Anti-peptide Antibodies Detect Conformational Changes of the Inter-SH2 Domain of ZAP-70 Due to Binding to the ζ Chain and to Intramolecular Interactions

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**T cell receptor (TCR) triggering induces association of the protein tyrosine kinase ZAP-70, via its two src-homology 2 (SH2) domains, to di-phosphorylated Immunoreceptor Tyrosine-based Activation Motifs (2pY-ITAMs) present in the intracellular tail of the TCR-ζ chain. The crystal structure of the SH2 domains complexed with a 2pY-ITAM peptide suggests that the 60-amino acid-long inter-SH2 spacer helps the SH2 domains to interact with each other to create the binding site for the 2pY-ITAM. To investigate whether the inter-SH2 spacer has additional roles in the whole ZAP-70, we raised antibodies against two peptides of this region and probed ZAP-70 structure under various conditions. We show that the reactivity of antibodies directed at both sequences was dramatically augmented toward the tandem SH2 domains alone compared with that of the entire ZAP-70. This indicates that the conformation of the inter-SH2 spacer is not maintained autonomously but is controlled by sequences C-terminal to the SH2 domains, namely, the linker region and/or the kinase domain. Moreover, antibody binding to the same two determinants was also inhibited when ZAP-70 or the SH2 domains bound to the ζ chain or to a 2pY-ITAM. Together, these two observations suggest a model in which intramolecular contacts keep ZAP-70 in a closed configuration with the two SH2 domains near to each other.

ZAP-70 is a protein tyrosine kinase (PTK) essential for the initiation of the signaling cascade activated by T cell antigen receptor triggering (1). Overall, ZAP-70 displays two structurally and functionally distinct moieties, an N-terminal one composed of two SH2 domains and a C-terminal kinase domain tethered by an ~80-amino acid-long linker (2) (also referred to as Interdomain B, (IB); Ref. 3).

So far, the only known function of the region comprising the two SH2 domains (hereafter indicated as (SH2)_2) is to provide a means to recruit ZAP-70 to the plasma membrane by those TCRs engaged with the ligand (4, 5). This is achieved through the coordinated anchorage of the SH2 domains to di-phosphorylated tyrosine-containing motifs (D/E)XXY(L/I)XXY (I/L) called ITAMs (for Immunoreceptor Tyrosine-based Activation Motifs) present within the cytoplasmic tails of TCR subunits ζ and ε (6–8). Thereafter, ZAP-70 undergoes tyrosine phosphorylation culminating in the up-regulation of its catalytic activity which in turn is required for phosphorylating cellular substrates (9–12).

The x-ray crystal structure of the (SH2)_2 of human ZAP-70 complexed with a di-phosphorylated ITAM (2pY-ITAM) peptide (3) has revealed an unsuspected structural complementarity and immediate contiguity of the two SH2 domains needed to create a high affinity binding site for the 2pY-ITAM. Thus, while the C-terminal SH2 possesses a binding pocket for the first pY of the ITAM, the corresponding pocket for the second pY in the N-terminal SH2 is contributed, in part, by residues of the C-terminal SH2. Moreover, the 60 amino acids forming the inter-SH2 spacer (hereafter referred to as Interdomain A (IA)) bulges out the SH2 domains and for the most part is structured as a coiled-coil of two antiparallel α-helices which assists in the formation of an interface between the two SH2 domains. It has been speculated that the IA may mediate additional intra- or inter-molecular interactions required for regulating ZAP-70 (3). This idea stems from several considerations. First, coiled-coils are often found to intervene in protein-protein interactions (13, 14). Second, there is at least one highly suggestive example involving the spacer connecting the two SH2 domains of the p85 subunit of the phosphatidylinositol 3’-kinase (PI 3-kinase). This region, predicted to be a coiled-coil, mediates the interaction with the catalytic p110 subunit (15, 16), and occupancy of the two SH2 domains influences the enzymatic activity (17, 18). Moreover, binding of singly or doubly phosphorylated peptides to the tandem SH2-containing SH-PTP-2 activates the phosphatase activity (19, 20). Finally, it has been reported that the catalytic activity of p72^{tyk2}, a PTK homologue of ZAP-70, may be increased by binding to a 2pY-ITAM (21, 22). The latter examples are suggestive of allosteric regulation mediated by the SH2-containing region of the protein.

To explore what could be the structural role of the IA in the entire ZAP-70, anti-peptide antibodies directed at this region were generated. The use of these antibodies revealed that binding of ZAP-70 or its isolated (SH2)_2 to a 2pY-ITAM or the TCR-ζ chain influences the conformation of the IA. Additional experiments also indicated that conformational constraints are imposed on the IA by the regions of the protein downstream of the (SH2)_2. These two observations, in combination, suggest a structural model for the entire ZAP-70.
EXPERIMENTAL PROCEDURES

Antibodies, Cell Lines, and Vectors—Anti-human ZAP-70 4.06 and 2.06 polyclonal antisera were produced by immunizing rabbits (a total of four, two for each peptide) with the synthetic peptides described in Table I coupled via their C- and N-terminal cysteines (cysteine 117 of ZAP-70, respectively, to maleimide-activated keyhole lympet hemocyan-in as recommended (Pierce). A similar procedure was used to generate a murine anti-ζ (Dana Farber Cancer Institute, Boston, MA) antibody which is directed at the first 11 amino acid residues of the human ζ chain. The anti-kinase domain polyclonal antisera 21.11 has been previously described (23). The ZAP-4 antibody (24) was kindly provided by S. Ley (National Institute of Medical Research, Mill Hill, London, UK). The anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology, Inc., (Lake Placid, NY). The anti-human TCR Vβ (101.5.2) mAb was provided by E. L. Reinherz (Dana Farber Cancer Institute, Boston, MA). Anti-VSV-G epitope antisemur (kindly provided by M. Arpin, Institut Curie, Paris, France) reacts against an 11-amino acid determinant derived from a vesicular stomatitis virus glycoprotein (VSV-G) (25). WT15.8, a Jurkat cell line expressing a ZAP-70 containing a VSV-Tag at the C terminus (22) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 1-glutamine, penicillin, and streptomycin (Life Technologies Inc., Rockville, MD). The generation of the cDNA construct that encodes the amino acids 1–276 of ZAP-70, comprising the SH2(N+C) plus 22 residues of the IB, and the expression vector used (pB3J1) have been previously described (26). The prokaryotic vector expressing the (SH2)2 of ZAP-70 as a glutathione S-transferase fusion protein (a gift from L. Samelson, National Institutes of Health, Bethesda, MD) and the purification procedure of the protein have been reported previously (27). The peptide was purified from the glutathioneS-transferase with factor Xa on a glutathione-Sepharose column during the purification as recommended (Amersham Pharmacia Biotech). Gel filtration analysis revealed the absence of aggregated protein.

Synthetic Peptides—Peptides corresponding to human ZAP-70 sequences 106–117 and 117–130 to amino acids 1 through 11 of the human TCR ζ chain (an additional cysteine was added to the C terminus of the coding sequence for coupling to the high performance liquid chromatography and in part biotinylated at the N terminus (final sequence SGSGNQLYNELNLGRREEYDVLD) were synthesized as mono- (on Tyr62) and di-phosphorylated (on Tyr 51 and Tyr62) forms (by F. Baleaux, Dept. of Organic Chemistry, Institut Curie, Paris, France). Peptides corresponding to the first ITAM of the human TCR ζ chain (=1, residues 48–66 plus a 4-amino acid linker at the N terminus (final sequence SGSGNQLYNELNLGRREEYDVLD) were synthesized as mono- (on Tyr62) and di-phosphorylated (on Tyr51 and Tyr62) forms (by F. Baleaux, Dept. of Organic Chemistry, Institut Pasteur, Paris). Peptides were purified by reverse-phase high performance liquid chromatography and in part biotinylated at the N terminus by Biotin sulfo-NHS (Pierce). The purity and the molecular weight of the peptides were confirmed by ion electro-spray ionization mass spectrometry.

Activation and Immunoprecipitation—For activation, cells were stimulated with anti-TCR mAb 101.5.2 at 1:200 dilution of ascites for 2 min at 37 °C. Unstimulated or TCR-activated cells were solubilized on ice for 10 min by addition of 1 ml of lysis buffer containing 1% Nonidet P-40, 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, and 1 mM NaVO4. Precleared postnuclear lysates were incubated with or without the synthetic ITAM peptides at the indicated concentration for 90 min at 4 °C. After incubation, lysates were sub-


teminated by anti-IA antisera and ITAM peptides. These results suggested that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding. These results indicated that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is spontaneously or as a consequence of anti-IA Ab binding.

RESULTS

Antibodies Directed at the IA of ZAP-70 Do Not Recognize the Protein Bound to the ζ Chain—Two antisera (named 4.06 and 2.06) were raised against synthetic peptides corresponding to the amino acid sequences 106–117 and 117–130 of ZAP-70, respectively (Table I). The peptide segment 106–117 begins shortly after the end of the N-terminal SH2 domain, and, together, the two peptides cover ~40% of the IA (2, 3). To investigate whether the two antisera recognized native ZAP-70 and to compare their reactivity with that of other anti-ZAP-70 antisera directed at different regions of the molecule, immunoprecipitation experiments were carried out from lysates of Jurkat cells unstimulated or stimulated with an anti-TCR mAb (Fig. 1). Similarly to 21.11 and ZAP-4 anti-peptide antisera, specific for sequences contained within the kinase domain and the IB, respectively (Table I), 4.06 and 2.06 immunoprecipitated tyrosine-phosphorylated ZAP-70 (Fig. 1, pY-ZAP-70 in lanes 1–8). In this as well as in other experiments, 2.06 was found to be weaker than 4.06 (see also below). However, in striking contrast with the antisera 21.11 and ZAP-4, in TCR-activated Jurkat cells, 4.06 and 2.06 did not show co-immunoprecipitation of phosphorylated ζ chain (cf. pY-ζ in lanes 4 and 6) which, as expected, in activated Jurkat cells co-immunoprecipitated with ZAP-70. The lack of detection of the ζ chain with both anti-IA antisera was not due to a lower capacity to immunoprecipitate ZAP-70. Indeed, with the anti-IA antisera, ζ was not visible even when the signal of ZAP-70 was similar to that obtained with 21.11 and ZAP-4 (cf. lane 2 with lane 6 or lane 4 with lane 5). In addition, the ζ chain remained undetectable after longer exposure times (not shown). Phosphorylated ZAP-70, not associated to the ζ chain but observable with anti-IA antisera is likely to represent a fraction of the molecules which detached from ζ spontaneously or as a consequence of anti-IA Ab binding. These results suggested that the epitopes recognized by the anti-IA antisera are masked or structurally modified when ZAP-70 is complexed with the ζ chain.

Binding of 2pY-ITAM to ZAP-70 or to the Isolated (SH2)2 Domains Inhibits Recognition by Anti-IA Antibodies—The lack of recognition of ZAP-70-ζ complexes by anti-IA Abs could be due to an alteration of the corresponding epitopes consequent to the binding of the SH2 domains to the ITAMs. To directly test this hypothesis, we assessed whether a synthetic peptide corresponding to a 2pY-ITAM inhibited recognition of ZAP-70 by anti-IA Ab. For these experiments, we used a Jurkat cell line named WT15.8, stably expressing ZAP-70 tagged at its C terminus with a VSV sequence (12). The use of tagged ZAP-70 was preferred since the anti-tag antisera was found to be the strongest, thus allowing sensitive detection of ZAP-70. Cell lysates from unstimulated WT15.8 were incubated with increasing concentrations of mono- (pY) or di- (2pY) phosphoryl-

| Name | Immunizing peptide | Protein region |
|------|-------------------|---------------|
| 4.06 | 106–117 | Interdomain A |
| 2.06 | 117–130 | Interdomain A |
| ZAP-4 | 271–290 | Interdomain B |
| 21.11 | 483–499 | Kinase domain |

a The limits of the human ZAP-70 sequence are according to Ref. 2.

b Numbering of the human ZAP-70 sequence is according to Ref. 2.

cf Numbering of the human ZAP-70 sequence is according to Ref. 2.
ated ITAM peptides containing a biotin molecule at the N terminus and then reacted with streptavidin-agarose or with the anti-ZAP-70 antisera. In agreement with previous reports (5), only the 2pY-ITAM was able to bind ZAP-70, as shown by precipitation with streptavidin-agarose (Fig. 2, lanes 12 and 13). Moreover, the 2pY-ITAM, but not the pY-ITAM, inhibited the immunoprecipitation of ZAP-70 with the 4.07 antibody in a dose-dependent manner (Fig. 2, cf. lanes 4, 5 and 6 with lanes 1, 2, and 3). Quantitation of the ZAP-70 band allowed calculation to an ∼90% inhibition of ZAP-70 immunoprecipitation in the presence of 10 µM of 2pY-ITAM. Similar levels of inhibition were obtained when untagged ZAP-70 was immunoprecipitated from Jurkat cells or when non-biotinylated 2pY-ITAM was used. Moreover, similar results were reproduced with 2.06 antisera (data not shown). This effect was restricted to the anti-IA antibodies since no inhibition was detected with the anti-kinase domain antisera (lanes 10 and 11). From these results, we conclude that the binding of the 2pY-ITAM to ZAP-70 is the event that determines the loss of immunoreactivity.

One possible explanation for the above results is that the ITAM itself may sterically hinder the epitopes and prevent antibody binding. However, the three-dimensional structure of the (SH2)$_2$2pY-ITAM complex (3) shows that both amino acid segments lie on the opposite side of the (SH2)$_2$2pY-ITAM interface (see also “Discussion”). Moreover, it is difficult to imagine how the two sequences which are part of an extended structure may both be sterically hindered by such a short peptide. Thus, it is extremely unlikely that this mechanism can account for the observed changes in anti-IA Ab reactivity. Another possibility could be that upon ITAM binding, either the kinase domain or the IB or both interact with the IA and induce a conformational change or a masking effect resulting in loss of anti-IA Ab immunoreactivity. Alternatively, this effect may be simply due to a conformational change of the IA induced by 2pY-ITAM binding independently of the rest of the molecule. To discriminate between these two possibilities, experiments similar to those presented in Figs. 1 and 2 were performed using the (SH2)$_2$ moiety of ZAP-70. To this end, Jurkat cells were transiently transfected with a vector expressing the tandem SH2 domains of ZAP-70 (26) and subjected to immunoprecipitation with the 4.06 antisera after TCR-mediated activation or after addition of the 2pY-peptide. Fig. 3A (top panel) shows that, in cells transfected with (SH2)$_2$ activated with anti-TCR mAb, the TCR-ζ chain (with associated ZAP-70) was detected as a series of strongly phosphorylated bands when immunoprecipitated with the anti-ζ antiserum but remained undetected when using the 4.06 antisera. However, the (SH2)$_2$ was efficiently immunoprecipitated from the same cells with the 4.06, as shown by stripping and reprobing the blot with this antiserum (Fig. 3A, bottom panel, lanes 1 and 2). Moreover, as previously demonstrated (26), the (SH2)$_2$ molecule could be detected in part associated with the TCR-ζ chain. Indeed, in the anti-ζ immunoprecipitation, a band corresponding to a small fraction of the transfected (SH2)$_2$ was visible and increased in intensity after activation (Fig. 3A, bottom panel, lanes 3 and 4). Of note is that in these experiments no tyrosine phosphorylation of the (SH2)$_2$ was detected (the corresponding band migrates as an ∼33-kDa molecular species) even after TCR stimulation.

Thus, like intact ZAP-70, the (SH2)$_2$ζ complex also could not be detected by antibodies directed at the 106–117 IA sequence. This was also verified in an in vitro assay since the 2pY-ITAM, but not the pY-ITAM, could almost completely inhibit the immunoprecipitation of the transfected (SH2)$_2$ with the 4.06 antibody (Fig. 3B). Similar results were obtained when the (SH2)$_2$ was immunoprecipitated with the 2.06 antisera after incubation with the 2pY-ITAM peptide (data not shown). These experiments indicated that the IB and/or the kinase domain were not implicated in the ITAM-induced inhibition of anti-IA antibodies recognition. However, one could not rule out the possibility that in Jurkat cells a putative protein associated with the IA region upon ITAM binding and produced the inhibitory effect by steric hindrance. Fig. 3C shows that this explanation...
is unlikely since addition of 2pY-ITAM, but not pY-ITAM (lanes 3 and 2, respectively), to purified bacterially expressed (SH2)2 of ZAP-70, was sufficient to produce a marked reduction of recognition by 4.06 antibodies. Note that the recombinant protein was able to bind the 2pY-ITAM (lane 5). Together, these data strongly suggest that the loss of anti-IA epitope recognition is essentially due to a conformational change transmitted to the IA region by binding of the ZAP-70 (SH2)2 to a 2pY-ITAM.

The Epitopes Recognized by the Anti-IA Antibodies Are Conformationally Dependent on the IB and/or the Kinase Domain—During the course of the experiments involving the expression of isolated (SH2)2 of ZAP-70 in Jurkat cells, we consistently noted that this molecule was immunoprecipitated by the anti-IA Abs more efficiently than the whole ZAP-70. Experiments were therefore set up to determine the magnitude...
of this effect and to exclude possible artifacts due to overexpression of the (SH2)2. Thus, Jurkat cells were transiently transfected with different amounts of the expression plasmid containing the (SH2)2 to obtain different (SH2)2/endogenous ZAP-70 ratios. Immunoprecipitations were then carried out with the 4.06 and 2.06 antisera, and the bands corresponding to ZAP-70 and (SH2)2 were quantitated by immunoblotting using 4.06. Before the immunoprecipitation step, an aliquot of the total lysate was used to estimate the relative expression of both molecules. In the experiment shown in Fig. 4A, endogenous ZAP-70 was expressed at approximately a 10-fold excess compared with the transfected (SH2)2, (see lane 1 and, for quantitation, Experiment I in Table II). If the reactivity of the antisera against the two proteins was the same, then the (SH2)2/ZAP-70 ratio should remain constant in the immunoprecipitate. However, after immunoprecipitation with both 4.06 and 2.06 antisera, a relative increase in the signal of the transfected (SH2)2 over ZAP-70 was clearly evident (Fig. 4A, lane 2 and 3). Thus, while the amount of (SH2)2 was 10-fold lower than ZAP-70, after immunoprecipitation with 4.06, the signal obtained for the two proteins was nearly the same. This higher reactivity toward the (SH2)2 was even more dramatic for the 2.06 antiserum. This reagent immunoprecipitated ZAP-70 inefficiently compared with 4.06 (cf. lanes 2 and 3) but immunoprecipitated the (SH2)2 as efficiently as 4.06. The magnitude of these modifications in reactivity toward (SH2)2 compared with ZAP-70 can be quantitatively appreciated by confronting the (SH2)2/ZAP-70 signal ratios in the cell lysate and in the immunoprecipitates of 4.06 and 2.06. These ratios are reported in Table II for the experiment shown in Fig. 4A (Experiment I) and for two additional ones in which higher amounts of (SH2)2 compared with ZAP-70 were expressed. Independently of the initial amounts of (SH2)2 and ZAP-70 present, there is an increase of the (SH2)2/ZAP-70 ratio in the immunoprecipitates. It is clear that on average for the epitope recognized by 4.06 Abs there is a gain of reactivity of ~5-fold, whereas such a change can be estimated to be >100-fold for 2.06 (with this antiserum only, <1% of the total intact ZAP-70 is detected after a single immunoprecipitation). Fig. 4B also shows, from one of these experiments, a control of the structural intactness of the (SH2)2 versus endogenous ZAP-70. Both proteins were able to bind with similar capacity to the 2pY-ITAM peptide as demonstrated by the fact that their ratio after binding is comparable with the one seen in the total lysate (lane 1). This result excludes that the anti-IA antisera recognize a grossly altered population of (SH2)2 molecules unable to bind to 2pY-ITAM.

From these experiments, we conclude that the epitopes recognized by both anti-IA are conformationally dependent on (or hindered by?) the C-terminal moiety of the molecule, including the IB and the kinase domain. The most direct interpretation of these results is that, in the intact ZAP-70, the two halves of the molecules, the (SH2)2 and the IB/kinase domain, come into contact.

**DISCUSSION**

The major factors determining recognition of an intact protein by anti-peptide Abs are accessibility and conformational flexibility of the epitope (27, 28). Thus, differences in reactivity of site-specific anti-peptide Abs observed in proteins after ligand binding or due to changes in the milieu composition have been useful in revealing conformational changes (29, 30). In this paper, we show that the reactivity of anti-peptide Abs directed at sequences within the inter-SH2 spacer of ZAP-70 is dramatically decreased in two apparently distinct situations: the binding of the two SH2 domains to the 2pY-ITAM or the presence of the C-terminal moiety of ZAP-70 which includes the IB and the kinase domain. Antisera to two contiguous sequences of the inter-SH2 spacer reacted very strongly and equally well with the (SH2)2 alone, but moderate-to-low, depending on the epitope (106–117 > 117–130), toward the intact ZAP-70. Moreover, Ab binding to the (SH2)2 alone as well as to the entire ZAP-70 was inhibited by the interaction with a 2pY-ITAM peptide, and both proteins could not be co-immunoprecipitated with these antisera when complexed to the ζ chain.

In the crystal structure of the isolated (SH2)2 complexed with a di-phosphorylated ζ1-ITAM (the same used in our study) (3), the sequence corresponding to the peptide 106–117 begins after three residues from the end of the N-terminal SH2 domain as part of a type II reverse turn followed by a long β-strand. The contiguous 117–130 sequence, instead, assumes an α-helix configuration and represents part of the stem, composed of two antiparallel α-helices wrapped around each other, protruding at ~90° with respect to the SH2 domains axis. The positioning of the 2pY-ITAM peptide in the complex is on the opposite side and away from the two sequences, thus making highly unlikely the possibility that the peptide itself causes inhibition of Ab binding by steric hindrance. Moreover, since 2pY-ITAM-dependent inhibition was observed also with recombinant bacterially expressed (SH2)2 (Fig. 3C), we can exclude that an unknown protein present in T cells binds to the complex and hinders the sequences targeted by the Abs. In addition, lack of Abs detection of (SH2)2 associated to the ζ chain was not the consequence of a phosphorylation event (Fig. 3A) that might involve Tyr126 in the inter-SH2 spacer (10). Thus, the most likely explanation of our results is that in the isolated (SH2)2 the two determinants (and perhaps the entire IA) exist in a state of high conformational flexibility that facilitates binding of the anti-peptide antibodies (27, 28). In support of this, Hatada et al. (3) noted from isoelectrofocussing experiments that the uncomplexed (SH2)2 of ZAP-70 existed in multiple isoforms that could be converted to a single one after binding to the 2pY-ITAM. The 2pY-ITAM-dependent inhibition of antibody binding can therefore be explained by assuming that the determinants in question undergo a conformational change and/or they are partially or totally masked as a consequence of global changes coinciding with a stabilization of the entire (SH2)2 structure by the 2pY-ITAM. By inference, the same reasoning may apply to the intact ZAP-70 where loss of anti-IA Ab reactivity was also induced by binding to 2pY-ITAM or tyrosine-phosphorylated TCR-ζ. It appears, therefore, that when ZAP-70 binds to the ζ chain, the (SH2)2 region assumes a configuration that is not present in at least part of the unbound molecules (see also below). A recent work has described conformational changes of the ZAP-70 homologue p72syk when bound to a 2pY-ITAM peptide detected with anti-peptide antibodies directed at its C-terminal end (31). These data together with ours suggest that relatively important changes in the configuration of this family of kinases take place upon binding to antigen receptors.

The second information gained from our studies is that in the intact ZAP-70 the IA assumes a particular configuration imposed by the protein portion downstream of the SH2 domains. Thus, the inability of the anti-IA Abs, especially those directed at the 117–130 sequence, to recognize a large proportion of intact ZAP-70 might be due to the IB and/or the kinase domain restricting the conformational freedom of the IA or causing a steric hindrance. Independently of these two possibilities, our observation indicates that an intramolecular interaction must exist that links the (SH2)2 to the IB and/or the kinase domain and suggests that ZAP-70 exists in a closed rather than in an extended configuration.

The observation that both inter- (e.g., 2pY-ITAM binding) and intramolecular interactions of ZAP-70 lead to structural changes identified by similar effects (e.g., inhibition/loss of anti-IA Ab reactivity) is highly suggestive of comparable
configurations assumed by the (SH2)_2 in both instances. Thus, it is possible that the interaction of the (SH2)_2 (perhaps through the IA) with a distal part of the molecule helps maintain the (SH2)_2 in a relatively stable configuration which approaches the one assumed when bound to a 2pY-ITAM. Such a configuration may favor a fast kinetics of ZAP-70 binding to the 2pY-ITAM (due to an entropic gain) as the SH2 domains would already be oriented close to each other in such a way as to facilitate their targeting. This is particularly relevant in light of the fact that the constitution of a high affinity binding site for 2pY-ITAM necessitates the immediate proximity of the N-terminal SH2 and C-terminal SH2 (3). 2pY-ITAM binding kinetics experiments with intact ZAP-70 and (SH2)_2 alone should help verify this hypothesis. However, if the proposed model is correct, the closed configuration may be metastable since both anti-IA antiseria were able to recognize a population of molecules that transit through, or are constitutively in, an open configuration. Binding to the ζ chain may then impose further modification to the IA, independent of the regions downstream of the (SH2)_2.

A growing body of data suggests that inter-SH2 domain spacers may be important for contributing toward establishing the orientation of tandem SH2 domains and perhaps spacers may be important for contributing toward establishment of the (SH2)_2 in a relatively stable configuration. Thus, it is possible that the interaction of the (SH2)_2 (perhaps through, or are constitutively in, an open configuration. Binding to the ζ chain may then impose further modification to the IA, independent of the regions downstream of the (SH2)_2)

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