Binding and function of phosphotyrosines of the Ephrin A2 (EphA2) receptor using synthetic Sterile α Motif (SAM) domains

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*Running Title: Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2

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Background: Ephrin A2 (EphA2) Sterile α Motif (SAM) domains undergo phosphorylation at Y921, Y930 and Y960.

Results: Recruitment of Grb7 SH2 domain by EphA2 SAM is phosphorylation site specific.

Conclusion: Tyrosine phosphorylation of the EphA2 SAM domain has wide implications for the differential recruitment of binding partners.

Significance: SAM tyrosine phosphorylation imparts specificity to its adaptor protein interactions and network formation, easily studied in vitro.
ABSTRACT

The Sterile α Motif (SAM) domain of the ephrin receptor tyrosine kinase, EphA2, undergoes tyrosine phosphorylation; but the effect of phosphorylation on the structure and interactions of the receptor is unknown. Studies to address these questions have been hindered by the difficulty to obtain site specifically phosphorylated proteins in adequate amounts. Here, we describe the use of chemically synthesized and specifically modified domain-length peptides to study the behavior of phosphorylated EphA2 SAM domains. We show that tyrosine phosphorylation of any of the three tyrosines, Y921, Y930, and Y960 have a surprisingly small effect on the EphA2 SAM structure and stability. However, phosphorylation at Y921 and Y930 enables differential binding to the SH2 domain of the adaptor protein Grb7, which we propose will lead to distinct functional outcomes. Setting up different signaling platforms defined by selective interactions with the adaptor proteins thus adds another level of regulation to EphA2 signaling.

INTRODUCTION

Phosphorylation plays a major role in the regulation of protein function (1, 2). Although there are many cellular studies using phosphorylation deficient proteins, there are relatively few systems where the effect of phosphorylation on the structure and the interactions of a protein have been tested in vitro (3, 4). Biophysical studies of phosphorylated proteins have been hampered by low yields, difficulties in obtaining site specific phosphorylation or by the lack of a good phosphomimetic. Recent progress in peptide synthesis has made it possible to generate sizeable protein domains with the incorporation of phosphotyrosines at specific positions (peptides up to 100 residues can now be synthesized by several companies). Here, we report a biophysical study of synthesized and specifically phosphorylated protein domains. To our knowledge this is the first report of a biophysical study utilizing full length tyrosine phosphorylated domains which have been generated by chemical synthesis.

Eph proteins belong to the family of transmembrane protein receptor tyrosine kinases (RTKs) (5-7). Signaling through Eph receptors regulates key cellular functions including cell migration, axon guidance, and angiogenesis under physiological and pathological conditions such as cancer (8, 9). Phosphorylation is known to be central to the regulation of Eph receptor function. For example, increased EphA2 tyrosine phosphorylation is a characteristic of basal breast cancer cells (10) and is associated with increased apoptosis of cardiomyocytes (11). Apart from ligand binding, receptor activation involves the phosphorylation of specific residues of the juxtamembrane region and the kinase domain (12-14). In addition, in vivo studies and proteomics surveys have revealed that the tyrosines of the C-terminal SAM domain (present in all Eph receptors but none of the other RTK subfamilies) also undergo phosphorylation (15-18). The SAM domains are common protein-protein interaction modules that typically form homo or heterodimers and are present in a diverse set of proteins (19-21). The structures of several SAM domains have been solved; showing a relatively well conserved topology of five alpha-helices (22, 23). The EphA2 SAM domain has three tyrosines, Y921, Y930, and Y960; of which Y921 is absolutely conserved in Eph and many other SAM domains (Figure 1). By contrast to most RTK phosphorylation sites which occur in relatively unstructured protein domain linker regions or loops (23, 24), the three SAM domain tyrosines are part of the folded protein structure. The tertiary structure may thus provide an additional level of regulation. Biological studies showing the phosphorylation of all the three tyrosines have already been reported: Y921 and Y960 were found to be phosphorylated when an EphA2 kinase-SAM domain construct is expressed in E. coli (12, 25) and Y960 phosphorylation was identified in a colorectal carcinoma cell line (26). Y930 is phosphorylated in mouse lung epithelial cells; furthermore, the Y930F phosphorylation-defective variant inhibited both the kinase activity and vascular assembly. Similarly, phosphatase LAR was shown to dephosphorylate Y930 (and possibly other tyrosines), an event that appears to abrogate binding to the SH2 domain of the adaptor protein Nck2 and attenuates cell migration (Y930F had the same effect on cell migration) (26). There is no biological information on the role of Y921 phosphorylation in EphA2 (although binding to the SH2 domain of Vav3 has been proposed (27)). However, in vivo studies have also shown that the conserved SAM domain tyrosine (Y921 in EphA2) is responsible for recruiting SH2 domains of Grb7 and Grb10 to EphB1 and this interaction is deemed es-
sential for the regulation of cell migration (15, 17, 28, 29).

The extent of phosphorylation of protein’s tyrosines in cells is typically not easy to ascertain or to manipulate in a site specific manner. Thus, the experiments reported above, rely on the expression of proteins in which a single tyrosine has been mutated to phenylalanine (a sidechain that mimics the unmodified residue and cannot be phosphorylated). While these biological findings suggest the importance of particular sites for the interactions, this strategy can deliver false negatives, because an interaction may still persist upon mutating a single site if interactions with several phosphorylated tyrosines are possible. Similarly, it may be noted that the previous reports were not accompanied by a molecular level framework, which involve consideration of protein conformational changes and competing binding processes. Biophysical studies in vitro, as reported here, can provide a deeper insight and propose models for investigation at the cellular level. Specifically, the EphA2 SAM domain forms a heterodimer with the SAM domain of SH2 domain-containing inositol-5’-phosphatase (SHIP2) (23, 30, 31). Binding of EphA2 SAM to SHIP2 SAM inhibits receptor endocytosis and enhances activation of Eph kinase (31). In vivo studies have also shown (using Y to F mutations in the EphA2 SAM domain) that tyrosine phosphorylation is not required for SHIP2 recruitment (31); however, it is not clear whether phosphorylation could, in fact, be be detrimental to SHIP2 binding. Here we studied directly whether the phosphorylation adds another level of complexity to the regulation of Eph receptors by controlling SAM domain mediated interactions.

Using synthetic domains, we studied the effect of phosphorylation of the EphA2 SAM domain on its structure and interactions with SHIP2 SAM. Further, stimulated by reports on EphB1 recruiting the SH2 domain of Grb7 (15, 17), we examined interactions of the phosphorylated domains with Grb7 SH2. Unexpectedly, we show that the phosphorylation of the tyrosines of EphA2 SAM domain has little effect on the overall structure of the domain. EphA2 SAM phosphorylated at Y930 could simultaneously engage the Grb7 SH2 and SHIP2 SAM domains. In contrast, Y921 is located near the SHIP2 binding region and Grb7 SH2 and SHIP2 SAM compete for binding to phosphorylated Y921. Surprisingly, EphA2 SAM phosphorylated at Y960 does not interact with Grb7 SH2, but also has no effect on SHIP2 SAM binding. We discuss how this phosphorylation dependent specificity could give rise to different signaling platforms, regulating the function of EphA2 receptors.

EXPERIMENTAL PROCEDURES

Protein cloning, expression, and purification-

cDNA for human Grb7 was a gift from Prof. Jun-Lin Guan (University of Michigan). Residues 425-532, corresponding to the SH2 domain were amplified by PCR and subsequently cloned into a pET30 Xa/LIC vector using ligation independent cloning (EMD Biosciences, USA). The plasmid containing Grb7 SH2 was transformed into E. coli BL21 (DE3) cells and grown at 310 K in either Luria–Bertini (LB) medium or in M9 minimal medium supplemented with 15NH4Cl. Cultures were grown to an O.D. 600nm of 0.8 for both media and then induced with 0.2 mM IPTG. Bacterial cells were harvested after 24 hours of induction at 289 K.

Purification of the Grb7 SH2 domain was carried out using Ni2+ affinity chromatography. In brief, the harvested cells were re-suspended in lysis(binding buffer (50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 30 mM imidazole, 1 mM TCEP-HCl) supplemented with EDTA-free Complete™ protease inhibitor (Roche, USA). Cells were disrupted by sonication. After centrifugation, the cleared lysate was applied to Ni-NTA beads (Qiagen, USA). Following washing with binding buffer containing 45 mM imidazole, the bound protein was eluted with 50 mM sodium phosphate buffer (pH 7.4) with 250 mM imidazole and 1 mM TCEP-HCl. The eluted protein was concentrated and buffer exchanged into the NMR buffer (20 mM Tris, pH 6.8, 100 mM NaCl, 1 mM TCEP-HCl). The EphA2 and SHIP2 SAM domain constructs, their expression and purification have been described previously (20). The EphA2 and SHIP2 SAM proteins were also exchanged into the same NMR buffer.

Preparation of tyrosine phosphorylated peptides -

Phosphorylated peptides corresponding to residues 910- SEWLESIKQQpYTEHFMAAGFT-931, denoted pep.pY921, 916-WKMQQFTEHF-MAAGpYTAIEVVQ-937, pep.pY931, and 951-LPGHQKRIApYSLLGLKDQVTV-972, called...
Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2 pep.
Pep.pY960 as well as the equivalent unphosphorylated peptides were purchased from Genscript (Genescit, USA). The peptides were dissolved into the NMR buffer without further purification. Three domain-length EphA2 SAM peptides (residues 901 to 976) were synthesized (United Peptide, Inc. USA) – also referred to as simply EphA2 below (experiments were carried out with the SAM domains only in this paper). Each domain peptide has phosphorylated sidechains at Y921 (EphhA2.pY921), Y930 (EphA2.pY930), or Y960 (EphA2.pY960). These full-length phosphorylated peptides initially had poor solubility in water and were refolded by incubation in 8 M urea (20 mM Tris, pH 6.8, 100 mM NaCl, 1 mM TCEP-HCl) overnight and then were dialysed extensively against the NMR buffer. Peptide and protein concentrations were determined by UV absorbance with reference to predicted extinction coefficients.

Circular Dichroism (CD) spectroscopy - The secondary structure and the thermal stability of the phosphorylated domains were examined by CD spectroscopy using established protocols (32). Spectra were recorded on a 20 µM sample using a cuvette with path-length of 4 mm on an Aviv (model 215) instrument. The temperature scans were carried out in the range of 293-363 K, at 222 nm, with a step size of 2 K and 30 sec equilibration period and 30 sec recording time. All the experiments were carried out in triplicate and signal from the buffer was subtracted.

NMR spectroscopy - All experiments were run at 298 K on an 800 MHz spectrometer equipped with a TCI probe (Bruker Avance). 1D ¹H NMR (using WATERGATE) and homonuclear 2D ¹H NOESY experiments (mixing time of 300 ms) were recorded with 300 µM samples of the SAM domains. ¹⁵N-¹H HSQC experiments on Grb7 SH2 were recorded on the ¹⁵N-labeled protein itself, or on an 1:1 mixture with unlabeled EphA2 domains or after further addition of 2 molar equivalents of unlabeled SHIP2 SAM. The data were processed using nmrPipe (33) and the 2D spectra were visualized using Sparky (Goddard TD, Kneller DG, SPARKY3, University of California, San Francisco). The 1D ¹H NMR spectra were plotted using the software Origin (OriginLab, Northampton, MA).

Isothermal titration calorimetry (ITC) - Interaction of the short (unphosphorylated and phosphorylated) peptides with Grb7 SH2 and of the domain-length phosphorylated and refolded EphA2 peptides with Grb7 SH2 and/or the SHIP2 SAM domain was measured by ITC (MicroCal ITC200, GE Healthsciences) with established protocols. Typically 40 µM of the EphA2 protein was used in the chamber and 400 µM of the SHIP2 SAM domain was used in the syringe for titration. ITC experiments with Grb7 SH2 were performed using Grb7 SH2 in the chamber and titrating in the corresponding binding partner. The data were analyzed using Origin (OriginLab, Northampton, MA).

Solvent accessible surface area - The solvent accessible surface area for the tyrosine residues of EphA2 was calculated using the algorithm, SURFACE of the CCP4 suite (34). The complex structure of EphA2 SAM : SHIP2 SAM (PDB ID: 2KSO) was used and calculations were set up with a probe of 1.4 Å radius.

RESULTS

Chemically synthesized polypeptides share native-like folds and are stable - Biophysical studies demand large quantities of pure proteins. It is difficult to obtain proteins that are fully phosphorylated at one specific site by exposure to kinases in vitro. Here, we obtained SAM domain polypeptides which have specific sites fully and stably phosphorylated during their chemical synthesis (United Peptides Inc., USA). Initial NMR experiments with the synthesized domains dissolved in buffer showed that these proteins are not properly folded (data not shown). We carried out a chemical denaturation, followed by a refolding step to correctly fold the domains. In order to confirm that a near native SAM domain structure is obtained, we analyzed the conformations of the refolded proteins by both 1D ¹H NMR (Figure 2) and homonuclear 2D ¹H NOESY experiments (Figure 3). The NMR spectra show that all three specifically phosphorylated SAM domains (referred to as EphA2.pY921, EphA2.pY930 and EphA2.pY960) are well folded; as evident from the dispersed amide signals, resonances for the tryptophan side chains, and up-field shifted methyl signals (highlighted with boxes in Figure 2). The spectra show that the peptides adopt a very similar structure to that of the recombinant protein. Subtle differences are apparent in EphA2.pY921 and EphA2.pY930;
the two tyrosines that are partially buried in the wild type protein (with 17.6% and 32.9% solvent exposure calculated for Y921 and Y930, respectively) and likely have become more exposed upon phosphorylation. In addition, we characterized the secondary structure of the phosphorylated proteins by far-UV CD spectroscopy and found that they share within ± 15% a similar α-helical content to the recombinant EphA2 SAM domain. We also assessed the thermal stabilities of the phosphorylated proteins and of the recombinant EphA2 SAM by measuring the signal at 222 nm as a function of temperature; phosphorylation at any one of its three tyrosines does not dramatically destabilize the SAM domain fold (Figure 4 and Table 1).

The phosphorylated proteins still interact with SHP2 SAM - Isothermal titration calorimetry (ITC) measurements have shown that EphA2 SAM domain binds the SHP2 SAM domain with a micromolar affinity (23). The phosphorylated EphA2 SAM domains (phosphorylated at Y921, Y930 or at Y960) bind SHP2 SAM with an affinity that is comparable to that of the recombinant unphosphorylated protein (Representative ITC interaction data is shown in Figure 5). We report the dissociation constants (K_D) and the derived thermodynamic contributions of the individual interactions in the Table 2. The change in binding enthalpy is slightly more favorable for the phosphorylated proteins compared to the unphosphorylated protein; but the binding entropy is lower. It is likely that the change in enthalpy for this process is systematic effect because the synthetic proteins have shorter termini, compared to the recombinant protein. Thus, remarkably, the binding of EphA2 to SHP2 SAM is essentially insensitive to tyrosine phosphorylation. We, therefore, decided to study the interaction of the phosphorylated domains with pY binding proteins.

Binding of EphA2 SAM to Grb7 SH2 is phosphorylation site specific - SH2 domains of the Grb family are known to bind the conserved phosphorylated tyrosine of Eph SAM domains (Y921 for EphA2 SAM) (29). We tested the binding of several Grb SH2 domains with EphA2 SAM and carried out a complete study by ITC and NMR for the Grb7 SH2 - EphA2 SAM interaction. Neither the unphosphorylated nor EphA2 SAM phosphorylated at Y960 interact appreciably with Grb7 SH2. In contrast, both EphA2.pY921 and EphA2.Y930 bound Grb7 SH2 with similar affinities (Table 2). The binding of EphA2.pY921 is almost entirely enthalpic, whereas EphA2.pY930 binding is largely driven by a favorable entropic contribution. This is the first study to report interaction of phosphorylated EphA2 SAM domains with Grb7 SH2.

In order to examine the possibility whether the conformational restraints imparted by the protein fold play a role in the interactions, we measured the interaction of short phosphorylated peptides with Grb7 SH2. (Table 2). All the short phosphorylated peptides, including pep.pY960, interact with Grb7 SH2 with a similar affinity. Since the pY960 within the folded SAM domain did not bind Grb7 SH2, this observation suggests that binding at this site is conformation dependent. We also carried out binding experiments with unphosphorylated short peptides, none of which bound.

Differential effects of EphA2.pY complex formation with Grb7 SH2 on the interaction with SHP2 SAM - Our ITC data shows that the phosphorylated proteins, EphA2.pY921 and EphA2.pY930, can bind both Grb7 SH2 and SHP2 SAM with similar affinities. The question arises whether SHP2 SAM and Grb7 SH2 can bind EphA2.pY921 or pY930 simultaneously or whether the binding is mutually exclusive (and competitive). To answer these questions we carried out ITC and NMR experiments to examine the possibility of a tri-molecular interaction. ITC experiments (Table 3) show a slight decrease in binding affinity of EphA2.pY921 and EphA2.pY930 for SHP2 SAM in presence of Grb7 SH2, suggesting Grb7 SH2 influences the EphA2 - SHIP2 interaction. Since the binding affinities between Grb7 SH2 and SHP2 SAM are similar, the equilibrium cannot be shifted substantially unless one protein is in large excess concentration. In the case of EphA2.pY960, it is possible that this domain only interacts with Grb7 SH2 in presence of SHIP2 SAM. However, the binding affinity and thermodynamic contributions are identical (within the error limits) for SHP2 SAM binding to EphA2.pY960 whether Grb7 SH2 is present or not, underscoring the fact that EphA2.pY960 does not bind Grb7 SH2 (Table 3).

To gather additional support for these observations, we acquired 15N-1H HSQC spectra of labeled Grb7 SH2 in presence of unlabeled EphA2.
with or without SHIP2 SAM proteins (Figure 6). Binding of both EphA2.pY921 and EphA2.pY930 to Grb7 SH2 is characterized by a decrease of resonance intensity of Grb7 SH2. This change arises due to the formation of a larger molecular weight complex, as Grb7 SH2 is a dimer and the pY binding interface and the dimerization interface are different (35, 36) (data not shown). However, it is not clear to what extent, if any; pY binding alters the dimerization of Grb7 SH2 (35, 36, 37). Upon addition of SHIP2 SAM to the pre-mixed complex of Grb7 SH2 (labeled): EphA2.pY921, we saw a change in intensity of several but not all of the dispersed resonances compared to the spectrum of Grb7 SH2 bound to Eph.pY921 (Figure 6A). The changes occur at the pY binding interface (38, 39), suggesting that some of the EphA2.pY921 : Grb7 SH2 complex is dissociating, so that EphA2 can form a complex with SHIP2. When we added SHIP2 SAM to the EphA2.pY930 / Grb7 SH2 (labeled) mixture, we observed significant line broadening of most of the Grb7 SH2 resonances (Figure 6B); this is consistent with the formation of a large complex (the Grb7 domains would still dimerize).

Addition of unphosphorylated EphA2 SAM domain or EphA2.pY960 did not alter the spectrum of Grb7 SH2 (not shown), consistent with the ITC data suggesting that these SAM domains do not interact with the SH2 domain. Furthermore, when we added SHIP2 SAM to the pre-mixed complexes of Grb7 SH2 and EphA2.pY960, we did not see any significant changes to the Grb7 SH2 resonances (Figure 6C), highlighting that Grb7 SH2 does not bind EphA2.pY960 even when it is bound to SHIP2. The differential signaling output that results from these selective interactions is discussed below (and in Figures 7).

### DISCUSSION

The detailed characterization of posttranslational modifications such as tyrosine phosphorylation and their role in specific protein-protein interactions is a prerequisite to understanding the mechanistic basis of signaling processes that in turn regulate the great majority of cellular functions. We took advantage of the recent progress in peptide synthesis technology to obtain domain-length polypeptides with specific tyrosine phosphorylation. Following a refolding procedure, the NMR and CD spectroscopic studies of the phosphorylated SAM domains (EphA2.pY921, EphA2.pY930, and EphA2.pY960) demonstrate that the chemically synthesized domains adopt native-like structures that are stable. Our finding that, phosphorylation is not accompanied by a large conformational change in the domain structure was initially surprising given that both Y921 and Y930 are partially buried. However, both of the tyrosine residues are probably capable of maintaining interactions with the neighboring residues even after phosphorylation. For example, the tyrosine hydroxyl of Y921 is exposed to the solvent and makes hydrogen bond contacts with the sidechains of the conserved H954 (Figure 1); the phosphate group of Y921 may interact with H954 similarly and help to maintain the overall conformation of the domain. Taken together, our observations establish that the domain-length phosphorylated peptides are a good model system to study the impact of EphA2 SAM phosphorylation on the domain’s interaction with other proteins.

Unphosphorylated EphA2 SAM binds SHIP2 SAM (23, 31, 32); phosphorylation might alter the affinity of this interaction. Unexpectedly, ITC measurements show that both the phosphorylated and unphosphorylated EphA2 SAM domains share a similar affinity for SHIP2 SAM. We anticipated an effect with phosphorylation in case of phosphorylated Y921 and Y960 since these are located close to the binding interface with SHIP2 SAM. Adding negative charge to the EphA2 interface (which by itself is dominated by positively charged residues) would be expected to weaken binding of the negatively charged SHIP2 SAM interface. However, our recent refinement of the structure of the complex suggests that the complex can sample alternate configurations (23, 40). The equilibrium between these different configurations may be shifted in the EphA2.pY921 - and EphA2.pY960-SHIP2 complexes, but assessing this possibility is beyond the scope and interest of the current study. Overall, we can conclude that phosphorylation of the EphA2 SAM domain by itself is not involved in the regulation of EphA2 SAM – SHIP2 SAM domain interactions. However, phosphorylation could affect the interactions of the domain with other proteins, which would influence EphA2-SHIP2 interaction indirectly.

Tyrosine phosphorylation of receptor tyrosine kinases and the subsequent recruitment of Src-homology 2 (SH2) domain containing adaptor proteins is a central event in the signaling (26, 41,
Here, we report that the phosphorylated Y921 and Y930 of EphA2 SAM recruit Grb7 SH2. A 23-residue peptide containing phosphorylated Y960 binds Grb7 SH2 just as well as the other two peptides, but surprisingly, the Y960 phosphorylated folded domain has no affinity for Grb7 SH2. This observation suggests that binding at this site is conformation dependent. Grb7 family SH2 domains bind to peptides in extended or in hair-pin conformations (43); pep.Y960 (and the other short peptides) are unstructured/only very weakly structured by themselves in solution, as indicated by AGADIR prediction (44) and is therefore able to bind the Grb7 SH2. In the folded protein Y960 is located in the helix α5 of the EphA2 SAM domain, which is unlikely to undergo the unfolding that would be required to allow SH2 binding. Thus, protein conformational features can override the binding affinity that unstructured pY containing polypeptides may have for SH2 proteins (43). This is in accordance with observations on other systems (45, 46) and emphasizes the need for caution in the interpretation of data obtained using peptide libraries/protein fragments in the elucidation of cell signaling mechanisms.

Our study of EphA2 SAM and Grb7 SH2 domains should translate to other Eph-like SAM domains, as Y921 is highly conserved in Eph-like SAM domains. Furthermore, the SAM domain structures and the topology of its interaction/locational of the interacting surfaces are similar across Eph-like SAM domains (21). Indeed, our ITC data shows that a SHIP2 SAM derived peptide in which Y1213 is phosphorylated (the equivalent of the highly conserved EphA2 Y921), also binds to Grb7 SH2 (Table 1). Binding partners specific for SHIP2, pY1213 are yet to be identified in vivo, but proteomics studies have found this tyrosine to be phosphorylated (http://www.phosphosite.org/id=71200) in myelogenous leukemia. Thus it is likely that phosphorylation of the highly conserved tyrosine (Y921 in EphA2) has a similar function throughout the Eph receptor family; by itself maintaining interactions with a SAM domain binding partner and at the same time, allowing a competition for it with Grb SH2 adaptor proteins.

While future studies are needed to examine the specificity of SH2 domain binding (e.g. Grb7 vs. other family members or other adaptor proteins like Vav and Nck family), our in vitro study presents an important finding with respect to SH2 binding site selection on EphA SAM domains. In absence of other binding partners, both pY930 and the highly conserved pY921 in EphA2 bind the SH2 domain equally well in vitro, but in cells only binding to pY930 was inferred (17). However, we can rationalize this finding in the context of a competition of SH2 domain with SHIP2 binding near the pY921 site. As SHIP2 binds several other, if not all EphA SAM domains in the same region (close to Y921), the site preference of SH2 binding for the distal pY930 site may be common to 8 of the 10 EphA isoforms which have this second tyrosine. Conversely, Grb7 has been reported to bind at the highly conserved tyrosine in EphB1 because EphB-family SAM domains may not bind SHIP2 (23).

The ability of EphA2.pY930 to bind SHIP2 SAM and Grb7 SH2 simultaneously (opposite surfaces are involved) could lead to the formation of extended networks after binding of Grb7 SH2 to the dimerized EphA2 receptor that is still bound to SHIP2 SAM. In addition, SHIP2 SAM is expected to form a homo-dimer/trimer through a coiled-coil region that is located in the middle of the protein from predictions (47, 48); thus allowing further crosslinking of SHIP2 SAM bound EphA2 receptors. Continued association with SHIP2 is likely to be important since this interaction has been shown to inhibit EphA2 receptor endocytosis (31). By contrast, Y921 is close to the predominant SHIP2 binding site and our results show that Grb7 and SHIP2 compete with one another for the same binding region.

If indeed the affinities are similar, the level of network formation involving Grb7 SH2 would depend both on SAM domain phosphorylation and on the concentration of Grb7, leading to the proposal of a step-wise mechanism, as shown in Figure 7. The local concentrations of adaptor proteins of receptor tyrosine kinases are often increased upon receptor activation due to their recruitment by the clustered receptors at the plasma membrane (49). Grb7 (and other members of the Grb family) may also be localized to the plasma membrane via their plekstrin homology (PH) domain (50) (not shown in the model). Excess Grb7 would dissociate SHIP2 from EphA2 SAM and may help to release SHIP2 from the membrane, leading to receptor endocytosis and down-regulation. However, the overall system is likely to be complex since in addition to the EphA2 mediated localization; there are also several other mecha-
nisms for localization of SHIP2 to the membrane (51). A similar model of receptor clustering and signaling has been proposed for the LAT-Grb2-SOS1 system (52) and for another receptor tyrosine kinase, FGFR2, the concentration of Grb2 also plays a regulatory role (53, 54).

In vivo experiments are required to test our model regarding these differential roles of EphA2 SAM phosphorylation in the context of different cellular concentrations of Grb7 and SHIP2 and the formation of ternary complexes. The present study has established that chemically synthesized, full-length protein domains are valid, if not better, alternatives to proteins expressed using recombinant systems as posttranslational modifications can be introduced fully and in a site specific manner. This opens up avenues to probe other signaling systems and provide a detailed molecular insight into their mechanisms of signaling.

In summary, our study shows that the binding of adaptor protein, Grb7 SH2, to EphA2 SAM is dependent upon the phosphorylation state of specific tyrosine residues of the SAM domain. Further, binding of Grb7 to phosphorylated Y930 EphA2 SAM does not affect SHIP2 SAM binding (Figure 8). By contrast, phosphorylated Y921 cannot bind Grb7 and SHIP2 simultaneously.
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FIGURE LEGENDS

Figure 1: The tyrosines of EphA2 SAM. (A) Sequence alignment of selected SAM domains; the highly conserved tyrosine and histidine (Y921 and H894 in EphA2) residues are highlighted using red font and marked with asterisks. The three tyrosines of EphA2 SAM domain are highlighted by a yellow background and the secondary structure of this domain is shown above the alignment. (B) The location of the tyrosine and histidine residues of EphA2 SAM and SHIP2 SAM in the three dimensional structure. Structure of the EphA2 : SHIP2 SAM:SAM complex (PDB ID: 2KSO) is drawn using cylinder representation, EphA2 SAM is in purple, SHIP2 SAM in blue and the tyrosine and histidine residues are shown as sticks.
Y921 and Y930 are partially buried. The conserved tyrosine and histidine residues of both EphA2 SAM and SHIP2 SAM (Y921/H954 and Y1213/H1246, respectively) are involved in hydrogen bonds.

**Figure 2. The phosphorylated EphA2 SAM domains are well-folded.** 1D-1H NMR spectra of EphA2 SAM domains, (A) EphA2.pY921, (B) EphA2.pY930, (C) EphA2.pY960 and (D) unphosphorylated/recombinant EphA2. The chemically synthesized, phosphorylated EphA2 polypeptides and the recombinant EphA2 domain share a similar global fold.

**Figure 3. The phosphorylation of EphA2 SAM domains is not accompanied by large conformational changes.** 2D homonuclear 1H-NOESY spectra of (A) unphosphorylated EphA2 SAM, (B) EphA2.pY921, (C) EphA2.pY930, and (D) EphA2.pY960; the phosphorylated domains adopt near-native-like global folds.

**Figure 4. Phosphorylated SAM domains share similar secondary structure with the recombinant EphA2 SAM domain and are thermally stable.** (A-D) Far UV circular dichroism (CD) spectra of phosphorylated and unphosphorylated SAM domains; all the proteins are α-helical. (E-H) Thermal unfolding of the domains monitored at 222nm; the approximate mid-point of unfolding, Tm, is shown by arrow. Phosphorylation did not significantly destabilize the domains.

**Figure 5. Phosphorylation of EphA2 SAM does not affect its binding to SHIP2 SAM domain.** Interactions of (A) EphA2.pY921, (B) EphA2.pY930, and (C) EphA2.pY960 with SHIP2 SAM were measured by Isothermal Titration Calorimetry (ITC). The polypeptides bind SHIP2 SAM with micromolar affinities (K_D ~4 µM) similar to the recombinant EphA2 SAM (K_D ~5 µM). The derived thermodynamic parameters are listed in Table 1.

**Figure 6. Grb7 SH2 competes with SHIP2 SAM for binding to the EphA2 SAM domain phosphorylated at Y930.** Left panel: an overlay of part of the 1H-15N HSQC spectrum of a Grb7 SH2 (15N-labeled)/EphA2 phosphorylated protein mixture (blue) and in presence of SHIP2 (red) is shown on the left panels. Right panels show schematic representation of the complexes formed. (A) SHIP2 SAM competes with Grb7 SH2 for binding to EphA2.pY921; the overlaid spectra are similar suggesting that EphA2.pY921 bound to Grb7 SH2 cannot bind SHIP2 SAM simultaneously. However, broadening of only some resonances corresponding to the pY binding residues of Grb7 SH2 are observed due to intermediate NMR-timescale exchange that occurs in the competition. (B) EphA2.pY930 can bind both Grb7 SH2 and SHIP2 SAM simultaneously as evidenced by extensive line broadening of essentially all but the most flexible residues. This broadening occurs due to the formation of a large tri-molecular complex; since Grb7 SH2 is a dimer EphA2.pY930 and SHIP2 SAM are also likely to be dimerized. (C) The spectrum of EphA2.pY960 pre-mixed with Grb7 SH2 (15N-labeled) show no significant changes upon addition of SHIP2 SAM, demonstrating that this SAM domain does not bind Grb7 SH2.

**Figure 7. The proposed model for the differential regulation of the EphA2 receptor and SHIP2 SAM localization by Grb7 SH2 bound to phosphorylated EphA2 SAM.** (A) In the absence of Grb7 and irrespective of phosphorylation of the SAM domain, EphA2 SAM (dark blue) is bound to the SAM domain of SHIP2 (blue). Interaction of EphA2 SAM – SHIP2 SAM domain localizes SHIP2 to the plasma membrane. The extracellular and the TM regions are also likely to be dimerized throughout, as shown. Linear assemblies are predicted to be formed, as shown in panel below. EphA2 and SHIP2 are drawn as dimers and only the SAM and CC-domains (pink) are depicted. (B) Phosphorylation of Y921 and Y930 and EphA2:SHIP2:Grb7 complex at sub-stoichiometric Grb7 with respect to an EphA2:SHIP2 1:1 concentration; Grb7 SH2 dimer (green) binds to EphA2 at pY930 and provides maximum cross-linking forming arrays of EphA2:SHIP2 (lower panel). (C) When Grb7 SH2 is present at stoichiometric concentration, less cross-linking of EphA2-SHIP2 might give rise to linear chains. (D) For excess Grb7 SH2 there would be a competitive binding of the adaptor protein to pY921 of EphA2 (and also pY1213 SHIP2 SAM), which displaces SHIP2 protein from the membrane. This results in endocytosis and down regulation of the receptor.
Figure 8. Recruitment of Grb7 SH2 by EphA2 is specific to the phosphorylation of tyrosine residues of the SAM domain. The phosphorylated Y930 of the SAM domain of EphA2 can interact with Grb7 SH2 and the SAM domain of SHIP2 simultaneously; whereas, Grb7SH2 and SHIP2 SAM domains compete for the phosphorylated Y921. EphA2 SAM phosphorylated at Y960 does not bind Grb7SH2.
Table 1. Thermal stabilities of the recombinant and phosphorylated EphA2 SAM domains.

| Protein       | Thermal Stability (Tm) (K) |
|---------------|---------------------------|
| EphA2.pY921   | 351 ± 2.0                 |
| EphA2.pY930   | 352 ± 1.6                 |
| EphA2.pY960   | 337 ± 3.2                 |
| recombinant EphA2 | 345 ± 2.6              |
Table 2. Thermodynamics of binding of phosphorylated and unphosphorylated EphA2 SAM domains and peptides to SHIP2 SAM and Grb7 SH2.

| Protein in Cell | Titrant | K (µM) | ΔH (kcal/mol) | TΔS (kcal/mol) | ΔG (kcal/mol) | Comment |
|-----------------|--------|--------|---------------|---------------|---------------|---------|
| EphA2.pY921     | SHIP2  | 4.1 ± 0.5 | -4.9          | 2.5           | -7.4          |         |
| EphA2.pY930     | SHIP2  | 3.4 ± 0.4 | -5.1          | 2.4           | -7.5          |         |
| EphA2.pY960     | SHIP2  | 3.9 ± 0.2 | -4.7          | 2.7           | -7.4          |         |
| recombinant     | SHIP2  | 5.2 ± 0.3 | -2.5          | 4.7           | -7.2          |         |
| Grb7 SH2        | SHIP2  | 3.5 ± 0.1 | -1.95         | 18.4          | -7.3          |         |
| Grb7 SH2        | EphA2.pY921 | 2.6 ± 0.7 | -8.0          | -0.3          | -7.7          |         |
| Grb7 SH2        | EphA2.pY930 | 8.6 ± 4.3 | -2.5          | 4.4           | -6.9          |         |
| Grb7 SH2        | EphA2.pY960 | -        | -             | -             | -             | no interaction |
| Grb7 SH2        | recombinant EphA2 | - | - | - | - | no interaction |
| Grb7 SH2        | pep.pY921   | 3.2 ± 0.6 | -14.7         | -7.2          | -7.5          |         |
| Grb7 SH2        | pep.pY930   | 2.6 ± 0.4 | -4.8          | 2.8           | -7.6          |         |
| Grb7 SH2        | pep.pY960   | 3.0 ± 0.6 | -15.4         | -7.9          | -7.5          |         |
| Grb7 SH2        | all 3 of the unphosphorylated short peptides | - | - | - | - | no interaction |
Table 3. Thermodynamics of SHIP2 SAM competing for phosphorylated EphA2 SAM bound to Grb7 SH2 in comparison with the phosphorylated domains binding to SHIP2 SAM.

| In cell                 | Titrant | K (µM)  | ΔH (kcal/mol) | TΔS (kcal/mol) | ΔG (kcal/mol) |
|-------------------------|---------|----------|---------------|----------------|---------------|
| EphA2.pY921/Grb7 SH2    | SHIP2   | 6.5 ± 4.0| -4.1          | 3.0            | -7.1          |
| EphA2.pY930/Grb7 SH2    | SHIP2   | 6.8 ± 3.2| -4.4          | 2.7            | -7.1          |
| EphA2.pY960/Grb7 SH2    | SHIP2   | 4.5 ± 0.4| -5.2          | 2.2            | -7.4          |
Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2

FIGURE 1

A

EphB1
---TAFTVDDWLSAIK---MVQRDSFLTAGFTSLQLVTQMTESSDLRIGTLAGHKKILNSISHMRVQ---
EphB2
---TSFNTEDWLSAIK---MGQKESFANAGFTSFQVSMLEMIDLRGVTAGHKKILNSIQVMRAQ---
EphA2
---VPFRTVSEWLSIK---MQQTEHMAAGTAIEKTVQMTNDDIKRIGVRPLCQKRIAALSGLKDO---
EphA4
---SAVVSVDWLSAIK---MDYKDQFTAAGYTTLEAVHVNNQEDLARGITATQHKNKLLSVMATQ---
Ste11
---NDLFFVQLFLEIG---CTQYLDIJFICNLVEETEIKYLDLILIALGNKIDRLKILRKSQXVQ---
Byr2
---YTSKEVAELKSIQ---LEKYEIQFSQQNIEG-RHILNHLTLPILKDGLGIENATGKQFLRQDRYER---
CNKSR2
---WSPSQVDWMMKALDC---LQYIKNKREAQSG-DQILRAITHQESLEDLGVSRIQHQLILEAVDLLCAL---
SHIP2
---LGEAGMSAWLRAIG---LERYEEGLVNGWDDLEFLSDITEEDLEEAVQDPAHKRLLLDTLQLSK---

B

Y930
Y921
H954
Y960
Y1213
H1246
FIGURE 2
FIGURE 3

Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2
FIGURE 5

A  
B  
C  

Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2
Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2

FIGURE 6

A

EphA2.pY921

SHIP2 SAM

15N Grb7 SH2

B

EphA2.pY930

SHIP2 SAM

15N Grb7 SH2

C

EphA2.pY960

SHIP2 SAM

15N Grb7 SH2
Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2

FIGURE 7
Interaction of phosphotyrosine EphA2 SAM domains with Grb7 SH2
Binding and function of phosphotyrosines of the Ephrin A2 (EphA2) receptor using synthetic Sterile α Motif (SAM) domains
Susmita Borthakur, HyeongJu Lee, SoonJeung Kim, Bing-Cheng Wang and Matthias Buck

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