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Parallel Chemical Protein Synthesis on a Surface Enables the Rapid Analysis of the Phosphoregulation of SH3 Domains

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Abstract: *Analysis of postranslationally modified protein domains is complicated by an availability problem, as recombinant methods rarely allow site-specificity at will. Although total synthesis enables full control over posttranslational and other modifications, chemical approaches are limited to shorter peptides. To solve this problem, we herein describe a method that combines a) immobilization of N-terminally thiolated peptide hydrazides by hydrazone ligation, b) on-surface native chemical ligation with self-purified peptide thioesters, c) radical-induced desulfurization, and d) a surface-based fluorescence binding assay for functional characterization. We used the method to rapidly investigate 20 SH3 domains, with a focus on their phosphoregulation. The analysis suggests that tyrosine phosphorylation of SH3 domains found in Abl kinases act as a switch that can induce both the loss and, unexpectedly, gain of affinity for proline-rich ligands.*

The combination of solid-phase peptide synthesis and native chemical ligation (NCL) enables investigations of post-translational protein modifications at a level of precision not accessible by the currently applied recombinant methods.[1] This also applies to mechanistic investigations of "phosphoregulation", in which access to site-specifically phosphorylated proteins is required.[2] Recombinant methods afford folded phosphoproteins.[3] However, the limited availability of suitable protein kinases restricts the global analysis of alternative protein phosphoforms. For example, the SH3 domain of the Abl protein, a nonreceptor tyrosine kinase critically involved in chronic myelogenous leukemia (CML),[4,5] is found phosphorylated at each of its three tyrosine residues (Y^7, Y^{30}, Y^{52}).[6,7] However, readily available kinases, such as Lyn, Fyn, or Hck, phosphorylate at only two (Y^7 and Y^{52}) residues.[8]

Total chemical synthesis may offer unrestricted access to protein phosphoforms. However, parallel synthesis by NCL[9] is an arduous task, especially when the protein fragments to be ligated are sparingly soluble and difficult to purify. To streamline protein synthesis, native chemical ligation has been performed on polymeric supports.[10] The need for comparatively large amounts of HPLC-purified peptide fragments is a bottleneck for the analysis of alternative protein posttranslational modifications (PTMs). We introduced a method for the rapid chemical synthesis of protein domains, which were subsequently analyzed on microtiter plates.[11] The method involved a C-terminal hexahistidine tag for immobilization to nickel-coated surfaces. This proved problematic. The NCL was performed in solution prior to immobilization because of the sensitivity of the Ni–histidine complex to thiol additives. Solution steps are labor intensive. The weakness of the Ni-His linkage was also the reason why extended NCL based on the removal of ligation auxiliaries or desulfurization[12,13] was not explored. This limited the synthesis to SH3 domains, in which cysteine occurs naturally.

We sought a method that enables the rapid synthesis and functional analysis of kinase SH3 domains in all phosphoforms listed in the phosphosite database, regardless of the presence of cysteine. We considered a combination of hydrazone ligation for the immobilization of cysteinyl peptide hydrazides **1**, on-surface native chemical ligation with biotinylated peptide thioesters **2** with concomitant hydrazone reduction (for stabilization of the surface attachment), on-surface desulfurization, and a surface-based binding assay for functional characterization (Scheme 1 A).

In a proof of feasibility study, we embarked on the synthesis of the SH3 domain of the YSC84 protein in yeast. First, we evaluated the immobilization chemistry. Aldehyde-functionalized well plates were prepared by adding the polyethylene glycol mercaptoacetal linker **6** (accessed in 3 steps, Figure S1) to maleimide-activated black 96-well plates (Scheme 2). Acid treatment exposed the aldehyde in **7**. In the subsequent step, peptide hydrazides **1** (prepared by automated solid-phase synthesis, Scheme 1 B)[14] were used in crude form. We assumed that only the full-length product can react in subsequent NCL. The immobilization was performed for 1 h at pH 4 in the presence of aniline to favor the hydrazone ligation (Figure S2).[15] The NCL of immobilized peptide **3a** with peptide thioester **2a** (prepared by our previously published method with self-purification, which provides crude peptide thioesters of high purity, Scheme 1 C, see section 3.2 in the Supporting Information)[11,16] occurred at a Leu-Cys junction that allows a subsequent conversion into the natural Leu-Ala structure (Scheme 3). During ligation of **2a** and **3a**, $NaCNBH_3$ was included in the buffer to reduce the hydrazone to the more stable N-alkylated peptide hydrazide in the full-length peptide **4a**. A biotin quantification assay revealed a 144 pmol load of the A21C mutant of the YSC84 SH3 domain **4a** per well (Figure S5).[11] Given the maximal achievable loading (150 pmol per well) provided by the manufacturer, we inferred that both the immobilization and the subsequent NCL proceeded quantitatively. The immobilized peptide (**4a**,**b**) obtained upon NCL was subjected to metal-free desulfurization.[17] This step

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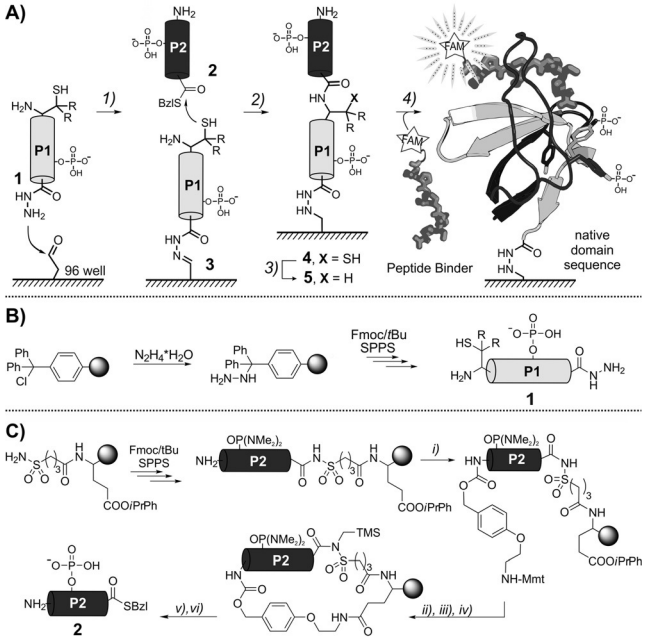
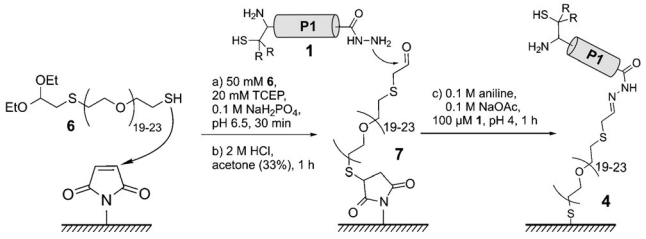
requires high concentrations of phosphines and *tert*-butylmercaptan or glutathione.[18] The biotin load remained unchanged, thus indicating the stability of the peptide hydrazide linkage (Figure S5).



Scheme 1. A) On-surface synthesis/analysis of protein domains by 1) immobilization of cysteine or penicillamine peptide hydrazides **1**, 2) native chemical ligation with peptide thioesters **2** and concomitant reduction of the hydrazone **3**, 3) radical desulfurization, 4) surface saturation binding analysis with fluorescein (FAM) labeled peptides. B) Synthesis of peptide hydrazides **1**. C) Self-purifying solid-phase synthesis (SPPS) of peptide thioesters **2**: i) 5 vol% NEt_3, 10 equiv $MmtNH(CH_2)_2OC_4H_4CH_2OCO_2C_4H_4NO_2$, ii) 1%TFA, iii) PyBOP, iv) $Me_3SiCH_2N_2$, v) NaSPh, BzlSH, and washing, vi) TFA cleavage from solid support. Bzl = benzyl, Fmoc = fluorenylmethoxycarbonyl, Mmt = monomethoxytrityl, PyBOP = benzotriazolyl-1-oxy-tripyrrolidinophosphonium hexafluorophosphate, TFA = trifluoroacetic acid, TMS = trimethylsilyl.



Scheme 2. Aldehyde functionalization of maleinimide-activated well plates. R = H, CH_3. TCEP = triscarboxyethylphosphine.

To explore the on-surface binding assay, the immobilized YSC84 SH3 domain was titrated with a known binder, the peptide FAM-ßAlaßAla-GTTNRGPPPLPPRA-NH_2 (**8**; $K_D = 1.3$ µM) of the yeast protein ACF2 (Figure S11).[19] The chemically synthesized YSC84 SH3 domain showed a $K_D = 1.2$ µM (Table 1). For comparison, we immobilized the

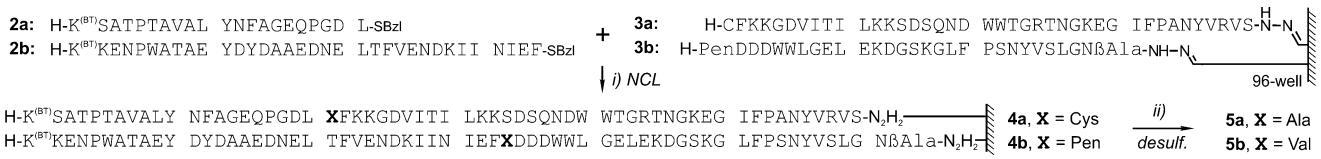
Table 1: Binding affinity (assessed as K_D in µM)[a] of immobilized cysteine or penicillamine mutants or desulfurized SH3 domains of YSC84 or AP1 for proline-rich peptides ACF2 and YIR003 (FAM-ßAlaßAla-RPKRRAPPPVPKKP-NH_2)

SH3 domain	ACF2, **8**	YIR003, **9**
A21C mutant of YSC84	1.9 ± 0.4	10 ± 2
desulfurized to native YSC84	1.2 ± 0.3 (1.1[b])	1.6 ± 0.4
V34Pen mutant of Abp1	2.8 ± 0.6	0.9 ± 0.4
desulfurized to native Abp1	2.6 ± 0.6	0.4 ± 0.2 (0.1[b])

[a] Determined by saturation binding analysis on the surface. [b] Determined by Landgraf et al.[19]

recombinant, GST-tagged SH3 domain on glutathione-coated well plates. Saturation binding analysis revealed a $K_D = 1.2$ µM. This is in agreement with the $K_D = 1.1$ µM determined previously by surface plasmon resonance measurements.[19] To further corroborate the results obtained in surface assays, we carried out binding experiments in solution. The $K_D = 1.4$ µM determined by a fluorescence polarization (FP) assay was in reasonable agreement with the data obtained by the surface saturation binding analysis (Figure S11). We then characterized the influence of cysteine mutants. The interaction of the synthetic A21C mutant with peptide **8** was weakened to $K_D = 1.9$ µM, as determined by both the surface-based assay (Table 1) and the FP-based assay in solution (Figure S11). The cysteine substitution had a greater effect on the interaction with the proline-rich fragment of YIR003 **9** and decreased the affinity by 84% (A21C-YSC84-SH3: $K_D = 10$ µM, wild-type YSC84-SH3; $K_D = 1.6$ µM). This highlights the importance of chemical methods that widen the scope of native chemical ligation beyond cysteine.

The N-terminal attachment of thiolated amino acids provides—after NCL and desulfurization—access to a variety of different ligation junctions. We explored the use of Pen ligation[18] in the on-surface synthesis of the SH3 domain of Abp1 (**5b** in Scheme 3). The Pen-terminated peptide hydrazone **3b** was treated with the 34-residue peptide thioester **2b**. The Pen ligation proceeded with a yield of 74% (Figure S6). Subsequently, the immobilized V34Pen mutant **4b** was



Scheme 3. On-surface synthesis of SH3 domains of YSC84 **5a** and AP1 **5b** from yeast. i) 100 µM **2**, 0.1 M Na_2HPO_4, 3 M GdnHCl, 50 mM sodium ascorbate, 50 mM $NaCNBH_3$, 20 mM TCEP, 50 mM sodium mercaptoethylsulfonate, pH 7; ii) 0.1 M Na_2HPO_4, 3 M GdnHCl, 0.2 M VA044 (2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride), 0.25 M TCEP, 80 mM *t*BuSH (for Cys) or glutathione (for Pen), pH 6.5, 50 °C, 5 h. GdnHCl = guanidinium hydrochloride.

desulfurized under radical conditions, thereby furnishing the wild-type SH3 domain **5b** of Abp1. The desulfurized protein had only slightly higher affinities for the peptide binders **8** and **9** than did the protein before desulfurization (Table 1). We inferred that penicillamine is a reasonably good mimic of valine.

With proof of feasibility demonstrated, we next turned our attention to the surface-aided synthesis and analysis of phosphorylated SH3 domains. As a consequence of its role in chronic myelogenous leukemia (CML), we explored the SH3 domain of the Abl protein, a nonreceptor tyrosine kinase which is highly tyrosine phosphorylated in leukemia cells.[6–8] The SH3 domain recognizes a proline-rich SH2 kinase interdomain *in cis*, which stabilizes a closed, inactive state of the Abl kinase (Figure 1 A, left). Interactions with proline-rich SH3 ligands *in trans* favor the opening to an active state (Figure 1 A, right).[20]

Phosphorylation at SH3 up-regulates Abl kinase activity, which has been linked to interference with the recognition of the proline-rich interdomain *in cis*.[8b,21] However, little is known about the effect of phosphorylation on interactions *in trans*. In addition, we studied the SH3 domain of the Arg (Abl2) protein, a closely related kinase that is believed to contribute to tumor progression.[5b,22] Phosphoproteomics revealed phosphorylation at each of the three tyrosine residues in both the Abl and the Arg SH3 domains (Figure 1 B).[6] By using the Trp^{36}-Cys^{37} site, we prepared eight different Abl SH3 domains (**6a**–**h**) by NCL with four different (phospho)peptide thioesters and two different (phospho)-peptide hydrazides (Figure S12). A similar analysis demanded two (phospho)peptide thioesters and four (phospho)peptide hydrazides when the Arg^{26}-Val^{27} site was used for construction of the Arg SH3 domains (**7a**–**h**) by Pen ligation and subsequent desulfurization.

We used four fluorescently labeled peptides for functional analysis of the phosphorylated Abl and Arg SH3 domains: two native ligands from the adaptor proteins 3BP1 and 3BP2,[23] a designed high affinity peptide p41,[24] as well as a peptide from the interdomain (ID) sequence between the SH2 and kinase domain of Abl.[25] The interaction of three of the peptides with the nonphosphorylated recombinant Abl SH3 domain has been previously quantified.[23,24] Similar values were determined by the on-surface assay with the immobilized, synthetic Abl SH3 domains (Table 2).

Rather unexpected results were obtained when the phosphorylated SH3 domains were studied. Monophosphorylation of the Abl SH3 domain was found to increase the affinities for the 3BP1 ligand two- to sixfold. Of note, double phosphorylation at Y^{30} and Y^{52} induced the highest affinity gains of one order of magnitude ($K_D = 3.0\ \mu M$ for $pY^{30}pY^{52}$ versus $K_D = 40\ \mu M$ for the nonphosphorylated Abl SH3 domain). By contrast, simultaneous phosphorylation at Y^7 and Y^{52} completely abolished the interaction with all the tested ligands (Table 2). These two tyrosine residues are located in proximity at the binding interface (Figure 2 A), and simultaneous phosphorylation may interfere with protein–ligand recognition.[24,25] In the case of the interaction with the

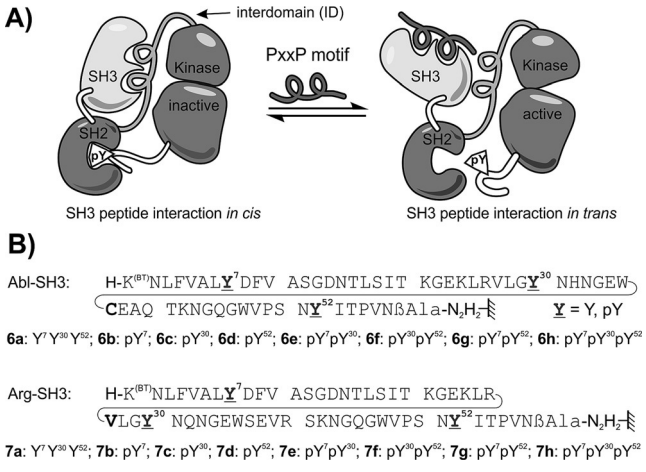


Figure 1. A) Activation of kinases of the Src family by interdomain displacement according to Moarefi et al.[20a] B) Sequences of synthesized Abl and Arg SH3 domain as well as different phosphoforms, with the corresponding numbering.

Table 2: Binding affinity (assessed as K_D in μM)[a] of phosphorylated (p) and nonphosphorylated (–) human SH3 domains of Abl and Arg for proline-rich sequences 3BP1 (FAM-ßAlaßAlaAPTMPPPLPP), 3BP2 (FAM-ßAlaßAlaPPAYPPPPVP), p41 (FAM-ßAlaßAlaAPTYSPPPPP), and the Abl interdomain peptide ID (FAM-ßAlaßAlaPAPKRNKPTVYGVSPNY).

SH3 domain[f]			SH3 ligands			
Y^7	Y^{30}	Y^{52}	3BP1	3BP2	p41	ID
Abl kinase						
–	–	–	40 ± 20 (34),[b] (24 ± 2)[e]	4.4 ± 0.8 (5)[b]	1.6 ± 0.8 (1.5)[c]	6 ± 1.5, (7 ± 4),[d] (12 ± 2)[e]
p	–	–	7 ± 3 (7 ± 4)[d], (10 ± 1)[e]	5 ± 2 (5.7 ± 0.6)[d]	12 ± 5 (16 ± 4)[d]	nad nad[d,e]
–	p	–	15 ± 11	13 ± 6	2.4 ± 0.8	nad
–	–	p	7 ± 2 (6 ± 3)[d]	4 ± 3 (3 ± 1)[d]	0.7 ± 0.2 (0.6 ± 0.3)[d]	nad nad[d]
p	p	–	13 ± 6	2.4 ± 0.5	1.5 ± 0.8	nad
–	p	p	3.0 ± 0.7 (2 ± 1)[d]	10 ± 3 (10 ± 6)[d]	0.6 ± 0.2 (0.7 ± 0.2)[d]	nad nad[d]
p	–	p	nad, nad[d]	nad, nad[d]	nad, nad[d]	nad, nad[d]
p	p	p	nad	nad	nad	nad
Arg kinase						
–	–	–	19 ± 5	4.1 ± 0.4	1.4 ± 0.5	nm
p	–	–	10 ± 4	4 ± 2	1.6 ± 0.9	nm
–	p	–	6 ± 2	7 ± 4	5 ± 2	nm
–	–	p	5 ± 2	2.4 ± 0.9	0.8 ± 0.5	nm
p	p	–	19 ± 10	4.4 ± 0.7	1.8 ± 0.9	nm
–	p	p	15 ± 3	6 ± 1	2.6 ± 0.7	nm
p	–	p	nad	nad	nad	nm
p	p	p	nad	nad	nad	nm

[a] Determined by fluorescence-detected saturation binding analysis on surface (see Figures S49–S55), errors are standard deviation. [b] Determined by Viguera et al.[23] [c] Determined by Pisabarro et al.[24] [d] Determined by fluorescence polarization in solution (Figures S22–S24). [e] Determined by Trp emission in solution (Figures S25–S28). [f] The numbering of the tyrosine residues reflects their position in the SH3 domain rather than in the protein. nad = no affinity detectable, nm = not measured.

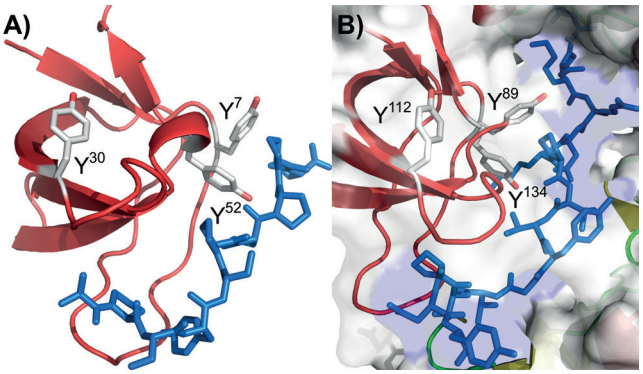


Figure 2. A) X-ray crystal structure (PDB: 1BBZ)[24] of the Abl SH3 domain with p41 (blue) bound to the RT loop. Tyrosines which were incorporated as phosphotyrosins are shown in stick representation. B) X-ray crystal structure (PDB: 2FO0)[25] of c-Abl tyrosine kinase. The SH3 domain (red) interacts with the SH2/kinase interdomain (blue) in downregulated Abl. The side chains of the corresponding tyrosine are shown (Y^{89} corresponds to Y^{7} in SH3 numbering, Y^{112} to Y^{30}, and Y^{134} to Y^{52}).

3BP2 ligand, notable effects were observed upon double phosphorylation. The involvement of the Y^{7} and Y^{30} residues led to enhanced binding, while 3BP2 recognition was attenuated upon phosphorylation of Y^{30} and Y^{52}. This is noteworthy. In the nonphosphorylated state, Abl SH3 will bind 3BP2 with higher affinity than 3BP1. Simultaneous phosphorylation at Y^{30} and Y^{52} will switch the preference to 3BP1, while combined phosphorylation of Y^{7} and Y^{52} switches the domain off. The detrimental effect of the latter phosphorylation mode was also observed for the designed peptide p41. Similar to the case of 3BP1, the p41 ligand was bound very tightly by the $pY^{30}pY^{52}$ protein, with a remarkably high affinity in the submicromolar range.

The analysis was repeated with the Arg SH3 domain. Again, simultaneous phosphorylation of Y^{7} and Y^{52} prevented interaction with any of the three examined peptide ligands. The Arg SH3 domain tolerated other phosphorylation modes and showed a fourfold increase in affinity for 3BP1 upon phosphorylation of Y^{52}.

The increase in affinity for 3BP1 and p41 observed for some phosphorylated Abl SH3 domains seemed to contradict previous results on the loss of SH3 function upon phosphorylation.[8,25] In these reports, phosphorylation at Y^{89} (corresponding to Y^{7} in SH3 numbering) was reported to induce a phenotype reminiscent of truncated Abl, in which the SH3 domain was lacking or malfunctioning.[4,5a] It was reasoned that the phosphorylated SH3 domain was no longer able to recognize the proline-rich interdomain between the SH2 and kinase domain (Figure 1 A). To test this hypothesis, we analyzed the binding of the interdomain peptide ID. In the crystal structure of c-Abl, this peptide occupies the SH3 binding cleft (Figure 2 B).[25] The nonphosphorylated Abl SH3 domain proved capable of recognizing the interdomain peptide ID *in trans* with a $K_D = 6\ \mu M$ (Table 2).

Remarkably, the phospho-SH3 domains had no affinity for ID. This, on the one hand, confirms the previous notion that phosphorylation interferes with the intramolecular recognition of the interdomain. On the other hand, our data show that phosphorylation does not generally hamper the interactions with proline-rich segments. Phospho-Abl SH3 remains functional and, in fact, changes the preference to other proline-rich protein segments *in trans* (such as in 3BP1).

Previously reported measurements of hydrogen–deuterium exchange by mass spectrometry suggested that phosphorylation at Y^{7} would disrupt interactions with Abl SH3 ligands.[8b] In contrast, our results revealed a gain of affinity for 3BP1, 3BP2 and p41 upon Y^{7} phosphorylation. To verify our findings by a solution phase assay, we synthesized five different Abl SH3 (phospho)forms in solution (Figure S15–S19). Synthesis on a preparative scale proved cumbersome because of the poor solubility of the peptide thioesters. Several rounds of optimization were required to provide sufficient amounts of SH3 proteins for functional analysis in solution. Such problems are not encountered when the synthesis and evaluation are performed as described in Scheme 1 because much lower amounts and concentrations are required for surface methods. The purified phosphoproteins were submitted to the FP assay in solution using fluorescence labeled ligands 3BP1, 3BP2, p41, and ID (Figure S22–S24). The dissociation constants conformed with the data obtained by the surface analysis (Table 2).

As a further control, we assessed the binding based on the emission of the tryptophan residues within the SH3 domains. This allowed the use of nonlabeled peptides. Again, Y^{7} phosphorylation was found to disrupt the interaction with ID′, while the affinity for 3BP1′ was improved (Table 2, Figure S25–28). ^{1}H NMR shift measurements further corroborated these findings and confirmed that both SH3 forms remained folded (Figure S29–S33).

Given the data from four independent assays it is tempting to speculate that phosphorylation of the Abl SH3 domain may serve two purposes: a) facilitating the opening/activation of the Abl kinase by inducing a loss of affinity for the internal ID peptide segment and b) fostering the recruitment of adaptor proteins by increasing the affinity for other proline-rich segments. Phosphorylation of Abl SH3 at Y^{7} (Y^{89} in full-length Abl) and Y^{52} (Y^{134} in Abl) by Hck and other Src family kinases has been identified and characterized.[8a,26] Our binding data suggest that these and potentially also other phosphorylation events could act as a switch that not only assists the activation of Abl kinase (as previously reported) but also facilitates the recruitment of adaptor proteins. Although the number of reports in which the phosphorylation of Y^{30} has been assigned by proteomic discovery-mode mass spectrometry is high (196 entries compared to 12 entries for Y^{7} and 221 entries for Y^{52}), there is, with the exception of our study, no report in which this phosphorylation event has been addressed by other means.[6]

In summary, we have established a method that provides rapid and robust access to arrays of phosphorylated protein domains. Hydrazone ligation enabled the immobilization of cysteinyl peptides, which were subsequently used in combined NCL/reduction reactions. The surface-linked peptide hydrazide linkage proved inert to the conditions used in metal-free desulfurization reactions. Both the peptide thioesters and the cysteinyl peptides were used as crude materials, which makes the approach convenient and fast. We demonstrated ligations

at cysteine, alanine (Cys→Ala), and valine (Pen→Val), and we expect a general applicability to any of the thiolated building blocks used to date in extended NCL.[12,27] One limitation we foresee is to assure protein folding on the surface, although this was not a problem for the tested SH3 domains.

The binding analysis of the different phosphoforms suggests that tyrosine phosphorylation can tune the recognition repertoire of SH3 domains. We showed that the phosphorylation of Abl SH3 (at Y^{30} and Y^{52}) enhances the affinity for specific proline-rich SH3 ligands by one order of magnitude. In contrast, simultaneous phosphorylation at Y^7 and Y^{52} or perphosphorylation switches the SH3 domain off. Given the important role and the high level of phosphorylation of the Abl kinase in chronic myelogenous leukemia and other cancer types,[4,5] it seems plausible to assume that phosphoregulation of the SH3 domain contributes to the rewiring of protein interaction networks during the development of cancer.

Acknowledgements

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Keywords: Abl · desulfurization · immobilization · native chemical ligation · protein phosphorylation

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[6] http://www.phosphosite.org shows evidence (high number of references) for phosphorylation of Abl SH3 at pY^7 (14 reports), pY^{30} (196 reports), pY^{52} (223 reports), and of Arg SH3 at pY^7 (10 reports), pY^{30} (1 report), pY^{52} (220 reports) in leukemia and other cancer cells (18.02.2016). The positions Y^7, Y^{30}, and Y^{52} (SH3 numbering) correspond to Y^{70}, Y^{93}, and Y^{115} in Arg and Y^{116}, Y^{139}, and Y^{161} in Abl.

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