Journal of Peptide Science
J. Peptide Sci. **9**: 77–89 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.439

JOURNAL OF PEPTIDE SCIENCE

Review

Surface Plasmon Resonance Spectroscopy in the Study of Membrane-Mediated Cell Signalling

HENRIETTE MOZSOLITS,[a] WALTER G. THOMAS[b] and MARIE-ISABEL AGUILAR[a]*

[a] Department of Biochemistry and Molecular Biology, PO Box 13D, Monash University, Clayton 3800, Victoria, Australia
[b] The Baker Heart Research Institute, PO Box 6492, St Kilda Rd Central, Melbourne 8008, Australia

Received 25 August 2002
Revised 29 August 2002

Abstract: Peptide–membrane interactions contribute to many important biological processes such as cellular signalling, protein trafficking and ion-channel formation. During receptor-mediated signalling, activated intracellular signalling molecules are often recruited into receptor-induced signalling complexes at the cytoplasmic surface of the cell membrane. Such recruitment can depend upon protein–protein and protein–lipid interactions as well as protein acylation. A wide variety of biophysical techniques have been combined with the use of model membrane systems to study these interactions and have provided important information on the relationship between the structure of these proteins involved in cell signalling and their biological function. More recently, surface plasmon resonance (SPR) spectroscopy has also been applied to the study of biomembrane-based systems using both planar mono- or bilayers or liposomes. This article provides an overview of these recent applications, which demonstrate the potential of SPR to enhance our molecular understanding of membrane-mediated cellular signalling.

Keywords: surface plasmon resonance spectroscopy; peptide–membrane interactions; signalling mechanisms; receptors

INTRODUCTION

Interactions between membranes and peptides/proteins are central to a number of cellular processes, including the insertion and folding of membrane proteins, the formation of ion channels, translocation of polypeptides through membranes, the interaction of peptide hormones with membrane receptors, signal transduction, the action of antimicrobial and cytotoxic peptides, lipolysis and blood coagulation [1,2]. In the context of signal transduction, the activation of many intracellular signalling molecules such as the myristoylated alanine-rich-kinase substrate (MARCKS) [3], protein kinase C [4], neuromodulin [5], secretory phospholipase A_2 [6], the HIV gag protein [7] and members of the Src tyrosine kinase family [3] involves recruitment of the protein into receptor-induced signalling complexes at the cytoplasmic surface of the cell membrane. This often involves protein–protein interactions as well as hydrophobic and electrostatic interactions between the positively charged face of a protein domain and the negatively charged membrane surface. Furthermore, discrete cholesterol-containing microdomains in the plasma membrane or so-called lipid rafts have also been shown to play a central role in the function of Ras proteins in signal transduction [8]. Indeed, the regulatory consequences

*Correspondence to: Dr Marie-Isabel Aguilar, Department of Biochemistry and Molecular Biology, PO Box 13D, Monash University, Clayton, Victoria 3800, Australia;
e-mail: mibel.aguilar@med.monash.edu.au

BIOGRAPHIES

Henriette Mozsolits

completed her doctoral studies in the Department of Biochemistry and Molecular Biology at Monash University in Melbourne under the supervision of Dr Mibel Aguilar. The title of her thesis was 'Surface Plasmon Resonance Spectroscopy for the Study of Peptide-Membrane Interactions'. She is presently a Research Scientist at the University of Pennsylvania, School of Medicine, USA.

Walter Thomas

is an NH&MRC Senior Research Fellow at the Baker Heart Research Institute in Melbourne. His research interests lie in the molecular processes that underlie the activation and deactivation of G protein coupled receptors with a particular focus on the angiotensin II receptor using a range of cellular and molecular approaches to investigate receptor signalling, phosphorylation, internalisation and protein–protein interactions. He also serves on the organizing committee of the Australian Peptide Conference.

Marie-Isabel (Mibel) Aguilar

is a Senior Lecturer in the Department of Biochemistry and Molecular Biology, Monash University in Melbourne. Her research focuses on peptide-membrane interactions and peptidomimetic design of enzyme inhibitors and T cell agonists/antagonists. The long-term aim of these studies is to increase our understanding of the conformational behaviour of peptides and proteins in biological environments and allow the rational design of peptide and protein based therapeutics. She also serves on the organising committee of the Australian Peptide Conference.

of the reversible membrane interactions of proteins classified as 'amphitropic' have been recently reviewed [9] and outline the critical importance of membrane interactions in signal transduction.

Apart from recruitment, subtle differences in the relative affinity of peptides and proteins for the phospholipids, as well as the orientation and degree of insertion of the peptide into the lipid bilayer, also contributes to the biological function of these proteins. Membrane-interacting peptides are commonly cationic in nature and undergo a transition from an extended conformation in aqueous solution to an amphipathic helical structure upon binding to the membrane [1,10]. Figure 1 shows some examples of interactions between peptides and membranes. The common structural feature of membrane-active peptides and proteins is the adoption of a stable secondary structure upon binding to the membrane surface. For example, for antimicrobial peptides, which act by perturbing the barrier function of the cell membrane, the induction of cationic, amphipathic α-helical structure is thought to play an essential role in their biological activity [11,12]. The orientation of a peptide at the membrane surface and/or the degree of insertion into the membrane interior could also play a role in the recruitment and assembly of signalling complexes.

To understand peptide–membrane interactions in detail, the role of numerous physicochemical parameters including peptide charge, hydrophobicity, amphipathicity and the degree of secondary structure and the angle subtended by the polar face, in the physicochemical interaction between peptides and the membrane have been analysed. A wide variety of biophysical techniques such as circular dichroism, nuclear magnetic resonance, fluorescence spectroscopy, Fourier transform infrared spectroscopy, attenuated total reflection Fourier transform infrared spectroscopy, immobilized artificial membrane chromatography combined with model membrane systems have been used to study biomolecular–membrane interactions [12–15]. These techniques have provided important information on specific structure–function relationships associated with peptide–membrane interactions.

To comprehend the role of membrane association in protein function, an analysis of the energetics of binding and insertion of peptides into the membrane, the complex interplay between membrane-induced conformational changes, and the associated variation in peptide or protein affinity during binding to a membrane surface is required. Hence, the complete characterization of the binding of peptides to lipid surfaces also requires the binding constants associated with the interactions to be determined. For example, the affinity of a specific protein for the

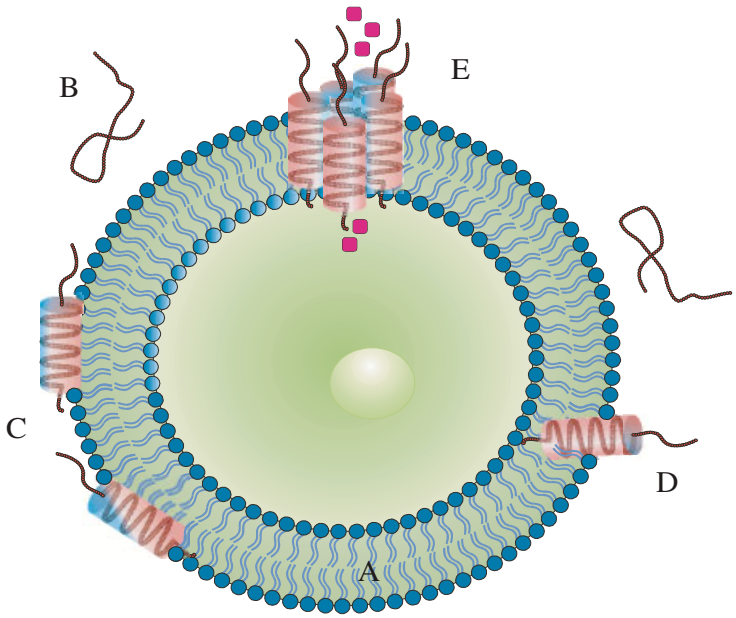

Figure 1 Schematic representation of bioactive peptide–membrane interactions. (A) the cell membrane, (B) the peptide is unstructured in an aqueous environment, (C) the peptide adopts an α-helical conformation upon binding to phospholipids, (D) the peptide inserting into the membrane, (E) ion channel formation.

membrane surface is a critical step in many signalling pathways, and selective binding to different phospholipids is central to understanding selective signalling events. It is also generally considered that the binding of membrane-active peptides to lipid membranes occurs via at least a two-step process [10,13]. Initially, the peptide binds electrostatically to the membrane localizing itself near the surface. The peptide then relocates on the surface or inserts further into the hydrocarbon region of the lipid membrane by hydrophobic interactions. Since the binding reaction is very fast, it is quite difficult to distinguish the two binding steps and therefore it is a challenge to study this process using conventional techniques [9].

In recent years, SPR spectroscopy has become a widely used technique to study antibody–antigen, DNA–DNA, DNA–protein, protein–protein and receptor–ligand interactions [16–18] and also to quantitate these interactions through the measurement of kinetic rate constants and affinity constants. More recently, SPR spectroscopy has also been applied to the study of biomembrane-based systems that involve planar mono- or bilayers or liposomes [19–24]. This article reviews the recent application of SPR to the study of the interaction of peptides involved in signalling pathways with biological membranes and demonstrates the potential of SPR to enhance our molecular understanding of membrane-mediated cell-signalling events.

SPR and Immobilized Membranes

SPR spectroscopy relies on the surface plasmon resonance phenomenon, which allows the real-time measurement of biomolecules binding to biomimetic surfaces without the application of a specific label as the method is dependent on the change in absorbed mass at the sensor surface. It is a surface-sensitive technique where the ligand is immobilized onto a solid support and the solute is kept in solution and the binding event can be readily detected and analysed. Figure 2 shows a schematic representation of an SPR system that consists of an SPR detector, light source, flow channel and a sensor chip. The technique can also be fully automated using the commercially available SPR instruments such as the BIACORE and IAsys instruments and large numbers of samples can be rapidly and conveniently analysed. Typically, in the BIACORE system ligands are immobilized on the surface of a sensor chip, which is covered by a thin gold layer (Figure 2) [16,25,26].

There are two commercially available chips that are suitable for studying membrane-based systems. BIACORE AB first released the HPA sensor chip, which consists of self-assembled alkanethiol

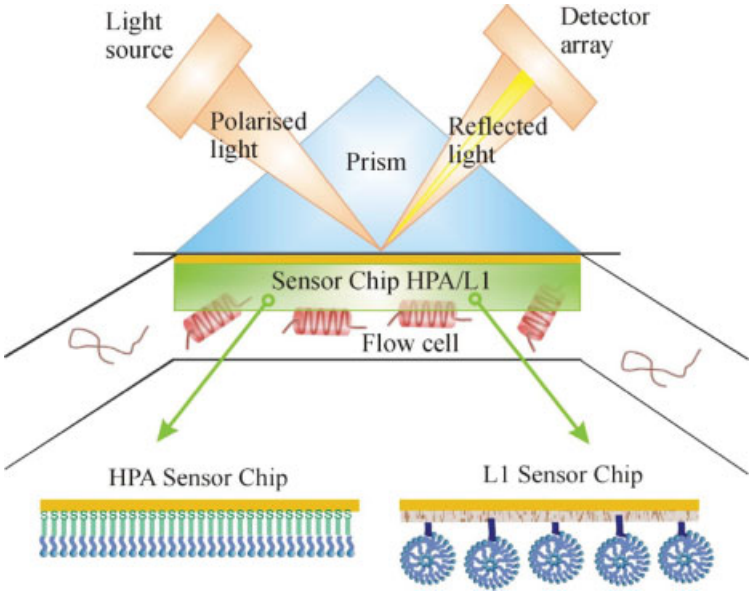

Figure 2 Binding of peptides onto the immobilized lipids (HPA) or liposomes (L1) surfaces in the BIACORE system.

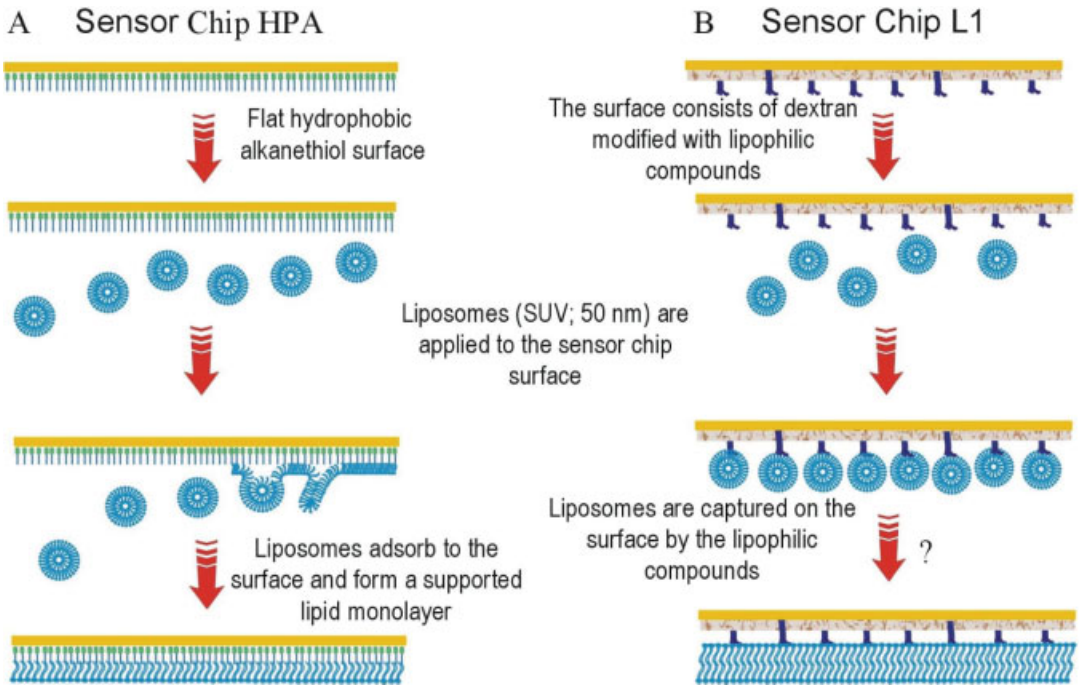

Figure 3 The preparation of a monolayer and a bilayer model membrane system on (A) an HPA and (B) an L1 sensor chip.

molecules covalently attached to the gold surface of the chip. As shown in Figure 3A, this chip can be used to prepare hybrid bilayer lipid membranes by the fusion of liposomes onto the hydrophobic surface [27]. The availability of the hydrophobic association (HPA) sensor chip significantly improved the preparation of solid supported lipid membranes to investigate membrane-mediated interactions. This sensor chip provides a hydrophobic surface on which liposomes fuse to form a hybrid bilayer membrane system similar to that prepared earlier by Plant *et al.* [28,29] by modification of a dextran-coated chip. There have since been a number of examples where this sensor chip has been applied to

the study of peptide–and protein–membrane interactions. These applications range from the analysis of protein–protein and protein–ligand interactions in a membrane environment to the study of the direct binding of peptides and proteins to a specific phospholipid surface [30].

For many applications, the covalent attachment of the hybrid bilayer formed with the HPA chip restricts the insertion of integral peptides and proteins. In order to provide a more appropriate experimental model for the characterization of these interactions, the vesicle capture (L1) sensor chip was introduced for analysis of model membrane systems by SPR. The L1 sensor chip is composed of a thin dextran matrix modified by lipophilic compounds on a gold surface, where the lipid bilayer system can be prepared through the capture of liposomes by the lipophilic compounds as shown in Figure 3B [21]. The immobilization of the biomimetic lipid surface onto the sensor chips is generally a fast and reproducible process. Both the HPA and L1 sensor chips can be conveniently applied to the study of membrane-based biomolecular interactions and to measure the binding affinity related to these interactions. An increasing number of examples of the use of this membrane surface in a wide range of biological applications has been discussed [30].

One issue that has yet to be completely resolved is whether the liposomes stay intact on the L1 sensor chip surface or fuse together to form a bilayer system tethered to the surface by the lipophilic anchors. These two possibilities were tested by atomic force microscopy and fluorescence microscopy [31]. Single liposomes could not be detected on the surface of the sensor chip and it was concluded that the captured liposomes (100 nm) fused together and formed a lipid bilayer on the surface of the L1 sensor chip. In contrast, others have shown by fluorescence microscopy using sulforhodamine-containing vesicles that the captured vesicles (100 nm) stayed intact on the L1 sensor chip surface forming an immobilized liposome layer [21]. Monitoring for the release of 5-carboxyfluoroscien, Cho and co-workers [32,33] also failed to detect any fluorescence following deposition of liposomes on the L1 chip, again suggesting that the liposomes remain intact. Regardless of the final orientation, this chip has been shown to be suitable for the generation of model membrane systems that provide a flexible lipid bilayer surface that closely resembles the surface of a cellular membrane.

Functional Reconstitution of Membrane-bound Receptors

Receptors bind specific ligands that initiate signalling cascades and the ability to study these interactions with purified receptor in a membrane environment is essential for unlocking the molecular details of these signalling events [34]. Prior to the release of the commercially available chips, the preparation of high-quality, lipid-membrane surfaces suitable for SPR-based biophysical studies of peptide and protein–membrane interactions was a significant technical challenge. The groups of Vogel *et al.* and Salamon and Tollin have pioneered these approaches and demonstrated the potential of SPR to provide important molecular insights into biomolecular membrane interactions. The supported lipid membrane initially developed by Vogel *et al.* [35] was prepared on a glass plate on which a thin gold film was first evaporated followed by the self-assembly of a layer of hydrophobic alkanethiol molecules that form covalent sulphur-gold bonds. In addition, their lipid bilayer systems possessed the flexibility, space and water required to keep incorporated membrane-spanning proteins in a functionally active form.

As a seminal example of the utility of such approaches, this tethered membrane system has been used to study the incorporation and function of the G protein-coupled receptor, rhodopsin, in membranes [36–38]. Rhodopsin is a membrane-bound receptor protein and its x-ray structure is the only one resolved for the G-protein-coupled receptor (GPCR) superfamily [39]. This large family of receptors activate heterotrimeric guanyl nucleotide binding proteins (G proteins) and comprise an extracellular amino terminus, seven transmembrane α-helical domains connected by alternating extracellular and intracellular loops and a cytoplasmic carboxyl terminal region, as depicted in Figure 4 for the angiotensin II receptor. The supported membranes described by Vogel's group were formed by the self-assembly of phospholipids and rhodopsin onto functionalized gold surfaces as depicted in Figure 5 and the formation of the surfaces and the biological function of rhodopsin were monitored by laterally and time-resolved surface plasmon resonance. The biological activity of the rhodopsin was maintained upon immobilization and this study demonstrated a potentially very useful method for the preparation of receptor-based biosensors.

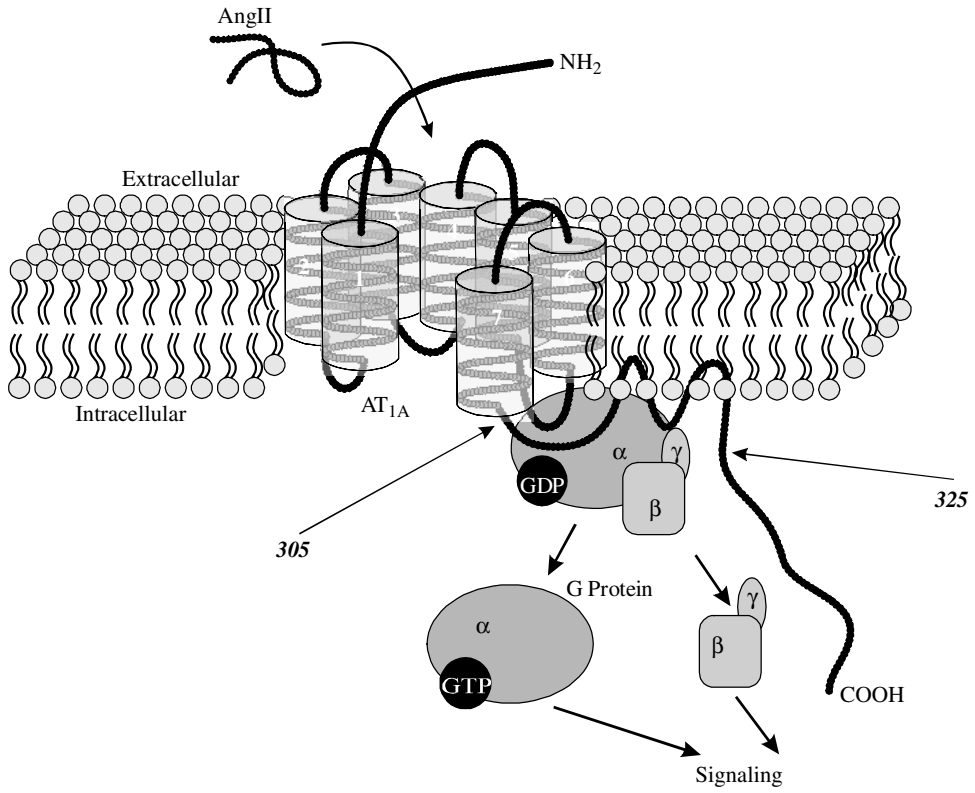

Figure 4 A schematic diagram of the AT_{1A} receptor showing the tethering of the carboxyl-terminal residues (305–325) to the membrane adjacent to transmembrane helix 7 and binding of the heterotrimericG proteins.

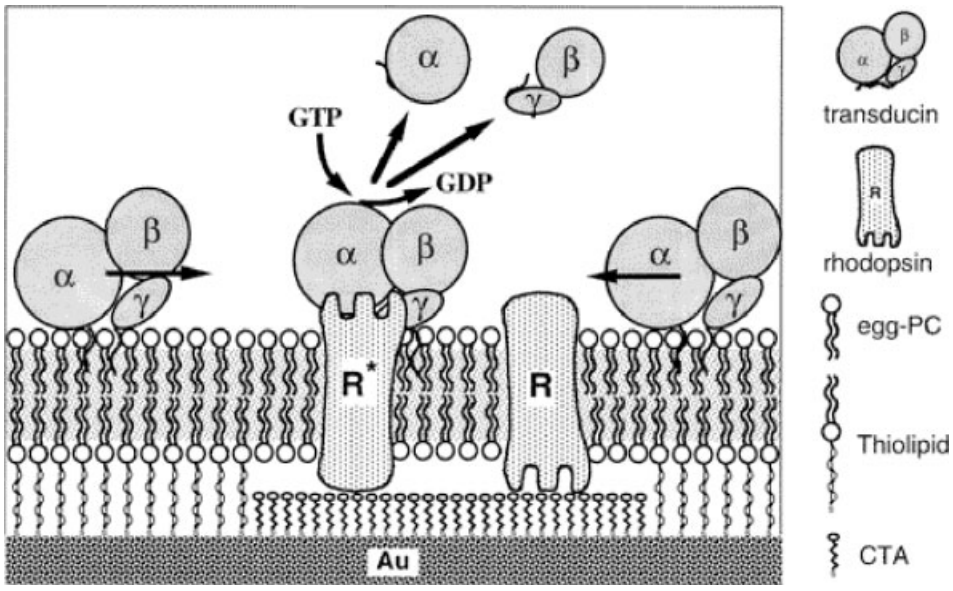

Figure 5 Schematic illustration of rhodopsin–transducin coupling on patterned supported membranes following a light flash. A gold surface (Au) serves as support for the membranes. It is functionalized with a patterned organic monolayer, consisting of alternating regions of carboxyl-exposing thiols (CTA, centre) and regions of hydrocarbon-exposing thiolipids (left and right). Rhodopsin (R) and phospholipids (egg-PC) are self-assembled onto this structured support, forming membranes which alternate between phospholipid bilayer domains (on CTA) and monolayer domains (on thiolipids). Reprinted with permission from Ref [37], Copyright 1998 American Chemical Society.

Salamon *et al.* have also developed SPR spectroscopy to study a wide range of biomolecular membrane interactions including protein–lipid binding, protein–protein interactions at a membrane surface and membrane receptor–ligand interactions [40–48]. Salamon *et al.* also demonstrated the use of SPR in the investigation of signal transduction by monitoring the binding and activation of G-protein (transducin or G_t) by bovine rhodopsin [47]. The supported egg PC lipid bilayer in which bovine rhodopsin was incorporated was also supported by a Teflon spacer over a silver film. It was found that transducin bound to the membrane-incorporated rhodopsin before and after photolysis, which can be monitored following the SPR spectral changes, and by estimating the average thickness of the protein/lipid film. After photolysis, the average thickness of the protein/lipid film increased, which could be related to the formation of metarhodopsin II. In addition, a further change can be seen in the SPR spectra upon addition of GTP resulting from the subsequent GTP/GDP exchange.

More recently, Tollin and co-workers developed a variant of SPR referred to as coupled plasmon-waveguide spectroscopy [42,46]. When applied to membrane-bound receptors for example, this technique allows changes in the structure of the receptor, both parallel and perpendicular to the lipid membrane, to be examined in response to the binding of ligand. In one example, it was found that binding of agonist to the human δ-opioid receptor caused an increase in the thickness and molecular packing density of the membrane, while antagonist binding did not show this effect. The results suggested differences in the degree of transmembrane helical reorientation upon ligand binding and provided direct evidence for the differential effects of agonists and antagonists upon membrane structure. These studies clearly provide important information on the structural changes, which occur in both the membrane and the receptor during the early stages of a signalling cascade.

Commercial biosensors are now being used to generate receptor-containing membrane preparations. For example, the binding of HIV envelope protein to the chemokine receptors CCR5 and CXCR4 which were embedded into retrovirus particles was recently demonstrated [49]. The virus particles containing the receptors were immobilized through amino coupling of the receptors to the F1 chip, and the functionality of the receptors was demonstrated with binding of specific antibodies and inhibition of binding by peptide inhibitors. This study demonstrated that a wide range of membrane proteins could be immobilized onto a biosensor surface using this viral carrier approach. More recently, Plant and colleagues used a custom-built instrument to generate hybrid bilayers derived from membrane vesicles of COS-1 cells over-expressing the CCR5 receptor [50]. The formation and morphology of the hybrid bilayer membrane was characterized by SPR and atomic force microscopy. The study also showed that the receptors were capable of specifically binding its ligand RANTES in a concentration-dependent manner (i.e. the 'right side out' orientation) and that the membrane preparations also contained 'inside-out' receptors which could be used to study intracellular signalling reactions. These results demonstrated a potential method for the rapid screening of potential receptor ligands.

Another approach to the immobilization of functional receptors was recently reported again using rhodopsin [51]. This method involved the covalent attachment of detergent-solubilized rhodopsin to an L1 chip via conventional amine coupling chemistry. This was then followed by injection of lipid/detergent-mixed micelles over the biosensor surface that resulted in the formation of a lipid bilayer after washing with buffer. The immobilized rhodopsin was shown to be functional by the dissociation of bound transducin following exposure to UV light. Furthermore, the rhodopsin could be recharged by injection of the agonist 9-*cis*-retinal, which demonstrated that both the agonist binding domain and the G-protein activating domains were accessible on the immobilized receptor. This procedure therefore provides a potentially routine method for the functional reconstitution and characterization of receptor signalling pathways.

Membrane-mediated cell signalling often involves proteins that have undergone post-translational lipid modification of critical cysteine residues. However, the preparation of recombinant lipid-modified proteins in high yield presents a significant technical challenge. A recent study by Bader *et al.* reported a method for the covalent coupling of lipidated peptides to recombinantly derived proteins [52]. They demonstrated their strategy using the modification of Ras constructs with different lipid molecules and used SPR to show that the modified Ras proteins can insert into artificial membranes prepared on an HPA chip. In addition, they demonstrated that the Ras proteins also inserted into biological membranes and exhibited comparable biological activity to the non-modified full-length protein in terms of cell differentiation. This study

thus demonstrated a very useful technique to generate large amounts of lipidated protein useful for biophysical and biochemical studies of signalling pathways, which are mediated by lipid-modified proteins.

Intracellular Membrane-tethering of Receptors

For many GPCRs, acylation of cysteine residues in the *C*-terminus and the subsequent insertion of the acyl group into the lipid bilayer is a dynamic agonist-regulated process that serves to tether the cytoplasmic tail to the inner face of the cell membrane. In contrast, other GPCRs, such as the angiotensin II type I receptor (AT_{1A}) do not contain cysteines in key sequence positions and are not acylated either basally or following stimulation but may interact with the membrane through a reversible amphipathic domain in the protein structure. The membrane tethering of the cytoplasmic *C*-terminus of the AT_{1A} receptor was studied through analysis of the membrane binding characteristics of a peptide (AT) corresponding to the proximal region of the AT_{1A} receptor carboxyl terminus (residues 305–325) and its LGJ analogue (possessing substitutions of four basic (Lys) residues putatively involved in molecular recognition) with different surface charged model bilayer membranes [53]. The capability of this positively charged, amphipathic α-helical region of the AT_{1A} receptor that is believed to be a focal point for receptor activation and deactivation was examined in relation to its ability to interact with lipid components in the cell membrane and thereby modulate local receptor attachment and structure. As illustrated in Figure 6, the AT peptide bound with high affinity to the negatively charged lipids (DMPG), but poorly to the zwitterionic lipids (DMPC). In contrast, the LGJ analogue, displayed poor association with both lipids, indicating that the anionic component is crucial to the interaction. CD analysis revealed that AT adopted helical structure and was partially inserted into the membrane, while LGJ adopted

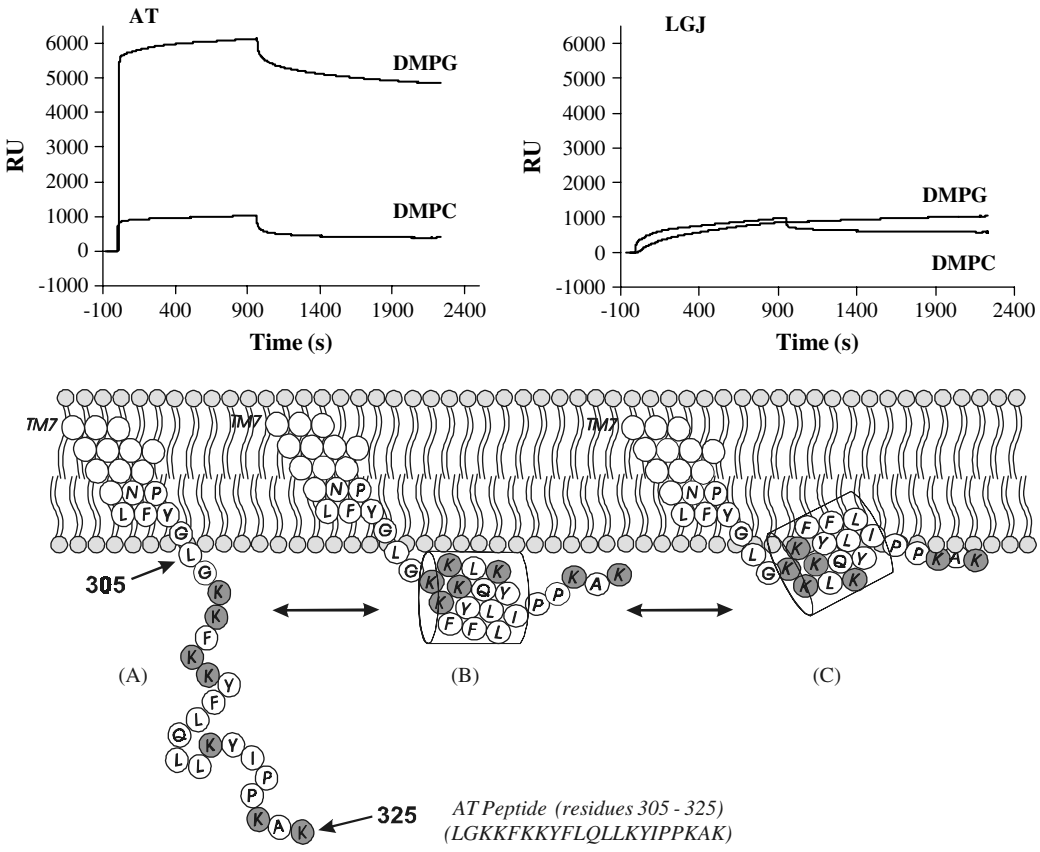

Figure 6 Sensorgrams (plots of RU (response units) *versus* time) for AT and LGJ peptides at 40 μM binding to DMPG and DMPC liposomes immobilized on the L1 sensor chip surface and model of the binding of AT peptide to anionic phospholipids in the membrane. Reprinted with permission from Ref [53], Copyright 2002, American Chemical Society.

a mixture of sheet and helix structure in DMPG and DMPC liposome solutions. These findings suggest that in intact AT_{1A} receptors, the proximal carboxyl-terminus associates with the cytoplasmic face of the cell membrane via a high affinity, anionic phospholipid-specific tethering that serves to increase the amphipathic helicity of this region [53]. Such associations may be important for receptor function and common for G protein-coupled receptors and a recent study has proposed that helix 8 of rhodopsin may act as a membrane-dependent conformational switch [54].

Interaction of Peripheral Membrane Proteins

The activation of signalling pathways via the binding of ligand to receptor often culminates in the recruitment of signal proteins to the cell membrane. What follows is a selection of examples where SPR has been used to study the membrane-binding features of several amphitropic, signalling proteins.

Recoverin is an amphitropic, *N*-myristoylated Ca^{2+}-binding protein that serves as a calcium sensor in visual transduction and the Ca^{2+}-dependent membrane association of recoverin is also dependent on the myristoyl modification (or Ca^{2+}-myristoyl switch). Lange *et al.* [55] used SPR to study the mechanism of the Ca^{2+}-myristoyl switch of recoverin and prepared a lipid-modified chip via the immobilization of biotinylated liposomes onto chips prederivatized with streptavidin. The binding of recoverin to either artificial liposomes or liposomes derived from membranes of the rod outer segment was dependent on both Ca^{2+} and the myristoyl group. The association kinetics for recoverin was fast and biphasic while dissociation was faster at lower Ca^{2+} concentrations. Overall, it was concluded that a small proportion of the total recoverin associates with the membrane at resting (dark) levels of free Ca^{2+}. In a complementary approach, another group studied the binding of Ca^{2+}-containing liposomes to immobilized recoverin [56]. Large changes in SPR signals were observed with a range of metals and they concluded that a large conformational change occurs upon metal binding followed by membrane binding.

A hybrid bilayer membrane was used to study the regulation of phospholipase D (PLD) activity in relation to its role in the release of phosphatidic acid and subsequent signalling pathways [57]. The isoform PLD1 was found to bind specifically to monolayers containing phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) but interacted weakly with surfaces containing phosphatidylserine or ($PI(3,4,5)P_2$). When correlated with the activity of the parent enzyme and mutants protein with changes in the putative pleckstrin homology (PH) domain, it was concluded that the functional PH domain regulates PLD by mediating its interaction with polyphosphoinositide-containing membranes, which may also be associated with a conformational change and regulation of the catalytic activity.

A further example of the use of SPR to probe the role of membrane interactions in signalling processes is a recent report [58] that studied the specific binding of Phox homology (PX) domains with phosphatidylinositol 3-phosphate (PtdIns-3-P) containing liposomes. The binding of a series of PX domain analogues to liposomes containing DOPC with and without PtdIns-3-P were measured and the results demonstrated a high affinity for PtdIns-3-P, suggesting that PtdIns-3-P may drive recruitment of proteins to the cytoplasmic or vacuolar membranes, where it is present in high amounts. Overall, these studies demonstrate the power of SPR in elucidating the molecular details of cellular signalling processes.

Neurocalcin is a member of the family of neuronal calcium sensors that belongs to the superfamily of EF-hand Ca^{2+}-binding proteins. Neurocalcin is myristoylated on its *N*-terminus and can associate with biological membranes in a Ca^{2+}- and myristoyl-dependent manner. The membrane binding properties of monoacylated neurocalcins modified with different acyl chains was studied by SPR and co-sedimentation [59]. The results indicated that neurocalcin was able to associate with membranes comprising brain-derived lipids irrespective of whether it was modified with lauric, myristic or palmitic acid, indicating that the Ca^{2+}-myristoyl switch can function with different lipid moieties.

Cho and colleagues have used the L1 chip to study the interaction of several peripheral membrane proteins with model membranes [32,33,60–64]. These studies particularly focused on the role of electrostatic and hydrophobic interactions between the protein and the membrane through analysis of a series of protein mutants. In one study, the role of cationic, aliphatic and aromatic residues in the membrane binding of five phospholipases A_2 was studied using liposomes composed of either 1,2-di-*O*-hexadecy-*sn*-glycero-3-phosphocholine (DHPC) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) [62]. The results allowed them to propose a model for the interfacial

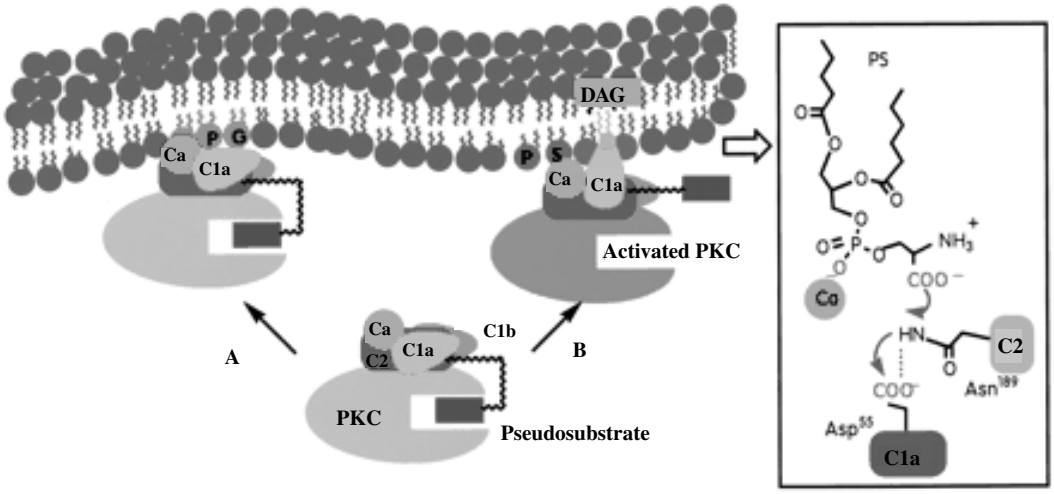

Figure 7 A proposed mechanism of the *in vitro* membrane binding and activation of conventional PKC based on SPR analysis of membrane interactions. In this model, the C1a domain and the C2 domain are tethered via a hydrogen bond between Asp55 and a C2 domain residue (e.g. Asn189). When the protein binds to PG-containing membranes (case A), the C1a-C2 tethering remains intact, and consequently PKC remains largely inactive. When the protein binds to PS-containing membranes (case B, see the inset), however, the carboxylate of PS releases Asp55 of the C1a domain from the tethering, resulting in the membrane penetration and DAG binding of the C1a domain and PKC activation Reproduced with permission from Ref [63], Copyright 2001, American Society for Biochemistry and Molecular Biology.

binding of peripheral proteins in which the protein binds initially to the membrane through electrostatic interactions, followed by penetration into the membrane via hydrophobic interactions.

This approach was also exploited in the study of the role of the C1 and C2 membrane targeting domains of protein kinase C-α (PKC-α) [32,63,64]. In particular, these studies investigated the role of Ca^{2+} in the membrane binding of these domains. Based on the differential binding of specifically mutated analogues, they proposed that the initial binding of PKC-α is driven by electrostatic interactions via the C2 domain which is mediated by a bound Ca^{2+} ion and specific residues in the C1a domain. This is then followed by disruption of the C1a tethering by a molecule of phosphatidylserine (PS) which in turn leads to membrane penetration and diacylglycerol binding of the C1a domain and PKC activation (see Figure 7). In order to further characterize the mechanism by which the C2 domains mediate the membrane targeting of phosphatidylinositol-specific phospholipases C-δ isoforms (PLC), the membrane-binding of the C2 domains of PLC-δ1, PLC-δ3 and PLC-δ4 was studied by SPR using the L1 chip and correlated with subcellular localization with time-lapse confocal microscopy [64]. It was found that the membrane binding of PLC-δ1-C2 and PLC-δ3-C2 involved Ca^{2+}-induced electrostatic interactions and PS coordination which in turn controlled the Ca^{2+}-dependent subcellular targeting to the plasma membrane. In contrast, PLC-δ4-C2 exhibited a Ca^{2+}-independent membrane binding and no selectivity for PS, which coincided with a prelocalization to the membrane prior to Ca^{2+} import and non-selective Ca^{2+}-mediated targeting to a range of cellular membranes. Together, these studies demonstrated that C2 domains of PLC-δ isoforms as Ca^{2+} dependent membrane-targeting domains and play a role in subcellular localization of these proteins.

Finally, the membrane-aggregating activity of annexin I has been examined using SPR [33]. Annexins are a family of proteins that reversibly bind membrane containing anionic phospholipids in a Ca^{2+}-dependent manner and these studies were aimed at delineating the mechanism of annexin I-mediated membrane aggregation. The binding of annexin I to immobilized liposomes was monitored and then followed by a secondary binding of another solution of liposomes to monitor liposome aggregation. The results revealed a direct correlation between the membrane aggregation activity and the relative affinity for the secondary membrane and support a model whereby annexin I first binds to specific phospholipids in the membrane and then interacts directly with the secondary membrane components via hydrophobic interactions. These

results can be compared to a recent study which used a quartz crystal microbalance technique to study the interaction of annexin A1 with solid-supported bilayers immobilized on gold electrodes [65]. They investigated the effect of cholesterol on the affinity and rate of membrane interactions with different phospholipids and further illustrate the molecular insight that can be obtained with membrane-based biosensors.

CONCLUSIONS

Characterization of biomolecular-membrane interactions is required to elucidate complex cellular processes. While SPR is an established approach to examine protein–protein interactions, recent studies described in this review, have demonstrated its utility for studying the signalling events mediated through specific protein: membrane interactions. In particular, the opportunity to reproduce an entire signalling cascade within an isolated membrane environment has the potential to transform our understanding of cellular signalling. Furthermore, the capacity to determine the membrane binding affinities of proteins or peptides and to characterize multiple binding steps in membrane interactions will provide new and important information, which will enhance our understanding of the molecular details of signalling events that occur both at the plasma membrane and in major cell organelles.

REFERENCES

1. Deber CM, Li SC. Peptides in membranes: helicity and hydrophobicity. *Biopolymers* 1995; **37**: 295–318.
2. Wimley WC, White SH. Peptides in lipid bilayers: structural and thermodynamic basis for partitioning and folding. *Curr. Opin. Struct. Biol.* 1994; **4**: 79–86.
3. Victor K, Jacob J, Cafiso DS. Interactions controlling the membrane binding of basic protein domains: phenylalanine and the attachment of the myristoylated alanine-rich C-kinase substrate protein to interfaces. *Biochemistry* 1999; **38**: 12 527–12 536.
4. Mosior M, McLaughlin S. Peptides that mimic the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes. *Biophys. J.* 1991; **60**: 149–159.
5. Wertz SL, Savino Y, Cafiso DS. Solution and membrane bound structure of a peptide derived from the protein kinase C substrate domain of neuromodulin. *Biochemistry* 1996; **35**: 11 104–11 112.
6. Berg OG, Yu BZ, Rogers J, Jain MK. Interfacial catalysis by phospholipase A2: determination of the interfacial kinetic rate constants. *Biochemistry* 1991; **30**: 7283–7297.
7. Zhou W, Parent LJ, Wills JW, Resh MD. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J. Virol.* 1994; **68**: 2556–2569.
8. Prior IA, Harding A, Yan J, Sluimer J, Parton RG, Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. *Nat. Cell. Biol.* 2001; **3**: 368–375.
9. Johnson JE, Cornell RB. Amphitropic proteins: regulation by reversible membrane interactions (review). *Mol. Membr. Biol.* 1999; **16**: 217–235.
10. Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim. Biophys. Acta* 1999; **1462**: 55–70.
11. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–395.
12. Blondelle SE, Lohner K, Aguilar M. Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity. *Biochim. Biophys. Acta* 1999; **1462**: 89–108.
13. Bechinger B. The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy. *Biochim. Biophys. Acta* 1999; **1462**: 157–183.
14. Haris PI, Chapman D. The conformational analysis of peptides using Fourier transform IR spectroscopy. *Biopolymers* 1995; **37**: 251–263.
15. Mozsolits H, Lee TH, Wirth HJ, Perlmutter P, Aguilar MI. The interaction of bioactive peptides with an immobilized phosphatidylcholine monolayer. *Biophys. J.* 1999; **77**: 1428–1444.
16. Fivash M, Towler EM, Fisher RJ. BIAcore for macromolecular interaction. *Curr. Opin. Biotechnol.* 1998; **9**: 97–101.
17. Malmqvist M, Karlsson R. Biomolecular interaction analysis: affinity biosensor technologies for functional analysis of proteins. *Curr. Opin. Chem. Biol.* 1997; **1**: 378–383.
18. Cooper MA. Optical biosensors in drug discovery. *Nat. Rev. Drug Discov.* 2002; **1**: 515–528.
19. Kuziemko GM, Stroh M, Stevens RC. Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. *Biochemistry* 1996; **35**: 6375–6384.
20. Heyse S, Stora T, Schmid E, Lakey JH, Vogel H. Emerging techniques for investigating molecular interactions at lipid membranes. *Biochim. Biophys. Acta* 1998; **1376**: 319–338.

21. Cooper MA, Hansson A, Lofas S, Williams DH. A vesicle capture sensor chip for kinetic analysis of interactions with membrane-bound receptors. *Anal. Biochem.* 2000; **277**: 196–205.
22. Wang W, Smith DK, Moulding K, Chen HM. The dependence of membrane permeability by the antibacterial peptide cecropin B and its analogs, CB-1 and CB-3, on liposomes of different composition. *J. Biol. Chem.* 1998; **273**: 27 438–27 448.
23. Saenko E, Sarafanov A, Ananyeva N, Behre E, Shima M, Schwinn H, Josic D. Comparison of the properties of phospholipid surfaces formed on HPA and L1 biosensor chips for the binding of the coagulation factor VIII. *J. Chromatogr. A* 2001; **921**: 49–56.
24. Mozsolits H, Wirth HJ, Werkmeister J, Aguilar MI. Analysis of antimicrobial peptide interactions with hybrid bilayer membrane systems using surface plasmon resonance. *Biochim. Biophys. Acta* 2001; **1512**: 64–76.
25. Morton TA, Myszka DG. Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Methods Enzymol.* 1998; **295**: 268–294.
26. Rich RL, Myszka DG. Advances in surface plasmon resonance biosensor analysis. *Curr. Opin. Biotechnol.* 2000; **11**: 54–61.
27. Cooper MA, Try AC, Carroll J, Ellar DJ, Williams DH. Surface plasmon resonance analysis at a supported lipid monolayer. *Biochim. Biophys. Acta* 1998; **1373**: 101–111.
28. Plant AL, Brigham-Burke M, Petrella EC, O'Shannessy DJ. Phospholipid/alkanethiol bilayers for cell-surface receptor studies by surface plasmon resonance. *Anal. Biochem.* 1995; **226**: 342–348.
29. Hubbard JB, Silin V, Plant AL. Self assembly driven by hydrophobic interactions at alkanethiol monolayers: mechanisms of formation of hybrid bilayer membranes. *Biophys. Chem.* 1998; **75**: 163–176.
30. Mozsolits H, Aguilar MI. Surface plasmon resonance spectroscopy: An emerging tool for the study of peptide–membrane interactions. *Biopolymers* 2002; **66**: 3–18.
31. Erb EM, Chen X, Allen S, Roberts CJ, Tendler SJ, Davies MC, Forsen S. Characterization of the surfaces generated by liposome binding to the modified dextran matrix of a surface plasmon resonance sensor chip. *Anal. Biochem.* 2000; **280**: 29–35.
32. Stahelin RV, Cho W. Roles of calcium ions in the membrane binding of C2 domains. *Biochem. J.* 2001; **359**: 679–685.
33. Bitto E, Li M, Tikhonov AM, Schlossman ML, Cho W. Mechanism of annexin I-mediated membrane aggregation. *Biochemistry* 2000; **39**: 13 469–13 477.
34. Subrahmanyam S, Piletsky SA, Turner AP. Application of natural receptors in sensors and assays. *Anal. Chem.* 2002 **74**: 3942–3951.
35. Terrettaz S, Stora T, Duschl C, Vogel H. Protein binding to supported lipid membranes: investigation of the cholera toxin-ganglioside interaction by simultaneous impedance spectrosopy and surface plasmon spectroscopy. *Langmuir* 1993; **9**: 1361–1369.
36. Lang H, Duschl C, Vogel H. A new class of thiolipids for the attachment of lipid bilayers on gold surfaces. *Langmuir* 1994; **10**: 197–210.
37. Heyse S, Ernst OP, Dienes Z, Hofmann KP, Vogel H. Incorporation of rhodopsin in laterally structured supported membranes: observation of transducin activation with spatially and time-resolved surface plasmon resonance. *Biochemistry* 1998; **37**: 507–522.
38. Bieri C, Ernst OP, Heyse S, Hofmann KP, Vogel H. Micropatterned immobilization of a G protein-coupled receptor and direct detection of G protein activation. *Nat. Biotechnol.* 1999; **17**: 1105–1108.
39. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 2000; **289**: 739–745.
40. Salamon Z, Brown MF, Tollin G. Plasmon resonance spectroscopy: probing molecular interactions within membranes. *Trends Biochem. Sci.* 1999; **24**: 213–219.
41. Salamon Z, Wang Y, Tollin G, Macleod HA. Assembly and molecular organization of self-assembled lipid bilayers on solid substrates monitored by surface plasmon resonance spectroscopy. *Biochim. Biophys. Acta* 1994; **1195**: 267–275.
42. Salamon Z, Lindblom G, Rilfors L, Linde K, Tollin G. Interaction of phosphatidylserine synthase from *E. coli* with lipid bilayers: coupled plasmon-waveguide resonance spectroscopy studies. *Biophys. J.* 2000; **78**: 1400–1412.
43. Salamon Z, Meyer TE, Tollin G. Photobleaching of the photoactive yellow protein from *Ectothiorhodospira halophila* promotes binding to lipid bilayers: evidence from surface plasmon resonance spectroscopy. *Biophys. J.* 1995; **68**: 648–654.
44. Salamon Z, Tollin G. Surface plasmon resonance studies of complex formation between cytochrome C and bovine cytochrome C oxidase incorporated into a supported planar lipid bilayer. II. Binding of cytochrome C to oxidase-containing cardiolipin/phosphatidylcholine membranes. *Biophys. J.* 1996; **71**: 858–867.
45. Salamon Z, Tollin G. Surface plasmon resonance studies of complex formation between cytochrome C and bovine cytochrome C oxidase incorporated into a supported planar lipid bilayer. I. Binding of cytochrome C to cardiolipin/phosphatidylcholine membranes in the absence of oxidase. *Biophys. J.* 1996; **71**: 848–857.
46. Salamon Z, Tollin G. Optical anisotropy in lipid bilayer membranes: coupled plasmon-waveguide resonance measurements of molecular orientation, polarizability, and shape. *Biophys. J.* 2001; **80**: 1557–1567.
47. Salamon Z, Wang Y, Soulages JL, Brown MF, Tollin G. Surface plasmon resonance spectroscopy studies of membrane proteins: transducin binding and activation

by rhodopsin monitored in thin membrane films. *Biophys. J.* 1996; **71**: 283–294.
48. Soulages JL, Salamon Z, Wells MA, Tollin G. Low concentrations of diacylglycerol promote the binding of apolipophorin III to a phospholipid bilayer: a surface plasmon resonance spectroscopy study. *Proc. Natl Acad. Sci. USA* 1995; **92**: 5650–5654.
49. Hoffman TL, Canziani G, Jia L, Rucker J, Doms RW. A biosensor assay for studying ligand-membrane receptor interactions: binding of antibodies and HIV-1 Env to chemokine receptors. *Proc. Natl Acad. Sci. USA* 2000; **97**: 11 215–11 220.
50. Madhusudhana Rao N, Silin V, Ridge KD, Woodward JT, Plant AL. Cell membrane hybrid bilayers containing the G-protein-coupled receptor CCR5. *Anal. Biochem.* 2002; **307**: 117–130.
51. Karlsson OP, Lofas S. Flow-mediated on-surface reconstitution of g-protein coupled receptors for applications in surface plasmon resonance biosensors. *Anal. Biochem.* 2002; **300**: 132–138.
52. Bader B, Kuhn K, Owen DJ, Waldmann H, Wittinghofer A, Kuhlmann J. Bioorganic synthesis of lipid-modified proteins for the study of signal transduction. *Nature* 2000; **403**: 223–226.
53. Mozsolits H, Unabia S, Ahmad A, Morton C, Thomas WG, Aguilar MI. Electrostatic and hydrophobic forces tether the proximal region of the angiotensin II receptor (AT1A) carboxyl-terminus to the cell membrane. *Biochemistry.* 2002; **41**: 7830–7840.
54. Krishna AG, Menon ST, Terry TJ, Sakmar TP. Evidence that helix 8 of rhodopsin acts as a membrane-dependent conformational switch. *Biochemistry* 2002; **41**: 8298–8309.
55. Lange C, Koch KW. Calcium-dependent binding of recoverin to membranes monitored by surface plasmon resonance spectroscopy in real time. *Biochemistry* 1997; **36**: 12 019–12 026.
56. Ozawa T, Fukuda M, Nara M, Nakamura A, Komine Y, Kohama K, Umezawa Y. How can Ca^{2+} selectively activate recoverin in the presence of Mg^{2+}? Surface plasmon resonance and FT-IR spectroscopic studies. *Biochemistry* 2000; **39**: 14 495–14 503.
57. Hodgkin MN, Masson MR, Powner D, Saqib KM, Ponting CP, Wakelam MJ. Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-biphosphate-specific PH domain. *Curr. Biol.* 2000; **10**: 43–46.
58. Yu JW, Lemmon MA. All Phox homology (PX) domains from *Saccharomyces cerevisiae* specifically recognize phosphatidylinositol 3-phosphate. *J. Biol. Chem.* 2001; **276**: 44 179–44 184.
59. Beven L, Adenier H, Kichenama R, Homand J, Redeker V, Le Caer JP, Ladant D, Chopineau J. Ca^{2+}-myristoyl switch and membrane binding of chemically acylated neurocalcins. *Biochemistry* 2001; **40**: 8152–8160.
60. Cho W. Membrane targeting by C1 and C2 domains. *J. Biol. Chem.* 2001; **276**: 32 407–32 410.
61. Cho W, Bittova L, Stahelin RV. Membrane binding assays for peripheral proteins. *Anal. Biochem.* 2001; **296**: 153–161.
62. Stahelin RV, Cho W. Differential roles of ionic, aliphatic, and aromatic residues in membrane–protein interactions: a surface plasmon resonance study on phospholipases A2. *Biochemistry* 2001; **40**: 4672–4678.
63. Bittova L, Stahelin RV, Cho W. Roles of ionic residues of the C1 domain in protein kinase C-alpha activation and the origin of phosphatidylserine specificity. *J. Biol. Chem.* 2001; **276**: 4218–4226.
64. Ananthanarayanan B, Das S, Rhee SG, Murray D, Cho W. Membrane targeting of C2 domains of phospholipase C-delta isoforms. *J. Biol. Chem.* 2002; **277**: 3568–3575.
65. Kastl K, Ross M, Gerke V, Steinem C. Kinetics and thermodynamics of annexin A1 binding to solid-supported membranes: a QCM study. *Biochemistry* 2002; **41**: 10 087–10 094.

