FEBS Letters 461 (1999) 68–72 FEBS 22859

Bruton's tyrosine kinase (Btk) associates with protein kinase C μ

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Received 23 August 1999; received in revised form 15 October 1999

Abstract Bruton's tyrosine kinase (Btk) is considered an essential signal transducer in B-cells. Mutational defects are associated with a severe immunodeficiency syndrome, X-chromosome linked agammaglobulinemia (XLA). Here we show by coimmunoprecipitation that a member of the protein kinase C (PKC) family, PKCμ, is constitutively associated with Btk. Neither antigen receptor (Ig) crosslinking nor stimulation of B-cells with phorbol ester or H_2O_2 affected Btk/PKCμ interaction. GST precipitation analysis revealed association of the Btk pleckstrin/Tec homology domain with PKCμ. Transient overexpression of PKCμ deletion mutants as well as expression of selected PKCμ domains in 293T cells revealed that both the kinase domain and the regulatory C1 region are independently capable of binding to the Btk PH-TH domain. These data show the existence of a PKCμ/Btk complex in vivo and identify two PKCμ domains that participate in Btk interaction.

1. Introduction

During antigen receptor induced signal transduction of lymphocytes protein tyrosine kinases of the Src, Syk and Tec family are considered to play essential roles [1,2]. Receptor proximal signals are initiated via src kinase directed activation of Syk and ZAP70 kinases followed by the activation of phosphotyrosine/SH2 domain mediated signal cascades including the phosphatidylinositol second messenger system and serine kinases of the protein kinase C (PKC) family, which fuel several downstream targets finally leading to a proliferative response of T- [1,3] and B-cells [2]. Tec kinases are expressed in T- and B-cells and also in mast cells. Much attention has been focused on one member, Bruton's tyrosine kinase (Btk) [4]. The Btk gene is variously mutated in X-linked immunodeficient mice (xid) or in X-linked agammaglobulinemia patients (XLA) [4,5]. These patients lack circulating B-cells and immunoglobulins and are therefore very vulnerable to infectious diseases [6]. Accordingly, Btk function is considered to be associated with development and activation of B-cells e.g. by controlling the PLCγ mediated intracellular Ca^{2+} signalling [7]. Within B-cell receptor triggered Btk activation, src kinases and PI-3 kinase [8] are implicated. Aside from B-cell specific functions, Btk has been shown to be involved in FcεRI induced mast cell degranulation and cytokine production through c-Jun N-terminal kinase (JNK) activation [9,10].

Btk contains a SH2, SH3, a Tec homology (TH) domain and, within the amino terminal region, a pleckstrin homology (PH) domain. This recently identified novel signal module [11] was found to mediate membrane association via lipid binding and, in addition, mediates protein-protein interactions [12]. For Btk, PH domain mediated PIP_3 binding is essential for activation leading to PLCγ tyrosine phosphorylation [8,13]. The importance of the PH domain as a regulator of Btk function was shown by expression of a PH domain mutant which leads to enhanced membrane association of Btk resulting in a transformed phenotype, suggestive of an upregulated Btk function [14].

In the light of these data, studies reporting binding of the PH domain of Btk to the regulatory C1 region of PKC [15,16] are of interest. Binding of PKC has been mapped to the amino terminal region of the PH domain of Btk [16] which is considered to represent the lipid binding domain [17,18]. Both, phosphatidylinositol phosphates and PKC, have been shown to bind with similar affinities [19,16], providing an example that PH domain mediated functions can be regulated by both protein and lipid ligands. For Tec kinases, the physiological function of PKC association appears to be the phosphorylation dependent regulation of Tec kinase activity [20,15], pointing to a regulatory role of PKCs within Tec kinase mediated signalling pathways.

We have recently described a novel PKC isotype, termed PKCμ [21], which, although ubiquitiously expressed, shows particularly high expression in thymus and hematopoietic cells [22]. In addition to the conserved kinase- and regulatory domains in common to all PKC isoforms, PKCμ displays structural features like a hydrophobic amino terminal domain, an acidic regulatory domain [23] and a pleckstrin homology domain [11]. First evidence for involvement of PKCμ in diverse cellular functions stems from reports showing enhancement of constitutive transport processes in PKCμ overexpressing epithelial cells [24] and PKCμ activation during antigen receptor mediated signalling in B-cells [25].

In the present study, we provide biochemical evidence that PKCμ is constitutively associated with Btk and show by in vitro binding studies, that both the kinase domain and the regulatory C1 domain of PKCμ interact with Btk.

2. Materials and methods

2.1. Recombinant PKCμ, construction of mutants and cell lines

The production of Sf158 insect cells overexpressing PKCμ has been described previously [26]. A cDNA fragment coding for the Btk pleck-

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Abbreviations: PKC, protein kinase C; GST, glutathione *S*-transferase; Pdbu, phorbol 12,13-dibutyrate; Btk, Bruton's tyrosine kinase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; xid, X-chromosome linked immunodeficiency; JNK, c-Jun N-terminal kinase

PII: S0014-5793(99)01424-6

strin homology domain including the TH domain [27] (amino acid 1–174, Btk PH-TH) was amplified introducing an *Eco*RI and an *Xho*I site. The cDNA fragment was cloned in frame with the glutathione *S*-transferase (GST) in pGEX5X-3 and expressed as a bacterial fusion protein. The human B-cell line SKW 6.4 was maintained in RPMI 1640 medium supplemented with 5% FCS. GST fusion proteins were isolated according to the manufacturer's instructions (Pharmacia). In brief, fusion proteins were bound to glutathione sepharose and quantified upon Coomassie staining by densitometric scanning, calibrated against an albumin standard. The PKCμ deletion mutant $PKC\mu_{\Delta 1-340}$ was constructed by deleting the respective cDNA region by restriction enzyme digest, initiating translation at Met-340 as described [28]. $PKC\mu_{\Delta PH}$ and $PKC\mu_{\Delta AD}$ were created using a fusion PCR approach looping out the coding region from K417–G553 ($PKC\mu_{\Delta PH}$) and E336–D391 ($PKC\mu_{\Delta AD}$), respectively. Initial PCR to amplify the 5′-cDNA fragment was carried out with a forward primer annealing at nucleotide position 478 of the PKCμ cDNA and a reverse primer annealing at the 5′ and 3′-end of the cDNA region to be looped out. Amplification of the 3′-cDNA fragment was carried out using a reverse primer annealing at nucleotide position 3000 of the PKCμ cDNA creating a *Bam*H1 site and a forward primer annealing at the 5′- and 3′-end of the cDNA fragment to be looped out. Fusion PCR generated a 2130-bp cDNA fragment for the ΔPH and a 2350-bp cDNA fragment for the ΔAD mutant. PCR fragments were digested with *Hin*dIII and *Bam*H1 and used to replace the PKCμ wild-type cDNA fragment cloned in the expression vector pcDNA3 (Invitrogen). The presence of the deletions was verified by dideoxy sequencing of both strands. The PKCμ C1 region covering amino acids 1–327 and the kinase domain (amino acids 550–912 was PCR amplified adding 5′-*Eco*RI and 3′-*Xba*I restriction sites and cloned in pCDNA3. The construction of the kinase dead PKCμ mutant (K612W) has been described previously [29]. This mutant can be weakly immunoprecipitated by the carboxyl terminal PKCμ antibody. The R28P mutant of the Btk PH domain cDNA (Btk-mut) was constructed by PCR based mutagenesis exchanging Arg-28 (CGC) to Pro-28 (CCC) according to the manufacturer's instructions (Quick change site directed mutagenesis, Stratagene) and was verified by dideoxy sequencing. FLAG tagged JNK cloned in pCDNA3 was used as a control for specificity of GST Btk PH-TH domain precipitation.

2.2. Immunoprecipitation by antibodies and GST fusion protein precipitation of PKCμ

Cells were either left untreated or stimulated with phorbol ester (100 nM, 10 min), Anti-IgG or H_2O_2/pervanadate as described [30] before lysis at 4°C in lysis buffer (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM Na-orthovanadate, 150 mM NaCl, 10 μg/ml leupeptin, 0.5 mM PMSF, 1 mM NaF, 1 mM nitrophenylphosphate) by incubating 30 min. After centrifugation of cell debris (15 min, 15 000 rpm) GST precipitation was done in 1 ml lysate portions (500 000 Sf 158 cells or 50×10^6 Jurkat cells) by incubation with the indicated amounts of GST fusion proteins coupled to glutathione sepharose for 90 min at 4°C. For immunoprecipitation a PKCμ antibody directed against carboxyl terminal epitopes and a Btk rabbit antibody directed against amino terminal epitopes (both from Santa Cruz Biotechnology) were used. Immunocomplexes were harvested by incubation with protein G sepharose (Pharmacia, $30\mu l/5\times10^7$ cell equivalents) for 30 min at 4°C. Immuno- or GST-complexes were washed three times in lysis buffer and subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Western blot detection of PKCμ and Btk was performed using either Btk or PKCμ antibodies as recommended by the manufacturer. GST was detected using an anti-GST antibody (Pharmacia). Western blots were stained using an alkaline phosphatase based detection system according to standard conditions.

2.3. Generation of PKCμ specific antibodies

Purified PKCμ, produced from Sf158 insect cells was used to immunize Balb/c mice. Using PKCμ specific immune serum and several PKCμ domain GST fusion proteins the antibody binding epitope could be mapped within the amino terminal region (amino acids 1–327). This mouse antiserum was used to immunoprecipitate the amino terminal expressed PKCμ domain in 293T cells.

2.4. Transfection of 293T cells

Two million cells were seeded per 90-mm-diameter dish in 10 ml RPMI supplemented with 5% FCS and transfected with 10 μg of DNA and 50 μl Superfect reagent (Qiagen) according to the manufacturer's protocol. Cells were harvested 40 h upon transfection and analyzed by GST pull down assays or immunoprecipitation as described above.

3. Results and discussion

3.1. Btk associates with PKCμ

Btk is expressed in B-cells and involved in early B-cell receptor initiated signalling pathways [31] in a similar way as the tyrosine kinase Syk which has been reported to associate with PKCμ [25]. Therefore, we analyzed potential in vivo association of PKCμ and Btk by coimmunoprecipitation from B-cells. SKW 6.4 B-cells were shown by Western blot analyses to express PKCμ and Btk in detectable amounts (data not shown). Btk was immunoprecipitated from phorbol ester stimulated or untreated SKW 6.4 cells using a rabbit antibody directed against amino terminal epitopes (Fig. 1A, lanes 1,2). In Btk immunoprecipitates, PKCμ was hardly detectable. However, Btk was readily detectable in PKCμ immunoprecipitates from both, phorbol 12,13-dibutyrate (Pdbu) stimulated and non-stimulated SKW 6.4 cells using two different, PKCμ specific antibodies of rabbit and mouse origin, directed against carboxyl terminal and amino terminal regions, respectively (Fig. 1A, lanes 3,4,8,9). A previously noted characteristic of the carboxyl terminal PKCμ specific antibody is its weaker binding to Pdbu activated, compared to non-activated PKCμ [22], leading therefore to a reduced PKCμ detection and a slightly reduced Btk precipitation efficacy (Fig. 1A, lane 3 vs. 4). SKW 6.4 B-cells were either activated with anti-IgG or the substitute stimulus, H_2O_2-pervanadate [30], resulting in PKCμ activation (data not shown) to monitor potential changes in Btk association. However, no changes in Btk association were found (Fig. 1A, lanes 5,6). As a negative control, PKCμ immunoprecipitates from Molt 4 cells, which do not express detectable amounts of Btk (data not shown), but high amounts of PKCμ, were analyzed. As shown in Fig. 1A (lane 7), only the expected PKCμ specific band was detectable, verifying the specificity of immunoprecipitation and Western blot reagents.

The PH domain of Btk has been reported to mediate association with PKCs in vitro and in vivo [15,16]. Further on it has been shown that the region close to the PH domain, the TH domain (Tec homology) [27], is essential for Btk structure and function [32]. In order to identify whether this region is critical for the Btk interaction with PKCμ, we analyzed recombinant PKCμ for potential Btk PH-TH domain association. The Btk PH-TH domain was expressed as bacterial GST fusion protein (see Section 2). Immobilized Btk PH-TH domain GST fusion protein was used to precipitate PKCμ expressed in Sf158 cells. As shown in Fig. 1B, PKCμ can be detected by immunoblot analysis (upper panel) in precipitates of GST fusion proteins with the Btk PH-TH domain. Using different amounts of GST Btk PH-TH domain fusion protein, PKCμ can be readily detected with 1 μg, showing best detection using 10 μg of Btk PH-TH domain GST fusion proteins (Fig. 1B). The specificity of PKCμ binding to Btk PH-TH domain was shown by pull down analysis of high amounts of bacterial GST proteins producing no specific PKCμ immunoreactive signal (Fig. 1B, right lanes). Mutation of arginine-28 in Btk either to histidine, proline or cysteine has been

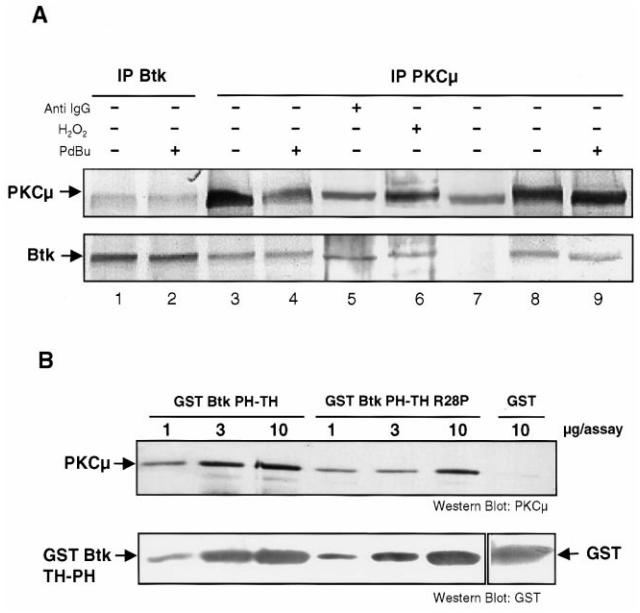

Fig. 1. Btk is associated with PKCμ. A: Btk coimmunoprecipitates with PKCμ. Shown are immunoprecipitates from lysates of 40×10^6 SKW 6.4 (lanes 1–6,8,9) or Molt 4 (lane 7) cells. Btk was immunoprecipitated using an amino terminal rabbit antibody. PKCμ was immunoprecipitated using a rabbit antibody raised against carboxyl terminal epitopes (lanes 3–7) or a mouse antiserum against amino terminal PKCμ regions (lanes 8,9). Detection was carried out with the carboxyl terminal antiserum. Cells were either left unstimulated or stimulated with phorbol ester (PdBu), anti-IgG or H_2O_2 as described in Section 2. B: The Btk PH-TH domain associates with recombinant PKCμ. PKCμ expressed in Sf158 cells was precipitated with the indicated amounts of Btk/Btk-mut GST PH-TH domain fusion proteins or with GST protein as a control. Bound PKCμ was detected by immunoblot analysis using a PKCμ antiserum and an alkaline phosphatase coupled secondary antibody. GST (26 kDa) or GST fusion proteins (40 kDa) were visualized using a goat anti-GST antibody.

reported to be associated with the XLA or xid phenotype in humans or mice [5], affecting PKC interaction [15]. Therefore we tested whether this mutation would affect PKCμ binding. A GST Btk PH-TH domain fusion protein carrying the R28P mutation indeed resulted in a decreased PKCμ pull down efficacy (Figs. 1B and 3B). These findings are consistent with previous observations [15] and point to a potential role of PKCμ in Btk mediated signalling pathways affected in the immunodeficiency XLA phenotype. Using GST PH-TH domain fusion proteins of Btk, endogenous PKCμ was similarly precipitated from extracts of phorbol ester stimulated or non-stimulated SKW 6.4 cells (data not shown).

3.2. The C1 region or the kinase domain of PKCμ are sufficient to mediate Btk PH-TH domain binding

The cysteine fingers in the C1 region of PKCs have been shown to represent the binding site for lipid second messengers as well as for regulatory proteins affecting protein kinase activity [33,34]. The PH domain of Btk has been reported to bind to the regulatory C1 domain of PKCε [16]. Although considerably different in primary structure, the C1 region of PKCμ shares the typical consensus sequence of zinc fingers responsible for phorbol ester or second messenger binding. We therefore used several PKCμ deletion mutants lacking the C1 region and other predicted functional domains to map the binding site of the Btk PH-TH domain. Fig. 2A displays the location of functional PKCμ domains.

Transfection of the PKCμ mutant $PKC\mu_{\Delta1-340}$ lacking the C1 region [35] in 293T cells resulted in the expression of an approximately 70-kDa PKCμ variant (wild-type 115 kDa) as shown by Western blot analysis (Fig. 2B). Two further deletions of PKCμ domains considered to be involved in PKCμ regulation were constructed: deletion of the predicted PKCμ PH domain [11] (K417–G553, $PKC\mu_{\Delta PH}$) and of an acidic region (E336–D391, $PKC\mu_{\Delta AD}$ for acidic domain), the latter of which is considered to participate in a phorbol ester independent activation [23], results in expression of 90-kDa or 100-kDa PKCμ mutants (Fig. 2B). A point mutation (K612W), creating a kinase deficient mutant, was also employed.

Btk PH-TH domain GST fusion proteins were used to precipitate PKCμ mutants from lysates of 293T cells transiently transfected with the respective expression constructs. As shown in Fig. 2C, PKCμ could be readily detected in all Btk precipitation analyses from 293T cells expressing wild-type PKCμ and PKCμ mutants. In contrast to binding of 14-3-3 proteins to the regulatory region [35], kinase activity

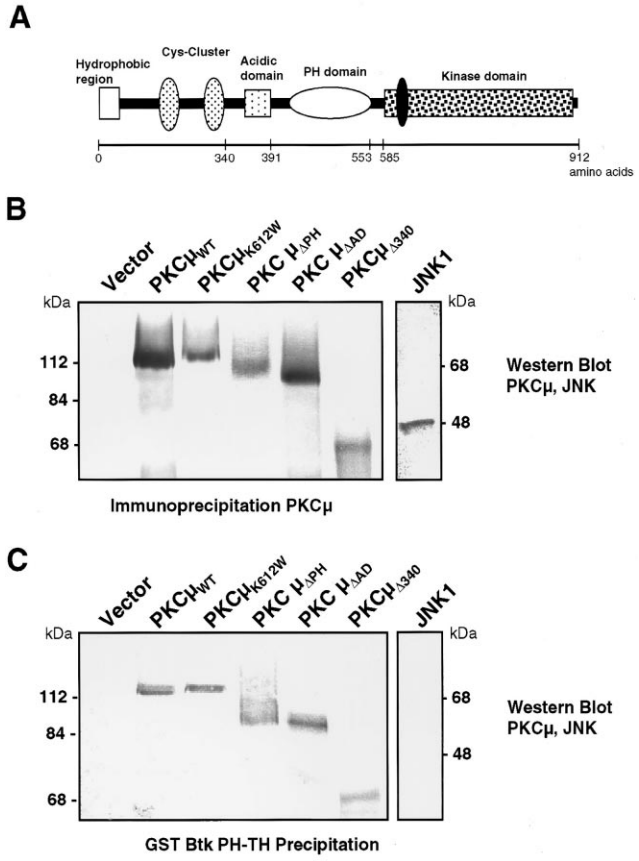

Fig. 2. Mapping of Btk binding domains in PKCμ. A: Schematic view of the known functional domains of PKCμ. B: Expression of PKCμ mutants. 293T cells were transfected with the indicated PKCμ mutants and detected by Western blot analysis using a carboxyl terminal rabbit antibody. C: Determination of the Btk PH-TH binding domain in PKCμ. A GST Btk PH-TH domain fusion protein was used to precipitate wild-type and the truncated PKCμ proteins upon transient overexpression in 293T cells. As a control FLAG-tagged JNK was transfected and analyzed in parallel. Expression was monitored using an anti-FLAG monoclonal antibody.

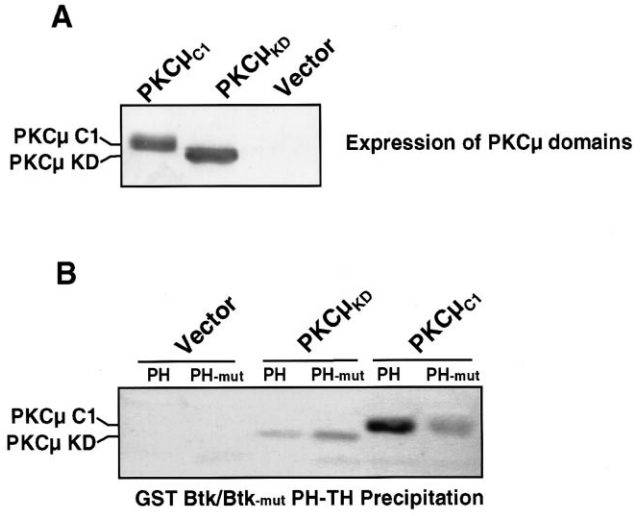

Fig. 3. BTK PH-TH GST fusion proteins bind to the kinase domain and the regulatory C1 region of PKCμ. A: Expression of PKCμ domains. The PKCμ kinase domain and the amino terminal region were transiently expressed in 293T cells. The kinase domain was detected using a carboxyl terminal antibody, the C1 domain with a polyclonal mouse antiserum. B: GST Btk PH-TH fusion proteins bind specific to the kinase domain and the regulatory region of PKCμ. Shown are precipitation analyses using the wild-type Btk PH-TH GST domain fusion protein (PH) or Btk carrying the R28P mutation (PH-mut). PKCμ was detected using a mix of the domain specific antibodies. As a control for specificity GST precipitations were included (data not shown).

was not essential to precipitate PKCμ by GST Btk PH-TH domain fusion proteins. The kinase deficient PKCμ mutant $PKC\mu_{K612W}$ was precipitated with similar efficacy like wild-type PKCμ (Fig. 2C, lower panel). FLAG-tagged JNK, which was also expressed at a significant level, was not precipitated and served as a control of specificity of GST Btk PH-TH pull down assay (Fig. 2B and C, right lanes).

In contrast to previous data identifying the C1 domain of phorbol ester responsive PKCs as the binding domain of the Btk PH domain [16], our findings using GST PH-TH domain fusion proteins point to a different association mode between Btk and PKCμ, involving its kinase domain too. To determine in detail which region of PKCμ mediates Btk PH-TH domain binding, the PKCμ C1 domain covering amino acids 1–327 including the cysteine rich zinc finger like structure and the kinase domain were expressed in 293T cells and analyzed for potential Btk binding by precipitation assays (Fig. 3A). As shown in Fig. 3B, the C1 region as well as the kinase domain of PKCμ could be precipitated by Btk GST PH-TH domain fusion proteins. No specific band was detectable by Western blot analysis in GST Btk TH-PH precipitates from 293T cells transfected with vector without insert (Fig. 3B, left lanes) or with the Syk tyrosine kinase as an additional control for specificity (data not shown). These data as well as the finding that JNK could not be precipitated by GST Btk-mut PH-TH fusion proteins (Fig. 2C) point to a specific interaction of PKCμ domains with Btk. The R28P mutation known to affect Btk function and binding to PKCs was analyzed for potential inhibition of PKCμ association. As shown in Fig. 3B, the R28P GST Btk PH-TH fusion protein did not significantly affect PKCμ kinase domain binding whereas the R28P fusion protein strongly reduces the precipitation efficacy of the PKCμ C1 domain (Fig. 3B, right lane). According to these data two independent binding sites of the Btk PH-TH domain can be postulated in PKCμ. These findings explain the partial inhibition of GST Btk-mut PH-TH domain binding shown in Fig. 1B, because only one of two binding sites is affected. The presence of two independent Btk PH-TH domain binding sites provides an explanation for the finding that in vitro precipitation of PKCμ by GST Btk PH-TH is only weakly competed by Pdbu containing phosphatidylserine micelles (data not shown), similarly as shown for other PKCs [16].

Although Btk is physically associated with PKCμ, the precise molecular mechanism of Btk PH-TH domain interaction with PKCμ and the physiological relevance of this interaction is currently unknown. cPKC mediated phosphorylation of Btk in vitro leads to reduced tyrosine autophosphorylation [15] and therefore has been suggested to negatively regulate Btk activity. Immunoprecipitates of Btk as well as GST PH-TH domain fusion proteins are no substrates for in vitro phosphorylation by recombinant PKCμ (F.J.J., unpublished observations) suggesting that PKCμ alone is not sufficient for effective serine phosphorylation of Btk. Vice versa, PKCμ is not considered as a substrate for Btk, as there is no significant tyrosine phosphorylation upon cellular stimulation (F.J.J., unpublished observations). As our data indicate no direct in vitro phosphorylation of either PKCμ or Btk, association mediated regulation of PKCμ and/or Btk kinase activity in vivo might be dependent on the presence of additional, currently unknown factors. Alternatively, PKCμ might serve as a scaffold protein generating a Btk activating signal complex. In favor of this hypothesis are the findings that the C1 domain of PKCμ serves as binding region for phosphatidylinositol 4-kinase and a phosphatidylinositol 4-phosphate 5-kinase [28] producing phosphatidylinositol phosphates which are involved in Btk membrane translocation which is a prerequisite for activation. Such a model would predict that the amino terminal region of Btk mediates PKCμ C1 domain association, whereas carboxyl terminal epitopes of the Btk PH-TH domain are responsible for constitutive association with the PKCμ kinase domain.

In B-lymphocytes, PKCμ is associated with the initial B-cell receptor signalling complex together with PLCγ1/2 and the tyrosine kinase Syk upon IgM stimulation [25]. As Btk is essential for PLCγ2 activation [36] a participation of Syk, Btk and PKCμ in the regulation of PLCγ triggered signalling pathways is conceivable. The role of PKCμ in the cellular signalling complex as a potential negative regulator of PLCγ [25] or regulator of Btk will be a subject to further research activities.

The demonstration of in vitro association of PKCμ with Btk points to a role in receptor proximal signalling events of lymphocyte antigen receptors. As, in addition to PKCμ, several other PKC isotypes also associate with Btk [16], they might be components of a partially redundant signalling system. This is underlined by the in vivo phenotype of the PKCß knock-out mouse, showing a similar, but milder immunodeficiency syndrome as compared to the Btk defective xid mouse [6,37]. It is therefore possible that the defects in signal transduction pathways involving PKCß are compensated in the PKCβ knock out mouse by other lymphocyte expressed PKC isotypes, probably including PKCμ.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Grant no. Jo227/4-3.

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