Enhanced Affinities and Specificities of Consolidated Ligands for the Src Homology (SH) 3 and SH2 Domains of Abelson Protein-tyrosine Kinase*

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The possible interrelationships between multiple domains of proteins involved in intracellular signal transduction are complex and not easily investigated. We have synthesized a series of bivalent consolidated ligands, which interact simultaneously with the SH2 and SH3 domain of Abelson kinase in a SH(32) dual domain construct, a portion of native Abelson kinase. Affinities were measured by quenching of intrinsic tryptophan fluorescence. Consolidated ligands have enhanced affinity and specificity compared to monovalent equivalents. Affinity is also dependent on the length of the linker joining the two parts, with an optimum distance similar to that expected from structural models of Abl SH(32). These results suggest that consolidated ligands may be generally useful reagents for probing structural and functional activities of multidomain proteins.

Src homology (SH) 3 domains are building blocks in many proteins involved in intracellular signal transduction. Detailed understanding of the pathways involving these domains is complicated by the substantial range of individual specificities in SH2 and SH3 domains, and their combination into large proteins that can form multiple homo- and heteromolecular associations. The reductionist approach of studying individual domains has been very successful (1–3). Nonetheless, the interrelationships between multiple domains has been very successful (1–3). Nonetheless, the interactions between the domains are still poorly understood. These interactions are likely to be of significance in explaining more fully the complete activities of the signal-transducing complexes. A detailed structural picture of the inter- and intramolecular organization of the domains is then a significant objective. Several cases of multiple SH2 and SH3 domain-containing constructs have been studied structurally (Lck SH(32) (Ref. 4), Grb2 SH(323) (Ref. 5), and Abl SH(32) (Ref. 6)), but there are technical limitations to current structural approaches. In the crystalline state, packing forces may be of the same magnitude as the weak interdomain forces, so there are limits to the interpretation of diffraction studies. Solution studies by NMR are only applicable in a molecular mass range less than ~30 kDa. For NMR, time-averaged NOE constraints in rapidly exchanging conformations are not readily interpretable. As a complement to direct structural methods, we propose the investigation of such multidomain complexes using “consolidated” ligands. These ligands, having multiple binding portions, may be expected to bind with high affinity and specificity when a linker between the two affinity segments is of the correct length, and there is little affinity of the linker itself. The consolidated ligands do not necessarily resemble any natural ligand. Such ligands are demonstrated here, binding to the SH(32) of Abelson kinase. It is reasonable to assume that consolidated ligands for dual SH2, dual SH3, or multiple combinations with other ligands can be produced using a similar approach.

The proposed consolidated ligands are similar in concept to affinity reagents, with the modification that the second functionality is a binding element rather than a reactive moiety. Consolidated ligands may also be useful reagents for studies of the cell biology of the signal transduction complexes (for example, in additional combination with antibodies or reporter groups like fluorescent tags), and may provide leads into possible classes of diagnostic and therapeutic agents in the many areas of pathology in which SH domains are involved. This approach will be useful where micromolar affinities of ligands to single SH domains provide insufficient affinity, and hence specificity, for pharmacological action (e.g. Refs. 7 and 8).

The protein target for this study is the regulatory apparatus, SH(32) (4), of human Abelson kinase (AbI). AbI was originally isolated as the gene product from abl of murine leukemia virus; the human abl gene has been isolated and shown, in cases of chronic myelogenous leukemia, to be a causative factor in a fusion following chromosomal translocation (reviewed in Ref. 9). Ligands of moderate affinity have been identified for the isolated SH2 (10) and SH3 domains (11, 12). For AbI SH3, a crystal structure of the complex of the ligand 3BP-1 is available (13), indicating that the ligand is in the so-called class I orientation (reviewed in Ref. 2). Using the general positioning of ligands to SH2 (1) as a framework, the likely orientation of the 2BP-1 ligand, PVY*ENVamide (10), was modeled. In an SH(32) model (Fig. 1), these ligands are closest at their C termini, and there is more than 20 Å separating them; about 32 Å separate the C terminus of the SH3 ligand from the N terminus of the SH2 ligand. An initial design of a consolidated ligand for both domains uses this model.

Consolidated ligand 1 (Table 1) then joins the C termini of the individual 2BP and 3BP ligands using a branch through the side chain of a lysine residue. By model building, the methylene segments of the lysyl side chain and the oligoglycyl linker are expected to provide sufficient separation between the C termini of the individual ligands. A series of consolidated ligands, with
different ligand segments for SH3, different lengths and orientations of the linker, and different analogs of the phosphotyrosyl residue in the ligand for SH2, were synthesized and tested for affinity to Abl SH(32), and in some cases to SH3 and SH2.

This design and synthesis of consolidated ligands is shown to be effective in Table I. Increases in affinity of approximately 2 orders of magnitude were observed compared to unbranched equivalents (I versus II, IV versus V). An order of magnitude increase was observed comparing the most strongly bound single ligand to the equivalent consolidated ligand (2BP1 versus IV). The affinity of a consolidated ligand changed when a subligand was modified proportionally to the change of affinity of separate ligand (I/3BP-1 versus IV/3BP-2). The simple linker chemistry used did not interfere with the subligands affinities to the individual SH3 or SH2 domains. Experiments with the single ligands as inhibitors show classical competitive binding. The values of affinities of ligands with variable linker lengths (II-G6, IV-G6, VI-G6, VII-G6) are most readily interpreted as indicating an optimal length of about G6, with little interaction between the linker and the SH(32) protein. Analogs of the 2BP1 ligand of IV, namely the H-phosphonate (VIII), and the pY of IV (IX) lead to decreases in affinity.

![Figure 1](image)

**FIG. 1.** Model of the orientations of SH2 and SH3 ligands in a consolidated ligand (I) on the Abl SH(32) protein. Coordinates of individual SH2 and SH3 domains (6, 18) were fitted to the approximate orientation of Lck SH(32) in the observed crystal structure (4). The peptide (I) was created and aligned to the expected binding positions based on modelling of SH2 ligands to Src SH2 (20) and SH3 ligands to Abl (16). The positioning of glycy1 and branched lysyl residues is arbitrary, other than the restrictions of the subligand positioning. Modelling and display used the Biosym system on an SGI Iris.

**Table I**

| LIGAND   | STRUCTURE   | AFFINITY nM SH(32) | AFFINITY nM SH2 | AFFINITY nM SH3 |
|----------|-------------|--------------------|-----------------|-----------------|
| 2BP-1    | PVY*ENVam   | 2,350(340)         | 2,020(230)      |                 |
| 3BP-1    | PPTMPPPPPLP | 56,000(900)        |                 |                 |
| 3BP-2    | PPAYPAAAAVP | 10,700(300)        | 10,500(200)     |                 |
| I        | (2BP-1)_G6  | 487(16)            |                 |                 |
| II       | (2BP-1)_G6  | 18,000(1,200)      |                 |                 |
| III      | (3BP-2)_Kam | 503(7)             |                 |                 |
| IV       | (3BP-2)_Kam | 249(5)             | 14,200(900)     | 5,370(340)      |
| V        | (2BP-1)_Kam | 22,100(1,700)      |                 |                 |
| VI       | (3BP-2)_Kam | 327(6)             |                 |                 |
| VII      | (3BP-2)_Kam | 389(23)            |                 |                 |
| VIII     | H-phosphonate analog of IV | 1,810(80) |                 |                 |
| IX       | desphospho-analog of IV | 13,800(920) | 6,050(82)       |
model of how the consolidated ligand binds is that there are specific contacts at the subligand sites, and a solvated linker region. Using a model derived from NMR studies of the SH3 and SH2 domains (6), assuming that the orientation for Abl is similar to that seen in Lck (4), the two subligands are correctly accommodated, and no specific interactions are forced on the linker segment (Fig. 1). The specificity of interaction is much increased also (Table I), so that IV will now discriminate between the complete SH(32) and its subdomains by more than an order of magnitude. The decreased affinity of IV compared to 2BP1 for the single SH2 domain is not readily explained. One possibility is that IV forms a partially folded structure reducing the population of ligand in a conformation suitable for binding to SH2.

The affinities in Table I might be expected, from simple physical chemical concepts, to be somewhat higher for the consolidated ligands. The thermodynamic treatment of the consolidation of fragment affinities has been addressed by J écques (24). Briefly, for affinities to SH2, SH3, and SH32, the free energy contributions may be related by $\Delta G^\ominus_{2BP1} = \Delta G^\ominus_2 + \Delta G^\ominus_3 + \Delta G^\ominus$ where the subscripts 2, 3, and 3 reflect the SH domain interaction, and the superscript i refers to the individual binding contributions, and $\Delta G^\ominus$ represents the change in probability of binding that results from the connection of the two ligands.

It may be assumed that the principal method by which apparent affinity increases for the ligands of this work is the reduction in degrees of translational freedom, when one subligand is bound (24). For perfect linker length and geometry, a consolidated ligand affinity up to the product of the individual association constants might be expected (i.e. $\Delta G^\ominus_{2BP1} = \Delta G^\ominus_2 + \Delta G^\ominus_3$). Increases in degrees of rotational freedom associated with each glycol residue, and with the lysyl methylenes reduce this affinity considerably, that is $\Delta G^\ominus$ represents a large, energetically unfavorable, increase in entropic contribution, and the overall free energy of interaction of the consolidated ligand is enhanced, but not dramatically, over that of its components.

There are many possible applications of these consolidated ligands and their analogs. The examples in Table I show that for Abl SH(32), the two ligand sites can be oriented as shown in Fig. 1, that the 3BP-1 and 3BP-2 ligands for SH3 (in I and IV) are bound in the same direction, and that the SH2 and SH3 binding sites do not interfere with each other. Applications are expected to other SH(32)-containing proteins for the purpose of similar mapping, or of obtaining high affinity and specificity of binding for investigational or therapeutic purposes (14). Some examples are dual and higher subligand consolidations for the adaptor protein Grb-2, and reagents like those shown here for Abl, although possibly linear rather than branched (15), for Src family tyrosyl kinases (16). A major challenge in the investigation of intracellular signal transduction involves the relatively transient nature of the signal transducing complexes formed. Highly specific reagents may permit trapping of, or selective interference with, these complexes.

The subligands in such consolidated ligands may be extended to other components of signal transducing complexes, for example the active sites of kinases, or binding sites of pleckstrin homology domains. For Abl, the consolidated ligands shown here are possible early leads to more complex ligands, which may have sufficient affinity and specificity to block the Bcr-Abl fusion kinase that is the predominant pathogenic agent in chronic mylogenous leukemia. The chemistry used in these consolidated ligands is relatively simple and was selected to provide flexible linkers, as has been done for the hirulogs (17). Linkers of the correct size and rigidity should provide greater affinities. The structure of the Abl SH(32)/consolidated ligand complex may permit rational design of linkers of an optimal rigidity and size, and also permit identification of additional interactions for increased affinity. Peptidomimetic and non-peptidic linkers are obviously practical, as are other subligands including lipids, steroids, carbohydrates, and nucleic acids. These consolidated ligands present novel opportunities for chemistry at the interface with biology.

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FIG. 2. Representative fluorescence titration data, using the methods described in the legend to Table I. Titration are for the branched consolidated ligand, IV, with the three constructs: SH(32), red; SH3, green; SH2, blue. Ligands were made as follows. Solid-phase syntheses of branched sequences as well as linear controls were carried out either manually or on a continuous-flow Millipore 9050 instrument, starting with Fmoc-PAL-PEG-PS resins (0.6 g scale, loading 0.16 mmol/g (normal) or 0.49 mmol/g (high-load)). Appropriate N-Fmoc-amino acids (4.0 eq) were used with Fmoc removal by piperidine-DMF (1:4, 2 × 8 min), and standard BOP/HOBt/NMM activation chemistry described elsewhere (Ref. 21, and references therein). Side chain protection was provided by a tert-butyl (tBu) ether and ester for Thr and Glu, respectively, and the N-terminal Pro residues (both branches) were protected by Boc. Syntheses of branched ligands began with incorporation onto PAL of the C-terminal lysine as its N-Fmoc, N-Dde deriva-

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