standard western-blotting technique. We use a 1X final concentration of Nu-PAGE LDS sample buffer \(Invitrogen) containing 50 mM DTT, incubate the samples at 70°C for 10

min, and then subject them to electrophoresis. The samples are separated either on 4-12% gradient Nu-PAGE Bis-Tris gels \(Invitrogen) or 3-8% gradient Nu-PAGE Tris-Acetate gels \(Invitrogen) according to the manufacturer instructions. **Chemical cross-linking of intact S2 cells** 1- Rinse the cells twice with room temperature phosphate-buffered saline \(PBS). 2- Harvest $5x10^6$ cells per condition in 1 ml of room temperature cross-linking buffer \(PBS pH8 for lysine-reactive cross-linkers or PBS pH 7.4, 2.5 mM EDTA for cysteine-reactive reagents). 3- Weigh and dissolve the chemical cross-linkers either in PBS for water soluble reagents or DMSO for the water-insoluble ones. 4- For each condition, add to the cells an equal volume of stock cross-linker solutions \(or vehicle only as a control) to reach the desired final concentrations. 5- Incubate for the time and at the temperature that you have previously optimized for your own protein. In the present study on Orai expressed in _Drosophila_ S2 cells, incubations were performed for 15 min at room temperature for the lysine-reactive cross-linker and for 20 min at room temperature for the cysteine-reactive reagents. 6- Stop the reaction by the addition of the quenching solution at a final concentration of 20 mM Tris pH 7.5 for the lysine-reactive cross-linkers or 25 mM DTT for the cysteine-reactive cross-linkers and incubate 10 min at room-temperature. 7- Rinse the cells twice with ice-cold phosphate-buffered saline \(PBS). 8- Solubilize the proteins in a lysis buffer containing detergents and analyze the size of the cross-linked products by standard a western-blotting technique. We lyse the cells in 85 µl of RIPA lysis buffer \(Upstate) supplemented with 1X Complete EDTA-free protease inhibitor mixture \(Roche) and pass them five times through a 26G needle. After 30 min of solubilization at 4°C under agitation, lysates are centrifuged \(16,000 x _g_, 10 min, 4°C) and the supernatants are collected.

References

Kedei, N. _et al_. Analysis of the native quaternary structure of vanilloid receptor 1. _J. Biol. Chem._ **276**, 28613-9 \(2001).