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This Application note describes how label-free interaction analysis using Biacore™ systems provided data to explain the mechanistic basis for the specific subcellular targeting properties of two peripheral proteins, cytosolic phospholipase A_2 ($cPLA_2$) and protein kinase C-α (PKC-α). In combination with live cell imaging, Biacore systems were used to characterize the membrane binding properties of these proteins. The results show that subcellular targeting of $cPLA_2$ and PKC-α can be explained in terms of their physicochemical lipid-binding properties.

Introduction

Understanding the plethora of complex pathways that transduce extracellular signals into controlled cellular responses remains a major scientific challenge. The normal function and pathological deregulation of cellular signaling pathways are of profound importance for many areas of biomedical science. While much progress has been made in identifying the large number of different biomolecules (including proteins, lipids, and nucleic acids) that comprise signaling cascades, many questions remain surrounding the mechanistic details of the intricate networks of the molecular interactions involved.

One important feature of cellular signaling involves the translocation of signaling proteins to specific cell membranes following cell activation. Peripheral membrane proteins are particularly important in this regard, and numerous studies have demonstrated the translocation of such proteins to specific membranes in response to activating signals (1). Although some of the conserved structural domains involved in this type of translocation have been identified, the underlying molecular mechanisms are not yet fully understood.

Cytosolic phospholipase A_2 ($cPLA_2$) is a lipolytic enzyme of pharmacological importance, which hydrolyzes membrane phospholipids and produces arachidonic acid (2). Protein kinase C-α (PKC-α) is a member of an important family of serine/threonine kinases involved in signal transduction pathways in a wide range of cellular functions (3).

Both $cPLA_2$ and PKC-α contain C2 (PKC conserved 2) domains, which have been shown to be important for calcium-dependent membrane targeting of peripheral proteins. These two proteins exhibit quite distinct lipid-binding preferences and calcium-dependent subcellular targeting specificities; $cPLA_2$ binds preferentially to phosphatidylcholine (PC) (4) and translocates to the perinuclear region in response to calcium (5), while, PKC-α translocates to the plasma membrane (6) and shows selective binding to phosphatidylserine (PS) (7). Stahelin *et al.* explored the possibility that these two characteristics may be functionally linked, by means of parallel studies using label-free interaction analysis to characterize membrane binding properties, and live cell imaging of fluorophore-tagged proteins to observe Ca^{2+}-dependent translocations (8). The results show that the subcellular targeting of C2 domains can be simply explained in terms of their physicochemical lipid-binding properties.

Methods

Label-free interaction analysis of protein binding to model membranes

Binding of C2 domains to model "plasma" and "perinuclear" membranes was assayed by coating vesicles of varying specific lipid composition onto sensor surfaces. For $cPLA_2$-C2 analysis, the sensor surface was coated with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) vesicles, representing specific and reference membranes, respectively. For PKC-α-C2 analysis, specific and reference surfaces were prepared with vesicles containing a 7:3 ratio of POPC/POPS, or a 7:3 ratio of POPC/POPG.

Control surfaces were prepared using BSA for $cPLA_2$-C2, or 100% POPC for PKC-α-C2 (since this is known to be an extremely low affinity binding). All interaction measurements were made at 24°C, with a 10 mM HEPES running buffer (pH 7.4) containing 0.1 M NaCl and 0.1 mM Ca^{2+}, and a flow rate of 60 µl/min to minimize mass transport effects. Association and dissociation rates were monitored for 90 s and 4 min, respectively.

A minimum of five concentrations were used for each protein, typically within a range of +/- 10-fold around the K_D. The immobilized vesicle surface was regenerated between measurements within the same series using 10 µl of 50 mM NaOH. After each measurement series was completed, the vesicle surface was removed by a 25 µl injection of 40 mM (3-[3-choamididopropyl]) dimethylammonio]-1-propane-sulfonate, prior to recoating with fresh vesicles.

Evaluation was carried out using a 1:1 Langmuir binding model to fit the data. All K_D values were defined in terms of the molarity of protein binding sites on the vesicle and not the molarity of phospholipids.

Live cell imaging of recombinant C2 protein translocation

The details of plasmid construction, cell transfection, fluorescent imaging, and data analysis are published elsewhere (8). Briefly, however, plasmid constructs under the control of an ecdysone-inducible promoter system were designed to express recombinant C2 proteins tagged with enhanced green fluorescent protein (EGFP). Following transfection of plasmid constructs into ecdysone receptor-expressing cells, recombinant C2/EGFP protein expression could then be controlled by addition of the insect hormone, ecdysone. Translocation of the C2 domain-containing proteins was then stimulated by the application of calcium (1 mM) via the cell culture medium, following activation of calcium uptake by ionomycin. The experimental design of these experiments is shown schematically in Figure 1.

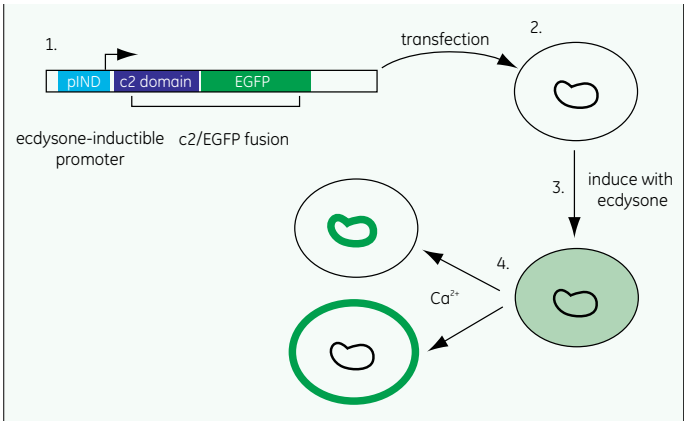


Fig 1. Experimental design for live cell imaging of EGFP-tagged C2 domain protein translocation. 1) Plasmids are constructed containing sequences coding for different C2 domains fused to EGFP and under the control of an ecdysone-inducible promoter. Ecdysone is an insect hormone and this promoter is therefore inactive by default in mammalian cells. 2) Plasmid DNA is transfected into mammalian cells that have been modified to express ecdysone receptor protein. 3) Ecdysone treatment induces the plasmid promoter to direct transcription of fusion gene mRNA and hence, EGFP-tagged fusion proteins. Protein is detected by confocal fluorescence microscopy. 4) After activation and calcium induction, translocation of EGFP-tagged C2 domains to the perinuclear or plasma membrane regions can be observed.

Results

Previous studies suggested that the calcium binding loops of the two peripheral proteins are involved in lipid binding specificity of PKC-α-C2 for PS and $cPLA_2$-C2 for PC, and identified amino acid residues that may be important for lipid binding. By immobilizing vesicles of different specific phospholipid composition on sensor surfaces and analyzing the binding of C2 domain mutants, the roles of key residues were addressed for both general phospholipid and specific headgroup recognition.

Table 1. Lipid-binding characteristics of PKC-α-C2 and mutants derived from Biacore assays. Data are shown for wild type PKC-α-C2 and alanine substitution mutations of Thr^{251} and Asn^{189}. For PKC-α-C2, the calculated K_D and rate constants are given, whereas the relative changes in affinity and association/dissociation rates are given for the mutants. The $(1/K_D^{PS}):(1/K_D^{PG})$ value is the inverse ratio of the affinity constants for binding to PS- and PG-containing vesicles and therefore indicates the degree of preferential binding to PS.

Protein	PKC-α-C2	T251A	N189A
Affinity to PS	K_D = 14 nM	↓ 18 x	↓ 5 x
Affinity to PG	K_D = 150 nM	↓ 16 x	≅ 1
$(1/K_D^{PS}):(1/K_D^{PG})$	10.7	9.6	1.9
Association rate^{PS}	$k_a = 3.2 \times 10^5\ M^{-1}s^{-1}$	↓ 3.5 x	↓ 2 x
Dissociation rate^{PS}	$k_d = 4.6 \times 10^{-3}\ s^{-1}$	↑ 5 x	↑ 2.5 x
Association rate^{PG}	$k_a = 3.6 \times 10^5\ M^{-1}s^{-1}$	↓ 3.5 x	↓ 1.1 x
Dissociation rate^{PG}	$k_d = 5.5 \times 10^{-2}\ s^{-1}$	↑ 4.3 x	↓ 1.1 x

Table 2. Lipid-binding characteristics of $cPLA_2$-C2 and mutants derived from Biacore assays. Data are shown for wild type $cPLA_2$-C2 and alanine substitution mutations of Leu^{39} and Tyr^{96}. For $cPLA_2$-C2, the calculated K_D and rate constants are given, whereas the relative changes in affinity and association/dissociation rates are given for the mutants. The $(1/K_D^{PC}):(1/K_D^{PS})$ value is the inverse ratio of the affinity constants for binding to PC- and PS-containing vesicles and therefore indicates the degree of preferential binding to PC.

Protein	$cPLA_2$-C2	L39A	Y96A
Affinity to PC	K_D = 11 nM	↓ 191 x	↓ 209 x
Affinity to PS	K_D = 120 nM	↓ 10 x	↓ 21.6 x
$(1/K_D^{PC}):(1/K_D^{PS})$	10.9	0.6	1.1
Association rate^{PC}	$k_a = 3.0 \times 10^5\ M^{-1}s^{-1}$	↓ 5.3 x	↓ 14.3 x
Dissociation rate^{PC}	$k_d = 4.6 \times 10^{-3}\ s^{-1}$	↑ 36.3 x	↑ 14.8 x
Association rate^{PG}	$k_a = 3.6 \times 10^4\ M^{-1}s^{-1}$	↓ 1.9 x	↓ 5 x
Dissociation rate^{PS}	$k_d = 5.5 \times 10^{-3}\ s^{-1}$	↑ 5.2 x	↓ 4.4 x

Mutational analysis of PKC-α-C2 domain lipid binding characteristics

For PKC-α-C2 analysis, liposomes were composed of either a 7:3 ratio of POPC/POPS or POPC/POPG. In these vesicles, PC phospholipids substitute for phosphatidylethanolamine (PE) in approximating the composition of the plasma membrane. Table 1 highlights some of the results obtained from this analysis.

As expected, PKC-α-C2 showed a strong preference for PS, with a relative affinity around 10-fold higher to POPC/POPS compared to POPC/POPG. Examination of individual rate constants also revealed that this specificity was almost entirely due to slower dissociation rate of complexes formed on PS-containing vesicles. The binding characteristics of single amino acid substitution mutants were also informative. Mutant T251A, for example, had the most dramatic effect of the mutations analyzed, showing an 18-fold reduction in affinity for POPC/POPS. Although this shows that Thr^{251} is an important residue for lipid binding, the effect on binding to POPC/POPG was very similar (a 16-fold reduction in affinity). The reduced affinity binding of T251A therefore maintains an approximately 10-fold preference for PS-containing vesicles, indicating that it does not confer significant PS-specificity. Mutant N189A, on the other hand, exhibited a 5-fold lower affinity for PS binding compared to wild type, but had no effect on PG binding. Mutation of Asn^{189} therefore reduces the preferential binding of PKC-α-C2 to PS compared to PG from 10-fold to 2-fold. This analysis was thus able to identify and distinguish between single PKC-α-C2 residues involved in either generic phospholipid, or specific PS binding.

It was also of interest to note that affinity effects caused by mutation of both Thr^{251} and Asn^{189} resulted from changes in both the association rate constant and dissociation rate constant (k_a and k_d, respectively). Other mutations, in two arginine residues in the calcium-binding loop, for example, affected only the association rate (data not shown). This indicates that while certain amino acids are involved exclusively in nonspecific electrostatic interactions with anionic lipids, others contribute to specific PS interactions that also maintain binding stability.

Mutational analysis of $cPLA_2$-C2 domain lipid binding characteristics

The PC-selective binding of $cPLA_2$ is known to derive exclusively from the C2 domain and several aliphatic and aromatic residues from its calcium binding loops have been shown to penetrate the membrane during binding. Label-free interaction analysis of the binding of $cPLA_2$-C2 and selected mutants to POPC and POPS was therefore undertaken to determine residues involved in PC selectivity.

As shown in Table 2, comparison of $cPLA_2$-C2 binding to vesicles consisting entirely of POPC or POPS revealed an 11-fold preference for PC in terms of K_D.

In contrast to PKC-α-C2, this selectivity appears to derive principally from a difference in association rate. Additional assays using mixed vesicles with increasing ratios of POPS to POPC showed that this reduced PC content correlated with decreasing affinity. Mutation of any of the four hydrophobic residues examined dramatically reduced the affinity for PC, by over two orders of magnitude. Table 2 shows the data obtained for mutations of one of the two aliphatic residues (Leu^{39}) and one of the two aromatic residues (Tyr^{96}). Mutation of both the aliphatic and aromatic type of residues greatly reduced the selectivity for PC binding (from 10.9 for wild type C2 down to 1.1 to 0.6 for the mutants), although the effects on individual rate constants were somewhat different. While mutation of the aromatic residues affected both k_a and k_d, the aliphatic mutations primarily affected k_d. Taken together, these results show that four hydrophobic residues in the calcium binding loops of $cPLA_2$-C2 are important for PC-selective binding, but that the aliphatic and aromatic amino acids display distinct properties at the protein/phospholipid interface.

Although extremely useful models for establishing the lipid-binding preferences of PKC-α and $cPLA_2$, the vesicles used in the described studies are considerably less complex than the biological membranes they were designed to represent. In order to put the data from Biacore assays into a more biological context, Stahelin *et al.* also examined the binding of selected C2 domain-derived proteins to complex vesicles. These consisted of multiple phospholipids and cholesterol, and were designed to mimic plasma and nuclear membranes as closely as possible, based on the current understanding of their detailed chemical compositions. This data (not shown here) was consistent with the results from the earlier Biacore assays in terms of the effects of mutations on relative affinities and lipid selectivity. The absolute affinities of PKC-α and $cPLA_2$ for their respective membrane mimics were, however, considerably stronger than those observed for the simple vesicles (30-fold and 5-fold higher, respectively). Another interesting difference from the initial vesicle-binding studies (where the C2 domains had comparable affinities for their respective targets) was that the affinity of PKC-α for the plasma membrane mimic was 4.5-fold higher than that of $cPLA_2$ for the nuclear membrane mimic.

Live cell imaging of subcellular protein translocations in response to calcium

The subcellular targeting patterns of isolated C2 domains from PKC-α and $cPLA_2$ have been to shown to duplicate those of the intact proteins in response to calcium. The question of whether this derives directly from the lipid-binding specificities of C2 domains, or is under the direction of specific adapter proteins, however, remains open. Having used label-free interaction analysis to obtain a detailed characterization of the binding of C2 domains and their mutants to model membranes, the behavior of these proteins was next examined in living cells.

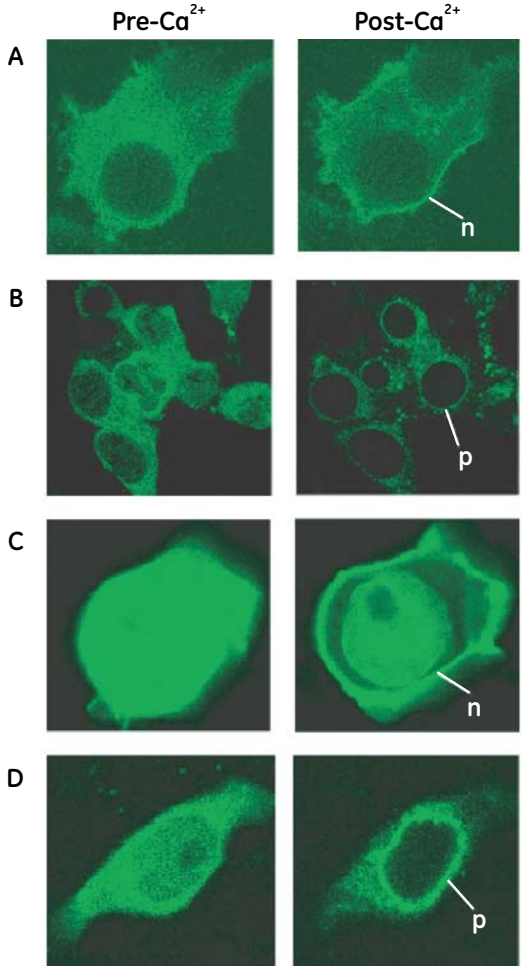


Fig 2. Calcium-dependent subcellular translocation of EGFP-tagged PKC-α, $cPLA_2$ and C2 domain swap chimera proteins. Images of cells expressing PKC-α (A), $cPLA_2$ (B), $cPLA_2$ containing a PKC-α C2 domain (C) and PKC-α containing a $cPLA_2$ C2 domain (D). Cells are shown before (left-hand panels) and after (right-hand panels) activation with Ca^{2+}. Accumulations of GFP signal at the plasma membrane (p) and perinuclear region (n) are indicated on the panels.

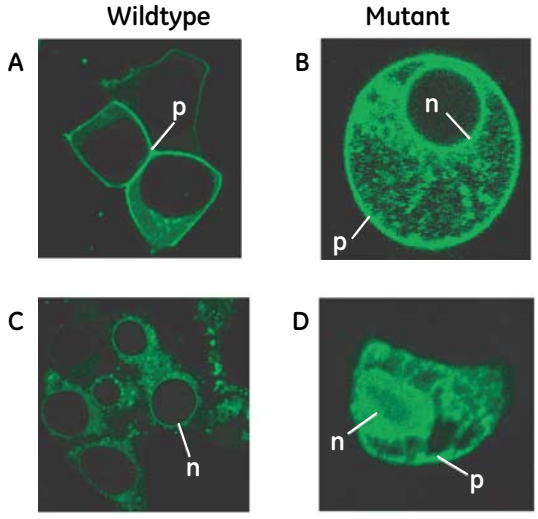


Fig 3. Post-calcium activated subcellular translocation of EGFP-tagged C2 domain proteins and their mutants. Images of cells expressing PKC-α-C2 (A), PKC-α-C2 N189A (B), $cPLA_2$-C2 (C), and $cPLA_2$-C2 Y96A (D). Accumulations of GFP signal at the plasma membrane (p) and perinuclear region (n) are indicated on the panels.

This was carried out by transient transfection of cells with plasmids designed to express EGFP-tagged proteins, and examination of dynamic subcellular protein localization by time-lapse confocal microscopy (Fig 1).

Initial experiments using isolated C2 domains (panels A and C, Fig 3) showed that while both PKC-α-C2-EGFP and $cPLA_2$-C2-EGFP proteins were initially dispersed throughout the cytoplasm of transfected cells, activation with calcium resulted in a dramatic translocation to the plasma membrane (PKC-α) or the perinuclear membranes ($cPLA_2$). The term "perinuclear" is used here because the resolution of the confocal imaging was not sufficient to discriminate between the endoplasmic reticulum/goli apparatus and the nuclear envelope itself. The movement of the $cPLA_2$-derived protein was significantly slower than that of its PKC-α -derived counterpart, but based on mag-indo-1 imaging, the protein translocation was synchronized with the intracellular Ca^{2+} accumulation in the different subcellular regions in both cases. When full-length EGFP-tagged PKC-α and $cPLA_2$ proteins were examined under the same conditions, they exhibited identical Ca^{2+}-induced translocation dynamics to those observed for the corresponding C2 domains (Fig 2).

To further examine the role of the C2 domains in subcellular targeting, full-length chimeric proteins were generated, in which the C2 domains of PKC-α and $cPLA_2$ were exchanged. This analysis showed that exchanging C2 domains reversed the normal targeting of the peripheral proteins, such that PKC-α containing a $cPLA_2$-C2 domain migrated to the perinuclear region, while $cPLA_2$ containing a PKC-α C2 domain translocated to the plasma membrane in response to Ca^{2+} (Fig 2).

Correlation of lipid binding characteristics with calcium-dependent subcellular targeting

Since binding characterization of the C2 domain mutants derived from label-free interaction analysis provided important data about the lipid-binding properties of PKC-α and $cPLA_2$, it was of interest to see how these data correlated with subcellular translocation behavior. Figure 3 shows the distribution of EGFP-tagged C2 domain proteins and their respective mutants after activation with calcium.

PKC-α-C2 showed a distinct translocation to the plasma membrane in response to Ca^{2+}, whereas the N189A mutant translocated to both the plasma and perinuclear membranes (Fig 3, A-B). This correlates with the 5-fold reduction in PS selectivity determined for this mutant in Biacore assays (Table 1) and an even stronger reduction in plasma versus nuclear membrane selectivity observed in the membrane mimic studies. Similarly, while $cPLA_2$-C2 translocated strongly to the perinuclear region after Ca^{2+} activation, the Y96A mutant translocated comparably to the plasma membrane and perinuclear regions (Fig 3, C-D). Again, this behavior was predicted from Biacore assays, which showed a 10-fold reduction in PC selectivity (Table 2) and an almost identical reduction in preferential binding to the nuclear membrane mimic.

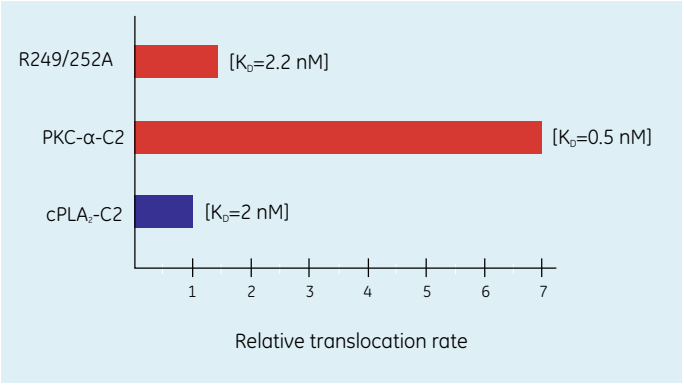


Fig 4. Relative membrane translocation rates for C2 domain proteins. Rates were calculated from the slopes of plots of EGFP intensity ratio (membrane/[membrane + cytosol]) versus time. In the figure, the translocation rate for cPLA$_2$-C2 is designated as 1, and the remaining rates are expressed relative to this value. Protein R249/252A is a double substitution mutation of $Arg^{249,252}$ derived from PKC-α-C2. The K_D values in parenthesis represent the affinities for binding to plasma membrane (PKC-α and R249/252A) or nuclear envelope (cPLA$_2$-C2) mimics derived from Biacore assays.

When membrane translocation rates were calculated for the proteins in the cell experiments, a good correlation was also observed to membrane binding data from Biacore assays. Since many of the mutants showed dual membrane targeting, these were excluded from this analysis because of ambiguity involved in determining their translocation rates. As shown in Figure 4, however, the translocation rate of PKC-α-C2 to the plasma membrane was around seven times faster than that of cPLA$_2$-C2 to the perinuclear region.

This was consistent with the 4.5-fold lower affinity (and 3.7-fold difference in k_d) of cPLA$_2$-C2 for the nuclear envelope mimic compared to that of PKC-α for the plasma membrane mimic, as measured in Biacore assays. PKC-α-C2 mutant R249/252A, which showed reduced affinity for POPS and the plasma membrane mimic, but maintained strong PS/plasma membrane selectivity, migrated to the plasma membrane more slowly than the wild type. The reduction in relative translocation rate was 4.4-fold, correlating closely with the 5-fold reduction in affinity (and 6-fold change in k_d) observed in binding to the plasma membrane mimic in Biacore assays. Taken together, the data strongly suggest that the subcellular targeting of PKC-α and cPLA$_2$ in response to Ca^{2+} influx is dictated by their selective binding properties to the specific lipid compositions of different cellular membranes.

Conclusions

Lipid-binding characteristics of PKC-α and cPLA$_2$ are an intrinsic part of their biological function. While detailed binding data is difficult to obtain from an in vivo system, purely in vitro approaches may be inadequate to fully describe complex biological mechanisms. Stahelin *et al.* circumvented these issues by combining the analytical power and flexibility of label-free interaction analysis with real-time studies of protein translocation in living cells.

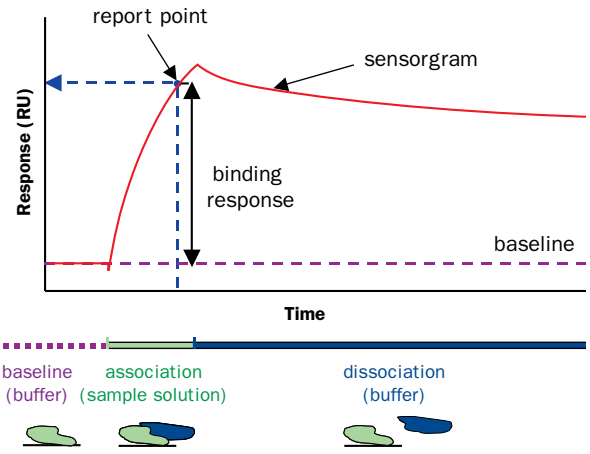
By using phospholipid vesicles, it was possible to screen proteins and establish their lipid-binding preferences. The marked selectivity of PKC-α-C2 for PS, and of cPLA$_2$-C2 for PC suggested that C2 domains are important for membrane translocation and lipid selectivity. The analysis of multiple mutations in the Ca^{2+}-binding loops of the C2 domains enabled the identification of individual residues with key roles in phospholipid affinity and selectivity. Moreover, the resolution of individual rate constants demonstrated that individual aliphatic residues contribute principally to binding stability (with mutations affecting mainly k_d), whereas aromatic residues appear to be involved in membrane association and dissociation steps (with mutations affecting k_a and k_d).

The Ca^{2+}-dependent subcellular translocation of C2 domain proteins and their mutants were consistent with data from Biacore assays and demonstrated a quantitative relationship between measurements in Biacore assays and subcellular translocation. Mutations that reduced phospholipid or membrane mimic selectivity, for example, also affected translocation in the cellular assays. The cellular behavior of the examined proteins was accurately predicted from their affinity and kinetic properties, as measured using Biacore system. In addition, the relative rate of translocation of C2 proteins was also found to be consistent with observed K_D and k_a values.

The work described here strongly suggests that the subcellular targeting properties of C2 domains of PKC-α and cPLA$_2$ can be largely accounted for by biophysical properties that dictate their lipid binding characteristics. The use of label-free interaction analysis using Biacore systems to supplement cell-based or animal techniques is thus a powerful approach to the study of membrane-binding proteins and their roles in signal transduction and other important biological processes.

Monitoring protein interactions with Biacore systems

Biacore systems monitor protein interactions in real-time using a label-free detection method. One of the interacting molecules is immobilized onto a sensor surface, while the other is injected in solution and flows over the sensor surface. As molecules from the injected sample bind to the immobilized molecules, this results in an alteration in refractive index at the sensor surface that is proportional to the change in mass concentration. Using the phenomenon of surface plasmon resonance (SPR), these changes are detected in real-time and data are presented as a sensorgram (SPR response plotted against time). Sensorgrams display the formation and dissociation of complexes over the entire course of an interaction, with the kinetics (association and dissociation rates) revealed by the shape of the binding curve.



The sensorgram provides real-time information about the entire interaction, with binding responses measured in resonance units (RU). Binding responses at specific times during the interaction can also be selected as report points. More information can be found at **www.gelifesciences.com/biacore**.

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For local office contact information, visit
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GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com/biacore

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