CHAPTER ONE

Chemokine Detection Using Receptors Immobilized on an SPR Sensor Surface

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Abstract

Chemokines and their receptors take part in many physiological and pathological processes, and their dysregulated expression is linked to chronic inflammatory and autoimmune diseases, immunodeficiencies, and cancer. The chemokine receptors, members of the G protein-coupled receptor family, are integral membrane proteins, with seven-transmembrane domains that bind the chemokines and transmit signals through GTP-binding proteins. Many assays used to study the structure, conformation, or activation mechanism of these receptors are based on ligand-binding measurement, as are techniques to detect new agonists and antagonists that modulate chemokine function. Such methods require labeling of the chemokine and/or its receptor, which

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can alter their binding characteristics. Surface plasmon resonance (SPR) is a powerful technique for analysis of the interaction between immobilized receptors and ligands in solution, in real time, and without labeling. SPR measurements nonetheless require expression and purification steps that can alter the conformation, stability, and function of the chemokine and/or the chemokine receptor. In this review, we focus on distinct methods to immobilize chemokine receptors on the surface of an optical biosensor. We expose the advantages and disadvantages of different protocols used and describe in detail the method to retain viral particles as receptor carriers that can be used for SPR determinations.

1. SURFACE PLASMON RESONANCE

Their ability to monitor the interaction between a molecule immobilized on the surface of a sensor and its interacting molecular partner in solution have made surface plasmon resonance (SPR) techniques powerful tools for analysis of the interplay between biological molecules (Rich & Myszka, 2006). SPR is based on the generation of an electromagnetic evanescent wave at a metal surface when polarized light strikes it at a critical angle of incidence (Homola, 2008). SPR biosensors measure the change in refractive index of the solvent during formation and dissociation of complexes near the assay surface (Rich & Myszka, 2000). Signals depend on mass alteration of the surface analyzed and are expressed as resonance units (RUs). The RU signal is thus directly proportional to mass variations on the sensor surface.

SPR-based techniques are commonly used for detailed analysis of the interaction between immobilized receptors and ligands in solution, in real time, and without analyte labeling. They allow determination of binding affinity and of the binding kinetics of a specific interaction, and are thus widely used in high-throughput screening procedures (Cooper, 2002). Alternative strategies for this type of analysis require fluorescent or radiolabeling of ligand to report its binding to the receptor; these approaches have extra time requirements and in some cases, due to altered binding properties of the partners, cause interference with molecular interactions.

The use of purified proteins facilitates SPR determinations. Experiments with membrane proteins offer additional complexity, as their expression and purification can alter conformation, stability and/or function outside the cell environment. Solubilization of membrane proteins solves the problem in some cases, although detergents must be selected carefully (le Maire,

Champeil, & Moller, 2000). Some lipid/detergent mixtures have been used in nuclear magnetic resonance (NMR) and crystallization experiments (Seddon, Curnow, & Booth, 2004); in structural and functional studies of the extremely light-sensitive G protein-coupled receptor (GPCR), rhodopsin, Reeves et al. used a mixture of the lipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine and the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (Reeves, Hwa, & Khorana, 1999).

Proteins are often expressed at low levels in the cell membrane. Overexpression can be attempted for a protein of interest if alterations in cell localization, protein conformation, and/or ligand binding can be ruled out with confidence. Proteins can also be overexpressed in heterologous systems such as in bacteria, although aggregation and correct posttransductional modifications must be monitored to avoid alteration in binding properties. In the case of the chemokine receptors and other GPCR, an additional problem is their complex structure. These receptors are formed by highly hydrophobic, seven-transmembrane domains that require a lipid bilayer to adopt a stable, functional conformation. Inclusion of these proteins in the lipid environment is no simple matter, and has been the object of several studies (Seddon et al., 2004; Soubias & Grawrisch, 2011).

A new technique combines plasmonic and fluorescence imaging and allows determination of membrane protein distribution in single living cells as well as evaluation of local binding kinetics in the context of the native environment (Wang et al., 2012). Other possibilities include use of a gold film bearing a periodic metallic nanopore array that supports free-standing lipid bilayers with membrane proteins that mimic a biological membrane (Maynard et al., 2009).

2. CHEMOKINE RECEPTORS: MEMBERS OF THE GPCR FAMILY

GPCR, the largest family in the human genome, include receptors for hormones, neurotransmitters, chemokines, calcium ions, and light (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003; Vassilatis et al., 2003). It is probably the most clinically relevant receptor class (Overington, Al-Lazikani, & Hopkins, 2006) and is thus the main focus of drug discovery research (Takakura, Hattori, Tanaka, & Ozawa, 2015). The structure of these receptors is complex, with a seven-transmembrane spanning hydrophobic α-helix, and requires a lipid environment to maintain

native conformation (Drake, Shenoy, & Lefkowitz, 2006; Tan, Brady, Nickols, Wang, & Limbird, 2004).

The members of the GPCR subfamily of chemokine receptors bind a group of low-molecular-weight pro-inflammatory cytokines (Rossi & Zlotnik, 2000). The chemokines were first described as specific mediators of leukocyte directional movement (Griffith, Sokol, & Luster, 2014); since then, they have been associated with a much wider variety of cell types and functions (Anders, Romagnani, & Mantovani, 2014; Guo et al., 2015; Maeda, 2015; Sozzani, Del Prete, Bonecchi, & Locati, 2015). Chemokines are essential elements in a variety of diseases characterized by inflammation and immune cell infiltration (Murdoch & Finn, 2000) such as asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis, colitis, Crohn's disease, and psoriasis. Two of these receptors, CXCR4 and CCR5, are the two main coreceptors for HIV-1 infection (Bleul, Wu, Hoxie, Springer, & Mackay, 1997), and some chemokine receptors also participate in tumor metastasis (Muller et al., 2001; Strieter, 2001) and transplant rejection (el-Sawy, Fahmy, & Fairchild, 2002).

The nearly 50 chemokines described to date are classified according to functional criteria as constitutive or inducible (Proudfoot, 2002). Constitutive chemokines are generally implicated in immune system homeostasis, whereas inducible chemokine expression is regulated mainly during inflammatory processes. In addition, several viruses encode highly selective chemokine receptor ligands that act as agonists or antagonists, and might thus have a role in viral dissemination or evasion of a host immune response (Alcami & Lira, 2010; Murphy, 2015). Given the physiological relevance of the chemokine receptors, several SPR assays have been developed to study their purification, solubilization, and reconstitution, to analyze their function, to evaluate allosterism, and to improve pharmacological studies.

3. CHEMOKINE RECEPTOR IMMOBILIZATION ON THE SENSOR CHIP

The first SPR experiments with chemokine receptors analyzed binding to β-arrestin 1 of C-terminal-derived CCR5 peptides immobilized on a CM5 sensor chip via an N-terminal cysteine thiol group (Huttenrauch, Nitzki, Lin, Honing, & Oppermann, 2002). For SPR, peptides and proteins, including the receptors, must be attached to the solid support of the sensor chip without altering native conformation or binding activity. This attachment must be stable over the course of a binding assay, and a sufficient

number of receptors must be presented to the solvent phase to interact with the ligand. It is also essential to avoid nonspecific binding of the sample, which influences RU detection and can mask the specific binding signal.

The most common strategy is covalent attachment to an *N*-hydroxysuccinimide/(1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (NHS/EDC)-derivatized dextran matrix, which allows coupling to amino groups. Low pH is sometimes necessary, although it increases possible receptor denaturation. Multiple amino groups on the receptor can lead to its random coupling and orientation on the sensor surface (O'Shannessy, Brigham-Burke, & Peck, 1992; Stein & Gerisch, 1996). Other modified matrices can be used to allow more specific, oriented receptor coupling (Alves, Park, & Hruby, 2005).

Another strategy is based on the noncovalent attachment via a histidine tag, avidin/biotin, or antibody-based designs that allow unidirectional coupling of membrane proteins (Fig. 1). These approaches offer several advantages, as receptor purification before capture is unnecessary, the orientation of the immobilized receptor is homogeneous due to directed capture through a probe, and high densities are achieved that enable study of low-molecular mass ligands; nonetheless, cell transfection, receptor solubilization and, when using avidin/biotin systems, specific receptor labeling might be necessary. The use of an anti-CXCR4 mAb immobilized to the gold surface of an immunochip facilitates CXCR4 concentration and correct orientation prior to reconstitution of a lipid bilayer environment on the chip (Stenlund, Babcock, Sodroski, & Myszka, 2003) (Fig. 1A). The

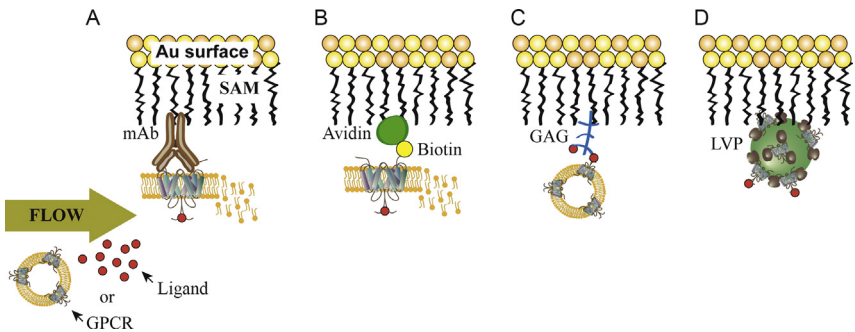

Figure 1 Strategies for GPCR attachment to dextran matrix. The interactive surface of a sensor chip consists of a self-assembled dextran monolayer (SAM) that bears functional groups to allow interaction of different compounds. For GPCR capture on the sensor chip surface, several strategies are used including direct binding to the dextran surface of monoclonal antibodies (A), avidin (B), GAG (C), or LVP (D).

structural and functional integrity of the captured receptor can be tested using conformation-sensitive monoclonal antibodies as well as its natural ligand. In the case of CXCR4, this strategy also illustrated the importance of the detergent in maintaining its structural integrity (Stenlund et al., 2003). The detergents CHS/DDM/CHAPS, combined with the lipids DOPC/DOPS at a 7:3 ratio, appear to be the best mixture for SPR studies of CCR5 and CXCR4 activity (Navratilova, Sodroski, & Myszka, 2005).

This antibody capture method was used to study natural ligand/small molecule binding to the chemokine receptors CXCR4 and CCR5. In these conditions, both receptors retained their ability to bind their ligands CXCL12 and CCL5, as well as the small molecule inhibitors, JM-2987 and TAK-779, respectively (Navratilova, Dioszegi, & Myszka, 2006), which demonstrates the value of this technique for biophysical and structural analyses. A similar approach was used to screen cocrystallization conditions for CCR5 and to characterize its binding to nine HIV gp120 variants (Navratilova, Pancera, Wyatt, & Myszka, 2006).

Some approaches use biotinylated ligands immobilized on streptavidin-functionalized sensor chips (Harding, Hadingham, McDonnell, & Watts, 2006) (Fig. 1B). Although biotin-labeled chemokines detect their receptors as effectively as commercially available antibodies and can be used, e.g., cell sorting (Le Brocq et al., 2014), biotinylation could alter the binding properties of these ligands, given their small size. Chemokines act *in vivo* by binding to cell surface glycosaminoglycans (GAG), which present them to their receptors for binding and subsequent biological activity (Hamel, Sielaff, Proudfoot, & Handel, 2009) (Fig. 1C). SPR methods are also used to determine GAG/chemokine-binding kinetics (Tanino et al., 2010), and sensor chip-immobilized GAG were used to retain chemokines at the SPR surface and to determine their ability to associate with viral chemokine-binding proteins (Alcami & Viejo-Borbolla, 2009).

Biosensors permit automated screening of receptor activity in distinct solubilization conditions, allow quantitation of the total amount of receptor captured on the surface, and assess receptor activity by assaying its response to conformation-sensitive ligands (Navratilova et al., 2005).

4. VIRAL PARTICLES AS CHEMOKINE RECEPTORS CARRIERS IN SPR

All methods described above are based on the capture of detergent-solubilized GPCR prior to reconstitution in a lipid environment

(Navratilova et al., 2005). Use of detergents and the need for purification are nonetheless variable elements that can affect functional characteristics of a receptor on the sensor surface. Given their intrinsic structural complexity, this is especially important in the case of GPCR. The chemokine receptors are found in many conformations at the cell membrane, including preformed homodimers, heterodimers, and even oligomers (Munoz, Holgado, Martinez, Rodriguez-Frade, & Mellado, 2012; Thelen, Munoz, Rodriguez-Frade, & Mellado, 2010). These distinct conformations and the way a given ligand modulates these structures dictates its functional effect. Receptor conformation is also influenced by the lipid composition of specific membrane regions and/or other cell surface molecules (Fallahi-Sichani & Linderman, 2009; Inagaki et al., 2012; Martinez-Munoz et al., 2014), which makes it difficult to establish reliable methods that reproduce native GPCR conformation(s) on the biosensor surface.

The use of viral particles (VPs) as receptor carriers could solve these problems (Fig. 1D). Particle immobilization eliminates the need to purify and reconstitute membrane proteins for ligand-binding studies; the technique allows functional characterization of interactions with membrane proteins that cannot be studied using standard equilibrium binding assays. Viruses incorporate plasma membrane proteins into their lipid envelope when they bud from the cell (Fig. 2). Both retro- and lentiviral particles (LVPs) have been immobilized for SPR determinations (Hoffman, Canziani, Jia, Rucker, & Doms, 2000; Suomalainen & Garoff, 1994; Vega et al., 2011); by modifying expression of the proteins of interest on the surface of the packaging cells, their use facilitates generation of adequate positive and negative controls for each assay.

4.1 Materials

- Plasmids pLVTHM, psPAX2, pMD2G, pCMV-dR8.91, pCMV-dR8.74 (Addgene; Cambridge, MA, https://www.addgene.org/Didier_Trono/)
- HEK-293 T cells (CRL-11268 ATCC; Manassas, VA)
- JetPEI (Polyplus-transfection SA; Illkirch, France)
- Aldehyde/sulfate latex beads (Invitrogen, Thermo Fisher Scientific; Waltham, MA)
- ^{35}S-methionine (Perkin Elmer; Waltham, MA)
- Biacore C1, F1, and CM5 sensor chips (GE Healthcare Life Sciences; Pittsburgh, PA)

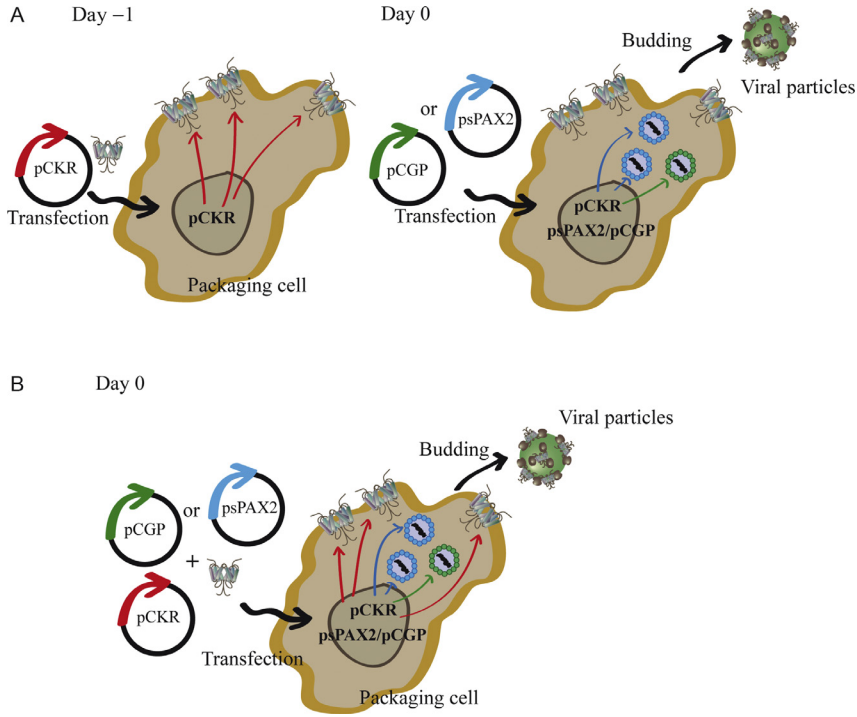

Figure 2 Generation of retroviral and lentiviral particles. Packaging cells are transfected with a chemokine receptor-containing plasmid (pCKR) in advance (A) or simultaneously (B) with the vectors needed to generate retroviral (pCGP) or lentiviral particles (psPAX2). During budding, viral particles incorporate the cell membrane as a coating that retains the structure and functional properties of the receptor.

- (Met)-free DMEM and DMEM media (Thermo Fisher Scientific; Waltham, MA)
- Anti-p24 antibody (Abcam; Cambridge, UK)

4.2 Method I: Generation of Retroviral Particles

To generate retroviral particles, murine leukemia virus (MLV) pseudotypes are produced by calcium phosphate-mediated transfection of HEK-293 T cells with a 3:1 ratio of receptor plasmid to pCGP, which encodes the MLV *gag* and *pol* genes. When a specific chemokine receptor must be evaluated, HEK-293 T cells transfected transiently or stably with the receptor should be used. At 4 h posttransfection, fresh medium supplemented with 10 m*M* *n*-butyric acid is added to increase protein expression; supernatant

is harvested at 48 h posttransfection, and cell debris is removed by low speed centrifugation and 0.45 μm filtration. The supernatant is pelleted (140,000 × *g*, 90 min) through 20% sucrose/PBS, washed in PBS, ultracentrifuged a second time (280,000 × *g*, 45 min) through 20% sucrose/PBS, and the pellet resuspended in 100 μl 10 m*M* Hepes, pH 7.4. The pseudotypes obtained can be stored at 4 °C or aliquoted and frozen at −20 °C. MLV pseudotypes must be analyzed by SDS-PAGE and Western blot for MLV gag and receptor expression, and purified by equilibrium density ultracentrifugation on a 15–45% sucrose gradient (217,000 × *g*, 16 h) (Fig. 2).

4.3 Method II: Generation of LVPs

Lentiviruses are also Retroviridae, but infect both dividing and nondividing cells (Frimpong & Spector, 2000). Lentiviral vectors are based on modified HIV-1 virus and are usually generated by transient cell transfection of several plasmids. The packaging unit (e.g., pCMV-dR8.91, pCMV-dR8.74, psPAX2) bears the genes that encode structural proteins and enzymes (gag, pol, tat, rev) needed to generate the LVP, the transfer vector (pLVTHM) bears the genetic material to be transduced into the target cell, and an envelope plasmid (pMD2G) derived from a heterologous virus, usually vesicular stomatitis virus (VSV), determines vector tropism. In the packaging unit, genes that encode crucial virulence proteins (env, vif, vpr, vpu, nef) are eliminated previously. In fact, only the packaging unit is needed to generate LVPs, which will not be infective. In this case, pMD2G plasmid can be excluded from the process, although viral protein and receptor levels must be titrated using alternative methods, as particles that lack the pMD2G plasmid will neither infect cells nor replicate, both necessary processes for LVP titration (see Section 4.4).

Although the packaging cell might express certain chemokine receptors (i.e., hCXCR4 in the case of HEK-293 T cells), a gene of interest can be included if needed in the transfer plasmid (pLVTHM) to increase receptor levels at the target cell surface and thus, at the LVP surface. It is also possible to express the receptor of interest using alternative plasmids under the control of more efficient promoters. In all cases, optimal conditions must be established for effective transfection of the packaging cells and expression of the desired protein in the LVP. Several procedures should be tested, including transfection of the plasmid containing the protein of interest prior to psPAX2 transfection, as well as the simultaneous transfection of

both plasmids (Fig. 2). LVP-containing supernatants should be collected initially at different time points and target protein expression on the LVP should be analyzed (see Sections 4.5 and 4.6) to establish the most effective, reproducible conditions.

As for retroviral particles, receptor expression is evaluated before LVP generation, and control LVPs are made using mock-transfected packaging cells. GPCR-expressing LVP can be attached to SPR biosensor surfaces by standard immobilization strategies. LVP can be produced by JetPEI-mediated transfection of HEK-293 T cells with pLVTHM or alternative transfer vectors, psPAX2 and pMD2G plasmids. At 4 h posttransfection, fresh medium is added (DMEM supplemented with 10% fetal calf serum, 2 m*M* L-glutamine and 1 m*M* sodium pyruvate), supernatant is harvested at 48 h posttransfection, and LVP processed as for retroviral particles. LVPs are aliquoted and stored at −80 °C. LVP must be analyzed by SDS-PAGE and Western blot for VSV envelope glycoprotein, VSV-G, and receptor expression prior to use in SPR determinations.

4.4 Method III: Titration of Viral Proteins

Prior to use in SPR experiments and to standardize the method, VPs should be titrated by PCR using specific primers for viral genes in the plasmids (Scherr, Battmer, Blomer, Ganser, & Grez, 2001). Virus can also be titrated by transducing HEK-293 T cells with serial dilutions of purified particles. In this case, HEK-293 T cells (3×10^4 cells/well) are plated in 1 ml complete DMEM in 24-well plates (day 1) and cultured (overnight, 37 °C, 5% CO_2), to adhere the plate. Serial dilutions of purified VP in 250 μl complete DMEM are prepared in parallel. On day 2, cells are counted ($6–8 \times 10^4$ cells/well) as a reference, and medium is aspirated from the plates before adding the VP dilutions; incubate (24 h) and add complete DMEM (1 ml/well). At 96 h after VP addition, detach transduced cells using 0.05% trypsin/EDTA. Evaluate the percentage of transduced cells by flow cytometry, measuring GFP expression (included in the transfer plasmid for LVP). VPs that lack VSV-G or alternative envelope plasmids cannot be titrated by transducing HEK-293 T cells, as the resulting particles are neither infective nor replicative. Titer is expressed as transforming units/ml, calculated as the number of cells transduced by a given volume of virions. This method cannot be used in packaging cells not transfected with pMD2G plasmid.

4.5 Method IV: Determination of Chemokine Receptor Levels on the VP

Chemokine receptor expression on the VP can be determined by Western blot using specific antibodies, by ELISA on VP-coated plates, or by flow cytometry. In this last case, small particle size limits measurement of the surface proteins by conventional techniques, and particles are coupled to latex beads to allow detection.

Aldehyde/sulfate latex beads have numerous aldehyde groups grafted to the surface of the polymer particle; this high density allows easy coupling of proteins and other materials to the latex particles in a one-step process. The sulfate groups on the microsphere surface maintain stability during covalent coupling. Use of fluorescently labeled ligands or conformation-dependent antibodies allows detection of the target GPCR in its native conformation on the VP surface. Latex beads are sonicated (5 min, room temperature—RT) and then incubated (15 min, RT) with the VPs. The reaction mixture is then diluted (1 ml PBS) and incubated (60 min, 4 °C, with continuous shaking). Finally, 100 µl glycine buffer (100 m*M* final concentration) is added to block free reactive groups. Incubate (30 min, RT, with continuous shaking), centrifuge (1950 × *g*, 3 min, RT), and wash once in PBS with 0.5% BSA. Resuspend beads in PBS with 0.5% BSA; 15 µl of beads are usually sufficient for eight different staining conditions. Chemokine receptors expressed on the surface of VPs bound to latex beads can then be stained with appropriate antibodies by standard techniques. As a negative control for flow cytometry, include uncoupled beads or beads coupled to VPs obtained from mock-transfected HEK-293 T cells. Protein expression in CKR-expressing and control VP can be monitored using GFP expressed by pLVTHM or by transfection with other GFP-bearing plasmids.

4.6 Method V: Quantitation of Receptor Number on VPs

To quantitate receptor number on the VPs surface, incubation media of the packaging HEK-293 T cells is removed 24 h posttransfection and cells are cultured in methionine (Met)-free DMEM supplemented with ^{35}S-Met (50 mCi/ml; 48 h, 37 °C, 5% CO_2). ^{35}S-Met-labeled VPs are collected, lysed, and proteins separated by SDS-PAGE. ^{35}S-Met incorporation can be visualized with a Phosphoimager, and relative receptor stoichiometry calculated using Quantity One software (BioRad, Hercules, CA) by normalizing the receptor of interest to a viral protein (such as p24 in lentivirus) with

a known number of Met residues and a known number of molecules in the virus; we assume 2100 molecules of p24/LVP (Briggs, Wilk, Welker, Krausslich, & Fuller, 2003). Values are interpolated on a regression curve for p24 data as internal standard. For this procedure, the packaging cells must be transfected with an envelope plasmid.

4.7 Method VI: Attachment of VPs to Biosensor Surfaces

For binding studies using purified receptor-bearing VPs, the virions must be retained on a derivative gold surface suitable for use in the optical biosensor (Fig. 3). A number of sensor surfaces are available from Biacore, each with different properties. A gold surface with a carboxylated alkane thiol (Biacore C1 chip) or a short carboxydextran matrix (Biacore F1 chip or CM5 sensor chip) must first be derivatized by a 10-min activation of surface carboxyl groups using a 1:1 mixture of EDC (1 *M*) and NHS (0.25 *M*). The buffer containing the VPs must be determined individually; pH range and ionic strength must be carefully controlled for immobilization and regeneration processes. Whereas MLV pseudotypes and LVPs are both injected in acetate buffer, composition and pH vary (0.1 *M* sodium acetate pH 5.5 and 10 m*M* sodium acetate pH 4.0, respectively); free surface carboxyl groups remaining after attachment are quenched with 1 *M* ethanolamine (pH 8.5, 5 μl/min, 7 min, RT).

5. SPR-BASED APPLICATIONS FOR CHEMOKINE RECEPTORS

Allosteric modulators are compounds that associate with receptors through binding sites different from those used by the specific ligands. They can modulate the endogenous agonists positively or negatively, or have no influence on receptor activity. Allosteric processes are particularly relevant in the case of the chemokine receptors, which can be homo- or heterodimers, or oligomers. In lymphoblasts that express CCR2, CCR5 and CXCR4 simultaneously, their specific antagonists (AMD3100 for CXCR4 and TAK779 for CCR5) affect ligand binding as well as CCL2-triggered cell migration toward CCR2 and CXCL12-triggered migration toward CXCR4 (Sohy, Parmentier, & Springael, 2007).

A SPR-based method was developed for high-throughput, label-free screening to identify ortho- and allosteric CCR5 ligands (Navratilova, Besnard, & Hopkins, 2011). The method is based on immobilization to the sensor gold surface of an antibody to a tag peptide (TETSQVAPA) included at the CCR5 C-terminal end. The antibody retains purified

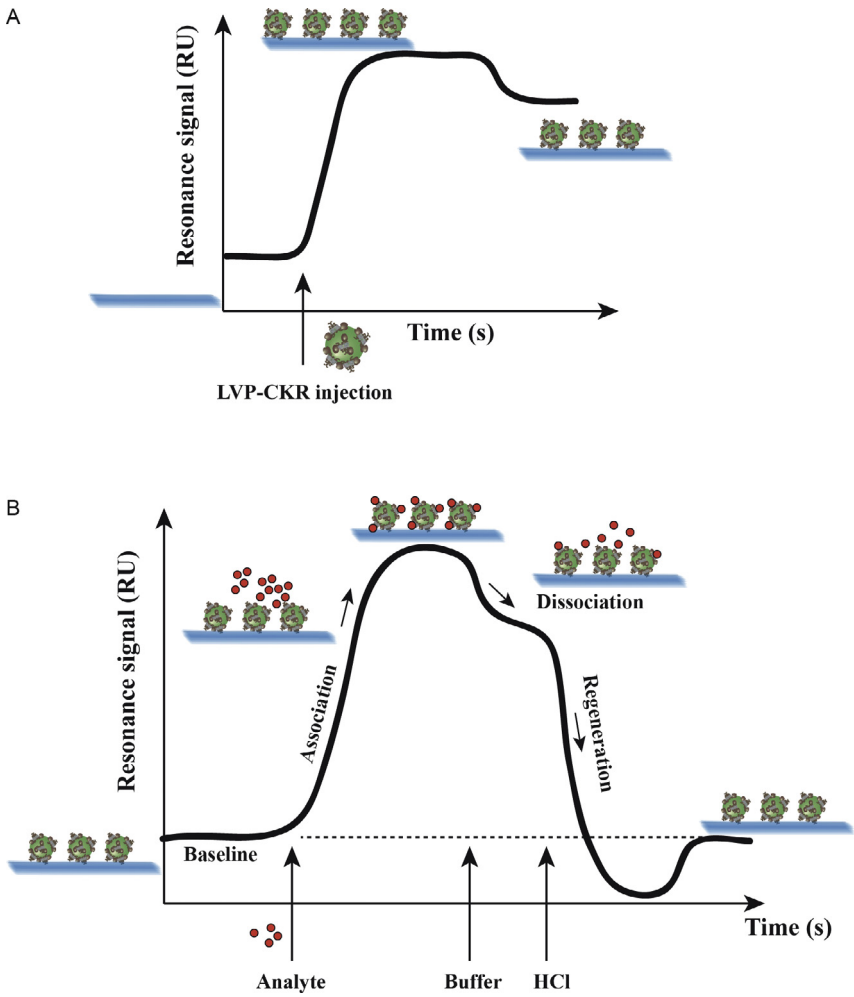

Figure 3 Scheme showing LVP attachment and ligand detection in a SPR device. As for purified proteins, LVPs are injected in the sensor in established salt and pH conditions to achieve maximum attachment to the dextran surface (A). The flow through the microfluidic chamber is maintained until the resonance signal stabilizes (baseline). The test analyte can now be injected (B) and changes in the resonance signal during the association and dissociation phases are recorded. Finally, the analyte is removed using appropriate buffers (regeneration) and the SPR-bound LVP is ready for further experiments.

CCR5. As a control, a reference chamber is used in which a CCR5 with a blocked binding site is retained in the same way. Although five CCR5-binding compounds were identified, with affinities in the 8.2–49 μ*M* range ligands (Navratilova et al., 2011), the method uses solubilized receptors and

therefore excludes the receptor's natural context. At least in theory, screening based on immobilized, receptor-bearing VPs would identify additional compounds that modulate chemokine responses *in vivo*, such as GAG, other chemokine receptors, or proteins that can bind to the receptor being studied.

As key mediators in many diseases, the chemokines have attracted considerable attention from researchers, and their detection in biological fluids could offer new possibilities for diagnosis of inflammatory and autoimmune disorders. A LVP bearing CXCR4 in its native plasma membrane context was used to develop a SPR method for rapid detection and quantification of analytes such as CXCL12 in biological samples, with no need for pretreatment (Vega et al., 2013); proof of concept was shown using a homemade biosensor (Mauriz et al., 2006). Gold chips are cleaned with trichloroethylene, acetone, and ethanol, immersed in piranha solution [H_2SO_4: H_2O_2 (3:1)], rinsed with water, ultrasonicated (5 min), and dried with an N_2 stream. The chip is placed over the flow cells and the prism is optically coupled to the chip using refractive index matching oil. A constant flow speed of 20 μl/min is maintained throughout the immobilization process. A carboxyl-terminated alkanethiol self-assembled monolayer (SAM) is then formed on the gold chip by flowing mercaptoundecanoic acid (0.05 m*M* solution in ethanol; 20 min); alkanethiol excess is rinsed with ethanol followed by water before the next immobilization step. The carboxylic surface is activated with 0.2 *M* EDC and 0.05 *M* NHS to form an NHS ester intermediate, followed immediately by injection of CXCR4-bearing LVP in acetate buffer (10 m*M* NaOAc). LVPs are thus coupled covalently to the ester via amine groups. The surface is blocked with 1 *M* ethanolamine pH 8.5. HCl (5 m*M*) is used to regenerate the surface completely, which permits more that 150 regeneration cycles with no notable signal loss relative to initial binding values (Fig. 3). The method allows detection of 5–40 n*M* CXCL12 in urine, is optimal in a 6.8–7.5 pH range, and although statistical significance must be corroborated with a larger sample cohort, it is clearly effective for detecting small molecules in urine samples.

6. CONCLUSIONS

SPR-based technologies provide an excellent method for monitoring interactions between ligands and their receptors. SPR studies of GPCR nonetheless present special difficulties, as these receptors are expressed at the cell membrane at relatively low levels and require a hydrophobic environment to maintain their native structure. In recent years, several protocols

have allowed successful solubilization, purification, reconstitution, and retention of correctly oriented GPCR at the surface of a sensor chip; these methods have demonstrated their value for evaluation of ligand-binding properties and have improved pharmacological studies. Many analyses have been developed to determine the role of solubilizing detergents, to select the best lipid composition for receptor reconstitution, and to orientate the receptor to increase the ligand-binding sites. Current evidence indicates that GPCR at the cell surface are not individual entities, but form dynamic oligomeric complexes that are modulated by receptor expression, ligand binding, the expression of other membrane proteins, and even the lipid composition. These factors obviously affect not only ligand binding, but also the pharmacological properties of the receptors. VPs offer new possibilities for these types of analyses. When a virus buds from the cell, it incorporates plasma membrane proteins into its lipid envelope, a unique means of reproducing native GPCR conformation(s) on the biosensor surface. Proof of concept using chemokine receptors demonstrates its validity and illustrates the potential for the use of VPs immobilized on SPR biosensors in ligand quantification, binding analysis, and drug screening.

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