Published in final edited form as:

Methods Mol Biol. 2015 ; 1233: 25–34. doi:10.1007/978-1-4939-1789-1_3.

Analysis of Epidermal Growth Factor Receptor Dimerization by BS³ Cross-Linking

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Abstract

Dimerization of receptor tyrosine kinases is a well-characterized process. It is imperative for the activation of many receptors, including the epidermal growth factor receptor (EGFR). EGFR has been shown to be regulated by a number of factors, including lipid raft localization. For example, alteration of the lipid raft localization of EGFR has been demonstrated to modify receptor dimerization. This protocol describes an assay to quantify EGFR dimers using BS³ cross-linking. BS³ cross-linking is well suited for this purpose because of its length, water solubility, and membrane impermeability. Although this protocol is written specifically for EGFR, the assay can be extrapolated in order to characterize dimerization of other receptor tyrosine kinases.

Keywords

Epidermal growth factor receptor; Dimerization; Lipid rafts; BS³; Cross-linking; DHA

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⁹This protocol describes an assay to assess dimerization of EGFR by cross-linking the dimers after ligand stimulation. As seen in Fig. 1, EGFR in unstimulated samples is observed as a monomer at 170 kDa. Upon stimulation with EGF, EGFR dimers are detected at a molecular weight of 340 kDa. Although this protocol is written specifically for EGFR, it can be modified in order to analyze other membrane receptor tyrosine kinases. For each receptor, tests are required to determine the optimal amount of FBS for serum deprivation conditions, the duration of serum deprivation, the concentration of ligand, and the duration of stimulation.

¹⁰Many receptor tyrosine kinases have been demonstrated to form homodimers and/or heterodimers. This protocol can be utilized to assess both homodimerization and heterodimerization. To assess heterodimerization, it will be necessary to perform Western blots for each receptor in the dimer. Since this protocol uses a PVDF membrane, the same membrane can be utilized for analysis of each individual receptor by stripping the membrane between probing for each receptor. Additionally, immunoprecipitation prior to Western blotting may be suitable. Oligomers can also be cross-linked using this method; however, alterations might be required depending on the size of the oligomers. Furthermore, this protocol utilizing BS³ can be used to cross-link receptor-ligand interactions. The described protocol is very versatile and can be easily adapted to assess numerous aspects of receptor tyrosine kinase function at the plasma membrane.

¹¹This protocol is specific for cross-linking proteins at the plasma membrane because BS³ is membrane impermeable. BS³ has been used to cross-link intracellular EGFR dimers [9], but the cells must be permeabilized during the time of BS³ incubation. Alternatively, disuccinimidyl suberate (DSS), a membrane-permeable equivalent to BS³, can be substituted. The drawback of DSS usage is that DSS is not water soluble. Therefore, it must first be dissolved in an organic solvent. Overall, alterations to the protocol would be required in order to assess intracellular dimers.

¹²Receptor dimerization can be largely affected by alterations to lipid rafts. An important aspect of lipid raft function is the fatty acid composition of the membrane. Treating cells with fatty acids, like DHA, changes the fatty acid composition of the membrane. DHA is incorporated into the plasma membrane of mouse colonocytes where it has been shown to perturb lipid raft mesodomains. In order to assess the effect of fatty acid treatment on receptor tyrosine kinase dimerization, cells must be pretreated with fatty acid(s) for 0–72 h prior to the dimerization experiment. For example, DHA can be complexed to fatty acid-free BSA and added to the cell culture at a concentration of 50 μM as described previously [10]. DHA is maintained in the culture media during the serum deprivation step to prevent its loss from the membrane, but DHA is not utilized during ligand stimulation to avoid non-membrane effects the fatty acid could potentiate. As seen in Fig. 2, cells treated with DHA have increased dimer formation compared to untreated cells [3]. However, linoleic acid (LA), an omega-6 fatty acid, does not perpetrate the same effect on EGFR dimerization as DHA. This data highlights the pivotal role of the fatty acid composition of the membrane on receptor tyrosine kinase activity.

1 Introduction

Receptor dimerization is often an indispensable step in receptor tyrosine kinase activation. For example, the epidermal growth factor receptor (EGFR) is a prototypical dimerization-activated receptor tyrosine kinase. Following ligand binding to EGFR, the receptor undergoes conformational changes that lead to the projection of the dimerization loop which facilitates the interaction with another ligand bound EGFR or other EGFR-family dimerization partner [1]. Dimerization is essential to enable the intracellular kinase domain of EGFR to become activated.

Signaling of many receptor tyrosine kinases is known to be dependent on the localization of these receptors to specific membrane domains, such as lipid rafts or caveolae. EGFR has been demonstrated to localize to lipid rafts (nanometer sized heterogeneous cholesterol enriched mesodomains), and the signaling capacity of EGFR is regulated by its lipid raft localization [2–4]. Perturbations to lipid rafts can alter EGFR localization and activity. It has been shown that disruption of lipid rafts increases EGFR clustering prior to ligand stimulation [5, 6], as well as increases EGFR dimerization upon ligand stimulation [2, 3]. Therefore, receptor dimerization status is a central part of studies on the lipid raft localization of EGFR.

Lipid rafts can be disrupted in a variety of different ways. A commonly used technique is to extract cholesterol, a major component of lipid rafts, from the membrane using methyl- β -cyclodextrin (M β CD). This is a very harsh treatment and has very strong effects on lipid rafts. In many of the studies conducted in our laboratory, we assess the effects of long-chain omega-3 fatty acids on lipid raft-mediated processes. Docosahexaenoic acid (DHA) is an omega-3 fatty acid consisting of 22 carbons and six double bonds. Due to the length and high degree of unsaturation of DHA, it is sterically incompatible with cholesterol [7]. Unlike M β CD, DHA subtly alters the size, composition, and function of lipid rafts without completely disrupting them. We have previously demonstrated that DHA shifts the localization of EGFR from lipid rafts into the bulk membrane [3]. The altered localization of EGFR upon DHA treatment leads to increased receptor dimerization upon stimulation with the EGFR-specific ligand, EGF. Interestingly, although EGFR dimerization and phosphorylation, which are considered hallmarks of receptor activation, are increased in DHA-treated cells, downstream signaling from the receptor is suppressed. These data suggest a central role for lipid rafts in regulating receptor tyrosine kinase activity.

Herein, we describe a method to assess receptor dimerization at the plasma membrane of adherent cells using bis[sulfosuccinimidyl] suberate (BS³) to cross-link dimers. BS³ contains two *N*-hydroxysulfosuccinimide (NHS) ester active groups on each end, connected by a 11.4 Å spacer arm of eight carbons. The NHS groups of BS³ rapidly react with primary amines on lysine residues and the N-terminal region of proteins. BS³ is water soluble up to approximately 100 mM at a pH of 7–9, which makes it ideal for use in live cell receptor cross-linking assays. Furthermore, the intermediate-length spacer arm works well for cross-linking EGFR dimers formed upon ligand stimulation. BS³ is noncleavable, so it cannot be utilized for reversible cross-linking. BS³ is well suited for studies on plasma membrane lipid rafts because it is membrane impermeable and will only label proteins on the cell surface.

2 Materials

2.1 Cell Culture

1. Cell incubator (33 °C and 5 % CO₂).
2. 150 mm culture dishes.
3. Young adult mouse colonocytes (YAMC); this is a non-malignantly transformed, conditionally immortalized cell line [8]. The cells express a temperature-sensitive mutant of the SV40 large T-antigen that is inducible with interferon (IFN)- γ . The temperature-sensitive protein is active at 33 °C but inactive at 37 °C. Therefore, unlike most mammalian cell lines, this cell line is maintained at 33 °C. This protocol is equally applicable for cell lines grown at 37 °C.
4. RPMI 1640 complete medium: To a 500 mL bottle add 532 μ L insulin, transferrin, selenous acid (ITS) Premix (BD Biosciences, CA, USA), 26.6 mL fetal bovine serum (FBS), and 5.3 mL Glutamax (Gibco, Grand Island, NY, USA). Immediately prior to use, add 1 μ L IFN γ (Gibco) per 10 mL medium.
5. RPMI 1640 serum deprivation medium: To serum-free, 1 % Glutamax RPMI 1640 medium, add FBS to a final concentration of 0.5 %. Also add 1 μ L IFN γ per 10 mL medium. This medium should not have ITS.

2.2 EGFR Stimulation

1. Murine EGF.
2. Ligand stimulation medium: Serum-free RPMI 1640 medium supplemented with 25 ng/mL EGF. This medium should not have any serum or ITS.

2.3 BS³ Cross-Linking

1. BS³ (Thermo Scientific, Rockford, IL, USA): Prepare 3 mM BS³ immediately prior to use by adding 8.58 mg BS³ to 5 mL of 1 $\times$ Ca²⁺-, Mg²⁺-free PBS. Prepare 5 mL per sample. Maintain on ice until ready to use.
2. 250 mM glycine (can be prepared in advance): Add 1.88 g of glycine to 100 mL 1 $\times$ PBS. Store at 4 °C.
3. 1 $\times$ Ca²⁺-, Mg²⁺-free PBS.

2.4 Cell Lysate Harvest

1. Rubber policeman.
2. Homogenization buffer (to be prepared the day of use): 50 mM Tris-HCl, pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 1 % triton X-100, 100 μ M activated sodium orthovanadate, 10 mM β -mercaptoethanol, Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:25. Maintain on ice until ready to use.

2.5 Protein Quantification

1. Coomassie Plus assay kit (Thermo Fisher Scientific, Rockford, IL, USA).
2. Bovine serum albumin (BSA).
3. Disposable borosilicate glass tubes.
4. 96-Well clear polystyrene plate.
5. Spectrophotometer plate reader.

2.6 Western Immunoblotting

1. Heating block.
2. Western blotting apparatus.
3. PVDF membranes.
4. 5× Western blot loading buffer: 0.25 % bromophenol blue, 0.5 M dithiothreitol (DTT), 50 % Glycerol, 10 % sodium dodecyl sulfate (SDS), 0.25 M Tris-HCl, pH 6.8.
5. Wash buffer: 0.1 % Tween-20 in 1× PBS (PBST).
6. Blocking solution and antibody diluent solution: 5 % BSA in PBST.
7. Enhanced chemiluminescent substrate.
8. High-molecular-weight protein marker (preferably prestained).
9. Rabbit monoclonal anti-EGFR antibody (Cell Signaling Technology, Danvers, MA, USA).
10. HRP-conjugated goat anti-rabbit IgG antibody.
11. Film or digital imaging system.

3 Methods

Prior to beginning the experiment:

It is necessary to have healthy cells growing in culture. Cells should be maintained in the log phase of growth by passaging the cells when the dish is approximately 70 % confluent. It is best to use early-passage cells to avoid any changes that could occur from prolonged culturing which might result in irreproducible results.

3.1 Day 1

1. Seed YAMC cells onto 150 mm dishes at 5.0×10^5 cells per dish in 15 mL of complete (full serum) media (*see* Note 1).
2. Incubate cells overnight in a 33 °C incubator with 5 % CO₂.

¹The number of cells seeded on day 1 should be enough for the dish to be approximately 90–95 % confluent after 48 h. It is important to determine the effect of serum starvation on the cell growth to calculate the number of cells to be seeded.

3.2 Day 2

1. On the following day, aspirate the media from the dishes and wash the cells one time with room temperature 1× PBS.
2. Add 15 mL of serum deprivation media (0.5 % FBS) to the dishes (*see* Note 2).
3. Incubate cells at 33 °C with 5 % CO₂ overnight (16–18 h).

3.3 Day 3

1. The following morning, remove the media and wash cells twice with room temperature 1× PBS.
2. Aspirate the PBS, place the dishes on ice, and add 15 mL ligand stimulation media, serum-freed media supplemented with 25 ng/mL mouse EGF (*see* Note 3).
3. Incubate cells for 1 h on ice (*see* Note 4).
4. Wash the dishes three times with ice-cold 1× Ca²⁺-, Mg²⁺-free PBS while maintaining the cells on ice (*see* Note 5).
5. Add 5 mL of 3 mM BS³ (in ice-cold 1× Ca²⁺-, Mg²⁺-free PBS) to each dish and incubate for 20 min on ice (*see* Note 6).
6. Quench the excess BS³ with 10 mL of 250 mM glycine in 1× PBS for 5 min at 4 °C.
7. Wash the dishes three times with ice-cold 1× PBS. Completely remove all of the PBS after the final wash.
8. Add 300 µL of homogenization buffer to each plate. Thoroughly scrape the entire cell layer with a rubber policeman.
9. Collect the scraped cell homogenate into 1.5 mL Eppendorf microcentrifuge tubes. Place the tubes on ice.
10. Lyse the cell homogenates by passing the cells through a 29G needle once. Flush the suspension very hard through the needle while maintaining the tube on ice to shear the cells.
11. Incubate the total cell suspension on ice for 30 min.

²The serum deprivation step will reduce receptor signaling and increase sensitivity of cells to stimulation the following day. Additionally, serum deprivation allows for the detection of effects that are specific to stimulation with the ligand of interest since FBS contains many growth factors that stimulate receptor tyrosine kinases, including EGFR. It may be necessary to reduce further the amount of serum or to increase the length of time that cells are serum deprived depending on the cell line utilized and the experimental design.

³The amount of EGF utilized in this protocol is an intermediate dose of EGFR ligand for stimulation of mouse colonic epithelial cells. In the literature the concentration of EGF utilized to stimulate EGFR ranges from less than 1 ng/mL to above 100 ng/mL. The amount of ligand should be adjusted according to the cell line and experiment. It is also necessary at this step to have an unstimulated control in order to observe ligand-dependent changes in receptor dimerization and to determine the status of EGFR dimers prior to stimulation.

⁴Incubation on ice during ligand stimulation allows for ligand binding and receptor dimerization but inhibits receptor endocytosis. This is important due to the membrane impermeability of BS³.

⁵It is important to thoroughly remove the amine-containing culture media from the dishes by washing before cross-linking with BS³.

⁶This volume of 5 mL of 3 mM BS³ solution is enough to just cover the bottom of the dish. Ensure that the entire dish is covered by the BS³ solution and that the dish is flat on the ice so that the solution does not collect on the side of the dish.

12. Centrifuge at $16,000 \times g$ at 4 °C for 20 min.
13. Transfer the supernatant (total cell lysate) to a new 1.5 mL microcentrifuge tube being careful not to disturb the pellet.
14. Mix the total cell lysate by pipetting up and down approximately five times.
15. Aliquot the lysate into 20–30 μL aliquots in 0.5 mL microcentrifuge tubes. Also, prepare one aliquot with only 10 μL to use for protein quantification.
16. Store the aliquots at $-80\text{ }^{\circ}\text{C}$ until ready to use.

3.4 Protein Quantification

1. Prepare samples in disposable borosilicate glass tubes by combining 2.5 μL of total cell lysate, 497.5 μL of double distilled water, and 500 μL of Coomassie Plus reagent.
2. Using BSA prepare standards of 0, 0.5, 1, 2, 4, 10, and 20 μg in a volume of 497.5 μL with double distilled water. Add 2.5 μL of homogenization buffer and 500 μL of Coomassie Plus reagent. Prepare samples and standards in triplicate in order to obtain a highly accurate protein concentration.
3. Vortex the samples for 5 s.
4. Add 300 μL of each sample to a designated well on a 96-well clear polystyrene plate. Use a plate reader spectrophotometer and quantify the absorption of the standards and samples at 595 nm.
5. Use the standards to calculate a standard curve and quantify the protein concentration of the samples.

3.5 Western Blotting

1. Prepare protein samples with 25 μg of protein. Bring all samples to a final volume of 20 μL with homogenization buffer or water. Add 5 μL of 5 $\times$ loading buffer. Heat samples on a heating block at 98 °C for 10 min.
2. Assess receptor dimerization by immunoblotting for EGFR. Follow a standard protocol for immunoblotting with the following slight alterations.
3. Using a 4–10 % tris-glycine gradient gel, SDS-PAGE should be run for approximately 4–5 h at 125 V (*see* Note 7).
4. Incubate the gel in transfer buffer for ~15 min before beginning the transfer.
5. Transfer onto a PVDF membrane overnight at 400 mA at 4 °C with a magnetic stirrer set to low speed (*see* Note 8).

⁷The 4–10 % gradient gel works well for separation of high-molecular-weight proteins/complexes. The separation is further improved by the long (4–5 h) migration time. It is required to have efficient separation of the monomers and dimers. It is best to have a colored molecular weight marker to ensure separation at high molecular weights.

⁸The long transfer time ensures efficient and complete transfer of proteins at high molecular weights.

6. Block the membrane with blocking buffer at room temperature for 1 h with slight agitation.
7. Probe the membrane with anti-EGFR rabbit monoclonal antibody at a dilution of 1:1,000 in antibody dilution buffer. Incubate with slight agitation overnight at 4 °C.
8. Wash the membrane three times for 10 min each with PBST and moderate agitation.
9. Incubate the membrane with HRP-conjugated goat anti-rabbit IgG at a dilution of 1:10,000 in antibody dilution buffer at room temperature with slight agitation for 1 h.
10. Wash the membrane three times for 10 min each with PBST and moderate agitation.
11. Incubate blot for 5 min with enhanced chemiluminescent substrate, remove substrate, and expose using film or digital imaging.
12. Quantify the intensity of the bands using image analysis software, such as Image J (NIH). Homodimers on the Western blot should be twice the molecular weight of the single receptor. The molecular weight of heterodimers can be calculated by adding the molecular weight of the individual receptors involved. Serum starved, unstimulated cells should be used as a control to determine the amount of dimer formation in unstimulated conditions.

Acknowledgments

This work was supported by RO1 CA168312 and P30ES023512-01.

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Fig. 1. Representative Western immunoblot. In unstimulated samples, no EGFR dimers are observed. Upon stimulation with EGF, a band of EGFR dimers (340 kDa) is observed at a molecular weight of twice the EGFR monomers (170 kDa)

Fig. 2.

Effect of fatty acid treatment on EGFR dimerization. YAMC cells were treated for 72 h with no fatty acid, linoleic acid (LA-18:2 9,12), or DHA (22:6 4,7,10,13,16,19) and serum deprived for the final 24 h. Cells were then stimulated with 25 ng/mL EGF and subjected to BS³ chemical cross-linking as described by the protocol. Cell lysates were assessed by Western blotting for EGFR. Figure reproduced with permission from Turk et al. 2012 [3]