Activation of Zap-70 Tyrosine Kinase Due to a Structural Rearrangement Induced by Tyrosine Phosphorylation and/or ITAM Binding

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ABSTRACT: The protein tyrosine kinase ZAP-70 is implicated in the early steps of the T-cell antigen receptor (TCR) signaling. Binding of ZAP-70 to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR ζ chain through its two src-homology 2 (SH2) domains results in its activation coupled to phosphorylation on multiple tyrosine residues, mediated by Src kinases including Lck as well as by autophosphorylation. The mechanism of ZAP-70 activation following receptor binding is still not completely understood. Here we investigated the effect of intramolecular interactions and autophosphorylation by following the kinetics of recombinant ZAP-70 activation in a spectrophotometric substrate phosphorylation assay. Under these conditions, we observed a lag phase of several minutes before full ZAP-70 activation, which was not observed using a truncated form lacking the first 254 residues, suggesting that it might be due to an intramolecular interaction involving the interdomain A and SH2 region. Accordingly, the lag phase could be reproduced by testing the truncated form in the presence of recombinant SH2 domains and was abolished by the addition of diphosphorylated ITAM peptide. Preincubation with ATP or phosphorylation by Lck also abolished the lag phase and resulted in a more active enzyme. The same results were obtained using a ZAP-70 mutant lacking the interdomain B tyrosines. These findings are consistent with a mechanism in which ZAP-70 phosphorylation/autophosphorylation on tyrosine(s) other than 292, 315, and 319, as well as engagement of the SH2 domains by the phosphorylated TCR, can induce a conformational change leading to accelerated enzyme kinetics and higher catalytic efficiency.

The protein tyrosine kinase (PTK) ZAP-70 is a component of two members of the Syk family of nonreceptor protein tyrosine kinases. It is exclusively expressed in T-cells and natural killer cells where it is involved in the early steps of the T-cell antigen receptor (TCR)[1] signaling (*1*). TCR triggering by antigen/major histocompatibility complex induces tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) present in its cytoplasmic ζ and ϵ domains (*2*, *3*). This event is essential for ZAP-70 recruitment to the plasma membrane and consequent up-regulation of its kinase activity (*4*−*7*). The mechanism of ZAP-70 activation following the ITAM recruitment has yet to be clarified. It has been shown that recruitment to the activated TCR is necessary for ZAP-70 activation, and there is a strong correlation between tyrosine phosphorylation of ZAP-70 and its kinase activation (*4*, *5*, *8*−*10*). Several studies report that ZAP-70 undergoes phosphorylation by Src kinases on key tyrosines present on the catalytic domain, identifying $p56^{lck}$ as a regulatory kinase which phosphorylates Tyr 493 on ZAP-70, increasing its kinase activity (*8*, *9*, *11*, *12*).

ZAP-70 is composed of three structural modules, an N-terminal one formed by its SH2 domains and their interconnecting region (hereafter called the $(SH2)_2$ region), followed by a second linker region called interdomain B and by the C-terminal kinase domain. A model in which binding of ZAP-70 to the phosphorylated ζ subunit activates its catalytic activity by relieving a negative regulation exerted by the $(SH2)_2$ region was proposed on the basis of the rigid structure predicted from the crystal data for this domain (*13*) and supported by the observation that ZAP-70 binding to the ITAMs *in vivo* results in its activation (*4*−*7*). Also, in previous examples, occupancy of the SH2 domains increased the catalytic activity of protein kinases and phosphatases (*14*−*17*) and binding of Syk to a diphosphorylated ITAM peptide resulted in increased kinase activity (*18*−*20*). Similarly, increased ZAP-70 autophosphorylation activity following binding to dimeric forms of phosphorylated ITAMs was reported (*10*, *21*). Moreover, reactivity of antibodies directed to the inter-SH2 region of ZAP-70 was altered either by deletion of the catalytic domain and interdomain B or by binding of the SH2 domains to a diphosphorylated ITAM peptide (*22*), suggesting the occurrence of intramolecular interactions mediated by the SH2-containing region. To study

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[1] Abbreviations: TCR = T-cell antigen receptor; SH2 = Src-homology 2; GST = glutathione *S*-transferase; $(SH2)_2$ = Interdomain A plus SH2 region; ZAP-70 255 3M = ZAP-70 255 Y292,315,319F mutant; ITAM = immunoreceptor tyrosine-based activation motif; Cdb3 = cytoplasmic domain of band 3; CD = circular dichroism.

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the regulation of ZAP-70 enzymatic activity, we previously expressed and characterized a truncated version of ZAP-70 lacking the $(SH2)_2$ region, named ZAP-70 255. We showed that ZAP-70 255 tyrosine phosphorylation was dramatically increased compared to ZAP-70, and observed an anomalous SDS−PAGE migration of the truncated protein (Magistrelli et al., in press). These findings are compatible with a model in which deletion of the $(SH2)_2$ region results in relief of a conformational constraint, potentially leading to a conformational change and consequent exposure of regions for tyrosine phosphorylation.

Here, we provide evidence that ITAM binding to the SH2 domains of ZAP-70 or autophosphorylation of the protein by incubation with ATP induces stimulation of its kinase activity, possibly relieving a conformational constraint in which the SH2 domains are implicated. Our study contributes insights to the regulation of ZAP-70 kinase activity and suggests a model for its activation following TCR binding *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies. COS1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), gentamicin, and nistatine. The following mouse antibodies were used: Z24820 (anti-human ZAP-70, Transduction Laboratories, Lexington, KY), 4G10 (anti-phosphotyrosine IgG2b, Upstate Biotechnology, Inc., Lake Placid, NY), and rabbit polyclonal antibodies sc-013 (anti-human Lck, Santa Cruz Biotechnology, Santa Cruz, CA).

Recombinant Protein Expression. Plasmids encoding GST-fused ZAP-70, ZAP-70 255 (lacking the first 254 residues), or ZAP-70 255 3M (with Tyr 292, 315, and 319 mutated to Phe), with a recognition cleavage site for the "prescission" protease (Pharmacia), were obtained as described in a separate report (Magistrelli et al., in press). The Lck complete cDNA was obtained by PCR on Jurkat total mRNA and subcloned into pMOS to obtain pMOS-Lck. An *Eco*RI−*Eco*RI polypeptide encoding for an Lck mutated form, Lck Y505F, was obtained by PCR on the complete Lck cDNA using a 5′ *Eco*RI primer (AATTGAATTCACCATGGGCTGTGGCTGCAGCTCACACC) and a 3′ *Eco*RI primer encoding a mutation of Tyr 505 to Phe (TTC). This polypeptide was then subcloned into a pMT2 vector to obtain pMT2-LckY205F.

COS1 cells were transiently transfected by the calcium phosphate method with pMT2-GST-ZAP-70, pMT2-GST-ZAP-70 255, or pMT2-GST-ZAP-70 255 3M, 60 μg per 5×10^6 cells, singularly or in combination with 5 μg of pMT2-LckY505F. Forty-eight hours after transfection, cells were lysed in buffer containing 25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 40 μg/mL benzamidine, 50 mM NaF, 40 μg/mL TPCK, 2 mM PMSF, 4 μg/mL leupeptin, 1 mM vanadate, 1% Triton, 100 mM NaCl, and 10% glycerol (lysis buffer).

For expression of recombinant ZAP-70 $(SH2)_2$ polypeptide, a *Bam*HI/*Eco*RI fragment encoding the 1−263 residues of ZAP-70 (1−263) was obtained by PCR using pBluescript ZAP-70 as a template and the oligonucleotides AATTGGATCCATGCCAGACCCCGCGGCGCACCTGC and AATTGAATTCTCATGAGGCGTTGCTGGCACTGCTG as primers. This fragment was subcloned into a *Bam*H1/*Eco*RI cut pGEX 6P-2 vector to obtain pGEX-SH2. The *E. coli* strain DH5α was transformed with the pGEX-SH2 plasmid, and protein expression was induced with 0.5 mM IPTG for 6 h at 30 °C.

ZAP-70, ZAP-70/L, ZAP-70 255, ZAP-70 255 3M, and the 1−263 polypeptide were purified by affinity chromatography on glutathione−sepharose resin (Pharmacia) and cleaved with prescission protease following the manufacturer's instructions. Proteins eluted from the resin were analyzed by sodium dodecylsulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and stained by Coomassie blue staining.

The purified proteins were analyzed by immunoblotting with anti-Ptyr antibodies using standard techniques and detected by the ECL technique (Amersham) carried out according to the manufacturer's instructions.

Synthetic Peptides. Peptides corresponding to ζ-ITAM (QNQLpYNELNLGRREEpYDVLD) and to the non-phosphorylated analogue were prepared according to standard methods of solid-phase synthesis (*23*). Phosphorylation of tyrosine residues was obtained following the methodology previously described (*24*). Peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and analyzed by electrospray ionization mass spectroscopy.

Enzyme Assay. ZAP-70 enzymatic activity on a gastrin peptide substrate (Bachem) was determined by adaptation of a spectrophotometric assay (*25*). Essentially, the kinase activity was followed by monitoring the decrease in absorbance at 340 nm due to NADH oxidation coupled to the ZAP-70 enzymatically produced ADP, by lactate dehydrogenase (LDH) and pyruvate kinase (PK). The composition of the coupled solution was 50 mM HEPES, pH 7.0, 10 mM $MnCl_2$, 50 μM ATP, 1.8 mM phospho(enol)pyruvate, 0.73 mM NADH, 8 U/mL PK, 24 U/mL LDH, and 10-100 μM gastrin. All measures for ZAP-70 kinetics were performed in quartz cuvettes (450 μL final volume) at a temperature of 37 °C. Enzymatic reactions were started by addition of 67 nM enzyme. In some experiments, the protein was preincubated at 37 °C for 20 min with 50 μM ATP, or 2.5 μM diphosphorylated or non-phosphorylated ITAM peptide, or 134 nM 1-263 polypeptide. For kinetic determination, the activity was measured in the linear part of the kinetic curve and the values of initial velocity were fitted to the Michaelis−Menten equation. To validate the spectrophotometric assay, kinetic parameters for the substrate gastrin determined with this method were compared with those calculated by RP-HPLC analysis of the reaction mixture.

Kinetics of ZAP-70 and ZAP-70/L autophosphorylation/cdb3 phosphorylation were performed by following ^{32}P incorporation on ZAP-70 or cdb3. Reactions were carried out at 37 °C in 50 mM HEPES, pH 7.0, 10 mM $MnCl_2$ buffer, using a 67 nM enzyme concentration, 6.7 μM ATP, 0.01 μM [γ-^{32}P]ATP, and 0.76 μM cdb3. The incorporated radioactivity was determined by SDS−PAGE analysis followed by autoradiography and imaging analysis on a Bio-Rad imaging densitometer (model GS-690) equipped with Molecular Analyst software (version 1.5).

Circular Dichroism Studies. CD spectra were recorded using a J-710 Jasco spectropolarimeter and collected over 40 min. Cuvettes with a path length of 0.01 cm were used.

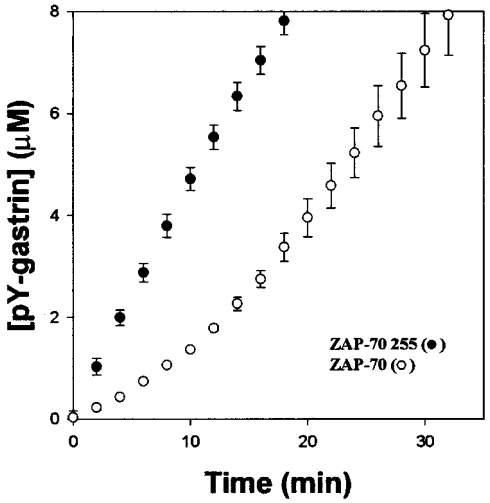

FIGURE 1: ZAP-70 but not ZAP-70 255 exhibits a lag phase. Time course of phosphorylation of gastrin (50 μM) after addition of 30 pmol of ZAP-70 (open circles) or ZAP-70 255 (full circles) to the reaction mixture (final volume 450 μL). Enzymatic reactions were started by addition of the purified enzyme preparations, and the decrease of absorbance at 340 nm, due to NADH oxidation coupled to ADP production, was monitored. pY-gastrin concentrations, corresponding to those of ADP, were calculated using an ϵ_{340} of 6220 M^{-1} cm^{-1}. Interassay variability ($N = 5$) of independent experiments from three different protein preparations is indicated by vertical bars.

Protein concentration was kept at 0.2 μM. Secondary structure analysis was performed by means of a Yang software package, model SSE338W. Deconvolution yielded the α-helix, β-sheet, β-turn, and random coil contents.

RESULTS

ZAP-70 WT but Not ZAP-70 Lacking Interdomain A and SH2 Domains Exhibits a Lag Phase Prior to Full Enzymatic Activation. Two different forms of ZAP-70 corresponding to the wild type and to a truncated ZAP-70 255 version lacking the first 254 residues which correspond to the $(SH2)_2$ region were transiently expressed in COS1 cells, purified to homogeneity, and characterized as described in a separate report (Magistrelli et al., in press). Preliminary characterization of the two forms showed evidence that deletion of the $(SH2)_2$ region resulted in increased phosphorylation and higher enzymatic activity. To understand the molecular basis for this behavior, their enzymatic activity was analyzed in parallel following the phosphorylation of a gastrin peptide substrate by a spectrophotometric assay.

Time-course experiments revealed a clear difference between ZAP-70 and ZAP-70 255 in the early stage of the kinetics. In fact, ZAP-70 was found to be very poorly active in the early phase of the reaction, showing a lag phase before reaching a linear rate of phosphorylation, which remained lower than that observed for ZAP-70 255 (Figure 1). The existence of the lag in ZAP-70 activation was verified in three independent protein preparations, and when the assay was repeated, as shown in Figure 1, a very low interassay variability was observed. These results might be explained by a time-dependent mechanism of ZAP-70 activation by autophosphorylation, potentially relieving a negative constraint exerted by the $(SH2)_2$ region, which is absent in the ZAP-70 255 truncated form. A K_m of 72 μM and a K_{cat} of 45 min^{-1} were calculated for ZAP-70 255 (Table 1), while, due to the nonlinearity of the reaction, it was impossible to calculate the kinetic parameters for WT ZAP-70.

Table 1: Determination of ZAP-70 Kinetic Constants for the Substrate Gastrin[a]

enzyme	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} μM^{-1})
ZAP-70/ITAM	4.7 (±0.3)	3.3 (±0.2)	0.70
ZAP-70/ATP	16.1 (±2.6)	4.0 (±0.2)	0.25
ZAP-70/L	15.9 (±0.8)	7.3 (±0.2)	0.46
ZAP-70 255/SH2/ATP	37.6 (±2.2)	16.4 (±0.4)	0.44
ZAP-70 255	72.6 (±17.9)	45.0 (±6.0)	0.62

[a] Kinetic constants were determined by measuring the initial velocity at different concentrations of gastrin, at an ATP concentration of 50 μM, at 37 °C. To obtain the activated form ZAP-70/ATP and (ZAP-70 255)/SH2/ATP, ZAP-70 and (ZAP-70 255)/SH2 were preincubated with 50 μM ATP at 37 °C for 20 min prior to addition of substrate. In all experiments the final enzymatic concentration was 67 nM. Experimental data were fitted to the Michaelis−Menten equation. In parentheses are indicated standard deviations.

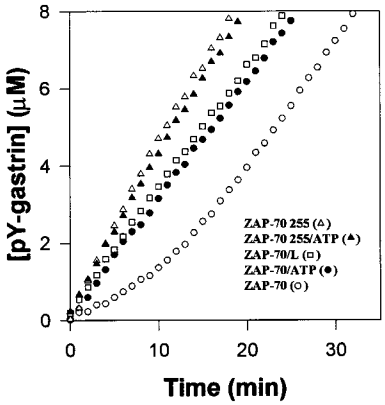

FIGURE 2: The lag phase is abolished by incubation with ATP or by Lck-mediated phosphorylation. Time course of phosphorylation of gastrin (50 μM) after addition of 30 pmol of ZAP-70 (open circles), ZAP-70 255 (open triangles), and ZAP-70/L (open squares) to the reaction mixture (final volume 450 μL). The latter represents a form of ZAP-70 phosphorylated *in vivo* by coexpression with Lck in COS1 cells. To study the effects of autophosphorylation, ZAP-70/ATP (full circles) and (ZAP-70 255)/ATP (full triangles) were obtained by 20 min of incubation of ZAP-70 and ZAP-70 255 at 37 °C in the reaction mixture containing 50 μM ATP prior to gastrin addition.

Preincubation with ATP or Phosphorylation by Lck Abolishes the Lag Phase and Result in a Higher Phosphorylation Rate. To verify whether progression to the fully active form could be due to activation by autophosphorylation, ZAP-70 was preincubated at 37 °C for 20 min with 50 μM ATP prior to the addition of the gastrin substrate (ZAP-70/ATP). This treatment resulted in loss of the lag phase, while the rate of phosphorylation remained comparable to that observed for the nonpreincubated ZAP-70 protein in the linear phase of the reaction (Figure 2). No effect was observed after preincubation of the ZAP-70 255 form (Figure 2). Preincubation of the protein without ATP, performed as a control, did not abolish the lag phase (data not shown).

Calculation of the kinetic parameters of ZAP-70/ATP allowed comparison with those obtained for ZAP-70 255. As shown in Table 1, ZAP-70 255 has both higher K_m and higher K_{cat} than the preincubated protein, resulting in a 2−3-fold increased catalytic efficiency, defined as the K_{cat}/K_m ratio, with respect to that of the WT protein activated by ATP preincubation. The 5-fold increase in the K_m of ZAP-

70 255 also suggests that deletion of the $(SH2)_2$ region induces a structural rearrangement that decreases its substrate affinity.

Lck tyrosine kinase is reported to phosphorylate ZAP-70 on tyrosine *in vivo*, resulting in its activation (*7*), and ZAP-70 phosphorylation by Lck at Tyr 493 was shown to increase its enzymatic activity (*8*, *9*). To study the effect of Lck phosphorylation on ZAP-70 kinetic behavior, we coexpressed it with a constitutively active form of Lck (Lck Y505F) in COS1 cells. As expected, anti-phosphotyrosine Western blot analysis of the purified protein, called ZAP-70/L, revealed higher phosphorylation compared to that of ZAP-70 expressed alone (data not shown). Lower than 1% Lck contamination was observed in the purified ZAP-70/L preparation by dot blot analysis with anti-Lck Ab, using recombinant Lck as standard (data not shown). Interestingly, phosphorylation by Lck was also found to abolish the lag phase observed in ZAP-70, resulting in a catalytic efficiency higher than that calculated for the ZAP-70 protein preincubated with ATP, but still lower than that calculated for the ZAP-70 255 form (Figure 2 and Table 1).

Analysis of ZAP-70 Autophosphorylation as Well as Phosphorylation of the Exogenous cdb3 Substrate Confirms the Existence of a Lag Phase in Its Activation, Which Is Not Observed for ZAP-70/L. To verify that these results were not due to an artifact associated with the use of the gastrin peptide as substrate, the kinetics of ZAP-70 phosphorylation were studied following the incorporation of ^{32}P on the cdb3 substrate, which is commonly used for ZAP-70 kinase assays (*26*). As shown in Figure 3 (panel A), a lag phase with progressive activation was observed not only for the cdb3 phosphorylation, but also in ZAP-70 autophosphorylation. When the ZAP-70/L protein was used as a control, we found that, in agreement with previous results obtained using the gastrin peptide, phosphorylation *in vivo* by Lck was able to abolish the lag phase in autophosphorylation as well as cdb3 phosphorylation (Figure 3, panel B). Plotting of data from densitometric analysis of the gel yielded curves very similar to those obtained by continuous measurement using the spectrophotometric method (Figure 3, panel C). These data suggest that the mechanism of progressive activation of ZAP-70 during the reaction is independent of the substrate used and might be relevant for its autophosphorylation *in vivo*.

Addition of Diphosphorylated ITAM Peptide Abolishes the ZAP-70 Lag Phase. ZAP-70 binding to the TCR phosphorylated ζ subunit results in its enzymatic activation (*4*, *7*). To verify whether ITAM binding *in vitro* could affect ZAP-70 kinetics, its activity was studied in the presence of diphosphorylated ITAM peptide at concentrations similar to those previously found to induce Syk activation (*18*, *20*). Addition of 2.5 μM diphosphorylated ITAM, but not of the corresponding non-phosphorylated peptide, was found to completely abolish the lag phase and resulted in activation of ZAP-70 (Figure 4). A comparable activation was observed when the protein was preincubated with ATP prior to the addition of the ITAM peptide (Figure 4), or when the ITAM peptide was directly added to the ATP preincubation mix (data not shown). As expected, no effect was observed after addition of the diphosphorylated ITAM peptide to the ZAP-70 255 form (data not shown). Calculation of the K_{cat} and K_m showed that addition of the ITAM peptide to ZAP-70 resulted in a catalytic efficiency equivalent to that of the

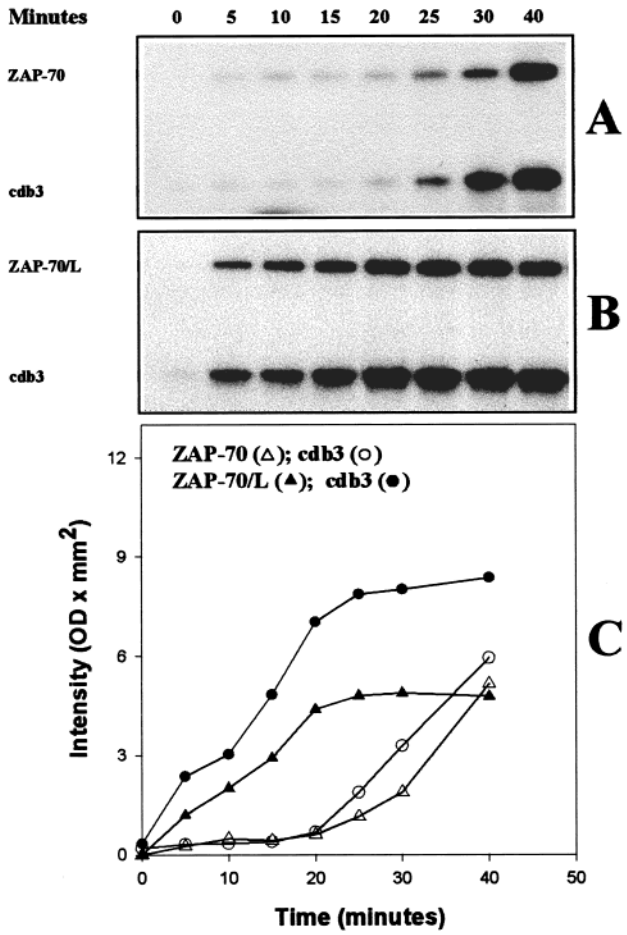

FIGURE 3: ZAP-70 but not ZAP-70/L exhibits a lag phase in autophosphorylation and in cdb3 phosphorylation. Time course of ZAP-70 WT (panel A) or ZAP-70/L (panel B) autophosphorylation and cdb3 phosphorylation. The mixtures, at a final volume of 150 μL, were incubated at 37 °C at an enzyme concentration of 67 nM, a cdb3 substrate concentration of 760 nM in the presence of 6.7 μM ATP, and 0.1 μM [γ-^{32}P]ATP. At the indicated times, 15 μL of the reaction mixture was withdrawn and boiled with 5 μL of sample buffer. The samples were then analyzed by SDS−PAGE, autoradiography (panels A and B), and densitometric analysis (panel C).

ZAP-70 255 form, essentially mediated by an increase in its substrate affinity (Table 1).

Preincubation with the Recombinant $(SH2)_2$ Domain Introduces a Lag Phase in ZAP-70 255 Kinetics. To study whether the lag phase observed for ZAP-70 and not for ZAP-70 255 could be linked to an intramolecular interaction mediated by the $(SH2)_2$ region, we reconstituted *in vitro* a full-length protein by incubating the truncated ZAP-70 255 with a recombinant polypeptide corresponding to the $(SH2)_2$ region. Preincubation at 25° of ZAP-70 255 with this polypeptide for 5 min prior to the start of the reaction was found to introduce a lag phase coupled to a decrease of its catalytic efficiency that made it almost comparable to that of ZAP-70 (Figure 5). In these experiments, a 1:2 stoichiometry of ZAP-70 255 to the $(SH2)_2$ polypeptide was used.

To verify whether this effect could be reversed by autophosphorylation, preincubation with the $(SH2)_2$ polypeptide was followed by 20 min of preincubation with ATP. As observed with ZAP-70, preincubation with ATP was found to abolish the induced lag phase and to activate (ZAP-70 255)/SH2 (Figure 5). A catalytic efficiency comparable

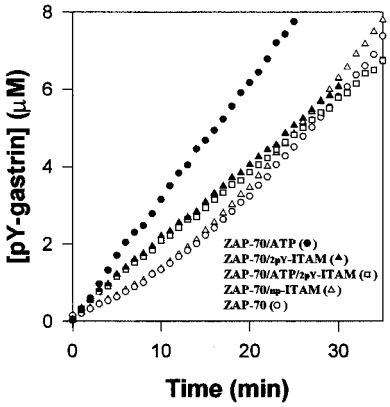

FIGURE 4: 2pY-ITAM-peptide but not the nonphosphorylated peptide abolishes the lag phase on ZAP-70. Time course of phosphorylation of gastrin (50 μM) after addition of 30 pmol of ZAP-70 to the reaction mixture alone (open circles), in the presence of non-phosphorylated 2.5 μM ITAM peptide (open triangles) or 2pY-ITAM peptide (full triangles). ZAP-70/ATP (full circles) was obtained by 20 min of incubation of ZAP-70 at 37 °C with 50 μM ATP prior to gastrin addition. ZAP-70/ATP/ITAM (open squares) was obtained by addition of 2.5 μM 2pY-ITAM peptide to the preincubated ZAP-70/ATP. Enzymatic reactions were started by addition of the substrate gastrin to the reaction mixture.

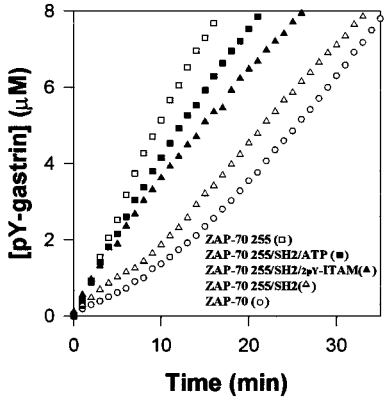

FIGURE 5: The SH2 domains added to ZAP-70 255 induce formation of a lag phase. Time course of phosphorylation of gastrin (50 μM) after addition of 30 pmol of ZAP-70 255 (open squares), ZAP-70 (open circles), or reconstituted (ZAP-70 255)SH2 (open triangles), which was obtained by incubation of 30 pmol of ZAP-70 255 with 60 pmol of the purified SH2 preparation at 25 °C for 5 min prior to the addition to the standard reaction mixture. To study the effect of autophosphorylation on the reconstituted full-length protein, (ZAP-70 255)/SH2 was preincubated for 20 min at 37 °C in the reaction mixture prior to gastrin addition (full squares). To analyze the effect of ITAM addition, (ZAP-70 255)SH2 was preincubated for 5 min with 2.4 μM 2pY-ITAM peptide (full triangles).

to that observed for ZAP-70/L was calculated for the reconstituted (ZAP-70 255)/SH2 after activation by autophosphorylation (Table 1). To test whether the lag phase and decrease of catalytic efficiency artificially induced for (ZAP-70 255)/SH2 could be abolished by interaction with the ITAMs, the diphosphorylated peptide was added to the reaction mix, again resulting in abrogation of the lag phase and in activation of the protein (Figure 5). Therefore, addition of the $(SH2)_2$ region to ZAP-70 255 was found to form a (ZAP-70 255)/SH2 complex functionally similar to full-length ZAP-70.

The Tyrosines 292, 315, and 319 Are Not Implicated in the Activation by Autophosphorylation. To investigate whether the tyrosines of the interdomain B were involved in the

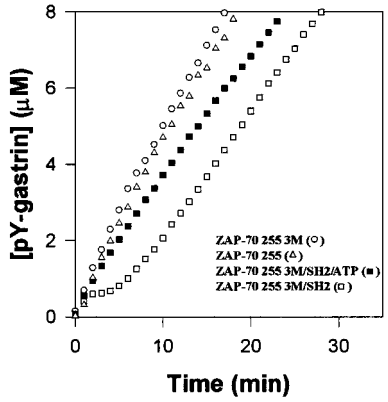

FIGURE 6: Triple mutant ZAP-70 255 3M behaves like ZAP-70 255. Time course of phosphorylation of gastrin (final concentration 50 μM) after addition to the reaction mixture of 30 pmol of ZAP-70 255 (open triangles), ZAP-70 255 3M (open circles), or (ZAP-70 255 3M)/SH2 (open squares). The reconstituted full-length triple mutant (ZAP-70 255 3M)/SH2 was obtained by a 5 min incubation at 25 °C of 30 pmol of ZAP-70 255 3M with 60 pmol of the purified $(SH2)_2$ preparation at a final volume of 15 μL. To study the effect of autophosphorylation on the reconstituted full-length triple mutant, (ZAP-70 255 3M)/SH2 was incubated for 20 min at 37 °C in the reaction mixture prior to addition of the substrate gastrin (full squares).

mechanism of activation by autophosphorylation, we used a mutant form of ZAP-70 255, called ZAP-70 255 3M, in which tyrosines 292, 315, and 319 are mutated to phenylalanine. Previous characterization of this mutant had shown that these tyrosines are not directly involved in the regulation of ZAP-70 catalytic efficiency (Magistrelli et al., in press). Accordingly, ZAP-70 255 3M was found to have a kinetic profile similar to that of ZAP-70 255 (Figure 6), which was not modified by preincubation with ATP (data not shown). When tested in the presence of the $(SH2)_2$ polypeptide, a lag in the initial part of the reaction associated with a reduction in the phosphorylation rate was observed (Figure 6). Similarly to what was observed for ZAP-70 255, abrogation of the activation phase and partial reactivation of the protein were obtained following preincubation with ATP, suggesting that the three mutated tyrosines are not implicated in this reactivation mechanism (Figure 6).

Upon Addition of ATP, Secondary Structure Changes Were Detected by Far-UV CD. To investigate whether the kinetic differences observed for the ZAP-70 different forms could be associated with conformational changes, ZAP-70, ZAP-70/L, and (ZAP-70 255)/SH2 were subjected to circular dichroism (CD) analysis. As shown in Figure 7, the CD spectra of all three forms have a broad negative band between 205 and 230 nm, consistent with a quite large contribution of α-helix to their secondary structure. Analysis of CD spectra predicted a composition of 60% α-helix, 15% β-structure, and 25% random coil for ZAP-70 and (ZAP-70 255)/SH2, again suggesting that the reconstituted complex is structurally similar to ZAP-70, as previously observed from the enzymatic point of view. Interestingly, 60% α-helix, 30% β-structure, and 10% random coil were predicted for ZAP-70/L, suggesting that phosphorylation by Lck induced a secondary structure difference that can be evidenced as an increase in its predicted β-structure component. The CD spectra of the three forms after 20 min of incubation with ATP were then analyzed. As shown in Figure 7, the CD spectrum of ZAP-70/L was not substantially changed, while

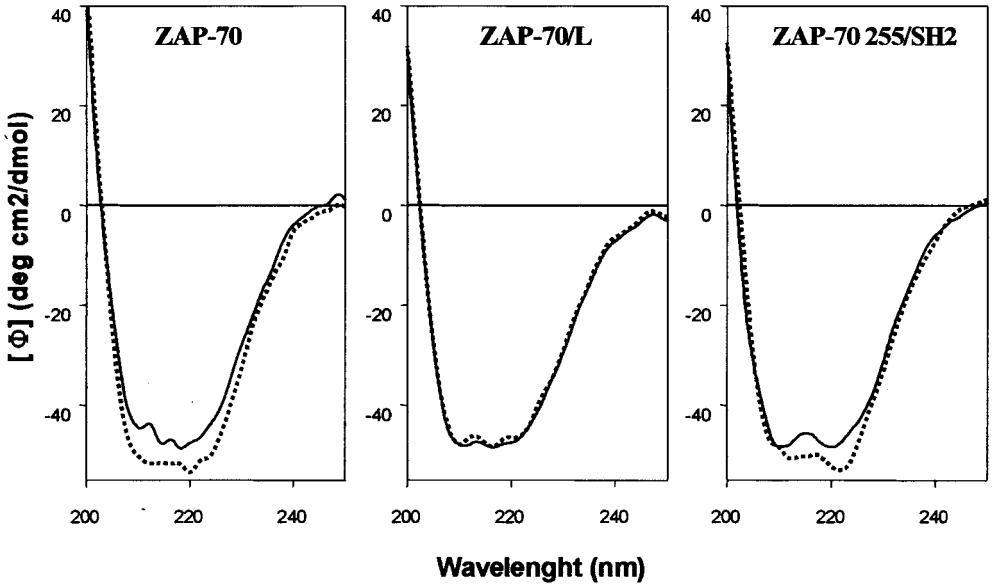

FIGURE 7: Circular dichroism spectra. CD spectra of untreated (dotted curves) *vs* ATP (50 μM) preincubated (continuous curves) ZAP-70, ZAP-70/L, and (ZAP-70 255)/SH2. Protein concentrations used were 0.31, 0.16, and 0.20 μM, respectively.

those of ZAP-70 and (ZAP-70 255)/SH2 showed a shift to less negative values of molar ellipticity that made them more similar to that of ZAP-70/L (compare the dotted with the continuous curves in Figure 7). These results suggest that ZAP-70 undergoes a structural change upon addition of ATP which is similar to that induced by *in vivo* phosphorylation by Lck and correlates with an increased catalytic activity and loss of the initial lag phase in the enzymatic reaction.

DISCUSSION

ZAP-70 binding to the phosphorylated TCR results in its phosphorylation on tyrosine and activation, suggesting that engagement of its SH2 domain may result in increased enzymatic efficiency. In a parallel study, we characterized a ZAP-70 deletion mutant lacking the $(SH2)_2$ region and showed that it displays increased tyrosine phosphorylation associated with anomalous SDS−PAGE migration (Magistrelli et al., in press). These studies also evidenced differences in the catalytic efficiency of ZAP-70 and its truncated version ZAP-70 255, supporting a model in which ZAP-70 could be activated through an allosteric mechanism upon binding of its SH2 domains to the ζ chain of the TCR. Here we analyzed more in detail the kinetics and regulation of ZAP-70 kinase activity using a continuous spectrophotometric assay to measure gastrin phosphorylation. A lag in gastrin substrate phosphorylation was observed for ZAP-70 but not for the ZAP-70 255 form. Absence of the lag phase was found to be associated with an increase of the enzyme processivity (K_{cat}), which was however coupled to an increase in the K_m, overall resulting in a small but reproducible (about 2−3-fold) increase of its catalytic efficiency. Similar results were obtained by following the kinetics of ^{32}P incorporation into cdb3. Moreover, these experiments also evidenced a lag phase in the kinetics of ZAP-70 autophosphorylation, suggesting that these results are not substrate-specific but reflect the kinetics of enzyme activation following its phosphorylation. In agreement with an allosteric model of activation, our data showed that the lag phase exhibited by full-length ZAP-70 can be abolished by incubation with diphosphorylated ITAM peptide. Conversely, incubation of ZAP-70 255 with a recombinant polypeptide encompassing the first 254 residues was found to reconstitute a complex functionally similar to the intact protein. Our results indicate that an interaction takes place between the two parts of the molecule, the $(SH2)_2$ region and the interdomain B/kinase region. These data fully complement our previous report showing that the reactivity of antibodies raised against residues in the interdomain A was decreased in the full-length ZAP-70 protein as compared to a polypeptide corresponding to the $(SH2)_2$ region, and that binding of ZAP-70 or its isolated $(SH2)_2$ domains to a diphosphorylated ITAM peptide modified the antibody reactivity, possibly due to a change in its structural arrangement (*22*). These observations suggest that an intramolecular interaction of the $(SH2)_2$ region with the downstream residues of ZAP-70 keeps the molecule in a "closed" configuration, which undergoes significant changes upon binding to the ITAM peptide (*22*). The data reported here also directly prove that the interaction between the $(SH2)_2$ and the downstream region of ZAP-70 exerts a negative regulation on its activity, which can be relieved through occupancy of the SH2 domains by a diphosphorylated ITAM peptide.

Although only a moderate increase in ZAP-70 catalytic efficiency is observed under the activation conditions *in vitro*, relief of an intramolecular constraint imposed by the $(SH2)_2$ region upon TCR binding might significantly affect ZAP-70 function *in vivo*, by temporally regulating its activity and ability to bind signal transducers and/or by changing its affinity for physiological substrates.

Lck was previously shown to recognize and phosphorylate Tyr 493 of ZAP-70 *in vitro*, leading to its increased enzymatic activity (*8*). Here we show that both autophosphorylation and Lck-mediated phosphorylation result in abrogation of the lag phase and ZAP-70 activation *in vitro*, possibly through an indirect effect mediated by Lck ability to up-regulate ZAP-70 autophosphorylation. Analysis of the CD spectra of the different ZAP-70 forms suggests that this activation is associated with secondary structure changes in the molecule that could result from a structural rearrangement.

Comparison of the ATP activation curves for ZAP-70 and the reconstituted ZAP-70 255/SH2 may contribute to the comprehension of this mechanism of allosteric regulation. Indeed, while ZAP-70 exhibits a typical lag phase which reaches linearity through a progressive increase in phosphorylation rate, (ZAP-70 255)/SH2 as well as the (ZAP-70 255 3M)/SH2 complex reproducibly show a biphasic curve of activation. The comprehensive shape of their kinetic curve is compatible with a mechanism in which the more active form ZAP-70 255 generates, upon interaction with the $(SH2)_2$ moiety, a less active complex which in turn starts to be activated by autophosphorylation with a behavior very similar to that of ZAP-70. This would suggest that the tyrosine(s) involved in the activation mechanism are not initially phosphorylated in the ZAP-70 255 molecule. It also indicates that the (ZAP-70 255)/SH2 complex is not dissociated upon autophosphorylation or ITAM addition, which would result in activity equal to that of ZAP-70 255, but rather undergoes a structural rearrangement similarly to what takes place in the WT form. Altogether, these observations suggest that the $(SH2)_2$ region is able to form a stable interaction with the rest of the molecule, even when produced as a separate polypeptide. As a result, upon autophosphorylation or ITAM binding, it would not dissociate from but rather change its orientation relative to the catalytic moiety.

On the basis of these observations, we would like to propose a mechanism to explain the allosteric activation *in vitro*. In this model, the $(SH2)_2$ region is able to interact with the downstream region of ZAP-70, through a mechanism which could be mediated by interaction with the interdomain A region, as previously suggested by analysis of the $(SH2)_2$ conformational changes mediated by intramolecular interactions (*22*). Engagement of the SH2 by binding to the ITAM peptide or introduction of negative charges due to autophosphorylation would result in a conformational change relieving the negative constraint. The interaction of the $(SH2)_2$ region with the downstream part of ZAP-70 would be kinetically favored by its intramolecular nature and should be expected to be weak due to its necessity of being easily reversed by ITAM binding. Autophosphorylation could represent an alternative mechanism for activation. In the Src model of intramolecular regulation of activity, phosphorylation of a C-terminal residue results in intramolecular interactions of the SH2 domain with the tail of the molecule and of the SH3 domain with an as yet undefined region (*27*). Activation of the molecule can be achieved by release of these SH2- and SH3-mediated interactions as well as by autophosphorylation at Tyr 419 in the active site (*28*, *29*). Similar results were obtained for the Src family member Hck (*30*). Autophosphorylation can be either an inter- or an intramolecular event, depending upon the kinase. Previous reports suggested that binding of ZAP-70 to the phosphorylated TCR ζ subunit enhanced its ability to autophosphorylate through a transphosphorylation mechanism (*10*). However, this is the first time that autophosphorylation of ZAP-70 and ITAM binding are directly shown to regulate its catalytic efficiency over a substrate. On the other hand, both binding to diphosphorylated ITAM peptides and autophosphorylation were previously shown to induce conformational changes in Syk associated with increased tyrosine kinase activity (*18*).

Overall, the data presented here show that ZAP-70 can be induced into a more active state by a structural rearrangement involving engagement or deletion of its $(SH2)_2$ region and/or tyrosine phophorylation. These results provide an *in vitro* model for the characterization of ZAP-70 activation *in vivo* and propose a structural basis for this mechanism that will be confirmed by further studies.

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