Chemical crosslinkers enhance detection of receptor interactomes

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Crosslinking in the Analysis of Receptor Interactomes: To Link or Not to Link?

To biochemically study receptor-protein interactions, one must be able to isolate receptors and their interactomes from the lipid plasma membrane. This process is challenging because of the need to keep diverse protein-protein interactions intact during the extraction and purification of the protein complex. Additionally, the structure and subcellular localization of the receptor in the membrane dictates the chemical conditions required for protein extraction (Thomas and McNamee, 1990). Large polypeptide membrane spanning receptors, such as ligand gated ion channels, demand strong detergent-based solubilization in order to ensure extraction of all receptor subunits. The stringency of these detergent conditions, however, can lead to a loss in numerous receptor-protein associations. Receptors that are embedded in lipid-rich and cholesterol heavy regions of the plasma membrane (such as rafts) require unconventional solubilization methods. Such lipid-rich and cholesterol heavy regions of the plasma membrane (such as rafts) require unconventional solubilization methods such as immunoprecipitation (IP) or pulldown assays. These methods rely on the specificity of the antibody or capturing bait and may bias toward abundant proteins and stable protein-protein interactions. In the absence of stringent controls, standard IP experiments can produce substantial false positive results. Finally, current biochemical methods used to detect protein interactions lack cellular spatial specificity; consequently, when a true interaction is discovered the subcellular localization of the interaction is unknown.

New strategies have emerged for enhancing the detection of protein interactions. Methods such as protein fragment complementation and chemical crosslinking (CC) can stabilize transient or labile protein interactions in vivo and in vitro (Box 1), and therefore enable the identification of many proteins within the interactome (Kluger and Alagic, 2004; Morell et al., 2007). Conventionally CC has been used in the study of extracellular interaction networks (interactomes) in cells, the capture of receptor interactomes and their dynamic properties remains a challenge. In particular, the study of interactome components that bind to the receptor with low affinity can rapidly dissociate from the macromolecular complex is difficult. Here we describe how chemical crosslinking (CC) can aid in the isolation and proteomic analysis of receptor-protein interactions. The addition of CC to standard affinity purification and mass spectrometry protocols boosts the power of protein capture within the proteomic assay and enables the identification of specific binding partners under various cellular and receptor states. The utility of CC in receptor interactome studies is highlighted for the nicotinic acetylcholine receptor as well as several other receptor types. A better understanding of receptors and their interactions with proteins spearheads molecular biology, informs an integral part of bench medicine which helps in drug development, drug action, and understanding the pathophysiology of disease.

Keywords: nicotinic acetylcholine receptor, chemical crosslinking, mass spectrometry, protein-protein interaction, signaling network, interactome
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BOX 1 | Technical Toolbox.

- To crosslink solubilized membrane proteins in vitro with BS3, add 2 mM BS3 to the enriched receptor fraction for 2 h at 4°C and mix (Figure 1B).
- To crosslink proteins in vivo, add 2.5 mM DSP to cultured cells for 2 h at 4°C (Figure 1C). The chemical reaction with DSP can be terminated by the addition of 50 mM Tris-HCl pH 7.5 at 4°C.
- Clever use of DSP crosslinking enables a range of experiments on receptor-protein interactions including an analysis of receptor interactomes at the cell surface, inside recycled vesicles, or in response to ligand stimulation (Figures 2A,B).
- A crosslinked receptor interactome can be purified using standard methods for immunoprecipitation and mass spectrometry (Figure 2C).

Interactomes appear better detected by CC at various stages of the receptor preparation and purification method (Vasilescu et al., 2004). Interactions that are generally too weak or too transient to be discovered in standard pulldown or IP assays alone, can be stabilized by covalent crosslinkers during the membrane solubilization process (Bond et al., 2009; Nordman and Kabbani, 2012). The common use of stringent chemical detergents such as radio-immunoprecipitation assay (RIPA) buffers, which interfere with many types of protein–protein interactions, can also benefit from the addition of covalent crosslinkers which are generally unperturbed by the RIPA reagent. Moreover, CC can be effectively combined with affinity purification protocols such as the IP prior to the mass spectrometry analysis (Vasilescu et al., 2004). To eliminate non-specific interactions of proteins during CC, the assay requires optimization before the start of the study. It is also not uncommon to run non-crosslinked samples in parallel during the course of a study (Kim et al., 2012).

A number of crosslinkers have been used to study receptor–protein interactions in cells (Brenner et al., 1985; Shinya et al., 2010; Miteva et al., 2013). These compounds are characterized by differences in their spacer arm as well as the composition of the two amine binding groups that recognize and covalently bind specific functional groups on target proteins (Figures 1B,C; Sinz, 2003; Trakselis et al., 2005). Table 1 lists crosslinkers that have been used to study receptor binding to intracellular proteins. The choice of a spacer arm length, between 5 and 25 Å, is experimentally important because it enables the identification of receptor–protein interactions at specific distances. A recent study utilized agarose beads whose surface was covalently linked with a cleavable chemical crosslinker by spacers of varied lengths to study the interactome of the post-synaptic density isolated from the rodent cortex (Yun-Hong et al., 2011). Experiments successfully

FIGURE 1 | Crosslinking the nAChR interactome. (A) A flow chart showing the methods for isolation, crosslinking, and proteomic analysis of nAChR interactomes from brain tissue or neural cells. The experimental design should take into the consideration the choice of the crosslinker as well as the

Frontiers in Pharmacology | Neuropharmacology January 2014 | Volume 4 | Article 171 | 2
Table 1 | A summary of crosslinkers used in the analysis of receptor protein interactions.

| Chemical name | Membrane permeable | Reversible toward | Receptor interactome applications | Spacer arm |
|---------------|--------------------|-------------------|-----------------------------------|------------|
| DSS           | Y                  | N                 | Amine                             | 11.4       |
| DSP           | Y                  | Y by DTT          | Amine                             | 12.0       |
| BS3          | N                  | Amines            | Aldosea et al. (2000), Nordman and Kabbani (2012) | 11.4 |
| MBP          | Y                  | N                 | Sulphydryl                        | <5         |
| ANB-NOS      | Y                  | N                 | Amines                            | 77         |
| Sulfos-AED   | Y                  | by DTT            | Amines Yun-Hong et al. (2011)     | 23.6       |

*Denotes photo-reactive linkers.

**IUPAC Names:** DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidyl propionate); BS3, bis(sulfosuccinimidyl) suberate; MBP, 4-maleimidobenzylamine N-hydrochloride; ANB-NOS, 6-(bis[2-aminophenoxy]sulfonanilido) hexane-1,3,5-triisothiocyanate; SAED, sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetoxyethyl)-1,3,5-triisothiocyanate.

Crosslinking has also enabled detection of changes in receptor function for the angiotensin receptor (Quian et al., 2001) and has been useful in determining interactions impacted by post-translational modification (Cao et al., 1999; Connolly, 1999; Ehlers, 2000). When combined with cell surface labeling, CC has been effective in determining changes in receptor glycosylation. Differential glycosylation of cell surface human and rat β2 adrenergic receptors (Lefkowitz and Shenoy, 2005) contributed to learning, memory, and goal driven behavior (Changeux, 2012). Recent evidence also reveals that nAChRs operate by coupling to intracellular proteins such as heterotrimmeric G proteins (Kabiani et al., 2013). Chronic nicotine exposure gives rise to neural adaptations such as an up-regulation of specific nAChRs through cell-delimited post-translational mechanisms (Sallette et al., 2005; Colombo et al., 2013). These receptor mechanisms are a hallmark of nicotine addiction yet it is still unclear which signaling pathways and mechanism regulate nAChR assembly and trafficking inside the cell. Proteomic studies, based on yeast-two-hybrid as well as conventional IP experiments have led to the identification of several intracellular proteins that bind nAChR subunits in the brain (Kabiani et al., 2007; Paula et al., 2009; Nordman and Kabbani, 2012; McClure-Begley et al., 2013). Directed protein interaction screens have also enabled discovery of proteins responsible for nAChR trafficking and assembly (Lin et al., 2002; Lau and Hall, 2001; Quian et al., 2001, Shenoy et al., 2006).
In the hippocampus, α7 nAChRs are expressed pre- and post-synaptically, contributing to GABA and glutamate neurotransmission (Liu et al., 2006; Londo et al., 2012). α7 receptors are also found to mediate the growth of axons (Hancock et al., 2008; Nordman and Kabbani, 2012) and dendrites (Campbell et al., 2011) in the developing hippocampus. Using the membrane impermeable and irreversible crosslinker BS3, we have defined dynamic changes in α7 interaction within solubilized membrane fractions from differentiated PC12 cells and hippocampal neurons (Figure 1B; Nordman and Kabbani, 2012). We show that α7 receptors are directly coupled to a G protein pathway consisting of Gαo, Gprin1, and GAP-43 in growing cells (Nordman and Kabbani, 2012; Figure 1C). In these studies, CC was vital to the detection of changes in receptor interaction with signaling molecules and heterotrimeric G proteins. The CC method was also able to enhance the detection of small signaling molecules such as receptor kinases in both Western blots and mass spectrometry experiments (Hu et al., 2010; Nordman and Kabbani, 2012). For example, using BS3 to crosslink the α7 nAChR network after nicotine activation, we identified rapid changes to the calcium-mediated signaling pathway of the receptor, which consisted of a dynamic association between GAP-43 and calmodulin (CaM) in the growing neurite (Figure 2C; Nordman and Kabbani, 2012). In particular, activation of the α7 nAChR was found to promote a rapid association between the receptor and CaM-bound GAP-43. This interaction was rapidly reversed by ligand inactivation of the α7 nAChR, showing that receptor association with CaM-bound GAP-43 was driven by nAChR channel function and calcium elevation in the cell (Nordman and Kabbani, 2012). These findings on dynamic associations of CaM and GAP-43 within the α7 nAChR interactome could not have been detected using standard IP assays alone underscoring the utility of the method in identifying changes in calcium bound CaM kinase II and subunits of the NMDA glutamate receptor within the postsynaptic density of hippocampal neurons (Garzoni et al., 2002), thus underscoring the utility of the method in identifying protein interactions that mediate nAChR trafficking, localization, and signaling. (A) Cell surface receptors can be selectively labeled with an anti-α7 nAChR monoclonal Ab. Cell surface labeling at 4°C can be combined with DSP in order to crosslink the receptor interactome. Alternatively, antibody labeling and crosslinking can be used to examine changes in the receptor interactome between internalized and cell surface nAChRs. (B) Chemical crosslinking can be used to study the dynamics of nAChR-protein interactions under various ligand treatment conditions. (C) Experimental evidence on α7 nAChR interactions with GAP-43 and CaM in developing neural cells. BS3 was used to crosslink the α7 nAChR interactome from differentiating cells. An IP was utilized to purify the receptor, which was visualized by SDS-PAGE. Protein identity was confirmed using LC-ESI MS and Western blot. These experiments demonstrate dynamic changes in CaM/GAP-43 association with α7 nAChR in response to nicotine activation (Nordman and Kabbani, 2012).
the study of rapid calcium driven changes in protein coupling in cells.

LOOKING AHEAD
Proteomic and yeast-two-hybrid studies on receptor interactions have enabled a broad understanding on the diversity and function of receptor-protein interactions in cells. These studies have enabled an interaction-based framework for defining the mechanisms of receptor signaling. Receptor–protein interaction identification however is not sufficient for understanding how receptors operate in cells. In particular, important questions remain on the spatial specificity and temporal aspects of receptor expression and signaling in cells. For multi-subunit channel receptors such as the glutamate AMPA receptor, the addition of the membrane impermeable linker BS5 has proven effective in the analysis of receptor subunit composition at the cell surface (Royajazadeh et al., 2012). Similar approaches with the aim of detecting protein–protein interaction in living cells are now necessary. Advancement in the design and experimental utility of C2 such as photo-reactive amino acid analogs (Suchanek et al., 2005) promises to enhance the study of receptor–protein interactions in vivo.

ACKNOWLEDGMENT
This work was supported by a Wings for Life Spinal Cord Research Grant to Nadine Kabbani.

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