

AD _____

Award Number: DAMD17-03-1-0540

TITLE: Feasibility of SH2 Binding as a Prognostic and Diagnostic Indicator: Probing the Tyrosine Phosphorylation State in Breast Cancer by Src Homology 2 Domain Binding

PRINCIPAL INVESTIGATOR: Bruce J. Mayer

CONTRACTING ORGANIZATION: University of Connecticut Health Center
Farmington, CT 06030-0002

REPORT DATE: August 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20060315 026

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 01-08-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 07/15/04-07/14/05	
4. TITLE AND SUBTITLE Feasibility of SH2 Binding as a Prognostic and Diagnostic Indicator: Probing the Tyrosine Phosphorylation State in Breast Cancer by Src Homology 2 Domain Binding				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-03-1-0540	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bruce J. Mayer E-Mail: bmayer@neuron.uchc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Connecticut Health Center Farmington, CT 06030-0002				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: Improved molecular diagnostic methods that can classify tumors and predict their response to therapy have enormous potential to improve the effectiveness of breast cancer treatments. The overall goal of this project is to develop a novel molecular diagnostic method, termed SH2 profiling, that can classify cell samples based on their global protein tyrosine phosphorylation state. The first aim is to use an existing SH2 profiling method, based on far-Western blotting, to analyze fresh surgical breast cancer samples. The second aim is to develop a more high-throughput quantitative reversed-phase SH2 profiling format, and test its usefulness in classifying breast cancer sample. The third aim is to develop histochemical SH2 profiling methods that can be used to analyze archived, formalin-fixed tissue sections, and perform pilot retrospective studies to determine whether SH2 binding patterns have potential value. In the past year we have begun analysis of clinical breast cancer samples by both far-Western and reverse-phase protein array. In particular we have optimized and validated a robust reverse-phase array platform for SH2 profiling with throughput and reproducibility suitable for analysis of clinical tumor samples, and generated 45 new SH2 domain probes for use in SH2 profiling. We have performed SH2 profiling on eight clinical breast cancer samples, and performed hierarchical clustering analysis of these tumors based on their SH2 binding patterns. We have also found an intriguing correlation between SH2 binding pattern and the presence of inflammation in the tumor.					
15. SUBJECT TERMS Molecular Diagnostics, Prognostic Classification of Tumors, Phosphotyrosine, SH2 Domains, Signal Transduction, Protein Microarrays, Histochemical Analysis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 38	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code) 301-619-7325

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	10
References.....	10
Appendices.....	10

INTRODUCTION

The focus of this study is to test whether a novel molecular diagnostic approach, which we term SH2 profiling, can serve as a useful prognostic tool to classify breast cancer. SH2 domains are small protein modules that bind specifically to tyrosine-phosphorylated peptides. These domains play an important role in normal signal transduction, mediating the formation of multiprotein complexes in response to changes in tyrosine phosphorylation (Machida and Mayer, 2005; Pawson, 2004). There are ~116 SH2 domains in the human genome, and different SH2 domains recognize different tyrosine phosphorylated proteins. SH2 profiling is a method in which a battery of SH2 domain probes is used to provide a snapshot of the global state of tyrosine phosphorylation in a cell sample (Machida et al., 2003; Nollau and Mayer, 2001). Different breast cancers are likely to have different patterns of tyrosine phosphorylation, reflecting differences in the signal transduction pathways active in the tumor, and thus SH2 profiling has the potential to provide a biologically relevant means to classify these tumors. In this project we proposed to test different formats of SH2 profiling (far-western blotting, reverse-phase arrays, histochemical analysis of fixed tumor sections) using breast cancer samples to test the feasibility of these approaches and their potential utility as prognostic tools.

BODY

Task 1: Perform SH2 profiling on fresh surgical samples of invasive carcinoma of the breast. Correlate SH2 profile data with standard pathologic criteria and other known molecular markers.

Because of unforeseen difficulties in reconciling the human subjects research protocol approved by the UCHC Institutional Review Board with the requirements of the US Army Human Subjects Research Review Board, human subjects approval for this project was not received until August 2004. Since approval, we have been able to obtain a limited number of surgical samples from the University of Connecticut Health Center and Hartford Hospital. We have to date performed reverse-phase SH2 profiling and 1D far-western SH2 profiling on a total of 8 different tumors (for some tumors different histologically distinguishable regions of the same tumor were analyzed separately) as well as two adjacent normal breast tissues.

The reverse-phase far-Western array method (described in detail in Task 2, below) was the primary method used to analyze these breast cancer tumor samples. We have not yet attempted to correlate the resulting SH2 profiling patterns with clinical outcomes due to the low number of samples. However, as detailed below in Task 2c, we have noted in at least one case that the presence of binding sites for a particular SH2 domain (Eat2) correlates with the presence of infiltrating lymphocytes. A goal for the next year will be to profile a larger number of breast cancer samples. To this end we are now collaborating with Dr. Kevin Claffey at UCHC, who has already banked more than 30 additional frozen breast cancer tumor samples, along with associated histological sections. These samples will be profiled along with any additional samples accrued over the next year. Thus by the end of the funding period we should have an excellent sense of the feasibility of using SH2 profiling to classify breast cancers.

Task 2: Develop reverse-phase protein microarray assay to quantitatively analyze SH2 binding profiles of patient tumor samples

- a) Test approach using macroarrays of lysates of cell lines (months 1-6)
- b) Test miniaturization of assay to microarray format—detection by ECL (months 7-12)
- c) Apply to tumor samples of Aim 1 (years 2 and 3)
- d) Test fluorescent SH2 labeling and detection (year 2)

Task 2a: We have devoted considerable effort to developing and validating a robust, reproducible, and quantitative reverse-phase (RP) far-Western array platform that can be used to generate SH2 profiling data from complex protein mixtures (cell or tissue lysates) as well as purified phosphoproteins. The current assay format (which we term the rosette RP assay) uses a 96-well blotting apparatus. Protein samples are replica-spotted on membranes in register with the wells of the blotting apparatus, and then each well is incubated with a different SH2 domain probe. Thus the relative binding of many SH2 domains can be quantified for each sample. In the past year we have dramatically increased the throughput of this assay format by spotting an array of up to 20 protein samples per well. Thus on a single membrane, more than 1900 quantitative data points can be generated (for example, 120 different protein samples (6 wells) can be tested simultaneously for binding to 16 different SH2 domain probes).

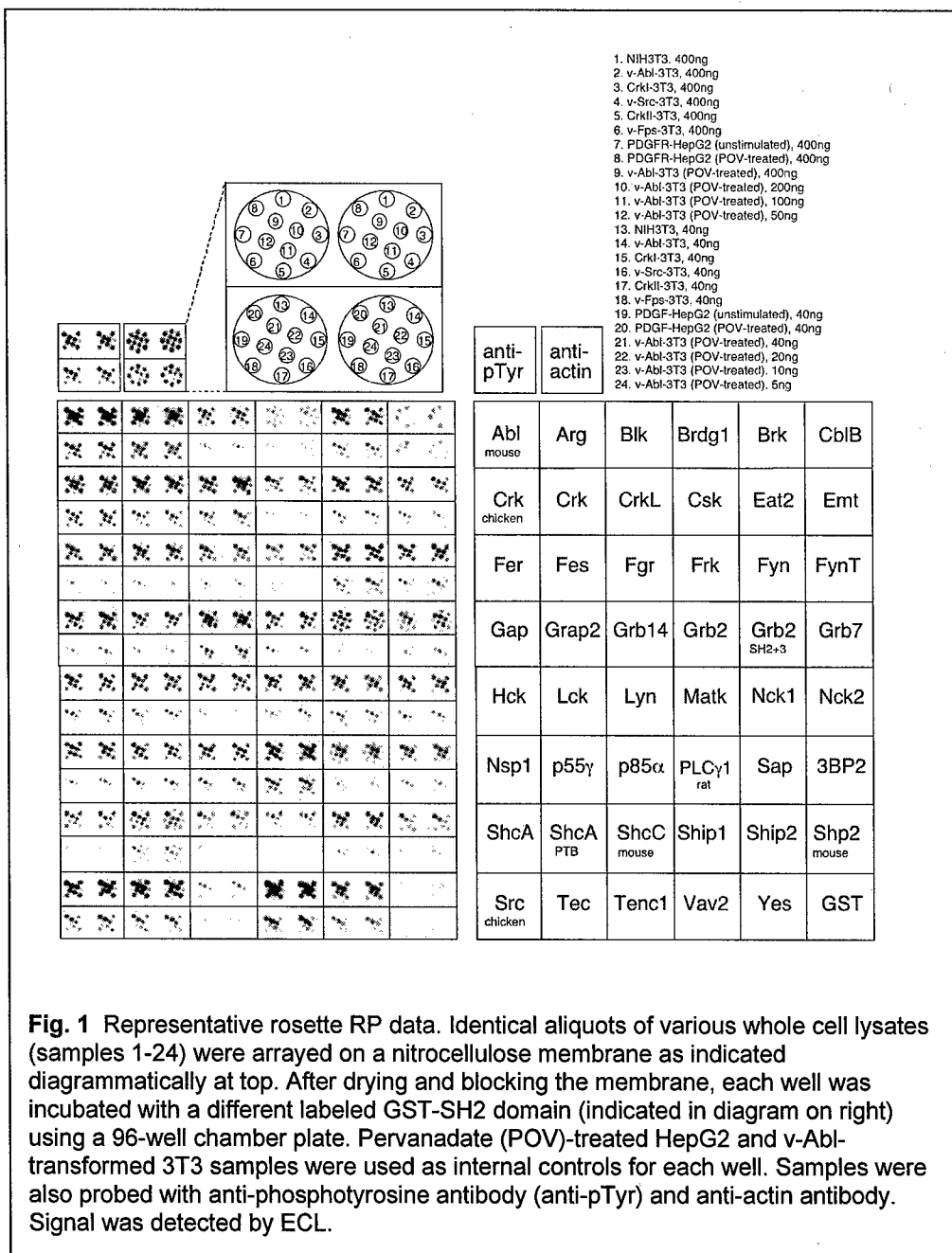


Fig. 1 Representative rosette RP data. Identical aliquots of various whole cell lysates (samples 1-24) were arrayed on a nitrocellulose membrane as indicated diagrammatically at top. After drying and blocking the membrane, each well was incubated with a different labeled GST-SH2 domain (indicated in diagram on right) using a 96-well chamber plate. Pervanadate (POV)-treated HepG2 and v-Abl-transformed 3T3 samples were used as internal controls for each well. Samples were also probed with anti-phosphotyrosine antibody (anti-pTyr) and anti-actin antibody. Signal was detected by ECL.

Small amounts of protein (generally less than 1 microgram whole cell lysate) are spotted on nitrocellulose membranes. Each well is then incubated with GST-SH2 domains pre-complexed with glutathione-HRP conjugate, membranes are washed and bound probe detected by enhanced chemiluminescence (ECL) on X-ray film. Scanned films are then quantified and background subtracted using ImageJ v1.33 software. Fig. 1 shows a representative experimental result. In this case 24 different samples (whole protein lysates from various tissue culture cell lines) were analyzed in duplicate for binding to 47 different SH2 domain probes. Lysates of pervanadate-treated v-Abl transformed NIH-3T3 cells were used as a positive control for SH2 binding. Two different dilutions of cell lysate (40 ng and 400 ng total protein) were analyzed to ensure signals were in the linear range. Other controls included a GST

probe (negative control for nonspecific binding of GSH-HRP-labeled probe to protein lysates), anti-pTyr (positive control for detection of phosphotyrosine in cell lysates), and anti-actin (positive control for spotting of cell lysates).

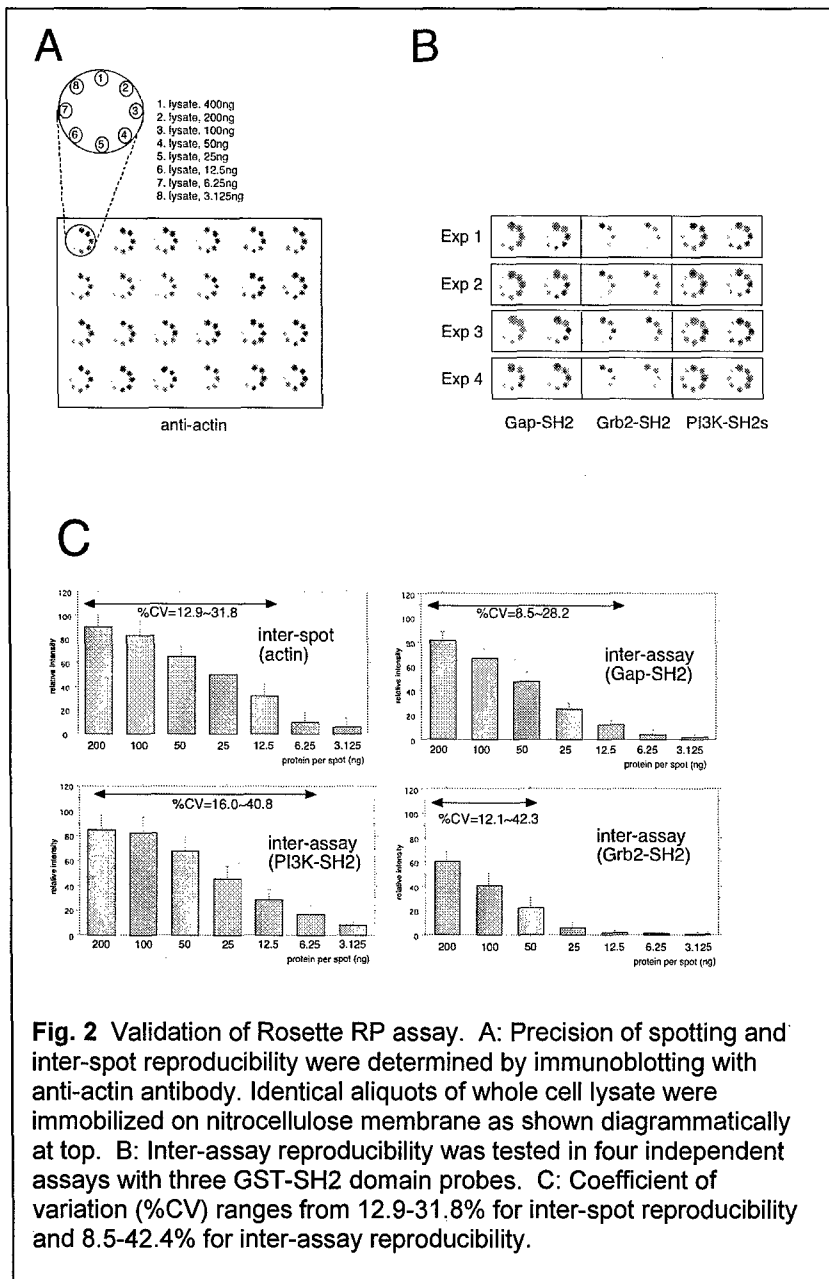


Fig. 2 Validation of Rosette RP assay. A: Precision of spotting and inter-spot reproducibility were determined by immunoblotting with anti-actin antibody. Identical aliquots of whole cell lysate were immobilized on nitrocellulose membrane as shown diagrammatically at top. B: Inter-assay reproducibility was tested in four independent assays with three GST-SH2 domain probes. C: Coefficient of variation (%CV) ranges from 12.9-31.8% for inter-spot reproducibility and 8.5-42.4% for inter-assay reproducibility.

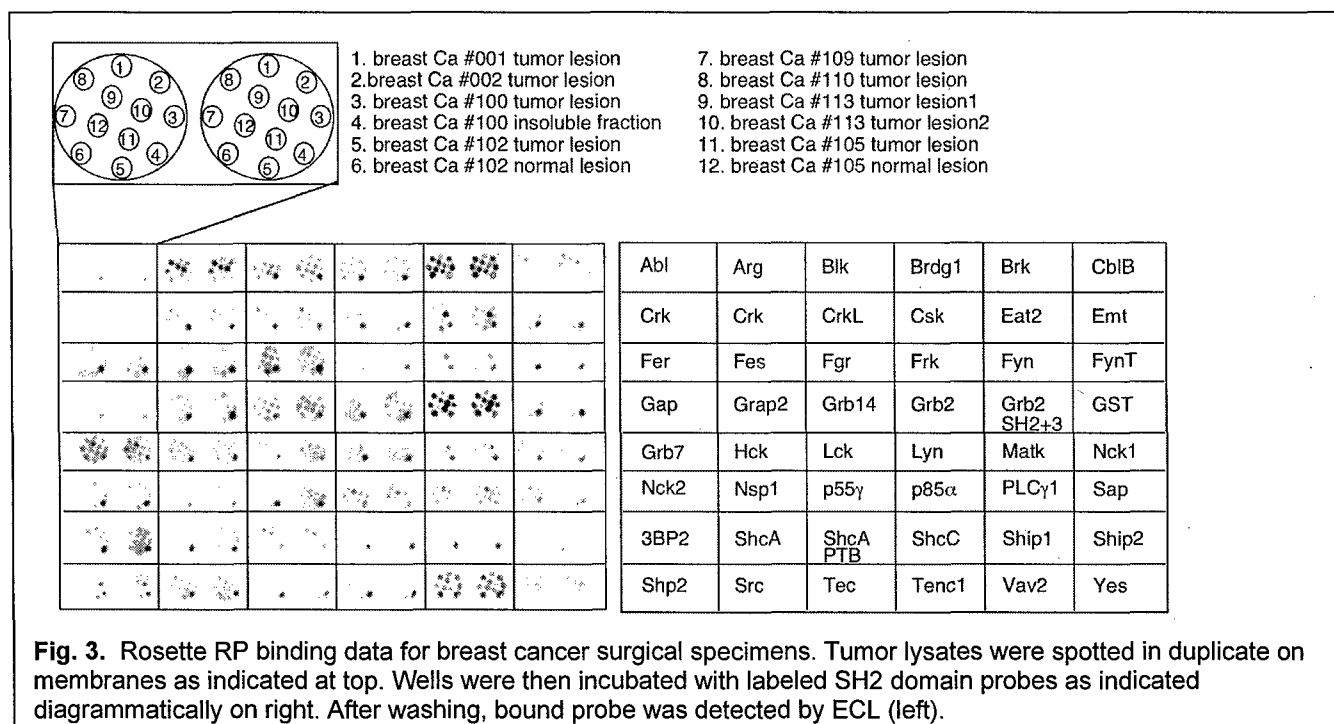
and the remaining 22 are currently under construction. By the end of the funding period, therefore, we should have in hand the entire complement of human SH2 domains for use in SH2 profiling.

Task 2b: We have not yet tested the miniaturization of this assay to a microarray (nitrocellulose-coated slide) format because the throughput of the established rosette RP format is sufficient for the number of clinical samples now available. The current format can also more easily accommodate large numbers of different SH2 probes (up to 96 at one time), whereas a slide-based microarray would require a separate slide for each probe, leading to lower throughput and reliability, and higher cost. Thus we felt that the effort to establish the

Reproducibility is a critical concern in the development of molecular diagnostic assays, thus we have extensively validated the rosette RP array system. We have found that inter-spot and inter-assay coefficients of variation range from 8.5% to 42.5% (Fig. 2). Almost all of the variation is due to spotting and detection errors, as inter-spot variation is virtually equivalent to the error between experiments (probe binding, washing, detection, and quantification performed on different days). Higher variation is seen with weaker signals (lower sample amounts); with more typical sample amounts (>100 ng), variation is generally less than 20%. We note that if the assay were moved to a true microarray format with robotic spotting and direct digital detection of bound probe, these spotting and detection errors are likely to decrease substantially. Such improvements will be a goal year 3 (Task 2b).

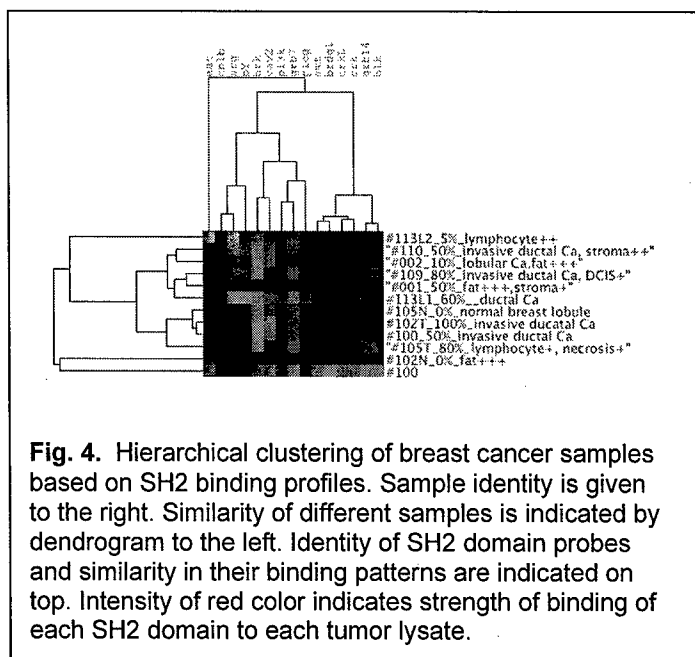
We have also devoted considerable effort in the past year to increasing the number of SH2 domain probes available for use in SH2 profiling studies. There are ~116 total SH2 domains in the human genome, and a much smaller number of phosphotyrosine-binding PTB domains (Pawson, 2004). During this past year we have generated new GST fusions for 45 previously unavailable SH2 domains, as well as three PTB domains. Thus we now have in the laboratory GST fusions for 94 out of 116 known SH2 domains (>80%),

microarray format was not yet justified. However the rosette RP array experiments are providing valuable information regarding sample preparation, blocking, binding and washing conditions, and data analysis that could easily be applied to the microarray format in the future. We have established a collaboration with Dr. Winfried Kruger in the UCHC molecular core facility to test robotic spotting of protein lysates on nitrocellulose slides. Dr. Kruger is also interested in constructing and testing microfluidic chambers that would allow multiple SH2 domain probes to be used on a single slide, circumventing many of the problems outlined above for the microarray format. These pilot experiments will be pursued in Year 3 of this award.



Task 2c: We have analyzed tissue lysates from eight surgical breast cancer samples, plus two adjacent normal samples. For one tumor (#113), lysates were prepared from two sections of the same tumor, one of which exhibited significant infiltration by inflammatory cells (lesion 2). Duplicate samples were profiled using the rosette RP assay described above in Task 2a using 48 different SH2 or PTB domain probes, plus anti-phosphotyrosine and anti-actin controls. Results are shown in Fig. 3. As can be readily seen, different SH2 domains vary widely in their binding patterns to tumor lysates, and more importantly different breast cancer samples exhibit different patterns of SH2 binding. In order to group the tumor samples based on similarity in their SH2 binding patterns, background-corrected quantitative binding data were then analyzed using a hierarchical clustering algorithm (Cluster v2.11 (Eisen et al., 1998) with “average linkage clustering” settings). The result was visualized as a “heat map” using Java TreeView (Saldanha, 2004) (Fig. 4). Clustering and bioinformatics studies were done in collaboration with Dr. Dong-Guk Shin and colleagues in the Department of Computer Engineering at UConn-Storrs.

While the number of tumor samples is too low to draw any general conclusions regarding the significance of SH2-based clustering, we feel that the data are promising in several ways. First, all breast tumors do not have similar profiles, and it is clear that some are quite distinct (for example, tumor #100, and tumor #113 lesion 2), suggesting they might have distinct biological properties. Tumor #113 is particularly interesting, in that two separate lesions of the same tumor were profiled. One particularly striking feature was very strong binding of the Eat2 SH2 to the lesion 2 lysate, whereas virtually no binding for this probe was seen for lesion 1. This result was confirmed by 1D far-Western blotting analysis with the Eat2 SH2 probe (Fig. 5). A very strong Eat2 SH2-



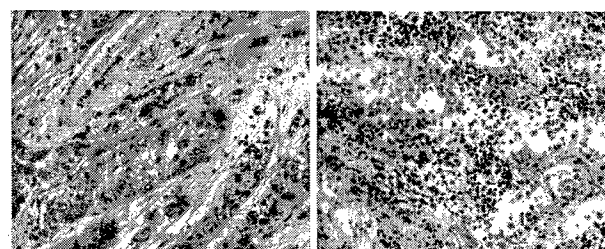
system in which GST-SH2 fusion proteins are expressed with a C-terminal “avitag”, which directs enzymatic biotinylation by the BirA product in *E. coli* (O’Callaghan et al., 1999). Each SH2 probe is thus tagged with a single biotin moiety, eliminating the disadvantages of heterogeneous multiple biotinylation seen when chemical biotinylation is performed. These singly-biotinylated probes could easily be stoichiometrically labeled using fluorophore-conjugated streptavidin for detection of binding in microarray formats using standard fluorescent array readers. We intend to test fluorescent labeling of SH2 domains when we perform pilot experiments of the microarray format (Task 2b), as this will allow us to quantify SH2 binding using the same fluorescent readers and software now used for nucleic acid microarray experiments.

Task 3: Establish conditions for using SH2 domains to probe sections of paraffin-embedded tumor samples. Perform retrospective study using archived tumors to test if positive staining with specific SH2 probes correlates with recurrence or survival.

- Establish histochemical staining method for detection and quantitation of binding of SH2 domains to sections of tumors (year 1)
- Analyze ~100 archived samples per year with at least 5 SH2 domain probes (ongoing; aim is to analyze at least 200 by end of year 2)
- Establish database with pathological criteria, patient outcomes; SH2 binding data when available (months 1-6)

binding band is seen for tumor #113 lesion 2, which is absent for the other tumor and normal samples. When frozen sections of lesions 1 and 2 from tumor 113 were examined by traditional histochemical staining, it is apparent that large numbers of infiltrating lymphocytes were present in lesion 2, but were largely absent in lesion 1 (Fig. 5). Thus the strong Eat2 signal in the SH2 binding profile correlates with inflammation. This is consistent with the role of Eat2 in mediating immunoreceptor signaling (Engel et al., 2003). Of course more work will be required to confirm this apparent correlation and its biological significance. But this result serves to highlight the potential of the SH2 profiling approach to sub-classify tumor samples in a biologically meaningful fashion.

Task 2d: We have not explicitly produced fluorescent SH2 probes, but we continue to exploit a



Tumor #113 lesion1

Tumor #113 lesion2

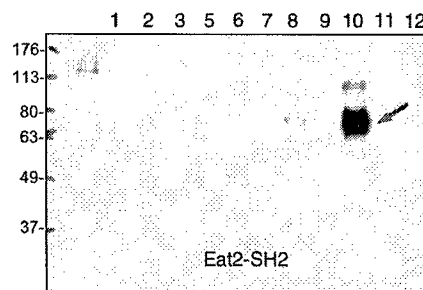


Fig. 5. Strong Eat2 SH2 binding correlates with inflammation. Top, frozen sections of lesions 1 and 2 of tumor 113 stained with H&E. Numerous infiltrating lymphocytes are observed in lesion 2. Bottom, 1D far-Western blot of tumor cell lysates (lane numbers correspond to sample numbers in Fig. 3), probed with Eat2 SH2 domain. Strong binding signal is observed only for sample 10 (red arrow), corresponding to tumor #113 lesion 2.

- d) Correlate SH2 binding with standard pathological criteria (months 6-24)
- e) Correlate SH2 binding with patient outcomes (recurrence, mortality) (years 2 and 3)

Work on Task 3 was initially delayed by lack of approval by the HSRRB to perform research on human anatomic samples. Once approval was obtained we performed pilot experiments to better define optimal conditions for histochemical analysis of tissue sections with SH2 domain probes, as outlined in the last progress report. In the past year these experiments have not been pursued further, as we felt that it was more important at this time to devote the limited manpower and resources available to the project to the development of the high-throughput reverse phase far western array, and to generate new SH2 domain probes, as outlined above in tasks 1 and 2. We are now poised, however, to begin the histochemical analyses of Task 3 in the near future.

Dr. Poornima Hegde, of the Department of Anatomic Pathology at UCHC, has continued her work assembling matched panels of invasive carcinoma of the breast, varying in Her2/Neu amplification and in their ER/PR status. These samples will be used in initial experiments to determine if binding of different SH2 domains correlates with these standard prognostic criteria. She has also helped with analysis of existing fresh frozen breast cancer samples (Task 2c), and sections from these existing tumors will be useful for validating tissue staining with SH2 domains (for example, we expect to see strong staining of sections of tumor #113, lesion 2 and not of lesion 1 when the Eat2 SH2 probe is used). We have continued to work with Ms. Laura Glynn of the UCHC General Clinical Research Center who is the study coordinator for all ongoing SH2 profiling studies, and who maintains a database (with patient identification coded) for cancer samples and associated clinical data.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization and validation of robust reverse-phase array platform for SH2 profiling with throughput and reproducibility suitable for analysis of clinical tumor samples
- Generation and purification of ~45 new SH2 domain probes in past year
- SH2 profiling of 8 clinical breast cancer samples
- Hierarchical clustering analysis of breast cancer samples based on SH2 binding patterns
- Preliminary correlation of specific SH2 binding (of Eat2 SH2 domain) with presence of inflammation in breast cancer sample

REPORTABLE OUTCOMES

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Profiling human SH2 domains. Abstract for poster presentation at Cold Spring Harbor Meeting on Protein Phosphorylation and Cell Signaling, May 18-22, 2005.

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Profiling global tyrosine phosphorylation patterns in breast cancer. A novel molecular diagnostic approach. Abstract for poster presentation at the 2005 Era of Hope meeting, June 8-11, 2005.

Review Article: Machida K and Mayer BJ. The SH2 domain: versatile signaling module and pharmaceutical target. *Biochim Biophys Acta Proteins and Proteomics* 2005; 1747:1-25

Invention Disclosure: Bruce J. Mayer, Kazuya Machida, and Peter Nollau, inventors. High-throughput multiplexed SH2 profiling methods. University of Connecticut disclosure #05-926, submitted May 5, 2005.

CONCLUSIONS

The work over the past year has established the feasibility of using SH2 profiling for analysis of clinical breast cancer samples, and suggest that SH2 profiling might provide useful information that can be used to classify breast cancer and to predict clinical outcomes. In the past year we have dramatically increased the throughput of the rosette RP SH2 profiling assay so that large numbers of clinical specimens can be analyzed simultaneously. Importantly, we have validated the inter- and intra-assay variability of the assay, and find that it is highly reproducible. We have continued to make progress in generating additional SH2 domain probes, so that at this time we have over 80% of the human complement of SH2 domains available for use in our profiling assays. Most importantly, we have now begun to use the SH2 profiling platform we have developed to analyze surgical breast cancer samples. We have obtained strong binding signals from clinical samples, and the profiles differ among the different tumors and can be used as the basis for clustering using an unsupervised hierarchical clustering algorithm. Although the small number of clinical samples currently available precludes correlation of SH2-based clustering with clinical parameters, we did note a striking increase in binding of the Eat2 SH2 domain to the one sample exhibiting extensive lymphocyte infiltration, suggesting that the SH2 binding patterns reflect meaningful biological differences in the different samples. Thus we are now poised to further exploit this novel method and to attempt to correlate specific binding patterns with clinical outcomes.

REFERENCES

- Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl Acad. Sci. USA.* 95:14863-14868.
- Engel, P., M.J. Eck, and C. Terhorst. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat. Rev. Immunol.* 3:813-821.
- Machida, K., and B.J. Mayer. 2005. The SH2 domain: versatile signaling module and pharmaceutical target. *Biochim Biophys Acta Proteins and Proteomics.* 1747:1-25.
- Machida, K., B.J. Mayer, and P. Nollau. 2003. Profiling the global tyrosine phosphorylation state. *Mol. Cell. Proteomics.* 2:215-233.
- Nollau, P., and B.J. Mayer. 2001. Profiling the global tyrosine phosphorylation state by Src Homology 2 domain binding. *Proc. Natl. Acad. Sci. USA.* 98:13531-13536.
- O'Callaghan, C.A., M.F. Byford, J.R. Wyer, B.E. Willcox, B.K. Jakobsen, A.J. McMichael, and J.I. Bell. 1999. BirA enzyme: Production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation. *Anal. Biochem.* 266:9-15.
- Pawson, T. 2004. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell.* 116:191-203.
- Saldanha, A.J. 2004. Java Treeview--extensible visualization of microarray data. *Bioinformatics.* 20:3246-3248.

APPENDICES

Research Abstract from Cold Spring Harbor Meeting on Protein Phosphorylation and Cell Signaling, May 18-22, 2005.

Research Abstract from 2005 Era of Hope meeting, June 8-11, 2005.

Review Article: Machida K and Mayer BJ. The SH2 domain: versatile signaling module and pharmaceutical target. *Biochim Biophys Acta Proteins and Proteomics* 2005; 1747:1-25

PROFILING HUMAN SH2 DOMAINS

Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer
Department of Genetics and Developmental Biology, University of
Connecticut Health Center, Farmington, CT

Tyrosine phosphorylation of proteins can create binding sites for downstream signaling molecules via phosphotyrosine-binding domains such as Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Approximately 116 SH2 domains are thought to exist in humans. We have generated GST fusions for almost the entire human complement of SH2 domains, with the aim of using them to profile the global tyrosine phosphorylation state of the cell (termed SH2 profiling). We have established a quantitative multiplex reverse-phase assay in which large numbers of protein samples (purified proteins or cell lysates) are immobilized on membranes and probed separately with multiple labeled SH2 domains. This high-throughput method enables more than 1,000 simultaneous SH2 binding reactions (number of protein samples x number of SH2 domains) in a single assay. We determined the phosphorylation dependency of binding for the human SH2 domains, and their binding patterns to lysates of cells after activation of protein-tyrosine kinase (PTK) receptors, cells transformed by activated PTK oncogenes, or human tumor samples. Approximately half of the GST-SH2 domains showed clear phosphorylation-dependent binding *in vitro*; for others detectable binding was not observed or fusion proteins were insoluble in bacteria. Since SH2 domains are highly homologous at the amino acid level, we asked whether their binding characteristics (by multiplex assay or far-western blotting) could be correlated with specific sequences. Binding characteristics were highly associated with phylogenetic relatedness based on multiple alignment analysis; as expected, SH2 domains from closely related family members showed similar patterns of binding to proteins from PTK-transformed cell samples. On the other hand, we observed distinct binding patterns for some SH2 domains found to have similar binding preferences by peptide library screening. This proteomic approach permits the rapid and comprehensive analysis of global patterns of SH2 domain binding to specific phosphoproteins, or to all phosphoproteins present in a cell. Such information reveals the connectivity of signaling pathways downstream of tyrosine phosphorylation, and may also be useful as a molecular diagnostic method for classifying malignancies.

PROFILING GLOBAL TYROSINE PHOSPHORYLATION PATTERNS IN BREAST CANCER. A NOVEL MOLECULAR DIAGNOSTIC APPROACH

Kazuya Machida, Christopher Thompson, and Bruce J. Mayer

Raymond and Beverly Sackler Laboratory of Genetics and Molecular Medicine,
Department of Genetics and Developmental Biology, University of Connecticut Health
Center, Farmington CT 06030-3301
bmayer@neuron.uchc.edu

The availability of improved prognostic indicators for breast cancer, making possible more accurate classification of tumors and prediction of clinical outcomes, would have enormous potential for improving treatment of the disease. The ability to predict a tumor's response to therapy, or its likelihood of metastasis or recurrence, would allow the patient and her physician to make more informed decisions about how to treat her cancer. The goal of this project is to develop novel molecular diagnostic methods to profile the global state of tyrosine phosphorylation in breast cancer cells. Because tyrosine phosphorylation controls many biological activities relevant to tumorigenesis, including proliferation, differentiation, motility, and invasion, it is likely that differences in tyrosine phosphorylation patterns will correlate with differences in the behavior of tumors in the body. We have developed a method, termed SH2 profiling, that uses a battery of Src Homology 2 (SH2) domains as probes for the presence of tyrosine phosphorylated proteins in a cell extract. SH2 domains are small protein modules that bind specifically to tyrosine phosphorylated peptides. There are ~115 SH2 domains in the human genome, and different SH2 domains bind to different classes of phosphorylated sites.

We have recently developed an improved high-throughput reverse-phase SH2 binding assay that can simultaneously obtain quantitative binding data for hundreds of tumor protein extracts and more than 50 different SH2 domain probes. We are now using a variety of bioinformatic approaches including unsupervised and supervised clustering algorithms to classify tumor samples based on their quantitative SH2 domain binding profiles. We are also applying SH2 profiling to the analysis of fixed, paraffin-embedded tumor samples. In this approach, SH2 domains are used as histochemical probes for breast cancer tumor sections and tissue microarrays. Such an approach will be important for establishing whether classifications based on SH2 profiling have prognostic value, as long-term clinical outcomes data are available for archived tumor samples.

Our preliminary data indicate that the SH2 profiling methods we have developed can successfully classify tumors based on their global tyrosine phosphorylation state, and are therefore likely to provide novel prognostic information not currently available by current methods. Compared with current genomic or proteomic profiling methods, SH2 profiling is relatively rapid, simple, and uses small amounts of tumor tissue. Once available in the clinic, such prognostic tests may allow each patient's treatment to be individually tailored to maximize therapeutic benefit while minimizing unnecessary suffering.

The U.S. Army Medical Research and Materiel Command under DAMD1703-1-0540 supported this work.



Review

The SH2 domain: versatile signaling module and pharmaceutical target

Kazuya Machida*, Bruce J. Mayer

Raymond and Beverly Sackler Laboratory of Genetics and Molecular Medicine, Department of Genetics and Developmental Biology,
University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3301, United States

Received 20 July 2004; received in revised form 29 September 2004; accepted 11 October 2004

Available online 2 November 2004

Abstract

The Src homology 2 (SH2) domain is the most prevalent protein binding module that recognizes phosphotyrosine. This ~100-amino-acid domain is highly conserved structurally despite being found in a wide variety of proteins. Depending on the nature of neighboring protein module(s), such as catalytic domains and other protein binding domains, SH2-containing proteins play many different roles in cellular protein tyrosine kinase (PTK) signaling pathways. Accumulating evidence indicates SH2 domains are highly versatile and exhibit considerable flexibility in how they bind to their ligands. To illustrate this functional versatility, we present three specific examples: the SAP, Cbl and SOCS families of SH2-containing proteins, which play key roles in immune responses, termination of PTK signaling, and cytokine responses. In addition, we highlight current progress in the development of SH2 domain inhibitors designed to antagonize or modulate PTK signaling in human disease. Inhibitors of the Grb2 and Src SH2 domains have been extensively studied, with the aim of targeting the Ras pathway and osteoclastic bone resorption, respectively. Despite formidable difficulties in drug design due to the lability and poor cell permeability of negatively charged phosphorylated SH2 ligands, a variety of structure-based strategies have been used to reduce the size, charge and peptide character of such ligands, leading to the development of high-affinity lead compounds with potent cellular activities. These studies have also led to new insights into molecular recognition by the SH2 domain.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Protein tyrosine kinase; Signal transduction; SH2 inhibitor; Phosphotyrosine mimetic

1. Introduction

The Src homology 2 (SH2) domain is the prototype for protein–protein interaction modules that mediate the formation of multiprotein complexes during signaling [1,2]. Unlike other binding modules such as the SH3 or PDZ domains, SH2 domains specifically function in protein tyrosine kinase (PTK) pathways, due to the dependence of their binding on tyrosine phosphorylation. They are evolutionarily conserved in many eukaryotes [3], and ~116 different examples (representing ~106 distinct proteins) are found in the human genome [4]. In addition to the

phosphorylated tyrosine (pTyr) residue, each SH2 domain recognizes several additional flanking residues, usually three to five amino acids C-terminal to pTyr, thereby acquiring selectivity for specific phosphorylated sites. For example, the autophosphorylated cytoplasmic domain of the PDGFβ receptor provides distinct binding sites for several specific SH2-containing proteins, including Y579 (*po*YIYV, where *po*Y denotes phosphotyrosine [5]) for Src and its relatives; Y740 (*po*YMDM) for phosphatidylinositol 3-kinase (PI3K); and Y771 (*po*YMAP) for Ras-GAP. Combinatorial phosphopeptide libraries have been used to probe the binding preferences of SH2 domains, leading to the realization that SH2 domains can be divided into classes based on their sequence and binding preferences [6,7].

In the cell, the specific association of SH2 domains and tyrosine phosphopeptides is used to mediate the reversible relocalization of proteins, which is important for efficient propagation of PTK signals [8]. SH2 domains are usually

Abbreviations: pTyr, phosphotyrosine; Cdep, 3'-carboxyl 4'-dicarboxyl phenylalanine; Fcp, 3'-aldehyde 4'-carboxyl phenylalanine; Dpp, 3', 4'-diphosphonophenylalanine; dpmF, diphosphonomethyl-phenylalanine

* Corresponding author. Tel.: +1 860 679 1835; fax: +1 860 679 8345.

E-mail address: machida@neuron.uhc.edu (K. Machida).

found in proteins together with other modules, either catalytic domains (for example, protein or lipid kinase and phosphatase domains), or other protein binding modules (for example SH3, PH, or PTB domains). Thus, depending on the nature of those other modules, proteins containing SH2 domains can participate in a wide variety of intracellular functions (reviewed in Refs. [2,8–11]). For instance, Grb2 (which contains two SH3 domains in addition to one SH2 domain) functions to recruit the downstream regulators SOS and Gab1 following receptor tyrosine kinase (RTK) activation. In this example, the Grb2 SH2 domain binds to sites on the autophosphorylated RTK, leading to Ras activation via SOS, which is bound to the N-terminal SH3 domain, while the PI3 kinase-Akt pathway is activated via Gab1, which is bound to the C-terminal SH3 domain [10]. When the other domains include an inhibitory module, as in the case of Cbl or SOCS, SH2-containing proteins can also act as negative regulators of signaling (discussed below, Section 2).

In addition to relocating proteins to specific sites (binding in *trans*), interplay between an SH2 and other domains in the same molecule (in *cis*) can also be important. SH2 domains of the Src and Abl families of nonreceptor tyrosine kinases engage in intramolecular interactions that maintain the kinase in an inactive configuration [12]. Association of the SH2 domains with extrinsic ligands relieves the autoinhibitory interaction, triggering catalytic activation. Similarly, activation by SH2 domain ligands has been reported for proteins containing tandem SH2 domains

such as SHP-1, SHP-2, PI3K, Syk, ZAP-70 and PLC γ probably because the global protein conformation is altered upon ligand binding [13–19]. In the case of nonreceptor tyrosine kinases, the SH2 and kinase domain also cooperate to enhance signals by facilitating processive phosphorylation of multiple tyrosines on substrate proteins [20–22].

SH2 domain function relies on two principles, tyrosine phosphorylation dependency and ligand specificity, whose structural basis is well understood. An SH2 domain consists of ~100 amino acids with two α -helices and seven β -strands, ordered $\beta\alpha\beta\beta\beta\beta\alpha\beta$, with a central anti-parallel β -sheet flanked by α -helices [23] (Fig. 1). Tyrosine-phosphorylated ligand peptides are recognized by two key regions, one the pTyr binding site (referred as the pTyr pocket), and a so-called “specificity determining region” which interacts with ligand residues C-terminal to pTyr. Based on X-ray crystal structure studies of the Src and Lck SH2 domains bound to high affinity ligands, the typical SH2 binding mode has been likened to a two-pronged plug engaging a two-holed socket, where the peptide backbone is extended and the side chains of pTyr and the Y+3 residue each project deeply into complementary pockets on the SH2 surface [24,25]. Different binding modes have also been observed; for instance, the Grb2 SH2 prefers ligands with a β -turn conformation of the peptide backbone, in which the Y+2 Asn residue plays a critical role [6,26] (various binding modes are reviewed in Ref. [27]).

Although the two-pronged model is a useful description of SH2 binding, the binding energies conferred by the pTyr

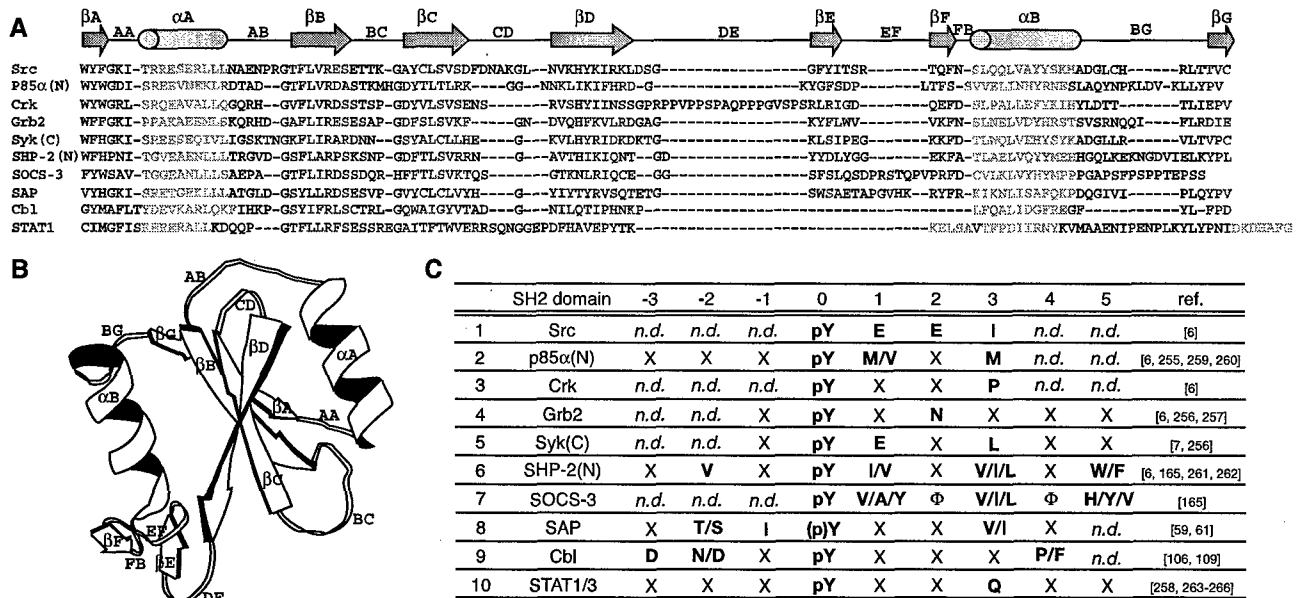


Fig. 1. Common architecture of SH2 domains. (A) Sequence alignment of various human SH2 domains. Typical secondary structural elements are depicted above the sequences and labeled according to standard nomenclature; α -helices are colored orange and β -strands blue. Divergent SH2 sequences for Cbl and STAT1 are also shown. (B) Ribbon diagram of prototypic SH2 domain. Secondary structural elements and loops are labeled. Figure was modified from crystal structure of Src SH2 domain [24]. (C) Phosphopeptide preferences of SH2 domains. Sequence preferences of SH2 domains described above were determined by combinatorial phosphopeptide library screening [6,7,59,61,109,165,255–258], mutagenesis [259–266] or structural analysis [106]. Positions are denoted as N-terminal (–3, –2, –1) or C-terminal (+1, +2, +3, +4, +5) relative to pTyr (0). X, any amino acid; Φ , hydrophobic amino acid. n.d., not determined (this position was not randomized or mutated in relevant reports).

pocket and the specificity-determining region are not equivalent. It is appropriate that the pTyr-dependent interaction should dominate, to ensure that the domain acts as a phosphorylation-dependent molecular switch. As a consequence, the selectivity of a single SH2 domain for its cognate peptide motif is not absolute. The *in vitro* dissociation constant for binding of an SH2 domain to its preferred pTyr peptide ranges from 0.2 to 1 μ M, over four orders of magnitude greater than the affinity for the unphosphorylated counterpart, but only moderately higher (~30–150-fold) than for nonspecific pTyr peptides [6,28–31]. It is important to remember that physiologically relevant ligands may not necessarily conform to the “optimal” high-affinity ligands obtained from *in vitro* screens [32]. Moderate-specificity interactions can be favored by high local concentration, or by the interaction of other domains of the two partners. In other cases, recognition of overlapping sets of target peptides by SH2 domains may be important to allow signals to amplify or diverge, creating multiplexed signaling networks.

The peptide binding specificity of an SH2 domain can be switched by point mutation of a key residue in the specificity determining region [33–35]. For instance, a Thr to Trp mutation at residue EF1 of the Src SH2 converted its peptide preference to that of Grb2 [34] (here and throughout, the established nomenclature for SH2 domain residues is used, where strands and helices are denoted by letter; see Fig. 1) [25]. More recently, it has been suggested that many SH2 domains, including the unmutated Src SH2, are able to bind quite well to typical Grb2 ligands [36]; however, in the case of the Grb2 SH2, the presence of the bulky Trp side chain prevents binding of extended peptides and thus selects exclusively for *poYxNx* motifs that adopt a β -turn confirmation [35]. Thus, SH2 binding specificity and binding mode appear to be more flexible than initially thought. Indeed, although SH2 binding is entirely dependent on tyrosine phosphorylation in most cases, ligands lacking pTyr have also been reported, including serine- or threonine-phosphorylated sites [37–40], phosphatidylinositol 3,4,5-trisphosphate [19,41], and unphosphorylated ligands [42–45]. This suggests that some SH2 domains might have evolved in an *ad hoc* fashion to recognize, with moderate affinity, various negatively charged ligands.

There are multiple examples of relatively divergent or atypical SH2 sequences, such as the SH2 domains of the Cbl, JAK, and STAT families (Fig. 1A). It has been proposed that SH2 domains can be divided into two groups, the Src type or the STAT type, on the basis of the secondary structure of the region between the central anti-parallel β -sheet and the α B helix [46]. The latter type is inferred to be evolutionarily more ancient. Putative SH2 proteins have also been found in plants, *i.e.*, *Arabidopsis*, as well as a more divergent type in yeast, although dedicated PTKs have not yet been identified in these species [46–48]. Until three-dimensional structures in complex with tyrosine-phosphory-

lated ligands are available for such domains, their membership in the SH2 family remains tentative.

Relative to single SH2 domains, the bidentate binding of tandem SH2 domains to bisphosphorylated peptide ligands (as in the binding of the p85 subunit of PI3K to activated PDGF β receptor doubly phosphorylated on Y740 and Y751) confers substantially higher affinity and specificity. Such an arrangement may indicate a requirement for unambiguous molecular recognition to elicit the appropriate biological response [49]. The increased specificity of multivalent binding can also be seen when SH2 domains act in concert with other binding module(s), for example an SH3 domain, to trap a ligand that bears binding sites for both domains [50–52].

Taken together, it is apparent that SH2 domains are diverse and versatile in nature, capable of either promiscuous or strict specificity depending on the modular domain arrangement and cellular context. It is worthwhile discussing in greater detail a few specific cases that illustrate these diverse functions. Thus, in the next section we describe recent work on three interesting families of SH2 proteins: SAP, Cbl and SOCS. In the section that follows, we highlight efforts to develop pharmaceutical SH2 inhibitors targeting PTK signaling in human disease.

2. SH2 domains in signal transduction

2.1. SH2 domain as blocker or adaptor?

X-linked lymphoproliferative disease (XLP) is a recessive hereditary disorder characterized by inadequate immune response to Epstein–Barr virus (EBV) infection, resulting in organ damage and death [53–55]. Abnormal T cell, B cell, and NK cell responses to virus infection have been reported in XLP, yet the mechanism of molecular pathogenesis has been elusive [55]. In 1998, three different groups independently identified the same gene (variously named *SAP/SH2D1A/DSHP*) which is mutated in XLP [42,56,57]. Surprisingly, the gene product SAP consists almost entirely of a single SH2 domain, flanked by a mere five N-terminal and 26 C-terminal residues lacking any recognizable functional motifs (Fig. 2A). In T cells, SAP was shown to bind to a tyrosine residue (Y281) in the cytoplasmic region of the receptor SLAM/CD150, suggesting a role in receptor signaling [42].

Unlike typical SH2 domains, the SH2 domain of SAP can bind the unphosphorylated Y281 site of SLAM with high affinity (K_d of 650 nM); the affinity increases only four- to fivefold upon phosphorylation [42,58,59]. Interestingly, the X-ray crystal structure of the ligand-bound form of SAP revealed that the binding could best be described as a “three-pronged plug and socket,” with three distinct regions mediating specific interactions. Phosphopeptide library screening indicated the consensus motif for SAP binding is $TIpoYxx(V/I)$ (where x represents any amino

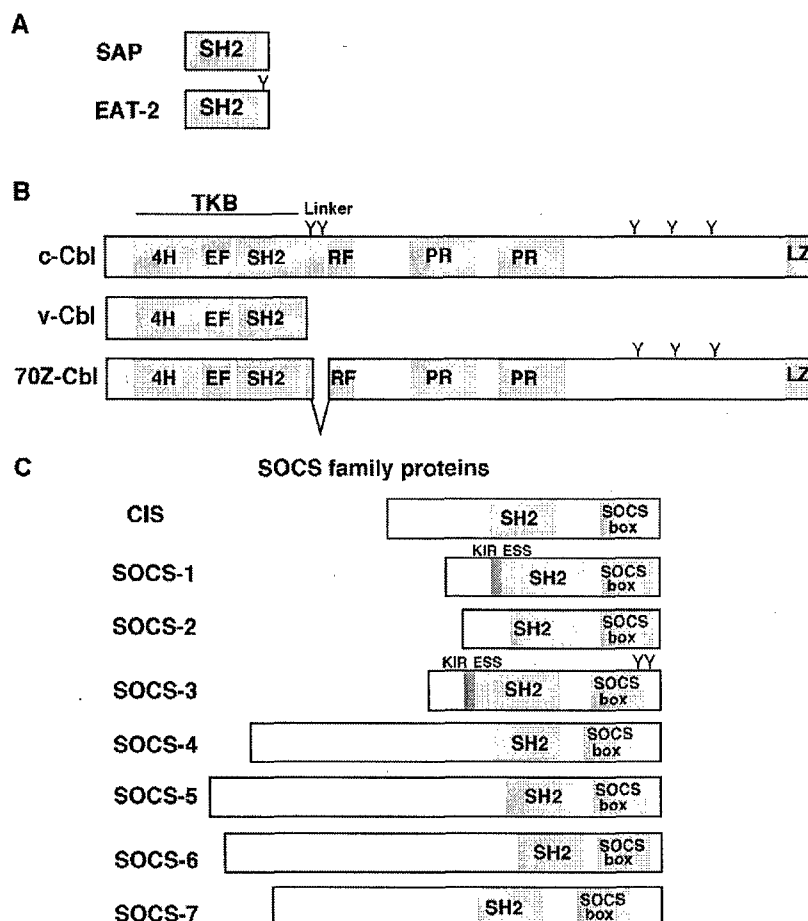


Fig. 2. Structure of SH2 domain-containing proteins. SH2 domain proteins discussed in this review are depicted diagrammatically. (A) SAP/SH2D1A consists of a single SH2 domain. EAT-2 also possesses a tyrosine phosphorylation site on its C terminus. (B) Cbl family proteins consist of multiple functional domains: a Cbl-N or Tyrosine Kinase Binding (TKB) domain, which contains a four-helix bundle (4H), an EF-hand (EF), and a variant SH2 domain (SH2); a linker region, which contains two putative tyrosine phosphorylation sites, Y368 and Y371 (denoted as YY); a Ring Finger domain (RF); Proline-Rich regions (PR); multiple tyrosine phosphorylation sites (denoted as YYY); and a Leucine Zipper region (LZ). (C) Eight members of the Suppressors Of Cytokine Signaling (SOCS) family are characterized by a central SH2 domain and a C-terminal SOCS box. SOCS-1 and SOCS-3 have additional motifs adjacent to the SH2 domain, the Extended SH2 Subdomain (ESS) and the Kinase Inhibitory Region (KIR).

acid); the Y-2, pTyr, and Y+3 positions are most important, consistent with the three-pronged model. Moreover, results suggested that any one of the three was dispensable for high-affinity binding, as long as the other two determinants matched the consensus [58–61]. Consistent with this, the tyrosine residue of the SLAM Y281 peptide can be replaced by any amino acid [61]; however, it should be noted that physiological ligands without tyrosine at the corresponding residue have not yet been identified.

How can this unique binding mode be explained structurally? The overall structure of SAP is similar to other SH2 domains such as Src [24]; the pTyr-pocket is on one side of the central anti-parallel β sheet while a hydrophobic groove on the other side interacts with the C-terminal part of the ligand peptide. However, three unique features were seen in the structure of SAP bound to the SLAM Y281 peptide [59,61]. First, the residues N-terminal to tyrosine make specific contacts with the SH2: a parallel β sheet interaction with strand β D of the

domain, assisted by hydrogen bonds from Thr at Y-2 of the ligand with Glu α 6 and with a buried water molecule. Second, the unphosphorylated Tyr residue associates with Arg β B5 (the “FLVRES” arginine, which is conserved in the pTyr pocket of almost all SH2 domains) via ordered water molecules instead of the usual phosphate oxygens. Interestingly, EAT-2, a molecule closely related to SAP, also binds the Y281 SLAM peptide in a three-pronged fashion. However, in this case binding is more phosphorylation-dependent, possibly because the more hydrophobic environment of the EAT-2 pTyr pocket relative to SAP (eliminating a buried water molecule) reduces interactions with unphosphorylated tyrosine [62]. Third, the C-terminal hydrophobic pocket is rather flexible and can adopt both open and closed configurations depending on the C-terminal residues of the ligand. This pocket is closed off by the EF loop when the groove is not occupied, creating a ridge to accommodate ligand peptides lacking a C-terminal “prong.”

How then does this simple SH2 protein play an important role immunoreceptor signaling relevant to XLP? Two models have emerged: SAP acts either as a competitor or as an adaptor (Fig. 3A). A single SH2 domain may serve as a natural competitor for other SH2-containing proteins by preventing their binding to sites such as Y281, Y307, and Y327 of SLAM [63]. SHP-2, a protein tyrosine phosphatase (PTP) with tandem SH2 domains, has been repeatedly coimmunoprecipitated with SLAM [42,58,64–66]. This interaction is disrupted by co-transfection of SAP, suggesting that SAP competes with SHP-2 for binding to phosphorylation sites on SLAM. Indeed, Y281 and Y327 match the peptide consensus for both SAP and SHP-2 (Fig. 1C) [59,61,67].

Second, SAP may have an adaptor function. Forced expression of SAP induces tyrosine phosphorylation of SLAM and coprecipitation of SLAM with SH2- or PTB-domain-containing proteins such as SHIP, Dok2, Dok1, Shc, and Ras-GAP. Thus, it appears that SAP may not compete with these other proteins for binding to SLAM, but instead facilitates their association to the receptor [66,68]. Importantly, this effect requires coexpression of FynT, a T cell-specific isoform of the Src family tyrosine kinase Fyn; on the other hand, it is apparently phosphatase-independent, because PTP inhibitors do not alter the tyrosine phosphorylation level. Moreover, FynT has been shown to bind to SLAM in a SAP-dependent manner. Taken together, these findings strongly suggest that SAP

functions as an adaptor, bridging FynT and SLAM. The mechanism was obscure, however, given the single-domain structure of SAP [69].

A potential solution to this conundrum was reported simultaneously by three groups in 2003: the SH2 domain of SAP could be shown by biochemical and structural approaches to bind directly to SH3 domain of FynT [67,70,71]. The binding interface overlaps with the canonical ligand binding region of the FynT SH3 domain but not that of the SAP SH2 domain, and the association was abolished by mutations in the SH3 domain that inactivate its binding to canonical proline-rich peptides [70]. The FynT SH3 domain does not bind the SAP SH2 via a polyproline motif, but instead through a small region of the β F strand and α B helix; mutation of Arg in the FB loop abolished binding to FynT without affecting binding to SLAM [70,71]. (Parenthetically, this contrasts with the other well-characterized example of trans SH2–SH3 interaction, in which a canonical SH3 binding mode was observed between the Abl SH3 domain and a polyproline motif in the long DE loop of the Crk SH2 domain [72]). Importantly, binding of SAP to the FynT SH3 domain appears to lead to kinase activation of FynT [73], presumably by abrogating SH3-mediated intramolecular autoinhibition as previously observed for other Src family tyrosine kinases.

It should be noted that the two models for SAP function described above are not mutually exclusive [74]. Although

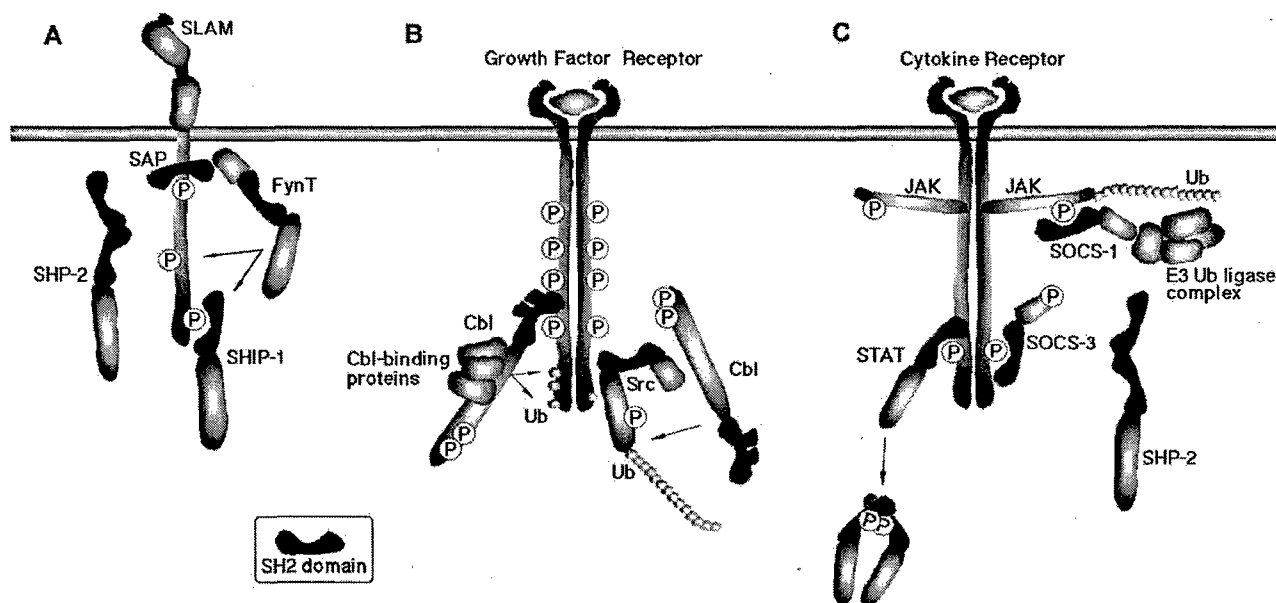


Fig. 3. SH2-mediated protein–protein interaction in signaling pathways. (A) SAP regulates SLAM-related immune receptor signaling. Two proposed models are illustrated. First, SAP prevents the association of SHP-2 with SLAM. Second, SAP couples FynT to SLAM by a direct interaction between the SAP SH2 and FynT SH3 domains, thus activating the kinase, leading to SLAM phosphorylation and recruitment of SH2-bearing downstream effectors such as SHIP-1. (B) Cbl down-regulates both activated RTKs and nonreceptor-type PTKs. Interaction between the activated RTK and Cbl TKB domain promotes ubiquitination and endocytosis of the receptor. The Cbl TKB can also bind *po*Y416 on the activation loop of Src and inhibit its kinase activity. Cbl can also associate with the Src SH3 via proline-rich motifs and promote ubiquitination of Src in a TKB-independent manner. (C) Substrate-specific interactions of SOCS SH2 mediate down-regulation of cytokine signaling by competing for STAT SH2 binding sites on the receptor, inhibiting JAK kinase activity, or linking to the ubiquitin–proteasome pathway via the SOCS box.

SAP is mutated in XLP patients, there is no obvious correlation between XLP disease phenotypes and SAP genotypes or the binding spectra of SAP SH2 domains to immunoreceptors [58,75]. Interestingly, introduction of several disease-specific missense mutations into SAP attenuates the association of SAP with FynT [67], suggesting a potential role of this interaction in the disease phenotype.

A number of intriguing questions remain. For example, what is the biological relevance of the phosphorylation-independent association of the SAP SH2 with the Y281 site of SLAM? A disease-specific mutant of SAP, T53I, can bind phosphorylated SLAM as well as wild-type (wt) SAP but has much lower affinity for the unphosphorylated form [58], suggesting phosphorylation-independent binding may be important. However, T53I SAP also has lower affinity for other partners, such as CD84, 2B4 (CD244), Ly9 (CD229), and FynT, which also may account for pathogenesis [58,67]. It will also be very interesting to know how binding of the FynT SH3 domain affects the binding preference of the SAP SH2 domain. Another open question involves competition between SAP and other partners for SH2 binding sites on the SLAM receptor. Why does SAP apparently compete with some partners for binding, such as SHP-2, but not with others such as SHIP? A plausible model is that SAP competes with SHP-2 for binding to Y281, but does not affect association of SHIP with Y307 and Y327 [42,58,64–66]. However, *in vitro* binding data indicate that SHP-2 and SHIP bind equally to the Y281, Y307, and Y327 sites of SLAM [67]. Possibly three-dimensional steric factors affect the accessibility of SH2 domains to these sites in the intact proteins. Alternatively, SHP-2 may regulate binding by dephosphorylating its SH2 domain binding site, as shown in the case of SHP-2 binding to IRS-1 [76]. The cytoplasmic domain of SLAM-related receptors contains a TxYxx(V/I) motif that has been dubbed the immunoreceptor tyrosine-based switch motif (ITSM), the function of which is proposed to switch depending on its SH2-containing binding partners, such as SAP, SHP-2 or SHIP [66,77].

Taken together, data suggest that the seemingly simple structure of SAP belies a complex function: by coupling FynT to SLAM, it mediates the tyrosine phosphorylation of sites that can recruit SHIP and other SH2 proteins. SAP (but not EAT-2, which appears to lack a FynT binding motif) has been shown to play a similar role for other immune receptors such as 2B4/CD244, which contain different spectra of SH2 binding sites [74,78–80]. Since SLAM can modulate production of Th1-type cytokines such as interferon γ and tumor necrosis factor [64,81], SAP deficiency may lead to abnormal Th1 responses. Indeed, impaired Th2 differentiation was observed in T cells from SAP $^{-/-}$ mice, which manifest up-regulation of interferon γ and down-regulation of IL-4, similar to SLAM $^{-/-}$ T cells [82–84]. The IL-4 defect is more drastic in SAP $^{-/-}$ T cells, however, suggesting

SAP may be involved in other SLAM-related receptor pathways.

Mice are not susceptible to EBV infection, so XLP must for now be studied in the less experimentally tractable human system. Although SLAM is generally thought to play a dominant role in mediating T cell cytotoxicity, a recent paper showed very low expression of SLAM and high expression of 2B4 on EBV-specific T cell lines from normal and XLP donors, suggesting a more dominant role for 2B4 in EBV infection [85]. Interestingly, the T cell lines from EBV-exposed and EBV-non-exposed XLP patients showed different interferon γ production levels following CD3 stimulation. Others found correlations of the expression of SLAM, 2B4 and SAP in T cells with clinical and immunological features of patients with EBV infection or tuberculosis [86,87]. Although XLP pathogenesis appears to be due to a disturbance of the Th1/Th2 cytokine balance, more clinically relevant studies are needed.

2.2. Cbl: inhibitor of active PTKs

The proto-oncogene Cbl was originally identified as the cellular counterpart of the retroviral oncogene v-Cbl, which induces B-cell lymphoma in mice [88]. The v-Cbl protein was found to have suffered a large C-terminal deletion compared to wt Cbl, which consists of an N-terminal domain (termed Cbl-N), a proline-rich region, and a C-terminus containing multiple potential sites of tyrosine phosphorylation (Fig. 2B). Cbl binds a number of signaling proteins containing SH2 and SH3 domains such as Crk, Grb2, Src and PI3K, and thus was initially thought to serve as an adaptor/scaffold protein [89–92]. However, three important findings have revealed a very different function for Cbl. First, Sli-1, the Cbl homologue of *C. elegans*, was found to act as a negative regulator of signaling downstream of the Let-23 RTK, indicating an inhibitory role [93,94]. Second, Cbl-N was found to associate with tyrosine-phosphorylated proteins [95,96]. Third, a RING finger motif was identified in the central part of the protein that could function as an E3 ubiquitin ligase [97–99]. These findings led to the hypothesis that Cbl functions as a terminator of PTK signaling, mediating the ubiquitination and subsequent degradation of tyrosine-phosphorylated proteins (Fig. 3B). Overexpression of Cbl induces ubiquitination of RTKs such as EGFR, PDGFR, CSF-1R and c-MET, and is also shown to inhibit activated nonreceptor tyrosine kinases such as Src [100–105].

The ability of the N-terminal domain to bind tyrosine-phosphorylated proteins was explained by the X-ray crystal structure of Cbl-N, which revealed the presence of a divergent SH2 domain [106]. Although not recognizable by sequence analysis, this module has the unmistakable features of an SH2 domain, with a few variations (notably the absence of the β E and β F strands and of a prominent BG loop). The larger Cbl-N region, also termed the tyrosine kinase binding (TKB) domain, consists of a four-helix

bundle, an EF hand calcium-binding motif, and the SH2 domain (Fig. 2B). This atypical SH2 domain serves as a reminder of the diversity of the SH2 domain family, which may have further unknown members that can only be recognized by secondary or tertiary structural analysis [46].

In the Cbl-N structure the four-helix bundle and EF hand contribute to the pTyr binding pocket of the SH2 domain, suggesting the three modules cooperatively recognize phosphorylated ligands, and indeed point mutation of any one of the three can abolish binding [106]. Interestingly, G306E, a point mutation in the β C strand of the SH2 domain first identified as a loss-of-function mutation in Sli-1, abrogates the phosphotyrosine-binding function of the domain, as well as the down-regulation of RTKs and the transforming ability of v-Cbl [107,108]. Based on the structure, it is postulated that substitution of glycine with glutamic acid could lead to formation of a buried salt bridge with Arg 294 in the conserved FLVRES motif in the β B strand of the SH2 domain, preventing its interaction with phosphotyrosine.

Screening of degenerate phosphopeptide libraries indicated a consensus of D(N/D)*xpo*Y for Cbl TKB binding, revealing the importance of N-terminal sequences and in particular the Y-2 residue (Fig. 1C) [109]. On the other hand, structural studies of Cbl-N bound to a ZAP-70 phosphopeptide indicated that the mode of interaction was similar to that of other SH2 domains, with the pTyr pocket and hydrophobic groove playing key roles. The groove is somewhat shallow, but specifically interacts with the Y+3 (Glu) Y+4 (Pro) residues of the ligand. Peptide residues N-terminal to pTyr were poorly ordered in the structure. Therefore, there is a discrepancy between biochemical and structural data regarding importance of N-terminal and C-terminal residues for ligand specificity.

This discrepancy can be rationalized by carefully considering the experimental approaches. While randomized phosphopeptide library screening is a powerful tool to determine ligand preferences, results can be skewed by the use of biased libraries in which particular residues are not randomized. For instance, preference in the Y+4 position was not determined in the case of Cbl-N. Furthermore, this method can only provide the relative preference at each position, and cannot score the effect of combinations of residues at different positions. To address this issue, the direct sequencing of preferred peptides by mass spectrometry (MS) has been proposed [110–112]. The importance of sequence combinations is supported by the recent identification in the c-Met family of RTKs of an unrelated consensus binding site for the Cbl TKB. A *Dpo*YR (Y1003) motif, which clearly does not satisfy the previous Cbl TKB consensus, can bind to the domain and is important for Cbl-mediated down-regulation of the receptor [113]. In this case, molecular modeling experiments suggest an intramolecular salt bridge between the Asp and Arg residues of the motif may stabilize it in a conformation favorable for binding the

pTyr pocket of the Cbl TKB domain. Nevertheless, both biochemical and structural data are consistent with other known physiological ligands for Cbl-N; for instance, SrcY416 (**DNE***p*oY**TAA**QQ) and EGFR Y1045 (**LQR***p*oY**SSDPT**) match the N-terminal and C-terminal consensus, respectively (Fig. 1C) [114].

Another structural analysis, in this case of the Cbl TKB domain in conjunction with the RING finger and linker region, provided further clues to Cbl function [115]. Surprisingly, the RING finger was found to anchor the TKB domain, and the linker region to stabilize the association, suggesting that deletion of the linker region may cause dramatic conformational instability of the TKB and RING finger [115]. The functional importance of this region is illustrated by the fact that 70Z-Cbl, which contains a deletion of only 17 amino acids in the linker region and RING motif (Fig. 2B), acquires oncogenicity [116]. Two tyrosine residues (Y368 and Y371) located in the middle of the linker region appear to be crucial. These tyrosines undergo phosphorylation under physiological conditions by Src and EGFR, and mutation of Y371 to Phe results in impaired ubiquitination of both PTKs [98,117]. Presumably, phosphorylation of Y368 and Y371 causes a conformational rearrangement of the TKB and RING domains that promotes the polyubiquitination of substrates such as Src and EGFR [118,119].

Src family kinases associate with Cbl via their SH3 domains, which bind proline-rich motifs of Cbl. Accumulating data suggest Cbl can inhibit Src and its relatives by two distinct mechanisms, one via the RING finger and another via the TKB domain [118,120]. Polyubiquitination of Src requires the kinase activity of Src and phosphorylation of Y371 in the linker region of Cbl, but not the Cbl TKB domain [117]. On the other hand, Cbl can also suppress Src kinase activity by the direct interaction of its TKB domain with phosphorylated Y416, within the activation loop of the Src catalytic domain [117]. Cbl is also known to associate with and down-regulate the ZAP-70 and Syk nonreceptor tyrosine kinases via its TKB domain, though the precise mechanism is not clear [103,104,121].

The mechanism of cell transformation by oncogenic Cbl is still not fully understood. Given that removal of the RING finger or linker region impairs the inhibitory role of Cbl (by abrogating its ubiquitin ligase activity), it was proposed that v-Cbl may act as a dominant negative, protecting phosphorylated substrates from endogenous Cbl and thereby leading to sustained PTK activation and cell transformation [122,123]. In this model, oncogenicity requires both an intact TKB domain and loss of the RING finger/ubiquitin ligase activity. Indeed, mutation of the TKB domain abolishes the transforming activity of v-Cbl [122]. However, the Y371F linker domain mutant of Cbl, which has lost the ability to ubiquitinate EGFR while retaining a functional TKB domain, fails to transform. Several other linker domain mutants similarly lose ubiquitin ligase activity yet are non-

oncogenic, while others, such as Y371A and Δ Y368, are as transforming as 70Z-Cbl [124]. These data indicate that dysfunction of the RING finger in conjunction with a functional TKB domain is not entirely sufficient for oncogenicity, suggesting additional regulation involving the linker region, e.g., interaction with other SH2-containing proteins or other regulators. On the other hand, it is now evident that oncogenic activation of RTKs, as in Tpr-Met and v-Fms, correlates with their inability to recruit Cbl as a result of mutation or deletion of TKB binding sites (reviewed in Ref. [125]).

2.3. Inducible suppressors: SOCS proteins

Although cytokine receptors do not possess intrinsic tyrosine kinase activity, their downstream signaling exploits SH2 domain-phosphotyrosine interactions. The cytoplasmic portions of these receptors constitutively associate with nonreceptor PTKs of the JAK family. Upon ligand stimulation, homo- or heterodimerization of receptors induces transactivation of JAKs, which in turn generates multiple tyrosine-phosphorylated sites on the receptor, providing binding sites for SH2 proteins such as Signaling Transducers and Activators of Transcription (STATs). STATs undergo tyrosine phosphorylation by JAKs, homodimerize via SH2-pTyr interactions, and subsequently translocate to the nucleus, where they induce transcription of cytokine-responsive genes [126]. In this system dissociation of STATs from the activated receptor is required. This is controlled by the affinity of STAT SH2 domain–ligand interactions, such that the affinity of the reciprocal STAT dimer is higher than that of the receptor for the STAT SH2 [127]. The importance of JAKs and STATs for cytokine signaling is indicated by the fact that knockout mice for these genes fail to respond to specific cytokines [128].

In addition to activation, suppression and termination of cytokine signaling are also essential for appropriate immune and inflammatory responses [129,130]. Negative regulation of cytokine signaling involves receptor internalization and degradation, tyrosine phosphatases, inositol phosphatases, Cbl (see Section 2.2), and Protein Inhibitors of Activated STATs (PIAS) [131–135]. Recently, the SOCS family of SH2 proteins has been added to this list [136–138]. Cytokine-inducible SH2 containing protein (CIS) was the first member of the family identified, as an immediate-early gene product induced by IL-2, IL-3 and erythropoietin (EPO) [139]. Upon EPO receptor stimulation, activated STAT5 binds the CIS promoter and induces its expression. In a classic negative feedback loop, CIS then binds to phosphorylated Y401 of the EPO receptor, the binding site for STAT5, thereby suppressing STAT5-mediated signaling [140]. Subsequently, three groups identified proteins similar to CIS, termed Suppressor Of Cytokine Signaling-1 (SOCS-1), JAK-Binding protein (JAB), and STAT-induced STAT Inhibitor-1 (SSI-1) [141–143]. We now know that there are more than 40 molecules in a family defined by the presence

of a “SOCS box,” which is part of an E3 ubiquitin ligase complex that can target proteins for degradation via the ubiquitin proteasome pathway [144]. Eight of these, referred to here as SOCS proteins, possess an SH2 domain N-terminal to the SOCS box (Fig. 2C) [145–147].

Although SOCS proteins are similar in structure, varying mainly in the length of the region N-terminal to the SH2 domain, their biological functions are diverse [138,148]. For example, SOCS-1 knockout mice exhibit constitutive activation of STAT-1 and overexpression of interferon- γ -induced genes, while SOCS-3 knockout mice exhibit severe erythrocytosis in various organs [143,149,150]. While the SOCS box is interchangeable among different family members [144], two factors may account for the functional diversity SOCS proteins. One is the kinetics of their regulation in response to cytokines. For instance, IL-6 stimulation of mouse M1 cells induces expression of SOCS-1, but not SOCS-2 and SOCS-3. On the other hand, IL-6 stimulation of mouse liver induces SOCS-1, -2, and -3 and CIS. SOCS-1 and SOCS-3 proteins decline to basal levels within 4–8 h, whereas CIS and SOCS-2 remain elevated for over 24 h [141].

A second factor is the contribution of sequences adjoining the SH2 domain. For instance, inhibition of JAK2 by SOCS-1 depends on two short sequences adjacent to the SOCS-1 SH2 [151–153]. One 12-amino-acid stretch N-terminal to SH2 domain, termed the Extended SH2 Subdomain (ESS), stabilizes the association of SOCS-1 with the kinase activation loop of JAK2. An additional 12-amino-acid N-terminal segment, termed the Kinase Inhibitory Region (KIR), is required to inhibit of JAK2 kinase activity (Fig. 2C). Interestingly, KIR may act as a pseudosubstrate for JAK2 [151,153]. A similar mode of inhibition has been observed in the Grb7, Grb10, Grb14 adaptor family proteins, which possess a PH domain and an SH2 domain. In the vicinity of their SH2 domains, a region termed BPS (between PH and SH2) or PIR (phosphotyrosine interacting region) binds the kinase domain of insulin receptor and suppresses its catalytic activity in cooperation with the SH2 domain [154–156]. Analogous to the auto-inhibition of Src family PTKs by their own SH2 and SH3 domains, the SOCS and Grb7 families thus represent different modes of PTK inhibition in which the SH2 domain plays a central role [157].

In the case of the SOCS family, further diversity in function is provided by SH2 domain specificity. While SOCS-3 has a similar domain composition to SOCS-1, the SOCS-1 SH2 binds JAKs rather than receptors, while the SOCS-3 SH2 binds receptors rather than JAKs [148]. The inhibitory effect of SOCS-1 on leukemia inhibitory factor (LIF) signaling is attenuated by replacement of its SH2 domain with that of SOCS-3 [153]. Interestingly, SOCS-1 loses its inhibitory activity by mutation of the invariant Arg in the conserved FLVRES motif in the β B strand of the SH2 domain, while SOCS-3 is tolerant to this mutation; for most SH2 domains, mutation of this

residue abolishes binding to tyrosine-phosphorylated peptides. Taken together, these findings imply important functional difference between SH2 domains specific to particular cytokine pathways.

Regulation of IL-6/gp130 receptor signaling by SOCS-3 illustrates the complicated interplay between SH2 proteins and their binding sites. IL-6 stimulation leads to tyrosine phosphorylation of sites on the cytoplasmic tail of gp130, including Y767, Y814, Y905, and Y915. All of these sites match the STAT3 SH2 binding consensus (YxxQ), suggesting the involvement of STAT3 in downstream signaling [158]. On the other hand, Y759 (Y757 in mice) has been shown to play an important role in inhibition of STAT3 signaling and activation of Ras-MAPK signaling, as point mutation of this residue enhanced the IL-6 response and reduced MAPK activation [159–161]. Knock-in mice in which this site is mutated (gp130^{Y759F}) exhibit T cell hyperresponsiveness and rheumatoid arthritis (RA)-like joint disease within the first year of age [162,163]. The SHP-2 tyrosine phosphatase is known to associate with phosphorylated Y759 in response to IL-6, suggesting SHP-2 is the negative regulator [159–161]. Recent data, however, demonstrate that SOCS-3 also inhibits IL-6 signaling via Y759, arguing that inhibition might in fact be due to SOCS-3 rather than SHP-2 [164]. As expected, the SH2 domains of SHP-2 and SOCS-3 favor similar motifs; Y759 (TVQpoYSTVVH) matches the consensus motifs for both (Fig. 1C) [161,165]. Indeed, *in vitro* data revealed both proteins can bind the Y759 peptide with similar affinity ($K_d=140$ nM for SOCS-3 vs. 170 nM for SHP-2), suggesting *in vivo* competition is likely [165].

One study demonstrated that the inhibitory effect of SOCS-3 was seen in cells lacking intact SHP-2, while conversely the forced localization of SHP-2 to the membrane in Y759F-expressing cells, in which association of SOCS-3 is prevented, also had an inhibitory effect [166]. Others also reported independent roles for SHP-2 and SOCS-3 in LIF-gp130 signaling using antisense oligonucleotides [167]. While these data indicate that SHP-2 and SOCS-3 independently regulate gp130 signaling via Y759, the physical and functional interaction of SOCS-3 with SHP-2 has also been reported [166,168]. *In vitro* binding experiments suggest that the SOCS-3 SH2 domain binds Y542 of SHP-2 [166], but since both molecules contain SH2 domains and potential tyrosine phosphorylation sites, and SHP-2 is known to engage in intramolecular SH2-pTyr interactions [169,170], it is difficult to define the precise binding mode for the complex. To complicate matters even further, Ras-GAP, an SH2-containing negative regulator of the Ras pathway, was shown to bind Y221 in the C-terminus of SOCS-3 in response to IL-2, resulting in activation of Ras-MAPK signaling [171].

Taken together, signaling downstream of the gp130 receptor is shaped by the tyrosine phosphorylation of binding sites for the SH2 domains of STAT3, SHP-2 and SOCS-3 (Fig. 3C). The physiological role of the dual

regulation by Y759 is not clear at this moment. Recently, Fischer et al. [172] showed that pre-stimulation of cells with IL-6 renders STAT3 activation less sensitive to further stimulation with IL-6. Interestingly, this desensitization was not observed in SOCS-3-deficient cells, but was unaffected in cells expressing mutant SHP-2, indicating different qualitative roles for SOCS-3 and SHP-2. Whereas the IL-6-induced anti-inflammatory responses of SOCS-3 knock-out macrophages and gp130^{Y759F} mutant macrophages were similar [173], mice lacking SOCS-3 in myeloid cells do not exhibit the severe RA-like inflammatory disease seen in gp130^{Y759F} transgenics [163,173,174]. Thus, SHP-2 and SOCS-3 appear to exert distinct regulatory roles on the STAT pathway *in vivo*, depending on the particular cell type.

3. SH2 domain inhibitors

Because of the important role of SH2 domains in signal transduction pathways, SH2-mediated protein-protein interactions are attractive therapeutic targets [175]. The fact that short pTyr-containing peptides (usually five to six amino acids) are sufficient to compete with larger protein ligands for SH2 domain binding has prompted researchers both in academia and industry to develop inhibitors targeting the SH2 domains of clinically relevant proteins, including Grb2, Src, Lck, Zap70, PI3K, and STAT3 [11,176,177]. A major difficulty in designing such inhibitors is the concentrated negative charge of SH2 ligands (from phosphate and acidic side chains), which is unfavorable for penetrating the cell membrane; in general, substitution of these negatively charged groups causes a dramatic loss of affinity [178,179]. Elimination or modification of the bridging oxygen of phosphotyrosine (to confer phosphatase resistance) also adversely affects binding. Moreover, because the pTyr pocket of the SH2 domain is so well conserved, any inhibitor with high affinity to this site cannot be selective for a particular SH2 domain, unless it also targets specificity-determining regions. For all these reasons, developing high-affinity SH2 inhibitors that are specific, stable, and cell-permeable is a formidable challenge. Despite these difficulties, however, great progress is being made, and in this section we review efforts aimed at developing inhibitors of the Grb2 and Src SH2 domains. Comprehensive reviews of this field are also available [176,180–183].

3.1. Inhibition of Grb2 SH2 domain

Since Grb2 is known to play a crucial role in signaling from RTKs to the Ras-MAPK cascade [184], Grb2 SH2 inhibitors would be expected to suppress cancer cell growth mediated by activated receptors such as HER2/Neu, EGFR, and c-Met. Grb2 is an adaptor protein possessing a single SH2 domain flanked by two SH3

domains. Upon RTK activation, the Grb2 SH2 domain preferably binds *poYxNx* motifs [7], such as Y317 of Shc and Y1139 of Her2/Neu, thus recruiting the Ras activator SOS to the membrane via the Grb2 SH3 domains [185,186]. Crystal structures of the Grb2 SH2 bound to its cognate peptide ligand have revealed that the ligand adopts a type I β -turn conformation [26], whereas more typical SH2 ligands adopt an extended mainchain conformation [24,25,187] (see Section 1). These two unique characteristics—the strong requirement for Asn at the *poY*+2 position of the ligand and the β -turn backbone conformation—suggested it would be possible to design highly specific inhibitors for the Grb2 SH2 domain.

3.1.1. Stepwise substitution of *poYxN* motif

A group from Novartis performed a systematic stepwise substitution at each residue of the parent tripeptide Ac-*poYIN*-NH₂, the shortest peptide retaining micromolar affinity to the Grb2 SH2 domain (blocked at the N and C termini with acetyl and amide groups, respectively) [188]. In this process, each position of the peptide is treated as a “module” which can be manipulated to mimic binding interactions observed in the crystal structure [26,176,180] (Fig. 4A). For instance, the *poY*+1 residue must adopt a right-handed 3_{10} helical backbone conformation to stabilize the type I β -turn and maintain multiple van der Waals contacts with the SH2 surface. To meet these criteria, various α,α -disubstituted amino acids known to stabilize the 3_{10} helical conformation were docked by computational modeling, and the most favorable were incorporated into the peptide and tested for binding affinity [189,190]. Modeling predictions were consistent with results from competitive binding assays; for example, aminocyclohexanecarboxylic acid (**Ac₆c**), predicted by modeling to have optimal van der Waals contacts, had the highest affinity among related analogs (Fig. 4B).

The same strategy was employed for other parts of the Ac-*poYIN*-NH₂ peptide to judiciously substitute peptide backbone and/or side chains. As Grb2 SH2 strongly favors Asn at the Y+2 position, any substitution at this position must fulfill strict local requirements, including a rigid backbone to stabilize the β -turn, preserving specific interactions with the Lys β D6 and Leu β E4, and steric accommodation of TrpEF1 of the Grb2 SH2. *cis*-2-aminocyclohex-3-ene carboxylic acid (**Achec**) was selected by substructure chemical database screening, and as predicted the 1*S*,2*R* diastereomer could substitute for Asn with little loss of affinity (Fig. 4B) [191]. The strict spatial requirements for this position are illustrated by the dramatic loss of binding potency seen with its diastereomer (**1*R*,2*S***)-**Achec**. Because the crystal structure indicated that the C-terminal part of Ac-*poYIN*-NH₂ makes van der Waals contacts with a hydrophobic patch on the surface of the SH2 domain, a lipophilic attachment was designed. Appending a C-terminal 3-naphthalen-1-yl-propyl functionality improved the binding affinity by 25-fold [192]. Modifications N-

terminal to pTyr, e.g., attaching 3-aminobenzyloxycarbonyl group (**3-amino-Z**) directly to the N-terminus, also led to dramatic improvements in binding potency (Fig. 4B).

All these substitutions were then combined to generate novel β -turn mimicking platforms, which were tested for in vitro binding affinity and for potency in cell-based assays [176,180]. One such compound, **CGP78850** (Fig. 4C), is 200-fold more potent than the parent Ac-*poYIN*-NH₂ peptide, and could block EGFR-Grb2 and Shc-Grb2 interactions in MDA-MB-468 human mammary carcinoma cells. More importantly, it also inhibited anchorage-independent growth of these cells, one of the defining properties of tumor cells [193]. CGP78850 was further modified into a prodrug in which the pTyr phosphate was esterified to improve cell permeability. The prodrug version reverted morphological transformation of Her2/Neu-expressing NIH-3T3 cells and inhibited hepatocyte growth factor (HGF)-induced tumor cell motility [193,194].

3.1.2. pTyr mimetics and macrocyclization

Burke and coworkers used the β -turn-mimicking platform of Novartis (above) as the starting point for two additional modifications, namely incorporation of pTyr mimetics and macrocyclization of the linear backbone. Because the cytosol has very high levels of constitutive tyrosine phosphatase activity, the stability of the phosphate linkage is a major concern in vivo. Early work indicated that substitution of pTyr with phosphonomethyl phenylalanine (**Pmp**), in which the pTyr bridging oxygen is substituted by a methylene group, led to a fivefold loss of binding potency in the case of ligands for the PI3K SH2 domain [195]. This was thought to be due to insufficient ionization of Pmp (because of its higher pK_a relative to pTyr at physiological pH) or loss of hydrogen bonds between the SH2 and the pTyr bridging oxygen [196,197]. When Pmp was incorporated into the β -turn platform, however, unexpectedly high affinity was reproducibly observed, comparable to that seen with pTyr (Fig. 4B) [180,198]. This group also tested pTyr mimetic groups lacking phosphate, such as mono- or dicarboxy phenylalanine derivatives, in the β -turn platform [199,200]. The monocarboxy series, e.g., carboxymethyl-phenylalanine (**cmF**, Fig. 4B), while useful in decreasing the net negative charge relative to pTyr, decreased affinity by almost 100-fold, underscoring the importance of two negative charges for molecular recognition by SH2 domains. Consistent with this, compounds containing dicarboxy pTyr mimetics such as *p*-malonylphenylalanine (**Pmf**, Fig. 4B) displayed comparable affinity to those with Pmp or pTyr.

In order to stabilize the β -turn conformation, the Burke group also attempted to constrain the linear peptide mimetic by macrocyclization [201–203]. Surprisingly, although the cyclic analogue exhibited approximately 100-fold enhancement in binding potency in vitro relative to its linear counterpart, it was not effective in whole cell assays [201].

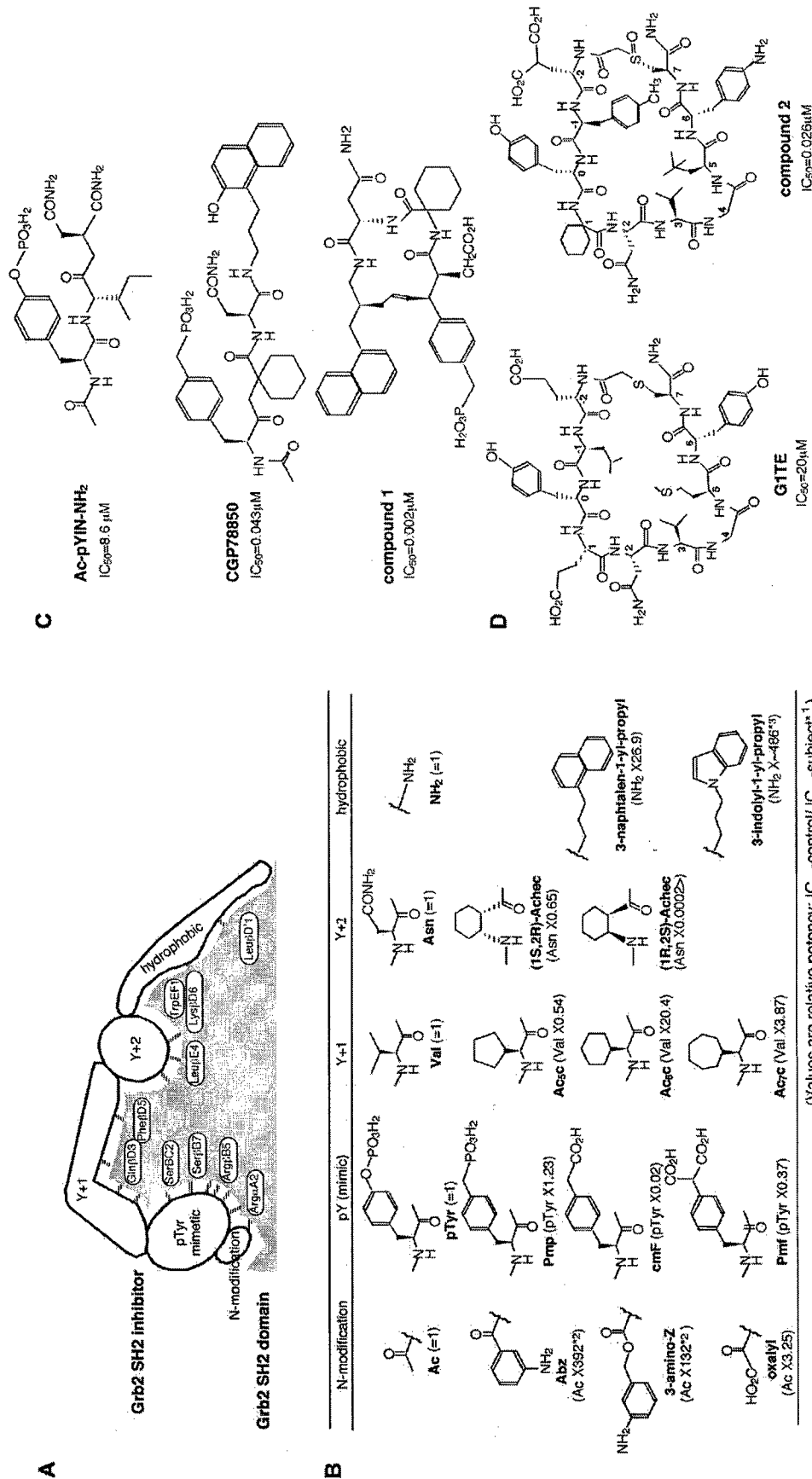


Fig. 4. Structure-based design of Grb2 SH2 domain inhibitors. (A) Schematic representation of binding mode of Grb2 SH2 domain with specific inhibitor. The inhibitor consists of five “modules” mimicking the parent tripeptide Ac-pY(I/V)N-NH₂. The modules for the pTyr mimetic and Y+2 position anchor to their respective binding pockets on the surface of the domain, while an acidic or lipophilic modification N-terminal to pTyr creates additional binding interactions, Y+1 module promotes β-turn mainchain conformation, and the C-terminal module interacts with hydrophobic surface of the domain. Key residues in the domain for each position are depicted. (B) Chemical structures of selected substituents for each position. ¹Relative potency (in parentheses) is defined as IC₅₀-control/IC₅₀-subject in equivalent inhibitor platform. ²³IC₅₀ values are compared in pTyr linear peptide platform [189]. Different values were reported in Pmp-based platform [213] (see text, Section 3.1.4). ³Direct comparisons of IC₅₀ values in the same platform are not available. Instead, 3-indolyl-1-yl-propyl, approximately 18 times more potent than 3-naphthalen-1-yl-propyl, has been reported [180,192,198,200]. (C) Chemical structures and IC₅₀ values obtained from competitive binding assays. Ac-pYIN-NH₂, minimum tripeptide retaining micromolar affinity to Grb2 SH2 domain [188]; **CGP78850**, Pmp-incorporating β-turn-mimicking inhibitor platform [193]; **compound 1**, β-turn-stabilizing macrocyclic inhibitor with carboxylic functionality (-CH₂COOH) at Pmp α position [202]. (D) Chemical structure and IC₅₀ values of cyclic non-phosphorylated Grb2 SH2 ligand peptides **GITE** and its modified form **compound 2** [205,209]. Abbreviations; Ac, acetyl group; Abz, anthranic acid; 3-amino-Z; 3-amino-benzoyloxy-carbonyl group; pTyr, phosphotyrosine; Pmp, phosphonomethyl phenylalanine; cmf, carboxymethylphenylalanine; Ac_nc, *cis*-2-amino-cyclohex-3-ene carboxylic acid.

However, when the pTyr α position was modified by a carboxymethyl ($\text{CH}_2\text{CO}_2\text{H}$) functionality (Fig. 4C, **compound 1**), this compound not only blocked the association of Grb2 with Her2/Neu in whole cell assays, but also displayed anti-mitogenic effects in MDA-MB-453 cells [202,204].

3.1.3. Non-phosphorylated ligand G1

Roller and coworkers employed a completely different strategy to identify novel unphosphorylated high-affinity ligands for the Grb2 SH2 domain [205]. They screened a combinatorial peptide phage-display library, where nine variable amino acids were flanked by cysteine residues (to promote disulfide-mediated cyclization). From this screen they isolated the cyclic peptide **CELYENVGMYC (G1)**, which potentially blocked binding of the Grb2 SH2 domain to a phosphorylated Shc Y317-derived peptide ($\text{IC}_{50}=10\text{--}25\mu\text{M}$) [205] (Fig. 4D, **GITE**: thioether-bridged analogue of G1). G1 is not tyrosine-phosphorylated but does contain a YxN motif. Mutational studies indicated the particular importance of this motif, as well as Glu at the Y-2 position (Fig. 4D) [205,206]. Structural analysis suggested that this cyclic peptide, for which ring closure is critical for binding, adopts a β -turn-like conformation similar to that found in natural (pTyr-containing) ligands, but utilizes a larger binding surface [206,207].

Relative to physiological Grb2 binding sites (e.g., Y317 in Shc and Y1139 in ErbB2), where the residue at the -2 position is Pro, Glu at this position of G1 is unique. A Glu to Ala substitution at this position of G1 caused a dramatic loss of affinity [205,206,208], while, on the other hand, substitution with unnatural amino acids containing acidic dicarboxylic side chains, e.g., γ -carboxyglutamic acid (Gla), significantly improved binding [208,209]. Tyrosine phosphorylation of G1 increases binding affinity by more than 100-fold. Interestingly, in this context an acidic residue at the -2 position is no longer beneficial, and the Glu to Ala substitution actually enhances binding, which suggests complementary roles for phosphotyrosine or an acidic residue at the -2 position. Although no structural comparison is yet available, a plausible interpretation is that the carboxyl group of Glu can engage in some of the same interactions as the pTyr phosphate. This suggests some degree of plasticity in the SH2 domain, allowing multiple modes of binding with different ligands.

Upon optimizing each position of G1, a high-affinity Grb2 SH2 inhibitor peptide was produced (Fig. 4D **compound 2**; $\text{IC}_{50}=26\text{ nM}$) [209]. Importantly, compound 2 effectively inhibited the association of Grb2 with ErbB2 in whole cell assays at low micromolar concentrations, demonstrating the potential usefulness of non-phosphorylated SH2 inhibitors in vivo [209]. A G1 analogue containing the pTyr mimetic Pmp (thereby acquiring additional negative charge), which was further modified to increase cell permeability by attaching a hydrophobic carrier peptide, also exhibited anti-mitogenic effects in

MDA-MB-453 cells (40% growth inhibition at $5\mu\text{M}$) [210]. In addition, a compound incorporating non-phosphorylated 3-aminotyrosine was recently reported to have low nanomolar affinity for the Grb2 SH2 [211]. Thus, it is apparent that there are multiple solutions to the tradeoff between the need for negatively charged pTyr mimetics for optimal affinity and the need to minimize charge to facilitate cell entry.

3.1.4. Impact of N-terminal modifications

Although the two different Grb2 inhibitor platforms (the β -turn mimicking tripeptide and cyclic peptide G1) were developed independently, some similarities are apparent (Fig. 4C and D). For instance, incorporating aminocyclohexanecarboxylic acid (**Ach/Ac6c**) into the Y+1 position (which helps stabilize the β -turn conformation) and structural constraint via macrocyclization are beneficial in both cases. It also is noteworthy that modifications N-terminal to pTyr significantly improve binding affinity for both platforms. An early hint was provided by the observation that a phosphopeptide (EpoYIN-NH_2) bearing an N-terminal fluorophore (anthranic group: **Abz**, Fig. 4B), which was used as a reference for ELISA-type assays, unexpectedly displayed a dramatic enhancement of affinity. This led to design of a 3-aminobenzoyloxycarbonyl (**3-amino-Z**) N-terminal group [188]. Structural studies revealed stacking interactions between the electron-rich anthranil moiety in the ligand and the electron-deficient guanidinium group of Arg αA2 of the SH2 [180,212]. *N*-oxalyl modification (COCOOH-) also modestly improved affinity in the β -turn platform (Fig. 4B). Molecular modeling dynamics simulation studies suggested additional electrostatic interactions between the acidic oxalyl functionality and the positively charged side chain of Arg αA2 [198,199,213].

N-terminal modifications also illustrate the complexities and subtleties inherent in "rational" inhibitor design. For instance, it is apparent that there are interesting compatibility issues between specific N-terminal modifications and pTyr mimetics. For example, the *N* α -3-amino-Z modification improves binding for pTyr-containing ligands, but not for Pmp-containing ligands [213], while the *N* α -oxalyl functionality is compatible with both pTyr and Pmp [198,213]. It is also remarkable that N-terminal modification by oxalyl or carboxymethyl ($\text{CH}_2\text{COOH-}$) functionalities brings an additional negative charge to the corresponding inhibitors; despite this, such compounds are often active in cell-based assays [201,202]. The affinity improvement observed in in vitro assays may not entirely account for this effect, since the unmodified counterparts, which are inactive in cell based assays, already bind with high affinity.

Importantly, N-terminal modification generates additional binding interactions not observed in the crystal structure of the Grb2 SH2 bound to peptides derived from natural ligands. In the case of the non-phosphorylated

cyclic G1 peptide, structural modeling analyses indicated acidic side chains at the Y-2 position make multiple interactions with arginines and serines at the pTyr pocket [208]. Do any natural ligands take advantage of similar N-terminal interactions? Rojas et al. [214] showed that a peptide encompassing the Shc-Y317 phosphorylation site (FDDPSYVNVQNL), which has acidic residues at the Y-3 and Y-4 positions, could inhibit Grb2-Shc interaction when delivered into cells without prior phosphorylation. Thus, under physiological conditions, it is likely that binding of Grb2 to sites such as Shc-Y317 would be less dependent on tyrosine phosphorylation than more typical SH2 interactions.

3.2. *Src inhibition and bone resorption*

Src is the prototype nonreceptor PTK, and its activation in human tumors such as breast cancer has been reported [215–217]. Src-deficient mice exhibit osteopetrosis, or hypertrophy of bones, indicating impaired osteoclast function [218]. Therefore, antagonizing Src function *in vivo* may inhibit tumor growth and be useful for treatment of osteoporosis and prevention of osteolytic changes related to tumor metastasis. However, because the Src protein is tightly regulated by multiple domains including SH2, SH3 and kinase domains, the consequences of inhibition of the Src SH2 domain are not entirely straightforward. Nonreceptor tyrosine kinases generally require SH2 domains for efficient substrate phosphorylation [20], so SH2 inhibition is likely to suppress phosphorylation and downstream signaling. In addition, it may suppress kinase-independent adaptor functions mediated by SH2 and SH3 domains of Src [219]. On the other hand, Src is normally held in an inactive conformation via an intramolecular interaction between its SH2 domain and a tyrosine-phosphorylated C-terminal residue, Y527. Abrogation of this interaction leads to activation of the kinase, and Y527 mutants of Src, e.g., Y527F, are known to be transforming. Hence, Src SH2 inhibitors are potentially valuable, but any clinical applications must take into account the potentially disastrous effects of inappropriate activation of Src.

Nevertheless, Src SH2 inhibitors have great potential to treat osteoporosis. One reason for optimism is that knock-in mice bearing a kinase-inactive K295M Src mutant exhibit partial recovery of the osteopetrosis phenotype, indicating that the Src SH2 and SH3 domains play a critical role in osteoclast function [219]. Until now, osteoporosis has generally been treated with two categories of drugs: bone resorption inhibitors such as bisphosphonate, and stimulants of bone formation, such as parathyroid hormone and prostaglandin E2 [220]. In recent years, several pharmaceutical companies including Glaxo, ARIAD and Aventis have been working to develop Src SH2 inhibitors, with the rationale that Src is a validated therapeutic target for bone resorption [182,221,222].

3.2.1. *Importance of the scaffold*

The Src SH2 binds to high-affinity ligands via a “two-pronged plug and socket,” where the side chains of pTyr and the Y+3 residue (generally Ile) are buried in distinct pockets [24,25,223]. There is no doubt that these two regions, with multiple specific contacts, are crucial for the SH2–ligand association. However, the linker region between pTyr and Y+3 (Y+1 and Y+2 residues of a peptide ligand) is also important for the design of Src SH2 inhibitors. This region makes contacts with the solvent-accessible hydrophobic surface of the SH2 domain and acts as a scaffold that appropriately orients the pTyr and Y+3 side chains into their respective pockets [181,224] (Fig. 5A). In the crystal structure of the Src SH2 domain bound to high affinity peptide, the mainchain of the Y+1 residue makes direct contact with His β D4 of the domain, while the side chains of Y+1 and Y+2 are relatively free [24]. Nevertheless, a series of analogs containing dipeptides or hydrophobic linkers between pTyr and the Y+3 Ile displayed more than 100-fold lower affinity than the wild-type peptide [179]. Single amino acid substitutions at Y+1 or Y+2 were tolerated relatively well, but D-amino acid substitution for Y+1 caused a major loss of affinity, indicating the importance of the backbone conformation in this position [179]. The hydrophobic surface here is mainly formed by Tyr β D5, which is known as a specificity-determining residue [6,33]. Mutational studies have shown that substitution of Tyr β D5 to Phe results in a 30-fold loss of affinity [225]. Surprisingly, this effect is greater than for a mutation of the conserved Arg α A2, known to make important electrostatic interactions in the pTyr pocket, which results in only a sixfold affinity loss [31]. Tyr β D5 makes van der Waals contacts with the side chain of Y+1, forming a platform for the extended ligand peptide backbone [24,225]. Therefore, selection of a scaffold that favorably interacts with this hydrophobic surface is a critical element in designing inhibitors.

The ideal scaffold would provide a rigid backbone, preserve existing hydrogen bonds with His β D4, Lys β D6 and Ile β E4, and make stacking interaction with the aromatic ring of Tyr β D5 [226]. Researchers at ARIAD have conducted structure-based design studies examining various types of scaffolds for Src SH2 inhibitors, including ureido-type peptide mimetics [227], disubstituted thiazoles [228], oxazoles [224] and benzamide [229]. Notably, X-ray crystallography revealed the benzamide template forms hydrogen bonds with Lys β D6, displacing water molecules found in the previous X-ray structure. Subsequently, a seven-membered bicyclic benzamide scaffold platform, which fully complements the convex surface created by Tyr β D5, has been developed [226] (Fig. 5B). Using an alternative approach, researchers at Aventis selected a caprolactam scaffold and also confirmed displacement of structured water molecules by X-ray crystallography. This platform, incorporating pTyr

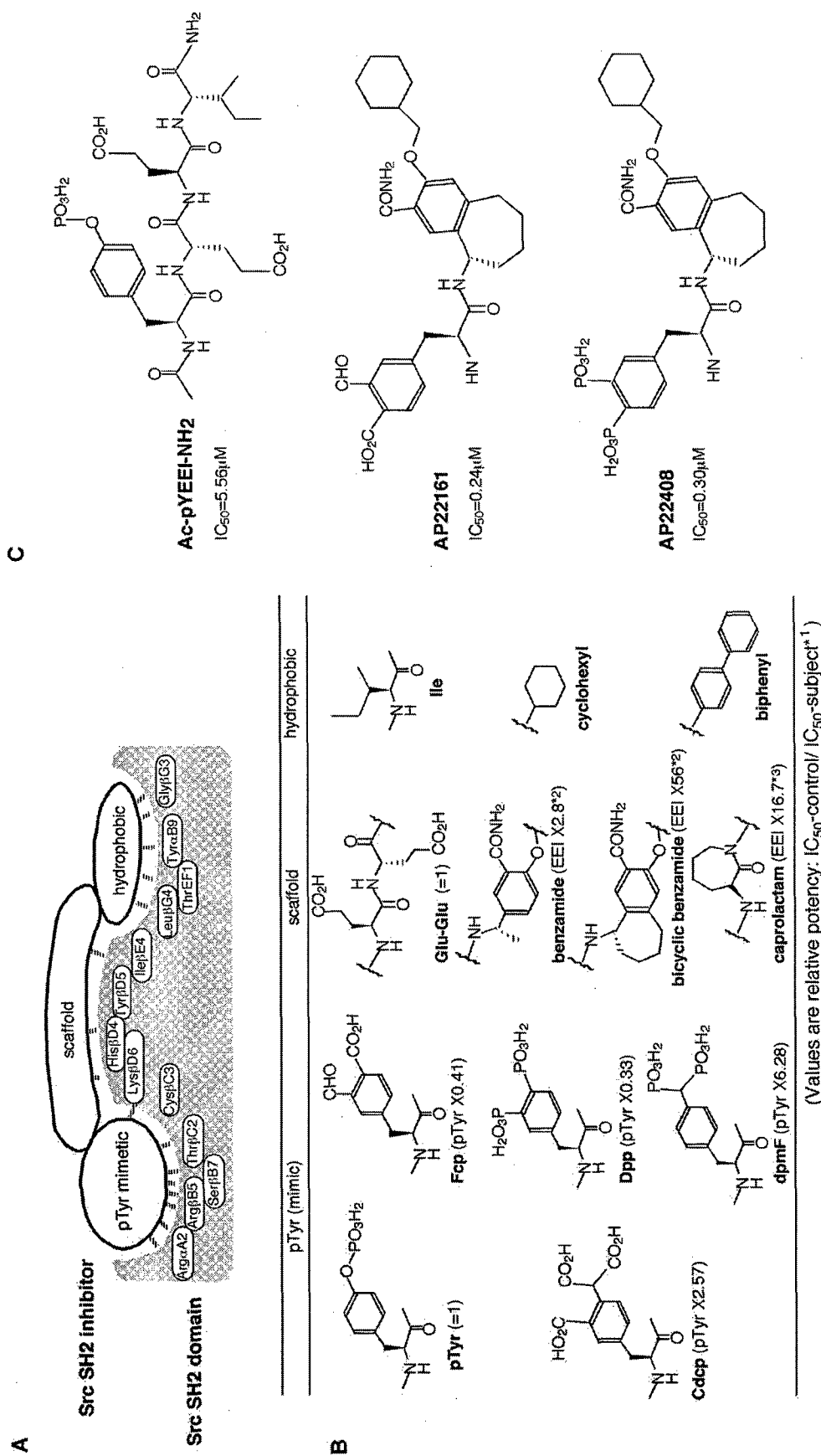


Fig. 5. Structure-based design of Src SH2 domain inhibitors. (A) Schematic representation of binding mode of Src SH2 domain with specific inhibitor. The inhibitor consists of three components: pTyr mimetic, central scaffold and hydrophobic moiety. The central scaffold interacts with a hydrophobic surface formed by TyrB5, and appropriately delivers pTyr mimetic and hydrophobic functionalities into their respective binding pockets. Key residues in the domain for each position are depicted. (B) Chemical structures of selected substituents for hydrophobic region, respectively [226,243,250]. * Relative potency is defined as in Fig. 4. Values for scaffolds are determined in combination with cyclohexyl (^{*}) or biphenyl (^{*}) modification for hydrophobic region, respectively [226,243,250]. (C) Chemical structures and IC₅₀ values by competitive binding assay for Src SH2 inhibitors. **Ac-po YEEI-NH₂**, a tetrapeptide with micromolar affinity to Src SH2 domain [233]; Cysteine-targeting **AP22161**, 3'-aldehyde 4'-carboxyl phenylalanine Fcp-incorporating bicyclic benzamide scaffold template exhibiting selective Src SH2 binding potency [233]; Bone-targeting **AP22408**, Dpp-incorporating bicyclic benzamide scaffold template exhibiting selective inhibition of osteoclasts [237]. Other bone-targeting high-affinity Src SH2 inhibitors carrying dpmf (IC₅₀=0.35) have also been reported [236,238].

and biphenyl functionalities, showed 15-fold higher affinity than the natural *poYEEI* peptide [230].

3.2.2. Cysteine-targeting pTyr mimetics

In order to improve selectivity for the Src SH2 domain versus those of other structurally similar Src family members, a series of cysteine-targeting pTyr mimetics have been designed [231–233]. The Src SH2 has a cysteine at β C3 which is conserved in Src proteins of different species, but not among other Src family proteins, where the β C3 residues are serine. Even among the entire complement of human SH2 domains Cys β C3 is rare, found only in SAP, EAT2, and Src (C. Thompson, unpublished observation). Bradshaw and Waksman [178] suggested this cysteine is particularly reactive since the highly basic environment of the pTyr pocket lowers the pK_a of the sulfhydryl. Therefore, an appropriately positioned electrophile such as an aldehyde group may form a reversible covalent bond with this group [231]. Various Cys β C3-targeting pTyr mimetics including 3'-formyl-pTyr, 3'-formyl-4'-Pmp and 3'-formyl-4'-carboxy-phenylalanine have been tested. The 3'-formyl-4'-Pmp template did not engage Cys β C3 because of the disposition of the aldehyde group [234]. For the 3'-formyl-pTyr template, X-ray structures confirmed hemithioacetal formation, but no inhibition was observed in cell-based assays. This failure was most likely due to some combination of loss of affinity through entropic effects at the pTyr pocket, phosphatase susceptibility, and poor cell permeability [231,232,234].

On the other hand, a 3'-formyl-4'-carboxy-phenylalanine-containing (Fig. 5B, **Fcp**) SH2 inhibitor, **AP22161** (Fig. 5C), showed dramatic improvement in binding potency. **AP22161** was shown to form a hemithioacetal by nuclear magnetic resonance (NMR) analysis, dependent on interaction of the formyl group with Cys β C3. It binds the Src SH2 100-fold better than that of Src family member Yes, and 1700-fold better than the more distantly related ZAP-70 SH2s [233] (Fig. 5C). Moreover, **AP22161** inhibited rabbit osteoclast-mediated resorption of dentine and showed partial morphological reversion of Src Y527F mutant-transformed rat fibroblasts [233]. The normal physiological role of this highly reactive cysteine is not clear. Mutation of Cys β C3 causes an eightfold improvement in affinity for consensus ligands, suggesting a negative role in the pTyr pocket [31]. In addition, Cys β C3 may play some role in the weak phosphatase activity reported for the Src SH2 domain [235].

3.2.3. Targeting SH2 inhibitors to bone

Another approach to enhance specificity is to target inhibitors to therapeutically relevant cells or tissues. Therefore, attaching functionalities that specifically target Src SH2 inhibitors to bone might promote the inhibition of bone resorption by osteoclasts while minimizing potential undesirable effects on other cells. To test this concept, two bone-directed bisphosphonate-incorporating pTyr mimetics,

4'-diphosphonomethyl-phenylalanine (termed as **dpmF** or **DMP**) and 3', 4'-diphosphonophenylalanine (**Dpp**), have been incorporated into benzamide templates to generate compounds such as **AP22408** [236–238] (Fig. 5B and C). Here dpmF and Dpp have dual functions, providing selectivity for bone and binding to the pTyr pocket. Although the multiple phosphates of dpmF and Dpp create additional negative charge relative to pTyr, inhibitors incorporating these groups effectively suppress in vitro bone absorption by rabbit osteoclasts at reasonable concentrations ($IC_{50} < 2 \mu M$), indicating that they can enter the cell efficiently [236–238]. While direct experimental evidence is not available, a plausible explanation involves accumulation of these inhibitors in bone matrix followed by uptake into osteoclasts by phagocytosis [181]. Interestingly, bisphosphonates themselves are known to inhibit osteoclast function and bone metastasis [239,240]. The osteoclast inhibitory abilities of Dpp- and dpmF-incorporating SH2 inhibitors, however, depend on their binding potency to the Src SH2 domain [237].

The two classes of Src SH2 inhibitors discussed here, cysteine-targeting and bone-targeting compounds, displayed almost equivalent in vitro binding potency ($IC_{50} = 0.1–0.4 \mu M$), while the IC_{50} values for the rabbit osteoclast resorption assay were very different, 43 μM for the cysteine-targeting compound and 2 μM for dpmF-incorporating compound [233,236]. To date, an inhibitor with both cysteine- and bone-targeting functionalities has not been reported. It is important to note that proof-of-concept studies have demonstrated that Src-specific SH2 inhibitors indeed suppress bone resorption by osteoclasts [233,236–238]. Since Src kinase inhibitors display similar activity, the functional importance of both the SH2 and the kinase domains of Src in osteoclasts has been convincingly demonstrated [241,242].

3.3. Structure-based design and binding mode

It is obvious that computational modeling, taking advantage of available crystal structure data, plays a key role in the structure-based design of SH2 inhibitors. For example, inhibitors containing various scaffolds were docked onto the Src SH2 domain, using the pTyr residue as an anchor based on the known binding mode of *poYEEI* high-affinity peptides, leading to the choice of benzamide scaffolds [229]. However, subsequent X-ray crystal structures demonstrated significant differences in the details of the actual mode of binding compared to modeling predictions. These differences suggest difficulties in de novo prediction of binding mode by computational modeling, and emphasize the importance of confirmation by structural analysis for each SH2 inhibitor [227,231]. Such structural studies sometimes reveal new and unexpected binding modes, which in turn provide valuable information for further inhibitor design. For instance, crystallizing the Src SH2 domain in citrate buffer

unexpectedly revealed a citrate ion stacking into the pTyr pocket [236]. Interestingly, additional binding interactions were seen, e.g., ionic bonds between citrate and Lys β D6, that had not been noted in previous structural studies, leading to the design of new pTyr mimetics such as dpmF and Dpp [236–238].

Researchers from Aventis reported the superimposition of crystal structure data for 11 SH2 inhibitors, comprising two different scaffold series, bound to the Src SH2 domain [243]. This valuable study revealed three different ligand-binding modes to the SH2 domain, depending on the mode of interaction with the Y+3 hydrophobic pocket of the Src SH2 domain. Thus, they suggested that the SH2 domain is able to adopt different binding modes, thereby increasing the flexibility of its interactions. This is most likely because the pTyr pocket can accept phosphate in different orientations, and also because ordered water molecules can bridge between the scaffold or hydrophobic regions of the ligand and the rigid surface of the SH2 domain.

3.4. Bioavailability of SH2 inhibitors and other challenges

In light of the major constraints on the bioavailability of SH2 inhibitors (cell permeability, stability, and antigenicity), together with simplicity of synthesis, it is clear that smaller, less charged compounds with minimal peptidic character are ideal [244,245]. In terms of size and peptidic character, Src SH2 inhibitors roughly the size of dipeptides have been developed with lipophilic scaffolds [182] (Fig. 5), while the Grb2 SH2 domain seems to require larger macrocyclic ligands to achieve high affinity (Fig. 4). The issue of negative charge is still challenging. Decreasing the charge of pTyr mimetics improves cell permeability, at the price of decreased affinity for the pTyr pocket. What is the minimum acceptable charge for binding to SH2 domains? Beaulieu et al. [246] tested various substitutions for acidic side chains of a *po*YEEI-OH peptide for their effect on binding of the Lck SH2 domain, and demonstrated low micromolar affinity for an inhibitor bearing a net charge of -2 at physiological pH. Gratifyingly, the bone-targeting Src SH2 inhibitor (bearing the highly charged bisphosphonate pTyr mimetic) is effective in cell-based assays; in this case, the disadvantage of high net negative charge is offset by the advantage of bone-specific targeting [236–238]. In order to rapidly screen potential SH2 inhibitors and drive future development, high-throughput assay platforms that can simultaneously evaluate both binding potency and cell permeability will be critical. The ARIAD group has developed a promising mammalian cell assay based on the two-hybrid system and used it to screen a panel of Src SH2 inhibitors [238,247].

A major rationale for developing both the Grb2 and Src SH2 inhibitors was their potential for treating human malignancies. Despite the spectacular recent success of

Imatinib in treatment of chronic myelogenous leukemia and other malignancies [248], achieving a significant survival benefit with new anti-tumor agents remains a daunting challenge. Nygren and Larsson [249] recently evaluated publicly available data for a total of 209 investigational anti-cancer drugs, of which only 12 were shown to have survival benefit in randomized controlled clinical trials. To date, Ras antagonists have demonstrated only limited efficacy, so the ultimate clinical success of Grb2 SH2 inhibitors, which target Ras signaling, is far from assured [249]. Furthermore, not surprisingly Grb2 inhibitors are ineffective in cells expressing mutationally activated Ras, because Ras is downstream of Grb2 in the signaling pathway [193]. It should be noted, however, that Grb2-SH2 inhibitors may block other pathway(s) normally mediated by the SH3 domains of Grb2, such as PI3K–Akt cell survival signaling. On the other hand, a Src SH2 inhibitor could partially revert cell transformation induced by Y527F Src, although a 10-fold higher concentration was needed relative to the Src kinase domain inhibitor PPI [238].

Moreover, a concern with Src SH2 inhibitors is their potential to inappropriately activate Src kinase by competing with the autoinhibitory interaction between the Src SH2 and the regulatory pTyr. The Aventis group reported that several high-affinity Src SH2 inhibitors did not activate Y527-phosphorylated Src in ELISA-based assays and yeast whole cell assays; the reasons for this are unclear [250]. One approach to this problem might be to design bidentate ligands that engage multiple domains of the target protein as a means to enhance specificity and sensitivity [50,251]. A tethered compound linking peptides that target the kinase active site and the SH2 domain of Src, thereby preventing substrate binding by both the SH2 and catalytic domains without stimulating kinase activity, was shown to enhance inhibitory potency compared to monovalent active site-directed compounds [252]. Despite these remaining unresolved issues, the use of SH2 inhibitors to specifically block phosphorylation-dependent protein–protein interactions remains an attractive concept.

4. Concluding remarks

We have highlighted three SH2-containing proteins and two groups of SH2 inhibitors to illustrate recent progress in understanding both the common features of SH2 domains and the differences that allow them to be adapted to so many cellular functions. These recent studies reveal a number of subtle twists on the common and accepted themes of SH2 function. For example, previously unappreciated binding modes were discovered, underscoring the plasticity of SH2 domains, which is likely to be relevant to interactions with physiological ligands. It is now apparent that the SH2 domain features an “adjustable fit” to accommodate various ligands, consistent with the notion that a single SH2 domain displays only modest specificity for various phosphopep-

tides in vitro assays [27]. It is of particular interest to know in which situations SH2 domains utilize ambiguous molecular recognition, which may favor propagation of multiple signals, versus strict molecular recognition (achieved by tandem domain arrangement or high local concentrations of ligand) leading to a single specific output.

The increasing availability of genome-wide and proteome-wide data will greatly impact both our understanding and possible exploitation of SH2 domain-mediated signaling. Phosphoproteomic analyses will produce unprecedented details about the identity, abundance, and kinetics of appearance of binding sites for different SH2 domains under various physiological conditions. Coupled with data on the binding preferences and abundance of all ~116 SH2 domains in the cell, we will be able to use computational modeling to predict which interactions will occur under different conditions, along with their subcellular localization and likely biological outputs. The first attempts have been made to profile the global patterns of SH2 domain binding sites in the cell [253,254], and such approaches are likely to become more quantitative and high-throughput in the future. In addition to helping understand normal cell signaling, such SH2 profiles may serve to highlight and classify aberrant signaling, as seen in immune disorders and cancer. Given the likely future development of highly specific and cell-permeable SH2 domain inhibitors, this raises the prospect of using such inhibitors to therapeutically manipulate signaling pathways in the cell. The first tentative steps down this road have already been taken, and we can expect the pace of progress to redouble in the near future.

Acknowledgments

We thank P. Nash, T. Pawson, and C. Thompson for discussion of the human complement of SH2 domains. We are particularly grateful to W.T. Miller for critical reading of this manuscript. The authors were partially supported by grants from the NIH (CA82258, CA094378 and CA107785) and the Department of Defense Breast Cancer Research Program (DAMD17-03-1-0540).

References

- [1] C.A. Koch, D. Anderson, M.F. Moran, C. Ellis, T. Pawson, SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins, *Science* 252 (1991) 668–674.
- [2] T. Pawson, G.D. Gish, P. Nash, SH2 domains, interaction modules and cellular wiring, *Trends Cell Biol.* 11 (2001) 504–511.
- [3] T. Kawata, A. Shevchenko, M. Fukuzawa, K.A. Jermyn, N.F. Totty, N.V. Zhukovskaya, A.E. Sterling, M. Mann, J.G. Williams, SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in *Dictyostelium*, *Cell* 89 (1997) 909–916.
- [4] T. Pawson, P. Nash, Assembly of cell regulatory systems through protein interaction domains, *Science* 300 (2003) 445–452.
- [5] R. Aasland, C. Abrams, C. Ampe, L.J. Ball, M.T. Bedford, G. Cesareni, M. Gimona, J.H. Hurley, T. Jarchau, V.P. Lehto, M.A. Lemmon, R. Linding, B.J. Mayer, M. Nagai, M. Sudol, U. Walter, S.J. Winder, Normalization of nomenclature for peptide motifs as ligands of modular protein domains, *FEBS Lett.* 513 (2002) 141–144.
- [6] Z. Songyang, S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, B.G. Neel, R.B. Birge, J.E. Fajardo, M.M. Chou, H. Hanafusa, B. Schaffhausen, L.C. Cantley, SH2 domains recognize specific phosphopeptide sequences, *Cell* 72 (1993) 767–778.
- [7] Z. Songyang, S.E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X.R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, et al., Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav, *Mol. Cell. Biol.* 14 (1994) 2777–2785.
- [8] T. Pawson, Protein modules and signalling networks, *Nature* 373 (1995) 573–580.
- [9] T. Pawson, Specificity in signal transduction. From phosphotyrosine-SH2 domain interactions to complex cellular systems, *Cell* 116 (2004) 191–203.
- [10] J. Schlessinger, M.A. Lemmon, SH2 and PTB domains in tyrosine kinase signaling, *Sci. STKE* 2003 (2003) RE12.
- [11] M.B. Yaffe, Phosphotyrosine-binding domains in signal transduction, *Nat. Rev., Mol. Cell Biol.* 3 (2002) 177–186.
- [12] S.R. Hubbard, M. Mohammadi, J. Schlessinger, Autoregulatory mechanisms in protein-tyrosine kinases, *J. Biol. Chem.* 273 (1998) 11987–11990.
- [13] R.J. Lechleider, S. Sugimoto, A.M. Bennett, A.S. Kashishian, J.A. Cooper, S.E. Shoelson, C.T. Walsh, B.G. Neel, Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor, *J. Biol. Chem.* 268 (1993) 21478–21481.
- [14] D. Barford, B.G. Neel, Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2, *Structure* 6 (1998) 249–254.
- [15] L. Shiue, M.J. Zoller, J.S. Brugge, Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE, *J. Biol. Chem.* 270 (1995) 10498–10502.
- [16] J.M. Backer, M.G. Myers Jr., S.E. Shoelson, D.J. Chin, X.J. Sun, M. Miralpeix, P. Hu, B. Margolis, E.Y. Skolnik, J. Schlessinger, et al., Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation, *EMBO J.* 11 (1992) 3469–3479.
- [17] T. Rordorf-Nikolic, D.J. Van Horn, D. Chen, M.F. White, J.M. Backer, Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit, *J. Biol. Chem.* 270 (1995) 3662–3666.
- [18] E.N. Neumeister, Y. Zhu, S. Richard, C. Terhorst, A.C. Chan, A.S. Shaw, Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins, *Mol. Cell. Biol.* 15 (1995) 3171–3178.
- [19] Y.S. Bae, L.G. Cantley, C.S. Chen, S.R. Kim, K.S. Kwon, S.G. Rhee, Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate, *J. Biol. Chem.* 273 (1998) 4465–4469.
- [20] K.M. Shokat, Tyrosine kinases: modular signaling enzymes with tunable specificities, *Chem. Biol.* 2 (1995) 509–514.
- [21] Z. Songyang, Recognition and regulation of primary-sequence motifs by signaling modular domains, *Prog. Biophys. Mol. Biol.* 71 (1999) 359–372.
- [22] B.J. Mayer, H. Hirai, R. Sakai, Evidence that SH2 domains promote processive phosphorylation by protein-tyrosine kinases, *Curr. Biol.* 5 (1995) 296–305.
- [23] J. Kuriyan, D. Cowburn, Modular peptide recognition domains in eukaryotic signaling, *Annu. Rev. Biophys. Biomol. Struct.* 26 (1997) 259–288.
- [24] G. Waksman, S.E. Shoelson, N. Pant, D. Cowburn, J. Kuriyan, Binding of a high affinity phosphotyrosyl peptide to the Src SH2

- domain: crystal structures of the complexed and peptide-free forms, *Cell* 72 (1993) 779–790.
- [25] M.J. Eck, S.E. Shoelson, S.C. Harrison, Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56lck, *Nature* 362 (1993) 87–91.
- [26] J. Rahuel, B. Gay, D. Erdmann, A. Strauss, C. Garcia-Echeverria, P. Furet, G. Caravatti, H. Fretz, J. Schoepfer, M.G. Grutter, Structural basis for specificity of Grb2-SH2 revealed by a novel ligand binding mode, *Nat. Struct. Biol.* 3 (1996) 586–589.
- [27] J.M. Bradshaw, G. Waksman, Molecular recognition by SH2 domains, *Adv. Protein Chem.* 61 (2002) 161–210.
- [28] J.E. Ladbury, M.A. Lemmon, M. Zhou, J. Green, M.C. Botfield, J. Schlessinger, Measurement of the binding of tyrosyl phosphopeptides to SH2 domains: a reappraisal, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 3199–3203.
- [29] J.M. Bradshaw, R.A. Grucza, J.E. Ladbury, G. Waksman, Probing the “two-pronged plug two-holed socket” model for the mechanism of binding of the Src SH2 domain to phosphotyrosyl peptides: a thermodynamic study, *Biochemistry* 37 (1998) 9083–9090.
- [30] E. Piccione, R.D. Case, S.M. Domchek, P. Hu, M. Chaudhuri, J.M. Backer, J. Schlessinger, S.E. Shoelson, Phosphatidylinositol 3-kinase p85 SH2 domain specificity defined by direct phosphopeptide/SH2 domain binding, *Biochemistry* 32 (1993) 3197–3202.
- [31] J.M. Bradshaw, V. Mitaxov, G. Waksman, Investigation of phosphotyrosine recognition by the SH2 domain of the Src kinase, *J. Mol. Biol.* 293 (1999) 971–985.
- [32] H.W. Kessels, A.C. Ward, T.N. Schumacher, Specificity and affinity motifs for Grb2 SH2–ligand interactions, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 8524–8529.
- [33] Z. Songyang, G. Gish, G. Mbamalu, T. Pawson, L.C. Cantley, A single point mutation switches the specificity of group III Src homology (SH) 2 domains to that of group I SH2 domains, *J. Biol. Chem.* 270 (1995) 26029–26032.
- [34] L.E. Marengere, Z. Songyang, G.D. Gish, M.D. Schaller, J.T. Parsons, M.J. Stern, L.C. Cantley, T. Pawson, SH2 domain specificity and activity modified by a single residue, *Nature* 369 (1994) 502–505.
- [35] M.S. Kimber, J. Nachman, A.M. Cunningham, G.D. Gish, T. Pawson, E.F. Pai, Structural basis for specificity switching of the Src SH2 domain, *Mol. Cell* 5 (2000) 1043–1049.
- [36] B. Gay, P. Furet, C. Garcia-Echeverria, J. Rahuel, P. Chene, H. Fretz, J. Schoepfer, G. Caravatti, Dual specificity of Src homology 2 domains for phosphotyrosine peptide ligands, *Biochemistry* 36 (1997) 5712–5718.
- [37] A.M. Pendergast, A.J. Muller, M.H. Havlik, Y. Maru, O.N. Witte, BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner, *Cell* 66 (1991) 161–171.
- [38] A.J. Muller, A.M. Pendergast, M.H. Havlik, L. Puil, T. Pawson, O.N. Witte, A limited set of SH2 domains binds BCR through a high-affinity phosphotyrosine-independent interaction, *Mol. Cell Biol.* 12 (1992) 5087–5093.
- [39] S.N. Malek, S. Desiderio, A cyclin-dependent kinase homologue, p130PITSLRE is a phosphotyrosine-independent SH2 ligand, *J. Biol. Chem.* 269 (1994) 33009–33020.
- [40] S.N. Malek, C.H. Yang, W.C. Earnshaw, C.A. Kozak, S. Desiderio, p150TSP, a conserved nuclear phosphoprotein that contains multiple tetratricopeptide repeats and binds specifically to SH2 domains, *J. Biol. Chem.* 271 (1996) 6952–6962.
- [41] L.E. Rameh, C.S. Chen, L.C. Cantley, Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins, *Cell* 83 (1995) 821–830.
- [42] J. Sayos, C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M.G. Roncarolo, H. Oettgen, J.E. De Vries, G. Aversa, C. Terhorst, The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM, *Nature* 395 (1998) 462–469.
- [43] A. Morrione, P. Plant, B. Valentinis, O. Staub, S. Kumar, D. Rotin, R. Baserga, mGrb10 interacts with Nedd4, *J. Biol. Chem.* 274 (1999) 24094–24099.
- [44] S.C. Pero, L. Oligino, R.J. Daly, A.L. Soden, C. Liu, P.P. Roller, P. Li, D.N. Krag, Identification of novel non-phosphorylated ligands, which bind selectively to the SH2 domain of Grb7, *J. Biol. Chem.* 277 (2002) 11918–11926.
- [45] N.P. Mahajan, S.H. Earp, An SH2 domain-dependent, Phosphotyrosine-independent interaction between Vav1, and the Mer receptor tyrosine kinase: a mechanism for localizing GEF action, *J. Biol. Chem.* 278 (2003) 42596–42603.
- [46] Q. Gao, J. Hua, R. Kimura, J.J. Headd, X.Y. Fu, Y.E. Chin, Identification of the linker-SH2 domain of STAT as the origin of the SH2 domain using two dimensional structural alignment, *Mol. Cell Proteomics* 3 (2004) 704–714.
- [47] A.J. MacLennan, G. Shaw, A yeast SH2 domain, *Trends Biochem. Sci.* 18 (1993) 464–465.
- [48] J.G. Williams, M. Zvelebil, SH2 domains in plants imply new signalling scenarios, *Trends Plant Sci.* 9 (2004) 161–163.
- [49] E.A. Ottinger, M.C. Botfield, S.E. Shoelson, Tandem SH2 domains confer high specificity in tyrosine kinase signaling, *J. Biol. Chem.* 273 (1998) 729–735.
- [50] D. Cowburn, J. Zheng, Q. Xu, G. Barany, Enhanced affinities and specificities of consolidated ligands for the Src homology (SH) 3 and SH2 domains of Abelson protein-tyrosine kinase, *J. Biol. Chem.* 270 (1995) 26738–26741.
- [51] T. Nakamoto, R. Sakai, K. Ozawa, Y. Yazaki, H. Hirai, Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase, *J. Biol. Chem.* 271 (1996) 8959–8965.
- [52] K. Alexandropoulos, D. Baltimore, Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, p130^{cas}-related protein, *Sin, Genes Dev.* 10 (1996) 1341–1355.
- [53] D.T. Purtilo, C.K. Cassel, J.P. Yang, R. Harper, X-linked recessive progressive combined variable immunodeficiency (Duncan’s disease), *Lancet* 1 (1975) 935–940.
- [54] D.T. Purtilo, H.L. Grierson, Methods of detection of new families with X-linked lymphoproliferative disease, *Cancer Genet Cytogenet* 51 (1991) 143–153.
- [55] T.A. Seemayer, T.G. Gross, R.M. Egeler, S.J. Pirruccello, J.R. Davis, C.M. Kelly, M. Okano, A. Lanyi, J. Sumegi, X-linked lymphoproliferative disease: twenty-five years after the discovery, *Pediatr. Res.* 38 (1995) 471–478.
- [56] A.J. Coffey, R.A. Brooksbank, O. Brandau, T. Oohashi, G.R. Howell, J.M. Bye, A.P. Cahn, J. Durham, P. Heath, P. Wray, R. Pavitt, J. Wilkinson, M. Leversha, E. Huckle, C.J. Shaw-Smith, A. Dunham, S. Rhodes, V. Schuster, G. Porta, L. Yin, P. Serafini, B. Sylla, M. Zollo, B. Franco, D.R. Bentley, et al., Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene, *Nat. Genet.* 20 (1998) 129–135.
- [57] K.E. Nichols, D.P. Harkin, S. Levitz, M. Krainer, K.A. Kolquist, C. Genovese, A. Bernard, M. Ferguson, L. Zuo, E. Snyder, A.J. Buckler, C. Wise, J. Ashley, M. Lovett, M.B. Valentine, A.T. Look, W. Gerald, D.E. Housman, D.A. Haber, Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 13765–13770.
- [58] M. Morra, M. Simarro-Grande, M. Martin, A.S. Chen, A. Lanyi, O. Silander, S. Calpe, J. Davis, T. Pawson, M.J. Eck, J. Sumegi, P. Engel, S.C. Li, C. Terhorst, Characterization of SH2D1A missense mutations identified in X-linked lymphoproliferative disease patients, *J. Biol. Chem.* 276 (2001) 36809–36816.
- [59] F. Poy, M.B. Yaffe, J. Sayos, K. Saxena, M. Morra, J. Sumegi, L.C. Cantley, C. Terhorst, M.J. Eck, Crystal structures of the XLP protein SAP reveal a class of SH2 domains with extended, phosphotyrosine-independent sequence recognition, *Mol. Cell* 4 (1999) 555–561.
- [60] S.C. Li, G. Gish, D. Yang, A.J. Coffey, J.D. Forman-Kay, I. Ernberg, L.E. Kay, T. Pawson, Novel mode of ligand binding by the SH2

- domain of the human XLP disease gene product SAP/SH2D1A, *Curr. Biol.* 9 (1999) 1355–1362.
- [61] P.M. Hwang, C. Li, M. Morra, J. Lillywhite, D.R. Muhandiram, F. Gertler, C. Terhorst, L.E. Kay, T. Pawson, J.D. Forman-Kay, S.C. Li, A “three-pronged” binding mechanism for the SAP/SH2D1A SH2 domain: structural basis and relevance to the XLP syndrome, *EMBO J.* 21 (2002) 314–323.
- [62] M. Morra, J. Lu, F. Poy, M. Martin, J. Sayos, S. Calpe, C. Gullo, D. Howie, S. Rietdijk, A. Thompson, A.J. Coyle, C. Denny, M.B. Yaffe, P. Engel, M.J. Eck, C. Terhorst, Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells, *EMBO J.* 20 (2001) 5840–5852.
- [63] V. Schuster, H.W. Kreth, X-linked lymphoproliferative disease is caused by deficiency of a novel SH2 domain-containing signal transduction adaptor protein, *Immunol. Rev.* 178 (2000) 21–28.
- [64] A.G. Castro, T.M. Hauser, B.G. Cocks, J. Abrams, S. Zurawski, T. Churakova, F. Zonin, D. Robinson, S.G. Tangye, G. Aversa, K.E. Nichols, J.E. de Vries, L.L. Lanier, A. O’Garra, Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells, *J. Immunol.* 163 (1999) 5860–5870.
- [65] J. Sayos, K.B. Nguyen, C. Wu, S.E. Stepp, D. Howie, J.D. Schatzle, V. Kumar, C.A. Biron, C. Terhorst, Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4, *Int. Immunol.* 12 (2000) 1749–1757.
- [66] L.M. Shlapatska, S.V. Mikhlap, A.G. Berdova, O.M. Zelensky, T.J. Yun, K.E. Nichols, E.A. Clark, S.P. Sidorenko, CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A, *J. Immunol.* 166 (2001) 5480–5487.
- [67] C. Li, C. Josef, C.Y. Jia, V.K. Han, S.S. Li, Dual functional roles for the X-linked lymphoproliferative syndrome gene product SAP/SH2D1A in signaling through the signaling lymphocyte activation molecule (SLAM) family of immune receptors, *J. Biol. Chem.* 278 (2003) 3852–3859.
- [68] S. Latour, G. Gish, C.D. Helgason, R.K. Humphries, T. Pawson, A. Veillette, Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product, *Nat. Immunol.* 2 (2001) 681–690.
- [69] A. Veillette, The SAP family: a new class of adaptor-like molecules that regulates immune cell functions, *Sci. STKE* 2002 (2002) PE8.
- [70] B. Chan, A. Lanyi, H.K. Song, J. Griesbach, M. Simarro-Grande, F. Poy, D. Howie, J. Sumegi, C. Terhorst, M.J. Eck, SAP couples Fyn to SLAM immune receptors, *Nat. Cell Biol.* 5 (2003) 155–160.
- [71] S. Latour, R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P.L. Schwartzberg, D. Davidson, A. Veillette, Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation, *Nat. Cell Biol.* 5 (2003) 149–154.
- [72] L.W. Donaldson, G. Gish, T. Pawson, L.E. Kay, J.D. Forman-Kay, Structure of a regulatory complex involving the Abl SH3 domain, the Crk SH2 domain, and a Crk-derived phosphopeptide, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 14053–14058.
- [73] M. Simarro, A. Lanyi, D. Howie, F. Poy, J. Bruggeman, M. Choi, J. Sumegi, M.J. Eck, C. Terhorst, SAP increases FynT kinase activity and is required for phosphorylation of SLAM and Ly9, *Int. Immunol.* 16 (2004) 727–736.
- [74] S. Latour, A. Veillette, Molecular and immunological basis of X-linked lymphoproliferative disease, *Immunol. Rev.* 192 (2003) 212–224.
- [75] J. Sumegi, D. Huang, A. Lanyi, J.D. Davis, T.A. Seemayer, A. Maeda, G. Klein, M. Seri, H. Wakiguchi, D.T. Purtilo, T.G. Gross, Correlation of mutations of the SH2D1A gene and Epstein–Barr virus infection with clinical phenotype and outcome in X-linked lymphoproliferative disease, *Blood* 96 (2000) 3118–3125.
- [76] T. Noguchi, T. Matozaki, K. Horita, Y. Fujioka, M. Kasuga, Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation, *Mol. Cell. Biol.* 14 (1994) 6674–6682.
- [77] S.P. Sidorenko, E.A. Clark, The dual-function CD150 receptor subfamily: the viral attraction, *Nat. Immunol.* 4 (2003) 19–24.
- [78] A. Veillette, S. Latour, The SLAM family of immune-cell receptors, *Curr. Opin. Immunol.* 15 (2003) 277–285.
- [79] P. Engel, M.J. Eck, C. Terhorst, The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease, *Nat. Rev., Immunol.* 3 (2003) 813–821.
- [80] R. Chen, F. Relouzat, R. Roncagalli, A. Aoukaty, R. Tan, S. Latour, A. Veillette, Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors, *Mol. Cell. Biol.* 24 (2004) 5144–5156.
- [81] G. Aversa, C.C. Chang, J.M. Carballido, B.G. Cocks, J.E. de Vries, Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN- γ production, *J. Immunol.* 158 (1997) 4036–4044.
- [82] C. Wu, K.B. Nguyen, G.C. Pien, N. Wang, C. Gullo, D. Howie, M.R. Sosa, M.J. Edwards, P. Borrow, A.R. Satoskar, A.H. Sharpe, C.A. Biron, C. Terhorst, SAP controls T cell responses to virus and terminal differentiation of TH2 cells, *Nat. Immunol.* 2 (2001) 410–414.
- [83] M.J. Czar, E.N. Kersh, L.A. Mijares, G. Lanier, J. Lewis, G. Yap, A. Chen, A. Sher, C.S. Duckett, R. Ahmed, P.L. Schwartzberg, Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 7449–7454.
- [84] N. Wang, A. Satoskar, W. Faubion, D. Howie, S. Okamoto, S. Feske, C. Gullo, K. Clarke, M.R. Sosa, A.H. Sharpe, C. Terhorst, The Cell Surface Receptor SLAM Controls T Cell and Macrophage Functions, *J. Exp. Med.* 199 (2004) 1255–1264.
- [85] R. Sharifi, J.C. Sinclair, K.C. Gilmour, P.D. Arkwright, C. Kinnon, A.J. Thrasher, H.B. Gaspar, SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease, *Blood* 103 (2004) 3821–3827.
- [86] H. Williams, K. Macsween, K. McAulay, C. Higgins, N. Harrison, A. Swerdlow, K. Britton, D. Crawford, Analysis of immune activation and clinical events in acute infectious mononucleosis, *J. Infect. Dis.* 190 (2004) 63–71.
- [87] V. Pasquinelli, M.F. Quiroga, G.J. Martinez, L.C. Zorrilla, R.M. Musella, M.M. Bracco, L. Belmonte, A. Malbran, L. Fainboim, P.A. Sieling, V.E. Garcia, Expression of signaling lymphocytic activation molecule-associated protein interrupts IFN- γ production in human tuberculosis, *J. Immunol.* 172 (2004) 1177–1185.
- [88] W.Y. Langdon, J.W. Hartley, S.P. Klinken, S.K. Ruscetti, H.C. Morse, 3rd, v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas, *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 1168–1172.
- [89] J.A. Donovan, R.L. Wange, W.Y. Langdon, L.E. Samelson, The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor, *J. Biol. Chem.* 269 (1994) 22921–22924.
- [90] R. de Jong, J. ten Hoeve, N. Heisterkamp, J. Groffen, Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia, *J. Biol. Chem.* 270 (1995) 21468–21471.
- [91] T. Fukazawa, K.A. Reedquist, T. Trub, S. Soltoff, G. Pancharoorthy, B. Druker, L. Cantley, S.E. Shoelson, H. Band, The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the c-cbl protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase, *J. Biol. Chem.* 270 (1995) 19141–19150.
- [92] D. Hartley, H. Meisner, S. Corvera, Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl, *J. Biol. Chem.* 270 (1995) 18260–18263.
- [93] C.H. Yoon, C. Chang, N.A. Hopper, G.M. Lesa, P.W. Sternberg, Requirements of multiple domains of SLI-1, a *Caenorhabditis*

- elegans* homologue of c-Cbl, and an inhibitory tyrosine in LET-23 in regulating vulval differentiation, *Mol. Biol. Cell* 11 (1995) 4019–4031.
- [94] G.D. Jongeward, T.R. Clandinin, P.W. Sternberg, sli-1, a negative regulator of let-23-mediated signaling in *C. elegans*, *Genetics* 139 (1995) 1553–1566.
- [95] M.L. Galisteo, I. Dikic, A.G. Batzer, W.Y. Langdon, J. Schlessinger, Tyrosine phosphorylation of the c-cbl proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation, *J. Biol. Chem.* 270 (1995) 20242–20245.
- [96] M.L. Lupher Jr., K.A. Reedquist, S. Miyake, W.Y. Langdon, H. Band, A novel phosphotyrosine-binding domain in the N-terminal transforming region of Cbl interacts directly and selectively with ZAP-70 in T cells, *J. Biol. Chem.* 271 (1996) 24063–24068.
- [97] C.A. Joazeiro, S.S. Wing, H. Huang, J.D. Levenson, T. Hunter, Y.C. Liu, The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase, *Science* 286 (1999) 309–312.
- [98] G. Levkowitz, H. Waterman, S.A. Ettenberg, M. Katz, A.Y. Tsygankov, I. Ahroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz, Y. Yarden, Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1, *Mol. Cell* 4 (1999) 1029–1040.
- [99] M. Yokouchi, T. Kondo, A. Houghton, M. Bartkiewicz, W.C. Horne, H. Zhang, A. Yoshimura, R. Baron, Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7, *J. Biol. Chem.* 274 (1999) 31707–31712.
- [100] S. Miyake, M.L. Lupher Jr., B. Druker, H. Band, The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 7927–7932.
- [101] G. Levkowitz, H. Waterman, E. Zamir, Z. Kam, S. Oved, W.Y. Langdon, L. Beguinot, B. Geiger, Y. Yarden, c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor, *Genes Dev.* 12 (1998) 3663–3674.
- [102] P.S. Lee, Y. Wang, M.G. Dominguez, Y.G. Yeung, M.A. Murphy, D.D. Bowtell, E.R. Stanley, The Cbl proto-oncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation, *EMBO J.* 18 (1999) 3616–3628.
- [103] Y. Ota, L.E. Samelson, The product of the proto-oncogene c-cbl: a negative regulator of the Syk tyrosine kinase, *Science* 276 (1997) 418–420.
- [104] S. Ota, K. Hazeki, N. Rao, M.L. Lupher Jr., C.E. Andoniou, B. Druker, H. Band, The RING finger domain of Cbl is essential for negative regulation of the Syk tyrosine kinase, *J. Biol. Chem.* 275 (2000) 414–422.
- [105] P. Peschard, T.M. Fournier, L. Lamorte, M.A. Naujokas, H. Band, W.Y. Langdon, M. Park, Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein, *Mol. Cell* 8 (2001) 995–1004.
- [106] W. Meng, S. Sawadkosal, S.J. Burakoff, M.J. Eck, Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase, *Nature* 398 (1999) 84–90.
- [107] C.B. Thien, W.Y. Langdon, EGF receptor binding and transformation by v-cbl is ablated by the introduction of a loss-of-function mutation from the *Caenorhabditis elegans* sli-1 gene, *Oncogene* 14 (1997) 2239–2249.
- [108] S. Miyake, K.P. Mullane-Robinson, N.L. Lill, P. Douillard, H. Band, Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain, *J. Biol. Chem.* 274 (1999) 16619–16628.
- [109] M.L. Lupher Jr., Z. Songyang, S.E. Shoelson, L.C. Cantley, H. Band, The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr292 negative regulatory phosphorylation site of ZAP-70, *J. Biol. Chem.* 272 (1997) 33140–33144.
- [110] K.D. Beebe, P. Wang, G. Arabaci, D. Pei, Determination of the binding specificity of the SH2 domains of protein tyrosine phosphatase SHP-1 through the screening of a combinatorial phosphotyrosyl peptide library, *Biochemistry* 39 (2000) 13251–13260.
- [111] M.A. Kelly, H. Liang, I.-I. Sytwu, I. Vlattas, N.L. Lyons, B.R. Bowen, L.P. Wennogle, Characterization of SH2–ligand interactions via library affinity selection with mass spectrometric detection, *Biochemistry* 35 (1996) 11747–11755.
- [112] S.W. Vetter, Z.Y. Zhang, Probing the phosphopeptide specificities of protein tyrosine phosphatases, SH2 and PTB domains with combinatorial library methods, *Curr. Protein Pept. Sci.* 3 (2002) 365–397.
- [113] P. Peschard, N. Ishiyama, T. Lin, S. Lipkowitz, M. Park, A conserved DpYR motif in the juxtamembrane domain of the Met receptor family forms an atypical c-Cbl/Cbl-b TKB domain binding site required for suppression of oncogenic activation, *J. Biol. Chem.* 279 (2004) 29565–29571.
- [114] A.Y. Tsygankov, A.M. Teckchandani, E.A. Feshchenko, G. Swaminathan, Beyond the RING: CBL proteins as multivalent adapters, *Oncogene* 20 (2001) 6382–6402.
- [115] N. Zheng, P. Wang, P.D. Jeffrey, N.P. Pavletich, Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin–protein ligases, *Cell* 102 (2000) 533–539.
- [116] C.E. Andoniou, C.B. Thien, W.Y. Langdon, Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene, *EMBO J.* 13 (1994) 4515–4523.
- [117] M. Yokouchi, T. Kondo, A. Sanjay, A. Houghton, A. Yoshimura, S. Komiya, H. Zhang, R. Baron, Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins, *J. Biol. Chem.* 276 (2001) 35185–35193.
- [118] A. Sanjay, A. Houghton, L. Neff, E. DiDomenico, C. Bardelay, E. Antoine, J. Levy, J. Gailit, D. Bowtell, W.C. Horne, R. Baron, Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility, *J. Cell Biol.* 152 (2001) 181–195.
- [119] C.K. Kassenbrock, S.M. Anderson, Regulation of ubiquitin protein-ligase activity in c-Cbl by phosphorylation-induced conformational change, and constitutive activation by tyrosine to glutamate point mutations, *J. Biol. Chem.* 279 (2004) 28017–28027.
- [120] H. Odai, K. Sasaki, Y. Hanazono, H. Ueno, T. Tanaka, K. Miyagawa, K. Mitani, Y. Yazaki, H. Hirai, c-Cbl is inducibly tyrosine-phosphorylated by epidermal growth factor stimulation in fibroblasts, and constitutively tyrosine-phosphorylated and associated with v-Src in v-src-transformed fibroblasts, *Jpn. J. Cancer Res.* 86 (1995) 1119–1126.
- [121] N. Rao, M.L. Lupher Jr., S. Ota, K.A. Reedquist, B.J. Druker, H. Band, The linker phosphorylation site Tyr292 mediates the negative regulatory effect of Cbl on ZAP-70 in T cells, *J. Immunol.* 164 (2000) 4616–4626.
- [122] D.P. Bonita, S. Miyake, M.L. Lupher Jr., W.Y. Langdon, H. Band, Phosphotyrosine binding domain-dependent up-regulation of the platelet-derived growth factor receptor alpha signaling cascade by transforming mutants of Cbl: implications for Cbl's function and oncogenicity, *Mol. Cell. Biol.* 17 (1997) 4597–4610.
- [123] C.B. Thien, W.Y. Langdon, Tyrosine kinase activity of the EGF receptor is enhanced by the expression of oncogenic 70Z-Cbl, *Oncogene* 15 (1997) 2909–2919.
- [124] C.B. Thien, F. Walker, W.Y. Langdon, RING finger mutations that abolish c-Cbl-directed polyubiquitination and down-regulation of the EGF receptor are insufficient for cell transformation, *Mol. Cell* 7 (2001) 355–365.
- [125] P. Peschard, M. Park, Escape from Cbl-mediated down-regulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases, *Cancer Cell* 3 (2003) 519–523.
- [126] J.E. Darnell Jr., STATs and gene regulation, *Science* 277 (1997) 1630–1635.
- [127] A.C. Greenlund, M.O. Morales, B.L. Viviano, H. Yan, J. Krolewski, R.D. Schreiber, Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process, *Immunity* 2 (1995) 677–687.

- [128] K.D. Liu, S.L. Gaffen, M.A. Goldsmith, JAK/STAT signaling by cytokine receptors, *Curr. Opin. Immunol.* 10 (1998) 271–278.
- [129] G.A. Rook, R. Hernandez-Pando, S.L. Lightman, Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance, *Immunol. Today* 15 (1994) 301–303.
- [130] W.S. Alexander, D.J. Hilton, The Role of Suppressors of Cytokine Signaling (SOCS) Proteins in Regulation of the Immune Response, *Annu. Rev. Immunol.* 22 (2004) 503–529.
- [131] G.J. Strous, P. van Kerkhof, R. Govers, A. Ciechanover, A.L. Schwartz, The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor, *EMBO J.* 15 (1996) 3806–3812.
- [132] M. Huber, C.D. Helgason, J.E. Damen, L. Liu, R.K. Humphries, G. Krystal, The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 11330–11335.
- [133] H. Ueno, K. Sasaki, K. Miyagawa, H. Honda, K. Mitani, Y. Yazaki, H. Hirai, Antisense repression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathway but not the ras pathway in epidermal growth factor receptor signaling, *J. Biol. Chem.* 272 (1997) 8739–8743.
- [134] M. David, H.E. Chen, S. Goelz, A.C. Lerner, B.G. Neel, Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1, *Mol. Cell. Biol.* 15 (1995) 7050–7058.
- [135] C.D. Chung, J. Liao, B. Liu, X. Rao, P. Jay, P. Berta, K. Shuai, Specific inhibition of Stat3 signal transduction by PIAS3, *Science* 278 (1997) 1803–1805.
- [136] S.E. Nicholson, D.J. Hilton, The SOCS proteins: a new family of negative regulators of signal transduction, *J. Leukoc. Biol.* 63 (1998) 665–668.
- [137] W.S. Alexander, Suppressors of cytokine signalling (SOCS) in the immune system, *Nat. Rev. Immunol.* 2 (2002) 410–416.
- [138] C.J. Greenhalgh, M.E. Miller, D.J. Hilton, P.K. Lund, Suppressors of cytokine signaling: relevance to gastrointestinal function and disease, *Gastroenterology* 123 (2002) 2064–2081.
- [139] A. Yoshimura, T. Ohkubo, T. Kiguchi, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, T. Hara, A. Miyajima, A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors, *EMBO J.* 14 (1995) 2816–2826.
- [140] F. Verdier, S. Chretien, O. Muller, P. Varlet, A. Yoshimura, S. Gisselbrecht, C. Lacombe, P. Mayeux, Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated Cis protein, *J. Biol. Chem.* 273 (1998) 28185–28190.
- [141] R. Starr, T.A. Willson, E.M. Viney, L.J. Murray, J.R. Rayner, B.J. Jenkins, T.J. Gonda, W.S. Alexander, D. Metcalf, N.A. Nicola, D.J. Hilton, A family of cytokine-inducible inhibitors of signalling, *Nature* 387 (1997) 917–921.
- [142] T.A. Endo, M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiyama, A. Yoshimura, A new protein containing an SH2 domain that inhibits JAK kinases, *Nature* 387 (1997) 921–924.
- [143] T. Naka, M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, T. Kishimoto, Structure and function of a new STAT-induced STAT inhibitor, *Nature* 387 (1997) 924–929.
- [144] B.T. Kile, B.A. Schulman, W.S. Alexander, N.A. Nicola, H.M. Martin, D.J. Hilton, The SOCS box: a tale of destruction and degradation, *Trends Biochem. Sci.* 27 (2002) 235–241.
- [145] S. Minamoto, K. Ikegame, K. Ueno, M. Narazaki, T. Naka, H. Yamamoto, T. Matsumoto, H. Saito, S. Hosoe, T. Kishimoto, Cloning and functional analysis of new members of STAT induced STAT inhibitor (SSI) family: SSI-2 and SSI-3, *Biochem. Biophys. Res. Commun.* 237 (1997) 79–83.
- [146] M. Masuhara, H. Sakamoto, A. Matsumoto, R. Suzuki, H. Yasukawa, K. Mitsui, T. Wakioka, S. Tanimura, A. Sasaki, H. Misawa, M. Yokouchi, M. Ohtsubo, A. Yoshimura, Cloning and characterization of novel CIS family genes, *Biochem. Biophys. Res. Commun.* 239 (1997) 439–446.
- [147] D.J. Hilton, R.T. Richardson, W.S. Alexander, E.M. Viney, T.A. Willson, N.S. Sprigg, R. Starr, S.E. Nicholson, D. Metcalf, N.A. Nicola, Twenty proteins containing a C-terminal SOCS box form five structural classes, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 114–119.
- [148] D.L. Krebs, D.J. Hilton, SOCS proteins: negative regulators of cytokine signaling, *Stem Cells* 19 (2001) 378–387.
- [149] R. Starr, D. Metcalf, A.G. Elefanti, M. Brysha, T.A. Willson, N.A. Nicola, D.J. Hilton, W.S. Alexander, Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 14395–14399.
- [150] J.C. Marine, C. McKay, D. Wang, D.J. Topham, E. Parganas, H. Nakajima, H. Penderville, H. Yasukawa, A. Sasaki, A. Yoshimura, J.N. Ihle, SOCS3 is essential in the regulation of fetal liver erythropoiesis, *Cell* 98 (1999) 617–627.
- [151] H. Yasukawa, H. Misawa, H. Sakamoto, M. Masuhara, A. Sasaki, T. Wakioka, S. Ohtsuka, T. Imaizumi, T. Matsuda, J.N. Ihle, A. Yoshimura, The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop, *EMBO J.* 18 (1999) 1309–1320.
- [152] T. Naka, T. Matsumoto, M. Narazaki, M. Fujimoto, Y. Morita, Y. Ohsawa, H. Saito, T. Nagasawa, Y. Uchiyama, T. Kishimoto, Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 15577–15582.
- [153] S.E. Nicholson, T.A. Willson, A. Farley, R. Starr, J.G. Zhang, M. Baca, W.S. Alexander, D. Metcalf, D.J. Hilton, N.A. Nicola, Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction, *EMBO J.* 18 (1999) 375–385.
- [154] E.G. Stein, R. Ghirlando, S.R. Hubbard, Structural basis for dimerization of the Grb10 SH2 domain: implications for ligand specificity, *J. Biol. Chem.* 278 (2003) 13257–13264.
- [155] E.G. Stein, T.A. Gustafson, S.R. Hubbard, The BPS domain of Grb10 inhibits the catalytic activity of the insulin and IGF1 receptors, *FEBS Lett.* 493 (2001) 106–111.
- [156] W. He, D.W. Rose, J.M. Olefsky, T.A. Gustafson, Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains, *J. Biol. Chem.* 273 (1998) 6860–6867.
- [157] H. Yasukawa, A. Sasaki, A. Yoshimura, Negative regulation of cytokine signaling pathways, *Annu. Rev. Immunol.* 18 (2000) 143–164.
- [158] T. Hirano, K. Ishihara, M. Hibi, Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors, *Oncogene* 19 (2000) 2548–2556.
- [159] M. Takahashi-Tezuka, Y. Yoshida, T. Fukada, T. Ohtani, Y. Yamanaka, K. Nishida, K. Nakajima, M. Hibi, T. Hirano, Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase, *Mol. Cell. Biol.* 18 (1998) 4109–4117.
- [160] F. Schaper, C. Gendo, M. Eck, J. Schmitz, C. Grimm, D. Anhof, I.M. Kerr, P.C. Heinrich, Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression, *Biochem. J.* 335 (Pt 3) (1998) 557–565.
- [161] D. Anhof, M. Weissenbach, J. Schmitz, R. Sobota, H.M. Hermanns, S. Radtke, S. Linnemann, I. Behrmann, P.C. Heinrich, F. Schaper, Signal transduction of IL-6, leukemia-inhibitory factor, and oncos-

- atin M: structural receptor requirements for signal attenuation, *J. Immunol.* 165 (2000) 2535–2543.
- [162] T. Ohtani, K. Ishihara, T. Atsumi, K. Nishida, Y. Kaneko, T. Miyata, S. Itoh, M. Narimatsu, H. Maeda, T. Fukada, M. Itoh, H. Okano, M. Hibi, T. Hirano, Dissection of signaling cascades through gp130 in vivo: reciprocal roles for STAT3- and SHP2-mediated signals in immune responses, *Immunity* 12 (2000) 95–105.
- [163] T. Atsumi, K. Ishihara, D. Kamimura, H. Ikushima, T. Ohtani, S. Hirota, H. Kobayashi, S.J. Park, Y. Sacki, Y. Kitamura, T. Hirano, A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis, *J. Exp. Med.* 196 (2002) 979–990.
- [164] S.E. Nicholson, D. De Souza, L.J. Fabri, J. Corbin, T.A. Willson, J.G. Zhang, A. Silva, M. Asimakis, A. Farley, A.D. Nash, D. Metcalf, D.J. Hilton, N.A. Nicola, M. Baca, Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 6493–6498.
- [165] D. De Souza, L.J. Fabri, A. Nash, D.J. Hilton, N.A. Nicola, M. Baca, SH2 domains from suppressor of cytokine signaling-3 and protein tyrosine phosphatase SHP-2 have similar binding specificities, *Biochemistry* 41 (2002) 9229–9236.
- [166] U. Lehmann, J. Schmitz, M. Weissenbach, R.M. Sobota, M. Hortner, K. Friederichs, I. Behrmann, W. Tsiaris, A. Sasaki, J. Schneider-Mergener, A. Yoshimura, B.G. Neel, P.C. Heinrich, F. Schaper, SHP2 and SOCS3 contribute to Tyr-759-dependent attenuation of interleukin-6 signaling through gp130, *J. Biol. Chem.* 278 (2003) 661–671.
- [167] J.L. Bartoe, N.M. Nathanson, Independent roles of SOCS-3 and SHP-2 in the regulation of neuronal gene expression by leukemia inhibitory factor, *Brain Res. Mol. Brain Res.* 107 (2002) 108–119.
- [168] J. Schmitz, M. Weissenbach, S. Haan, P.C. Heinrich, F. Schaper, SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130, *J. Biol. Chem.* 275 (2000) 12848–12856.
- [169] W. Lu, D. Gong, D. Bar-Sagi, P.A. Cole, Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling, *Mol. Cell* 8 (2001) 759–769.
- [170] P. Hof, S. Pluskey, S. Dhe-Paganon, M.J. Eck, S.E. Shoelson, Crystal structure of the tyrosine phosphatase SHP-2, *Cell* 92 (1998) 441–450.
- [171] N.A. Cacalano, D. Sanden, J.A. Johnston, Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras, *Nat. Cell Biol.* 3 (2001) 460–465.
- [172] P. Fischer, U. Lehmann, R.M. Sobota, J. Schmitz, C. Niemand, S. Linnemann, S. Haan, I. Behrmann, A. Yoshimura, J.A. Johnston, G. Muller-Newen, P.C. Heinrich, F. Schaper, The role of the inhibitors of interleukin-6 signal transduction SHP2 and SOCS3 for desensitization of interleukin-6 signalling, *Biochem. J.* 378 (2004) 449–460.
- [173] H. Yasukawa, M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, T. Hirano, K.R. Chien, A. Yoshimura, IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages, *Nat. Immunol.* 4 (2003) 551–556.
- [174] B.A. Croker, D.L. Krebs, J.G. Zhang, S. Wormald, T.A. Willson, E.G. Stanley, L. Robb, C.J. Greenhalgh, I. Forster, B.E. Clausen, N.A. Nicola, D. Metcalf, D.J. Hilton, A.W. Roberts, W.S. Alexander, SOCS3 negatively regulates IL-6 signaling in vivo, *Nat. Immunol.* 4 (2003) 540–545.
- [175] J.S. Brugge, New intracellular targets for therapeutic drug design, *Science* 260 (1993) 918–919.
- [176] C. Garcia-Echeverria, Antagonists of the Src homology 2 (SH2) domains of Grb2, Src, Lck and ZAP-70, *Curr. Med. Chem.* 8 (2001) 1589–1604.
- [177] J. Turkson, J.S. Kim, S. Zhang, J. Yuan, M. Huang, M. Glenn, E. Haura, S. Sebti, A.D. Hamilton, R. Jove, Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity, *Mol. Cancer Ther.* 3 (2004) 261–269.
- [178] J.M. Bradshaw, G. Waksman, Calorimetric investigation of proton linkage by monitoring both the enthalpy and association constant of binding: application to the interaction of the Src SH2 domain with a high-affinity tyrosyl phosphopeptide, *Biochemistry* 37 (1998) 15400–15407.
- [179] T. Gilmer, M. Rodriguez, S. Jordan, R. Crosby, K. Alligood, M. Green, M. Kimery, C. Wagner, D. Kinder, P. Charifson, et al., Peptide inhibitors of src SH3-SH2-phosphoprotein interactions, *J. Biol. Chem.* 269 (1994) 31711–31719.
- [180] H. Fretz, P. Furet, C. Garcia-Echeverria, J. Schoepfer, J. Rahuel, Structure-based design of compounds inhibiting Grb2-SH2 mediated protein-protein interactions in signal transduction pathways, *Curr. Pharm. Des.* 6 (2000) 1777–1796.
- [181] W.C. Shakespeare, SH2 domain inhibition: a problem solved? *Curr. Opin. Chem. Biol.* 5 (2001) 409–415.
- [182] T.K. Sawyer, R.S. Bohacek, D.C. Dalgarno, C.J. Eyermann, N. Kawahata, C.A. Metcalf III, W.C. Shakespeare, R. Sundaramoorthi, Y. Wang, M.G. Yang, SRC homology-2 inhibitors: peptidomimetic and nonpeptide, *Mini Rev. Med. Chem.* 2 (2002) 475–488.
- [183] R. Sundaramoorthi, N. Kawahata, M.G. Yang, W.C. Shakespeare, C.A. Metcalf III, Y. Wang, T. Merry, C.J. Eyermann, R.S. Bohacek, S. Narula, D.C. Dalgarno, T.K. Sawyer, Structure-based design of novel nonpeptide inhibitors of the Src SH2 domain: phosphotyrosine mimetics exploiting multifunctional group replacement chemistry, *Biopolymers* 71 (2003) 717–729.
- [184] T. Takenawa, H. Miki, K. Matuoka, Signaling through Grb2/Ash-control of the Ras pathway and cytoskeleton, *Curr. Top. Microbiol. Immunol.* 228 (1998) 325–342.
- [185] L. Buday, J. Downward, Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor, *Cell* 73 (1993) 611–620.
- [186] S.E. Egan, B.W. Giddings, M.W. Brooks, L. Buday, A.M. Sizeland, R.A. Weinberg, Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation, *Nature* 363 (1993) 45–51.
- [187] R.T. Nolte, M.J. Eck, J. Schlessinger, S.E. Shoelson, S.C. Harrison, Crystal structure of the PI 3-kinase p85 amino-terminal SH2 domain and its phosphopeptide complexes, *Nat. Struct. Biol.* 3 (1996) 364–374.
- [188] P. Furet, B. Gay, C. Garcia-Echeverria, J. Rahuel, H. Fretz, J. Schoepfer, G. Caravatti, Discovery of 3-aminobenzoyloxycarbonyl as an N-terminal group conferring high affinity to the minimal phosphopeptide sequence recognized by the Grb2-SH2 domain, *J. Med. Chem.* 40 (1997) 3551–3556.
- [189] C. Garcia-Echeverria, P. Furet, B. Gay, H. Fretz, J. Rahuel, J. Schoepfer, G. Caravatti, Potent antagonists of the SH2 domain of Grb2: optimization of the X+1 position of 3-amino-Z-Tyr(PO3H2)-X+1-Asn-NH2, *J. Med. Chem.* 41 (1998) 1741–1744.
- [190] C. Garcia-Echeverria, B. Gay, J. Rahuel, P. Furet, Mapping the X(+1) binding site of the Grb2-SH2 domain with alpha,alpha-disubstituted cyclic alpha-amino acids, *Bioorg. Med. Chem. Lett.* 9 (1999) 2915–2920.
- [191] P. Furet, C. Garcia-Echeverria, B. Gay, J. Schoepfer, M. Zeller, J. Rahuel, Structure-based design, synthesis, and X-ray crystallography of a high-affinity antagonist of the Grb2-SH2 domain containing an asparagine mimetic, *J. Med. Chem.* 42 (1999) 2358–2363.
- [192] J. Schoepfer, H. Fretz, B. Gay, P. Furet, C. Garcia-Echeverria, N. End, G. Caravatti, Highly potent inhibitors of the Grb2-SH2 domain, *Bioorg. Med. Chem. Lett.* 9 (1999) 221–226.
- [193] B. Gay, S. Suarez, G. Caravatti, P. Furet, T. Meyer, J. Schoepfer, Selective GRB2 SH2 inhibitors as anti-Ras therapy, *Int. J. Cancer* 83 (1999) 235–241.

- [194] B. Gay, S. Suarez, C. Weber, J. Rahuel, D. Fabbro, P. Furet, G. Caravatti, J. Schoepfer, Effect of potent and selective inhibitors of the Grb2 SH2 domain on cell motility, *J. Biol. Chem.* 274 (1999) 23311–23315.
- [195] S.M. Domchek, K.R. Auger, S. Chatterjee, T.R. Burke Jr., S.E. Shoelson, Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphono-peptide, *Biochemistry* 31 (1992) 9865–9870.
- [196] T.R. Burke Jr., K. Lee, Phosphotyrosyl mimetics in the development of signal transduction inhibitors, *Acc. Chem. Res.* 36 (2003) 426–433.
- [197] T.R. Burke Jr., M.S. Smyth, A. Otaka, M. Nomizu, P.P. Roller, G. Wolf, R. Case, S.E. Shoelson, Nonhydrolyzable phosphotyrosyl mimetics for the preparation of phosphatase-resistant SH2 domain inhibitors, *Biochemistry* 33 (1994) 6490–6494.
- [198] Z.J. Yao, C.R. King, T. Cao, J. Kelley, G.W. Milne, J.H. Voigt, T.R. Burke Jr., Potent inhibition of Grb2 SH2 domain binding by non-phosphate-containing ligands, *J. Med. Chem.* 42 (1999) 25–35.
- [199] T.R. Burke Jr., J. Luo, Z.J. Yao, Y. Gao, H. Zhao, G.W. Milne, R. Guo, J.H. Voigt, C.R. King, D. Yang, Monocarboxylic-based phosphotyrosyl mimetics in the design of GRB2 SH2 domain inhibitors, *Bioorg. Med. Chem. Lett.* 9 (1999) 347–352.
- [200] Y. Gao, J. Luo, Z.J. Yao, R. Guo, H. Zou, J. Kelley, J.H. Voigt, D. Yang, T.R. Burke Jr., Inhibition of Grb2 SH2 domain binding by non-phosphate-containing ligands. 2. 4-(2-Malonyl)phenylalanine as a potent phosphotyrosyl mimetic, *J. Med. Chem.* 43 (2000) 911–920.
- [201] Y. Gao, J. Voigt, J.X. Wu, D. Yang, T.R. Burke Jr., Macrocyclization in the design of a conformationally constrained Grb2 SH2 domain inhibitor, *Bioorg. Med. Chem. Lett.* 11 (2001) 1889–1892.
- [202] C.Q. Wei, Y. Gao, K. Lee, R. Guo, B. Li, M. Zhang, D. Yang, T.R. Burke Jr., Macrocyclization in the design of Grb2 SH2 domain-binding ligands exhibiting high potency in whole-cell systems, *J. Med. Chem.* 46 (2003) 244–254.
- [203] Z.D. Shi, C.Q. Wei, K. Lee, H. Liu, M. Zhang, T. Araki, L.R. Roberts, K.M. Worthy, R.J. Fisher, B.G. Neel, J.A. Kelley, D. Yang, T.R. Burke Jr., Macrocyclization in the design of non-phosphorus-containing Grb2 SH2 domain-binding ligands, *J. Med. Chem.* 47 (2004) 2166–2169.
- [204] Z.D. Shi, K. Lee, H. Liu, M. Zhang, L.R. Roberts, K.M. Worthy, M.J. Fivash, R.J. Fisher, D. Yang, T.R. Burke Jr., A novel macrocyclic tetrapeptide mimetic that exhibits low-picomolar Grb2 SH2 domain-binding affinity, *Biochem. Biophys. Res. Commun.* 310 (2003) 378–383.
- [205] L. Oligino, F.D. Lung, L. Sastry, J. Bigelow, T. Cao, M. Curran, T.R. Burke Jr., S. Wang, D. Krag, P.P. Roller, C.R. King, Nonphosphorylated peptide ligands for the Grb2 Src homology 2 domain, *J. Biol. Chem.* 272 (1997) 29046–29052.
- [206] F.D. Lung, Y.Q. Long, P.P. Roller, C.R. King, J. Varady, X.W. Wu, S. Wang, Functional preference of the constituent amino acid residues in a phage-library-based nonphosphorylated inhibitor of the Grb2-SH2 domain, *J. Pept. Res.* 57 (2001) 447–454.
- [207] Y.C. Lou, F.D. Lung, M.T. Pai, S.R. Tzeng, S.Y. Wei, P.P. Roller, J.W. Cheng, Solution structure and dynamics of G1TE, a non-phosphorylated cyclic peptide inhibitor for the Grb2 SH2 domain, *Arch. Biochem. Biophys.* 372 (1999) 309–314.
- [208] Y.Q. Long, J.H. Voigt, F.D. Lung, C.R. King, P.P. Roller, Significant compensatory role of position Y-2 conferring high affinity to non-phosphorylated inhibitors of Grb2-SH2 domain, *Bioorg. Med. Chem. Lett.* 9 (1999) 2267–2272.
- [209] P. Li, M. Zhang, M.L. Peach, X. Zhang, H. Liu, M. Nicklaus, D. Yang, P.P. Roller, Structural basis for a non-phosphorus-containing cyclic peptide binding to Grb2-SH2 domain with high affinity, *Biochem. Biophys. Res. Commun.* 307 (2003) 1038–1044.
- [210] P. Li, M. Zhang, M.L. Peach, H. Liu, D. Yang, P.P. Roller, Concise and enantioselective synthesis of Fmoc-Pmp(Bu(t))(2)-OH and design of potent Pmp-containing Grb2-SH2 domain antagonists, *Org. Lett.* 5 (2003) 3095–3098.
- [211] Y.L. Song, P.P. Roller, Y.Q. Long, Development of 1-3-aminotyrosine suitably protected for the synthesis of a novel nonphosphorylated hexapeptide with low-nanomolar Grb2-SH2 domain-binding affinity, *Bioorg. Med. Chem. Lett.* 14 (2004) 3205–3208.
- [212] J. Rahuel, C. Garcia-Echeverria, P. Furet, A. Strauss, G. Caravatti, H. Fretz, J. Schoepfer, B. Gay, Structural basis for the high affinity of amino-aromatic SH2 phosphopeptide ligands, *J. Mol. Biol.* 279 (1998) 1013–1022.
- [213] T.R. Burke Jr., Z.J. Yao, Y. Gao, J.X. Wu, X. Zhu, J.H. Luo, R. Guo, D. Yang, N-terminal carboxyl and tetrazole-containing amides as adjuvants to Grb2 SH2 domain ligand binding, *Bioorg. Med. Chem.* 9 (2001) 1439–1445.
- [214] M. Rojas, S. Yao, J.P. Donahue, Y.Z. Lin, An alternative to phosphotyrosine-containing motifs for binding to an SH2 domain, *Biochem. Biophys. Res. Commun.* 234 (1997) 675–680.
- [215] A.E. Ottenhoff-Kalff, G. Rijksen, E.A. van Beurden, A. Hennipman, A.A. Michels, G.E. Staal, Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product, *Cancer Res.* 52 (1992) 4773–4778.
- [216] N.N. Mazurenko, E.A. Kogan, I.B. Zborovskaya, F.L. Kissel'jov, Expression of pp60c-src in human small cell and non-small cell lung carcinomas, *Eur. J. Cancer* 28 (1992) 372–377.
- [217] T.K. Weber, G. Steele, I.C. Summerhayes, Differential pp60c-src activity in well and poorly differentiated human colon carcinomas and cell lines, *J. Clin. Invest.* 90 (1992) 815–821.
- [218] P. Soriano, C. Montgomery, R. Geske, A. Bradley, Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice, *Cell* 64 (1991) 693–702.
- [219] P.L. Schwartzberg, L. Xing, O. Hoffmann, C.A. Lowell, L. Garrett, B.F. Boyce, H.E. Varmus, Rescue of osteoclast function by transgenic expression of kinase-deficient Src in src^{-/-} mutant mice, *Genes Dev.* 11 (1997) 2835–2844.
- [220] L. Gil, Y. Han, E.E. Opas, G.A. Rodan, R. Ruel, J.G. Sedor, P.C. Tyler, R.N. Young, Prostaglandin E2-bisphosphonate conjugates: potential agents for treatment of osteoporosis, *Bioorg. Med. Chem.* 7 (1999) 901–919.
- [221] G.J. Pacofsky, K. Lackey, K.J. Alligood, J. Berman, P.S. Charifson, R.M. Crosby, G.F. Dorsey Jr., P.L. Feldman, T.M. Gilmer, C.W. Hummel, S.R. Jordan, C. Mohr, L.M. Shewchuk, D.D. Sternbach, M. Rodriguez, Potent dipeptide inhibitors of the pp60c-src SH2 domain, *J. Med. Chem.* 41 (1998) 1894–1908.
- [222] D. Lesuisse, G. Lange, P. Deprez, D. Benard, B. Schoot, G. Delettre, J.P. Marquette, P. Broto, V. Jean-Baptiste, P. Bichet, E. Sarubbi, E. Mandine, SAR and X-ray. A new approach combining fragment-based screening and rational drug design: application to the discovery of nanomolar inhibitors of Src SH2, *J. Med. Chem.* 45 (2002) 2379–2387.
- [223] L. Tong, T.C. Warren, J. King, R. Betageri, J. Rose, S. Jakes, Crystal structures of the human p56lck SH2 domain in complex with two short phosphotyrosyl peptides at 1.0 Å and 1.8 Å resolution, *J. Mol. Biol.* 256 (1996) 601–610.
- [224] J.L. Buchanan, R.S. Bohacek, G.P. Luke, M. Hatada, X. Lu, D.C. Dalgarno, S.S. Narula, R. Yuan, D.A. Holt, Structure-based design and synthesis of a novel class of Src SH2 inhibitors, *Bioorg. Med. Chem. Lett.* 9 (1999) 2353–2358.
- [225] J.M. Bradshaw, V. Mitaxov, G. Waksman, Mutational investigation of the specificity determining region of the Src SH2 domain, *J. Mol. Biol.* 299 (2000) 521–535.
- [226] W.C. Shakespeare, R.S. Bohacek, M.D. Azimioara, K.J. Macek, G.P. Luke, D.C. Dalgarno, M.H. Hatada, X. Lu, S.M. Violette, C. Bartlett, T.K. Sawyer, Structure-based design of novel bicyclic nonpeptide inhibitors for the src SH2 domain, *J. Med. Chem.* 43 (2000) 3815–3819.
- [227] M.S. Plummer, D.R. Holland, A. Shahripour, E.A. Lunney, J.H. Fergus, J.S. Marks, P. McConnell, W.T. Mueller, T.K. Sawyer,

- Design, synthesis, and cocrystal structure of a nonpeptide Src SH2 domain ligand, *J. Med. Chem.* 40 (1997) 3719–3725.
- [228] J.L. Buchanan, C.B. Vu, T.J. Merry, E.G. Corpuz, S.G. Pradeepan, U.N. Mani, M. Yang, H.R. Plake, V.M. Varkhedkar, B.A. Lynch, I.A. MacNeil, K.A. Loiacono, C.L. Tiong, D.A. Holt, Structure-activity relationships of a novel class of Src SH2 inhibitors, *Bioorg. Med. Chem. Lett.* 9 (1999) 2359–2364.
- [229] E.A. Lunney, K.S. Para, J.R. Rubin, C. Humblet, J.H. Fergus, J.S. Marks, T.K. Sawyer, Structure-based design of a novel series of nonpeptide. Ligands that bind to the pp60src SH2 domain, *J. Am. Chem. Soc.* 119 (1997) 12471–12476.
- [230] P. Deprez, I. Baholet, S. Bulet, G. Lange, R. Amengual, B. Schoot, A. Vermond, E. Mandine, D. Lesuisse, Discovery of highly potent Src SH2 binders: structure-activity studies and X-ray structures, *Bioorg. Med. Chem. Lett.* 12 (2002) 1291–1294.
- [231] P.S. Charifson, L.M. Shewchuk, W. Rocque, C.W. Hummel, S.R. Jordan, C. Mohr, G.J. Pacofsky, M.R. Peel, M. Rodriguez, D.D. Sternbach, T.G. Consler, Peptide ligands of pp60(c-src) SH2 domains: a thermodynamic and structural study, *Biochemistry* 36 (1997) 6283–6293.
- [232] K.J. Alligood, P.S. Charifson, R. Crosby, T.G. Consler, P.L. Feldman, R.T. Gampe Jr., T.M. Gilmer, S.R. Jordan, M.W. Milstead, C. Mohr, M.R. Peel, W. Rocque, M. Rodriguez, D.W. Rusnak, L.M. Shewchuk, D.D. Sternbach, The formation of a covalent complex between a dipeptide ligand and the src SH2 domain, *Bioorg. Med. Chem. Lett.* 8 (1998) 1189–1194.
- [233] S.M. Violette, W.C. Shakespeare, C. Bartlett, W. Guan, J.A. Smith, R.J. Rickles, R.S. Bohacek, D.A. Holt, R. Baron, T.K. Sawyer, A Src SH2 selective binding compound inhibits osteoclast-mediated resorption, *Chem. Biol.* 7 (2000) 225–235.
- [234] W.C. Shakespeare, R.S. Bohacek, S.S. Narula, M.D. Azimioara, R.W. Yuan, D.C. Dalgarno, L. Madden, M.C. Botfield, D.A. Holt, An efficient synthesis of a 4'-phosphonodifluoromethyl-3'-formyl-phenylalanine containing SRC SH2 ligand, *Bioorg. Med. Chem. Lett.* 9 (1999) 3109–3112.
- [235] R.J. Boerner, T.G. Consler, R.T. Gampe Jr., D. Weigl, D.H. Willard, D.G. Davis, A.M. Edison, F. Loganzo Jr., D.B. Kassel, R.X. Xu, et al., Catalytic activity of the SH2 domain of human pp60c-src; evidence from NMR, mass spectrometry, site-directed mutagenesis and kinetic studies for an inherent phosphatase activity, *Biochemistry* 34 (1995) 15351–15358.
- [236] R.S. Bohacek, D.C. Dalgarno, M. Hatada, V.A. Jacobsen, B.A. Lynch, K.J. Macek, T. Merry, C.A. Metcalf III, S.S. Narula, T.K. Sawyer, W.C. Shakespeare, S.M. Violette, M. Weigele, X-Ray structure of citrate bound to Src SH2 leads to a high-affinity, bone-targeted Src SH2 inhibitor, *J. Med. Chem.* 44 (2001) 660–663.
- [237] W. Shakespeare, M. Yang, R. Bohacek, F. Cerasoli, K. Stebbins, R. Sundaramoorthi, M. Azimioara, C. Vu, S. Pradeepan, C. Metcalf III, C. Haraldson, T. Merry, D. Dalgarno, S. Narula, M. Hatada, X. Lu, M.R. van Schravendijk, S. Adams, S. Violette, J. Smith, W. Guan, C. Bartlett, J. Herson, J. Iulucci, M. Weigele, T. Sawyer, Structure-based design of an osteoclast-selective, nonpeptide src homology 2 inhibitor with in vivo antiresorptive activity, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 9373–9378.
- [238] S.M. Violette, W. Guan, C. Bartlett, J.A. Smith, C. Bardelay, E. Antoine, R.J. Rickles, E. Mandine, M.R. van Schravendijk, S.E. Adams, B.A. Lynch, W.C. Shakespeare, M. Yang, V.A. Jacobsen, C.S. Takeuchi, K.J. Macek, R.S. Bohacek, D.C. Dalgarno, M. Weigele, D. Lesuisse, T.K. Sawyer, R. Baron, Bone-targeted Src SH2 inhibitors block Src cellular activity and osteoclast-mediated resorption, *Bone* 28 (2001) 54–64.
- [239] Y. Sohara, H. Shimada, M. Scadeng, H. Pollack, S. Yamada, W. Ye, C.P. Reynolds, Y.A. DeClerck, Lytic bone lesions in human neuroblastoma xenograft involve osteoclast recruitment and are inhibited by bisphosphonate, *Cancer Res.* 63 (2003) 3026–3031.
- [240] T. Yoneda, N. Hashimoto, T. Hiraga, Bisphosphonate actions on cancer, *Calcif. Tissue Int.* 73 (2003) 315–318.
- [241] M. Missbach, M. Jeschke, J. Feyen, K. Muller, M. Glatt, J. Green, M. Susa, A novel inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption in vitro and in rodent models in vivo, *Bone* 24 (1999) 437–449.
- [242] C.B. Vu, G.P. Luke, N. Kawahata, W.C. Shakespeare, Y. Wang, R. Sundaramoorthi, C.A. Metcalf, T.P. Keenan, S. Pradeepan, E. Corpuz, T. Merry, R.S. Bohacek, D.C. Dalgarno, S.S. Narula, M.R. van Schravendijk, M.K. Ram, S. Adams, S. Liou, J.A. Keats, S.M. Violette, W. Guan, M. Weigele, T.K. Sawyer, Bone-Targeted pyrido[2,3-*d*]pyrimidin-7-ones: potent inhibitors of Src tyrosine kinase as novel antiresorptive agents, *Bioorg. Med. Chem. Lett.* 13 (2003) 3071–3074.
- [243] G. Lange, D. Lesuisse, P. Deprez, B. Schoot, P. Loenze, D. Benard, J.P. Marquette, P. Broto, E. Sarubbi, E. Mandine, Principles governing the binding of a class of non-peptidic inhibitors to the SH2 domain of src studied by X-ray analysis, *J. Med. Chem.* 45 (2002) 2915–2922.
- [244] M.A. Navia, Protein-drug complexes important for immunoregulation and organ transplantation, *Curr. Opin. Struct. Biol.* 6 (1996) 838–847.
- [245] J.C. Verhoef, H.E. Bodde, A.G. de Boer, J.A. Bouwstra, H.E. Junginger, F.W. Merkus, D.D. Breimer, Transport of peptide and protein drugs across biological membranes, *Eur. J. Drug Metab. Pharmacokinet.* 15 (1990) 83–93.
- [246] P.L. Beaulieu, D.R. Cameron, J.M. Ferland, J. Gauthier, E. Ghro, J. Gillard, V. Gorys, M. Poirier, J. Rancourt, D. Wernic, M. Llinas-Brunet, R. Betageri, M. Cardozo, E.R. Hickey, R. Ingraham, S. Jakes, A. Kabcenell, T. Kirrane, S. Lukas, U. Patel, J. Proudfoot, R. Sharma, L. Tong, N. Moss, Ligands for the tyrosine kinase p56lck SH2 domain: discovery of potent dipeptide derivatives with mono-charged, nonhydrolyzable phosphate replacements, *J. Med. Chem.* 42 (1999) 1757–1766.
- [247] R.J. Rickles, P.A. Henry, W. Guan, M. Azimioara, W.C. Shakespeare, S. Violette, M.J. Zoller, A novel mechanism-based mammalian cell assay for the identification of SH2-domain-specific protein-protein inhibitors, *Chem. Biol.* 5 (1998) 529–538.
- [248] B.J. Druker, Perspectives on the development of a molecularly targeted agent, *Cancer Cell* 1 (2002) 31–36.
- [249] P. Nygren, R. Larsson, Overview of the clinical efficacy of investigational anticancer drugs, *J. Intern. Med.* 253 (2003) 46–75.
- [250] E. Mandine, V. Jean-Baptiste, B. Vayssiere, D. Gofflo, D. Benard, E. Sarubbi, P. Deprez, R. Baron, G. Superti-Furga, D. Lesuisse, High-affinity Src-SH2 ligands which do not activate Tyr(527)-phosphorylated Src in an experimental in vivo system, *Biochem. Biophys. Res. Commun.* 298 (2002) 185–192.
- [251] D.J. Maly, I.C. Choong, J.A. Ellman, Combinatorial target-guided ligand assembly: identification of potent subtype-selective c-Src inhibitors, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 2419–2424.
- [252] A.A. Profit, T.R. Lee, J. Niu, D.S. Lawrence, Molecular rulers: an assessment of distance and spatial relationships of Src tyrosine kinase Sh2 and active site regions, *J. Biol. Chem.* 276 (2001) 9446–9451.
- [253] K. Machida, B.J. Mayer, P. Nollau, Profiling the global tyrosine phosphorylation state, *Mol. Cell. Proteomics.* 2 (2003) 215–233.
- [254] P. Nollau, B.J. Mayer, Profiling the global tyrosine phosphorylation state by src homology 2 domain binding, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 13531–13536.
- [255] L.C. Cantley, Z. Songyang, Specificity in recognition of phosphopeptides by src-homology 2 domains, *J. Cell Sci., Suppl.* 18 (1994) 121–126.
- [256] K. Muller, F.O. Gombert, U. Manning, F. Grossmuller, P. Graff, H. Zaegel, J.F. Zuber, F. Freuler, C. Tschopp, G. Baumann, Rapid identification of phosphopeptide ligands for SH2 domains. Screening of peptide libraries by fluorescence-activated bead sorting, *J. Biol. Chem.* 271 (1996) 16500–16505.

- [257] H. Gram, R. Schmitz, J.F. Zuber, G. Baumann, Identification of phosphopeptide ligands for the Src-homology 2 (SH2) domain of Grb2 by phage display, *Eur. J. Biochem.* 246 (1997) 633–637.
- [258] M. Wiederkehr-Adam, P. Ernst, K. Muller, E. Bieck, F.O. Gombert, J. Ottl, P. Graff, F. Grossmuller, M.H. Heim, Characterization of phosphopeptide motifs specific for the Src homology 2 domains of signal transducer and activator of transcription 1 (STAT1) and STAT3, *J. Biol. Chem.* 278 (2003) 16117–16128.
- [259] W.J. Fantl, J.A. Escobedo, G.A. Martin, C.W. Turck, M. del Rosario, F. McCormick, L.T. Williams, Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways, *Cell* 69 (1992) 413–423.
- [260] A. Kashishian, A. Kazlauskas, J.A. Cooper, Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo, *EMBO J.* 11 (1992) 1373–1382.
- [261] R.D. Case, E. Piccione, G. Wolf, A.M. Benett, R.J. Lechleider, B.G. Neel, S.E. Shoelson, SH-PTP2/Syp SH2 domain binding specificity is defined by direct interactions with platelet-derived growth factor beta-receptor, epidermal growth factor receptor, and insulin receptor substrate-1-derived phosphopeptides, *J. Biol. Chem.* 269 (1994) 10467–10474.
- [262] G. Huyer, Z.M. Li, M. Adam, W.R. Huckle, C. Ramachandran, Direct determination of the sequence recognition requirements of the SH2 domains of SH-PTP2, *Biochemistry* 34 (1995) 1040–1049.
- [263] N. Stahl, T.J. Farruggella, T.G. Boulton, Z. Zhong, J.E. Darnell Jr., G.D. Yancopoulos, Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors, *Science* 267 (1995) 1349–1353.
- [264] C. Gerhartz, B. Heesel, J. Sasse, U. Hemmann, C. Landgraf, J. Schneider-Mergener, F. Horn, P.C. Heinrich, L. Graeve, Differential activation of acute phase response factor/STAT3 and STAT1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. I. Definition of a novel phosphotyrosine motif mediating STAT1 activation, *J. Biol. Chem.* 271 (1996) 12991–12998.
- [265] P.J. Coffey, W. Kruijer, EGF receptor deletions define a region specifically mediating STAT transcription factor activation, *Biochem. Biophys. Res. Commun.* 210 (1995) 74–81.
- [266] H. Shao, X. Xu, M.A. Mastrangelo, N. Jing, R.G. Cook, G.B. Legge, D.J. Tweardy, Structural requirements for signal transducer and activator of transcription 3 binding to phosphotyrosine ligands containing the YXXQ motif, *J. Biol. Chem.* 279 (2004) 18967–18973.