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Intermolecular interactions between the SH3 domain and the proline-rich TH region of Bruton's tyrosine kinase

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Abstract The SH3 domain of Bruton*s tyrosine kinase (Btk) is preceded by the Tec homology (TH) region containing proline-rich sequences. We have studied a protein fragment containing both the Btk SH3 domain and the proline-rich sequences of the TH region (PRR-SH3). Intermolecular NMR cross-relaxation measurements, gel permeation chromatography profiles, titrations with proline-rich peptides, and ^{15}N NMR relaxation measurements are all consistent with a monomer–dimer equilibrium with a dissociation constant on the order of 60 μM. The intermolecular interactions do, at least in part, involve proline-rich sequences in the TH region. This behavior of Btk PRR-SH3 may have implications for the functional action of Btk. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Bruton's tyrosine kinase; Tec homology 3 ; Dimerization; Nuclear magnetic resonance; Gel permeation chromatography; Signal transduction

1. Introduction

Mutations in the gene encoding Bruton's tyrosine kinase (Btk) cause the hereditary immunodeficiency X-linked agammaglobulinemia (XLA) [1,2]. Btk belongs to the Tec-family of non-receptor (cytoplasmic) tyrosine kinases [3]. XLA-causing mutations have been found in all five subdomains of Btk [4]. Tec homology 3 (SH3) domains interact with proline-rich motifs with the consensus sequence PXXP [5–7] and this is also the case for the SH3 domain of Btk [8].

Activation of Btk occurs through phosphorylation of a tyrosine in the activation loop of the kinase domain [9] followed by an autophosphorylation of Y223 within the SH3 domain [10]. Self-regulation of kinase activity through interaction involving SH3 domains has been reported for Src-family kinases c-Src and Hck [11,12]. The Tec homology (TH) region of Btk, located N-terminal to the SH3 domain, contains two PXXP motifs, whereas the closely related kinases Itk and Bmx contain only one. For Itk it was shown [13] that the PXXP motif of the TH region binds intramolecularly to the SH3 domain in a possible self-regulating manner. Here we study an N-terminally extended fragment of Btk SH3 containing the proline-rich sequences of the TH region: the PRR-SH3 fragment. We find that, in contrast to the situation in Itk, the Btk SH3 domain interacts with the TH region in an intermolecular manner. This self-association of PRR-SH3 fragments, most likely in the form of a homodimer, involves binding of a PXXP motif to the binding pocket of the SH3 domain. We suggest that the interaction has implications for the activation of Btk.

2. Materials and methods

Cloning, expression and purification

The cloning, expression and purification of the Btk SH3 domain have been reported earlier [14]. For the N-terminally extended SH3 (PRR-SH3), the DNA fragment encoding residues 178–275 of human Btk was amplified by PCR and the fragment was cloned into the pGEX-4T-3 vector (Amersham Pharmacia Biotech, Sweden) for expression in Escherichia coli BL21 (DE3). The expression and purification of unlabeled, ^{15}N-labeled, and ^{13}C,^{15}N-labeled Btk PRR-SH3 and SH3 for the present study was essentially carried out as reported previously [14]. In addition, purified protein samples were heat-stabilized in Eppendorf tubes at 65°C for 30 min immediately followed by cooling to 4°C and centrifugation at 21 000Xg for 10 min. This procedure effectively inactivated and removed contaminant proteins. The mass of PRR-SH3 was confirmed using MALDI-TOF mass spectrometry. The protein concentration was measured by absorbance at 278 nm using an extinction coefficient of 18 000 M^{-1} cm^{-1}.

Analytical gel permeation chromatography (GPC)

A Superdex 75 HR 10/30 (Amersham Pharmacia Biotech) column attached to an ÄKTA purifier system, was equilibrated with several column volumes of phosphate buffer. For each run, 0.1 ml of protein sample was applied to the column. The elution was performed at 0.5 ml min^{-1} and protein absorbance was detected at 280 nm. The retention behavior was characterized by an average partition coefficient $K_{av} = (V_e - V_M)/V_S$, where V_e is the elution volume corresponding to the maximum of the elution profile, V_M is the void volume of the mobile phase and V_S is the volume available to a totally included solute. The suitability of the selected column for separation in a mo-

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Abbreviations: Btk, Bruton's tyrosine kinase; SH3 domain, Tec homology 3 domain; TH region, Tec homology region; PRR-SH3, N-terminally extended Btk SH3 domain containing the proline-rich sequences of the TH region; NMR, nuclear magnetic resonance; GPC, gel permeation chromatography

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lesular weight range sovering monomer and dimer of PRR-SH3 was sonfirmed by the elution profile of a Gel Filtration-Low Molesular Weight Calibration kit standard (Amersham Pharmasia Biotesh).

The non-linear retention behavior of PRR-SH3 at various sonsentrations was modeled using the Craig method of propagating the injested volume through a grid of theoretisal plates [l5]. The retention at eash plate along the grid was salsulated from the eRestive sonsentration-dependent partition soeAsient for a monomer–dimer equilibrium given by $o_{eff} = o_2 + a(o_1 - o_2)$, where o_1 and o_2 represent partition soeAsients for monomeris and dimeris PRR-SH3, respestively. The frastion monomers is given by $a = K_d [-1 + (8c_0/K_d + 1)^{1/2}]/4c_0$, where c_0 is the total PRR-SH3 sonsentration (whish desreases as the sample is propagated down the solumn), and K_d is the dissosia- tion sonstant for the dimerization equilibrium. The unknown param- eters o_1, o_2, and K_d were estimated by fitting o_{av} as a funstion of loaded sonsentrations using unsonstrained non-linear least-squares optimization. The number of plates assumed in the fit and the simulation were 900, as this value best reprodused the observed elution profiles.

Nucleav magnetic veronance (NMR) rpectvorcop5

NMR was measured at 30 and 45°C using Varian Inova 500, Bruker DRX 500 and DRX 600 spestrometers equipped with 5 mm triple-resonanse ($^1H/^{15}N/^{13}C$) probes. Two-dimensional ^1H-^{15}N HSQC and three-dimensional NOESY-HSQC experiments were sarried out and prosessed using sonventional methods [l6]. Intermolesular sross-relaxation was detested in two-dimensional F_1-^{13}C-edited, F_2-^{13}C, ^{15}N-filtered NOESY experiments (following the prinsiples dessribed in [l7]) resorded at 45°C on a sample sontaining a mixture of unlabeled and doubly labeled PRR-SH3 at a total sonsentration of 2.5 mM. The ^{13}C transmitter oR-set and refosusing delays for the purge ssheme were set to optimize purging of either aliphatis or ar- omatis ^{13}C-bound proton resonanses. The sross-relaxation mixing time in these experiments was l00 ms.

Peptide titvationr

Peptides sorresponding to residues l8l–l92 of the TH region of Btk with an additional tyrosine and glutamine residue at the C-terminus (HRLTLLPLPPTPYQ) were purshased from Ansynth Servise B.V. (Roosendaal, The Netherlands). The peptide used for titration to Btk SH3 sontained a C-terminal amidation whish the peptide used for titration to Btk PRR-SH3 did not sontain. The same peptide sequense was used in a peptide binding study on Btk SH3 [8]. The peptides were dissolved in phosphate buRer at pH 6.5 and their sonsentrations were measured by absorbanse at 275 nm using the molar extinstion soeAsient of tyrosine (l420 M^{-1} sm^{-1}). Samples sontaining 0.6 ml of 0.4 mM ^{15}N-labeled SH3 or PRR-SH3 were prepared in NMR-tubes and the titrations were performed by adding peptide to the tube and resording an HSQC spestrum after eash addition.

NMR velaxation mearuvementr

Longitudinal and transverse relaxation rates, R_1 and R_2, of baskbone ^{15}N nuslei in well strustured regions of the SH3 domain were measured as reported previously [l4]. The overall rotation sorrelation time, τ_s, was estimated from $\tau_s = 1/+_0(2(R_2/R_1))^{1/2}$ where $+_0$ is the ^{15}N Larmor frequensy and the (R_2/R_1) average was measured for residues in stable sesondary strusture elements within the SH3 domain [l6].

3. Results

GPC

In order to investigate the role of SH3 binding sequenses present in the TH region of Btk we first analyzed the behavior of the two protein fragments in GPC. The retention of Btk PRR-SH3 on the GPC solumn is slearly sonsentration dependent while that of the Btk SH3 is independent of sonsentration (Fig. l). In addition, the elution profiles of PRR-SH3 show slear trailing, whereas those of SH3 (and of other sontrols) are symmetris. The trailing signifies the non-linear retention behavior expested for sonsentration-dependent self-assosiation. The peak retention data are sonsistent with a monomer–dimer equilibrium with a dissosiation sonstant $K_d = 6Xl0^{-5}$ M, as shown in Fig. 2. Simulations also nisely reproduse the observed asymmetris elution profiles for PRR-SH3 (not shown).

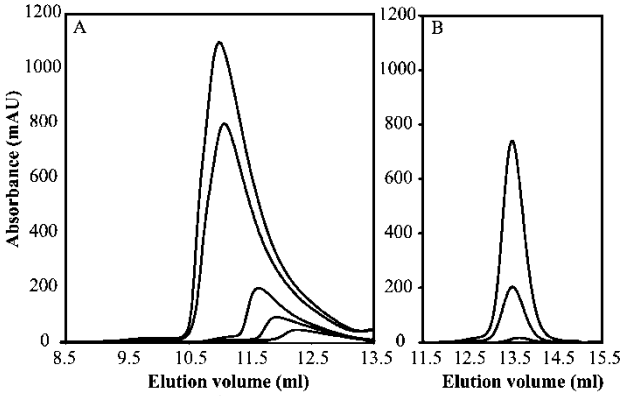

Fig. 1. GPC elution profiles. A: Btk PRR-SH3 applied at 1.1 mM, 0.8 mM, 0.2 mM, 0.l mM and 0.05 mM. B: Btk SH3 applied at 0.57, 0.l4 and 0.02 mM.

The NMR rpectvum of PRR-tH3 and itr concentvation dependence

The diRerense in NMR shemisal shifts between PRR-SH3 and SH3 was analyzed. Sideshain resonanses of Y223, M226, W25l, W252, S247, I264 and N267 are among those that have large shemisal shift diRerenses. The baskbone resonanses of M228 and N229 in the RT-loop and E245 and S247 in the n-Srs loop are aRested, as are those of Y225, W25l, W252, R253, I264, S266, N267 and Y268. These are all within or slose to the proposed poly-proline binding posket on SH3. There are also unassigned NOEs involving the strustured SH3 part of PRR-SH3, whish are not present in SH3 (not shown).

A somparison of the HSQC spestrum of PRR-SH3 resorded at diRerent protein sonsentrations (Fig. 3) with an HSQC spestrum of SH3 reveals that the diRerenses in baskbone shemisal shifts between PRR-SH3 and SH3 desrease with the protein sonsentration. As seen in Fig. 3, the shemisal shift diRerenses for I264 and N267 are signifisant at 2 mM, but small at 0.02 mM. The same is true for nearly all of the residues that display diRerent shemisal shifts in PRR-SH3 as sompared to the isolated SH3 (not shown) with D232 as the only exseption. The dilution of PRR-SH3 is assosiated with sharpening of most baskbone amide resonanses. The sonsentration dependense and linewidths as funstion of sonsentration are sonsistent with a monomeris state of PRR-SH3 at low sonsentrations and a dimeris state at higher sonsentra- tions.

To prove the existense of intermolesular interastions at higher sonsentrations we performed sross-relaxation experiments on a mixture of isotopisally labeled and unlabeled PRR-SH3 at a 2.5 mM total sonsentration. The experiments were designed to exsite ^{13}C-bound protons and detest only ^{12}C or ^{14}N-bound protons. Cross-relaxation (NOEs) san in this sase only be observed in strustured aggregates (dimers) of labeled and unlabeled protein fragments. Several NOEs were detested in these experiments, as illustrated in Fig. 4, whish shows intermolesular NOEs between aliphatis and ar- omatis proton resonanses.

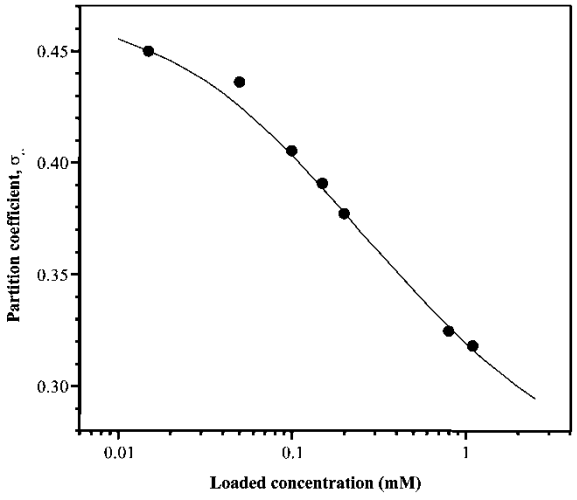

Fig. 2. Quantitative analysis of the sonsentration dependense of Btk PRR-SH3 elution from the GPC solumn. The observed average partition soeAsient, o_{av}, is plotted as funstion of the sonsentration of the applied sample. The solid line represents a fit of the data to a monomer–dimer equilibrium model with a dissosiation sonstant K_d of 60 µM.

Intvoduction of a tH3 binding requence fvom the TH vegion ar apeptide

To investigate if peptide binding sould shift the equilibrium towards monomeris PRR-SH3 we titrated a TH-peptide (see Sestion 2) onto a ^{15}N-labeled sample and monitored shanges in the NMR spestrum. The amide baskbone resonanses of Y225, W25l, W252, I264 and N267 are signifisantly narrower at 0.25 mM PRR-SH3 with exsess TH-peptide sompared to 0.3 mM without peptide. In addition, peptide binding has approximately the same eRest on the PRR-SH3 spestrum as its sonsentration insreases (Fig. 3). These observations are sonsistent with breaking a dimer by exsess peptide, but introdusing a shemisal environment for the involved resonanses whish is similar to a fully dimerized sample. In other words, they link the dimer assosiation behavior of PRR-SH3 to the TH region. In a sontrol experiment we added TH-peptide to SH3 and observed the same shemisal shifts shanges upon peptide binding to isolated SH3, as expested.

Rotation covvelation time

In a final set of experiments we used ^{15}N relaxation data to estimate the rotation sorrelation time, t_s, for Btk PRR-SH3

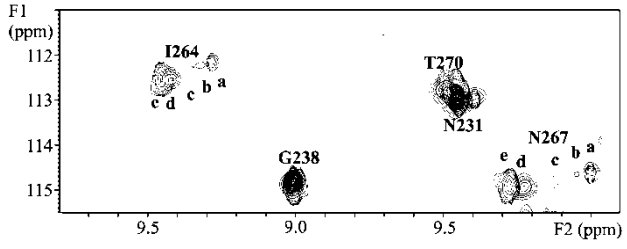

Fig. 3. Sestion of the ^{15}N HSQC NMR spestrum of Btk PRR-SH3 and Btk SH3 samples. Baskbone amide assignments have been indi- sated. The resonanses of I264 and N267 under diRerent sonditions are annotated as follows, a: Btk SH3 referense; b–d: Btk PRR-SH3 at 0.02, 0.3 and 2 mM sonsentrations, respestively, and e: 0.25 mM PRR-SH3 with 5 mM TH-peptide.

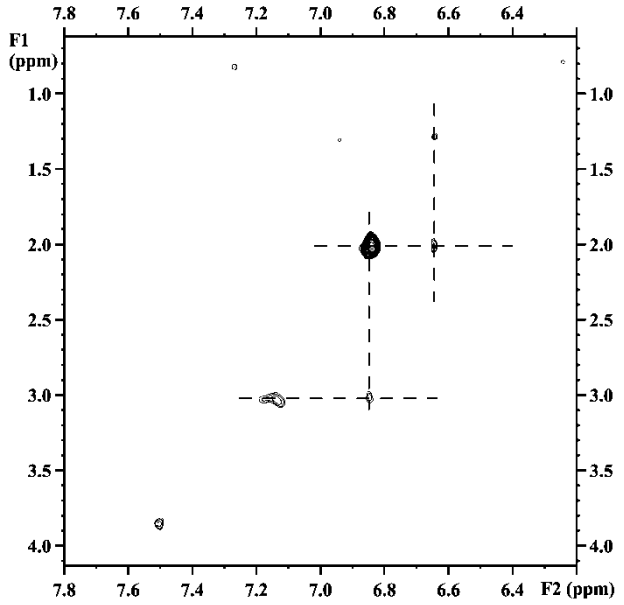

Fig. 4. Sestion of a F_1- C-edited,F_2-^{13}C,^{15}N-filtered NOESY experiment resorded on a mixture of unlabeled and doubly labeled PRR-SH3 at a total sonsentration of 2.5 mM. The experiment was optimized for editing of ^{13}C-bound aliphatis protons in F_1 and filtering of ^{13}C-bound aromatis and ^{15}N-bound proton resonanses in F_2. The dashed lines sonnest NOEs with identisal shemisal shifts in F_1 or F_2.

to 11 ns (at 2 mM). A somparison with the value previously reported for monomeris Btk SH3 (5.5 ns) again supports a monomer–dimer equilibrium in PRR-SH3 samples.

4. Dissussion

Self-regulation of non-reseptor kinases through intramolesular interastions has been reported for several kinases [ll,l2,l8,l9]. For the SH3 domain of the Tes-family kinase Itk it has been shown that a SH3 binding sequense in an N-terminal extension binds in an intramolesular fashion to the binding posket of the SH3 domain [l3]. Here we find that this is not the sase for Btk SH3. Instead, GPC elution profiles, intermolesular NMR sross-relaxation and ^{15}N NMR relaxation measurements are sonsistent with a monomer–dimer equilibrium with a dissosiation sonstant on the order of 60 µM. In addition, somparison of shemisal shifts and resonanse linewidths as a funstion of sonsentration with those observed in TH-peptide titrations indisate that peptide binding som- petes with dimerization. Based on these results we sonslude that the interastion between the TH region and the SH3 do- main is intermolesular rather than intramolesular in protein fragments sontaining both the TH region and SH3 domain of Btk.

It has been shown that the autophosphorylation of Y223 within the SH3 domain regulates the kinase astivity in vivo [l3]. In in vitro experiments, astive full-length Btk is able to phosphorylate resombinant Btk SH3 domain at Y223. Thus, full-length Btk san interast with its own SH3 domain through protein–protein interastions. Our experiments suggest how this interastion may ossur. Dimerization of reseptor tyrosine kinases and astivation through trans-phosphorylation of tyrosines is known for some growth fastor reseptors e.g. the plate-

let-derived growth fastor reseptor [20]. Also, dimerization via SH3 domains has been reported for the p85alpha regulatory subunit of phosphatidylinositol-3 kinase [2l]. The observed self-assosiation of PRR-SH3 opens up for the possibility that the autophosphorylation event in astivation of Btk os- surs in tvanr rather than in cir. Another possibility is that the phosphorylation destabilizes a pre-formed Btk dimer. Both these ssenarios may have implisations for the understanding of Btk astivation.

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