Brief Definitive Report

The Protein Product of the c-*cbl* Protooncogene Is Phosphorylated after B Cell Receptor Stimulation and Binds the SH3 Domain of Bruton's Tyrosine Kinase

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Summary

X-linked agammaglobulinemia, a B cell immunodeficiency, is caused by mutations in the Bruton's tyrosine kinase (*Btk*) gene. The absence of a functional Btk protein leads to a failure of B cell differentiation and antibody production. B cell receptor stimulation leads to the phosphorylation of the Btk protein and it is, therefore, likely that Btk is involved in B cell receptor signaling. As a nonreceptor tyrosine kinase, Btk is likely to interact with several proteins within the context of a signal transduction pathway. To understand such interactions, we have generated glutathione *S*-transferase fusion proteins corresponding to different domains of the human Btk protein. We have identified a 120-kD protein present in human B cells as being bound by the SH3 domain of Btk and which, after B cell receptor stimulation, is one of the major substrates of tyrosine phosphorylation. We have shown that this 120-kD protein is the protein product of c-*cbl*, a protooncogene, which is known to be phosphorylated in response to T cell receptor stimulation and to interact with several other tyrosine kinases. Association of the SH3 domain of Btk with $p120^{cbl}$ provides evidence for an analogous role for $p120^{cbl}$ in B cell signaling pathways. The $p120^{cbl}$ protein is the first identified ligand of the Btk SH3 domain.

X-linked agammaglobulinemia (XLA) patients have mutations in the Bruton's tyrosine kinase (*Btk*) gene (1–3) and consequently suffer from a lack of mature B cells in their peripheral blood. The identification of Btk as a member of the nonreceptor tyrosine kinase family, a family of proteins already established as being involved in hematopoietic signal transduction, fits well with the disease phenotype of XLA. The Btk protein is expressed in early and mature human B cell lines but is absent in terminally differentiated plasma cell lines, which is consistent with the requirement of a functional Btk protein for normal B cell differentiation (4–6). There is now evidence from a variety of sources to suggest that Btk is one of the many tyrosine kinases involved in B cell receptor (BCR) signal transduction. Stimulation of the BCR has been shown to lead to the tyrosine phosphorylation of Btk and an increase in its kinase activity (7–10).

The Btk protein contains Src homology (SH) regions 2 and 3, which are noncatalytic domains present in a wide variety of signal transduction molecules (11). Analysis of Src-related tyrosine kinases suggests that these domains are involved in intermolecular recognition and the formation of heteromeric protein complexes. Signal transduction pathways are likely to be controlled by the formation of these protein complexes (12). SH2 domains recognize tyrosine residues that have been phosphorylated by activated tyrosine kinases (13). Deletion analysis has shown that the SH3 domains of Grb2 and phospholipase C-γ are required for cellular localization (14). SH3 domains mediate protein–protein interactions via recognition of specific proline-rich peptide sequences (15, 16) and to date, although the sequence PXXP is always present, most SH3 domain ligands contain multiple prolines. Using a yeast hybrid trapping method and a fusion protein system, the SH3 domains of Fyn, Lyn, and Hck, Src-related tyrosine kinases known to be involved in signal transduction pathways in B cells, have been shown to bind to proline rich motifs present in the Btk protein (17). The association between Btk and these tyrosine kinases also implicates Btk in B cell signaling pathways.

The protein targets of the SH2 and SH3 domains of Btk are unknown. To address this problem, we have made glutathione *S*-transferase (GST) fusion protein constructs containing the human Btk SH2 and SH3 domains. Using these fusion proteins, we have identified several polypeptides in human B cells that are bound by the SH3 domain of Btk in vitro.

The protein product of c-*cbl* ($p120^{cbl}$) is present in early B lineage and myeloid cells (18), and the SH3 domains from

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a large number of proteins, including those of Fyn, Grb2, Lck, Fgr, Nck, and PLCγ1, have already been shown to bind p120^{cbl} (19, 20). The p120^{cbl} protein therefore appeared to be a good candidate for one of the polypeptides bound by the SH3 domain of Btk and we show here that this is indeed the case. The p120^{cbl} protein is also expressed in T cells and is rapidly phosphorylated after stimulation of the TCR; consequently, it is thought to be involved in this signaling pathway (19). We have found that p120^{cbl} is rapidly phosphorylated after stimulation of the BCR, suggesting that it is also involved in the BCR signaling pathway.

Materials and Methods

GST Fusion Proteins. The human *Btk* cDNA was used to amplify the region encoding the SH3 domain from nucleotides 787–951, including all of the designated SH3 domain (1) with five additional amino acids at the COOH-terminal end, by PCR using the primers SH3F (5′-CTGAGGATCCGTTGTGGCCCTTTATGATTAC) and SH3R (5′-TATCAGAATTCTGCTTCAGTGACATAGTTACTAG) at 58°C with Taq polymerase (Biopro™; Bioline, London, UK) according to the manufacturer's instructions. The PCR product was cloned into the expression vector pGEX-2T (Pharmacia Biotech, Uppsala, Sweden) and the insert was sequenced to ensure that no PCR mutations had been included. The fusion protein was induced and purified as described (21) using 0.75 ml glutathione Sepharose (GS) beads (Pharmacia Biotech) with 800 ml of bacterial culture. The protein concentration was estimated after SDS-PAGE and staining with Coomassie blue.

Cell Lines and Cell Lysis. A human Burkitt's lymphoma B cell line, Daudi, and a human T cell line, Molt-4, were grown in RPMI 1640, 10% FCS, 1.5 μg/ml gentamycin at 37°C to a density of 0.5–1.0 × 10^6/ml. Pelleted cells were washed in prewarmed RPMI 1640 and either lysed immediately or stimulated by incubation with goat F(ab′)$_2$ fragment to human IgM (Organon Teknika, Durham, NC). 1–5 × 10^7 cells were lysed in 1 ml of 1% NP40, 20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1 mM DTT, 10 mM NaF, 1% aprotinin, 20 μM leupeptin, 100 μM sodium orthovanadate, 1 mM PMSF, at 4°C for 10 min. Lysates were clarified using centrifugation and incubated overnight at 4°C with 12 μg fusion protein (attached to GS beads), or with 10 μl Agarose-conjugated anti-phosphotyrosine mAb (4G10; Upstate Biotechnology, Inc., Lake Placid, NY) per ml of cell lysate. The immobilized proteins were pelleted from the cell lysate, using a 15-s centrifugation at 6,000 rpm in a microfuge, and washed five times with cold lysis buffer and once with PBS. The immobilized proteins were resolved in reducing conditions by SDS-PAGE.

Western Analysis. The electrophoresed proteins were transferred onto Hybond C nylon membranes (Amersham International, Amersham, UK) using a semidry blotter. The membranes were blocked with 5% nonfat milk/PBS for 1 h at room temperature, then rinsed briefly twice in 0.05% Tween-20/PBS (PBS-T). The membranes were then incubated in a 1:100 dilution of anti–c-*cbl* antiserum (C-15; Santa Cruz Biotechnology Inc., Santa Cruz, CA) according to the manufacturers instructions. A 1/1,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti–rabbit IgG antiserum was used as a second layer. The antigen/antibody reactions were visualized using the enhanced chemiluminescence detection system (Amersham) with exposure time periods ranging from 30 s to 20 min. Tyrosine phosphorylation analysis was performed as described above using the mouse monoclonal 4G10 antiserum, with the incubation in 0.5% nonfat milk/PBS-T and using HRP-conjugated rabbit anti–mouse IgG2b antiserum as a second layer.

Metabolic Labeling. The cells were washed twice in methionine- and cysteine-free RPMI 1640 medium, suspended at 10^7 cells per ml in this media. [^{35}S]methionine/cysteine (1 mCi/5 × 10^8 cells, Trans-label; ICN, UK) was added and the cells incubated at 37°C for 4 h. The cells were washed twice with RPMI 1640 and then lysed as described above. The metabolically radiolabeled proteins were separated in reducing conditions by SDS-PAGE and were visualized by autoradiography.

Results and Discussion

To identify proteins that are associated with the *Btk* gene product, we produced the human Btk SH3 domain as a GST fusion protein (SH3-GST) and used it to immobilize polypeptide ligands from human B cell lysates. Metabolically radiolabeled Daudi cells were solubilized in lysis buffer and the clarified supernatants were incubated with SH3-GST immobilized to glutathione-Sepharose. The samples were washed, and the bound proteins were separated by SDS-PAGE and then autoradiographed. A large number of polypeptides were bound by SH3-GST but not by GST alone, notably those with apparent molecular masses of ~50, 63, 65, and 120 kD (Fig. 1 *a*). The extent of tyrosine phosphorylation of these polypeptides was then determined by stimulating Daudi cells through the BCR for a range of time periods before cell lysis and incubation with SH3-GST. The immobilized samples were immunoblotted with an antiphosphotyrosine mAb. Stimulation through the BCR led to tyrosine phosphorylation of at least two of the Btk SH3 ligands that were not phosphorylated in unstimulated cells (Fig. 1 *b*). In particular, a 120-kD polypeptide showed a very high level of tyrosine phosphorylation after stimulation of Daudi cells (Fig. 1 *b*).

The product of the c-*cbl* protooncogene is expressed in early B lineage, B and T cell lines, and myeloid cells (18) and, since the SH3 domains from several proteins have already been shown to bind p120^{cbl} (19, 20), this protein appeared to be a good candidate for the 120-kD polypeptide bound by the SH3 domain of Btk. This was confirmed by incubating lysates of metabolically radiolabeled B and T cell lines with SH3-GST and, after autoradiography, immunoblotting the immobilized proteins with anti–c-*cbl* antibody. In both cell types, p120^{cbl} was clearly seen to be one of the major proteins bound by the SH3-GST (Fig. 2). Other candidates for the phosphorylated 120-kD polypeptide included PLCγ1, as well as GAP and PI-3 kinase p110. Immunoblotting with the appropriate antsera showed that none of these proteins were bound by SH3-GST (results not shown).

The binding of p120^{cbl} to SH3 domains of other proteins has been shown to be via the COOH-terminal region of the protein, which includes a large proline-rich region (20). SH3 domains have been shown to bind the PXXP motif (22), and p120^{cbl} contains 17 of these motifs. Further characterization of SH3 ligand binding has shown that additional prolines are important in stabilizing the left-handed type II polyproline helix conformation bound by the SH3 domain (16, 22, 23), and almost all of the PXXP motifs within p120^{cbl} can

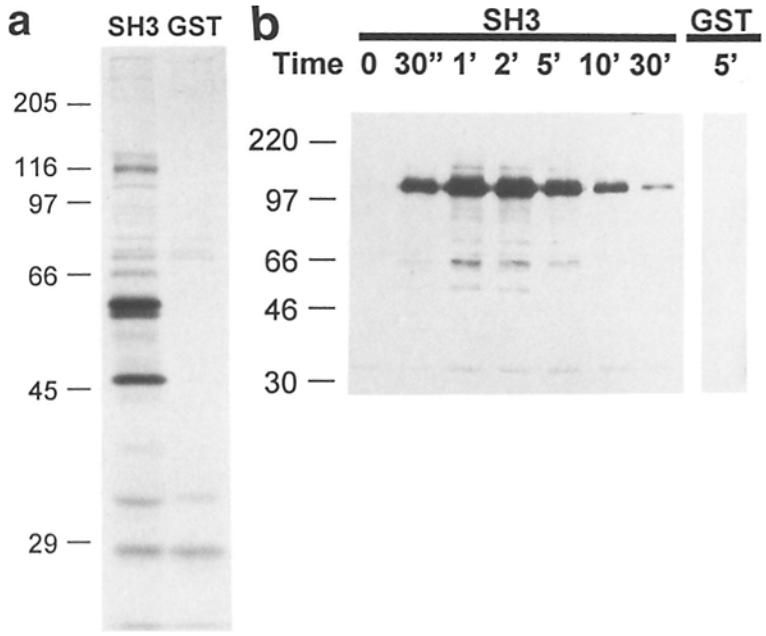

Figure 1. The Btk SH3 domain binds several B cell proteins in vitro. (*a*) Daudi cell lysates radiolabeled with [^{35}S]methionine/cysteine, were incubated with SH3-GST or GST (see Materials and Methods). Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. (*b*) Daudi cells were stimulated through the BCR for a range of time periods before lysis and incubation with SH3-GST and GST proteins. Bound proteins were visualized by immunoblotting with antiphosphotyrosine mAb. The molecular mass markers used are shown in kilodaltons.

be extended to motifs consisting of three or more proline residues. The proline-rich region of p120^{cbl} therefore provides a large number of potential binding sites for the SH3 domains of Btk and other proteins. The SH2 domains of Fyn, Lck, Blk, and other proteins have also been found to bind to p120^{cbl}; however, these associations depend on the phosphorylation of the p120^{cbl} protein (19). We found no evidence for any association between the SH2 domain of Btk and the p120^{cbl} protein (results not shown).

The p120^{cbl} expressed in T cells is rapidly phosphorylated after stimulation of the TCR and, consequently, it is thought to be involved in this signaling pathway (19). To address the possibility that p120^{cbl} performs a similar role in BCR signaling, BCR-stimulated Daudi cells were lysed and tyrosine-phosphorylated proteins were immunoprecipitated with Agarose-conjugated antiphosphotyrosine mAb and immunoblotted with anti–c-*cbl* Ab (Fig. 3). The p120^{cbl} was immunoprecipitated with antiphosphotyrosine mAb within 10 s of BCR stimulation, and this high level of phosphorylation was maintained for 2 min. The extent of phosphorylation of this 120-kD polypeptide was markedly reduced after 30 min (Fig. 3). The phosphorylation of p120^{cbl} was also shown by immunoprecipitating p120^{cbl} from whole-cell lysate with anti–c-*cbl* Ab followed by immunoblotting with antiphosphotyrosine mAb (data not shown). We have obtained evidence that p120^{cbl} is one of the major substrates for tyrosine phosphorylation detected after BCR stimulation. Whole-cell lysates depleted of Btk SH3 domain ligands by SH3-GST precipitation were immunoblotted with anti–c-*cbl* antibody and antiphosphotyrosine mAb. Depletion of p120^{cbl} from

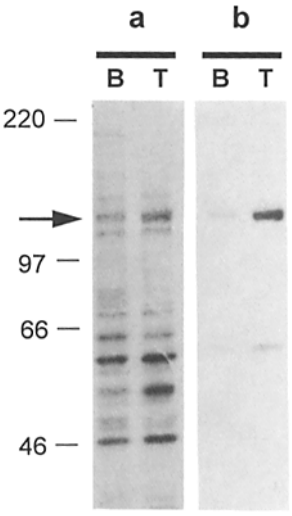

Figure 2. The SH3 domain of Btk binds p120^{cbl} in vitro. Daudi cell lysates, B cells (*B*) and Molt-4 cell lysates, T cells (*T*) radiolabeled with [^{35}S]methionine/cysteine were incubated with SH3-GST. (*a*) Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. The p120^{cbl} protein is indicated by an arrow. (*b*) The proteins bound by the immobilized fusion protein were subsequently analyzed by immunoblotting with anti–c-*cbl* antibody. This antibody recognizes the p120^{cbl} protein and a 60-kD protein. The molecular mass markers used are shown in kilodaltons.

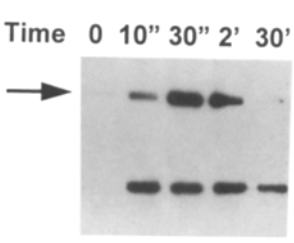

Figure 3. p120^{cbl} is rapidly phosphorylated on tyrosine after BCR stimulation. Daudi cells stimulated through the BCR for a range of time periods were lysed and incubated with Agarose-conjugated antiphosphotyrosine mAb. The immunoprecipitated proteins were analyzed by immunoblotting with the anti–c-*cbl* antibody. The p120^{cbl} protein is indicated by an arrow. The molecular mass markers used are shown in kilodaltons.

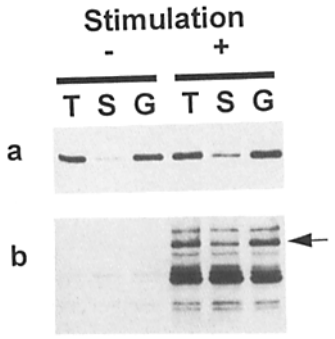

Figure 4. Phosphorylated p120^{cbl} is depleted by binding to the SH3 domain of Btk after BCR stimulation. BCR-stimulated and unstimulated Daudi cells were lysed, incubated with either SH3-GST or GST, and the subsequent depleted lysates were analyzed by immunoblotting with anti-c-*cbl* antibody (*a*) and antiphosphotyrosine mAb (*b*). Track T is total cell lysate; S is cell lysate depleted of proteins that bind to the Btk SH3 domain; G is cell lysate depleted of proteins binding to the GST protein. In *b*, the p120^{cbl} protein is indicated by an arrow.

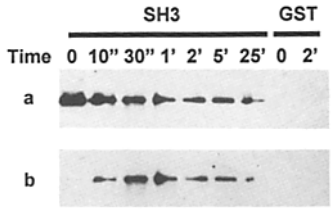

Figure 5. The amount of p120^{cbl} protein bound in vitro by the Btk SH3 domain decreases after BCR stimulation. Daudi cells were stimulated through the BCR for a range of time periods, lysed, and incubated with GST fusion proteins. The proteins bound by the immobilized fusion protein were analyzed by immunoblotting with the anti-c-*cbl* antibody (*a*) and the antiphosphotyrosine mAb (*b*). To confirm that equivalent amounts of SH3-GST were used in each sample the lower part of the SDS-PAGE gel was stained with Coomassie blue (results not shown).

whole-cell lysates by the Btk SH3 domain was seen clearly in both unstimulated and stimulated cells, when compared to lysates after incubation with the GST protein (Fig. 4 *a*). The depletion by the SH3-GST of one of the major substrates for tyrosine phosphorylation was also observed (Fig. 4 *b*); this substrate migrates to the same position as p120^{cbl} (Fig. 4, *a* and *b*).

Thus, p120^{cbl} is rapidly phosphorylated after stimulation of the BCR in a manner analogous to that observed for p120^{cbl} in T cells after stimulation through the TCR (19), suggesting that p120^{cbl} plays a role in BCR signaling. This provides yet another example of the similarities between TCR and BCR signaling pathways (24, 25). Previous observations have suggested that regulation of p120^{cbl} phosphorylation is necessary to ensure controlled cell growth (26). Mutations within p120^{cbl} were found to induce tyrosine phosphorylation of p120^{cbl} and also to promote transformation of NIH 3T3 fibroblasts and tumor formation in mice (26). The phosphorylation of p120^{cbl} after BCR and TCR activation may link these receptors to signaling pathways, which results in the controlled cell growth of B and T cells.

The amount of p120^{cbl} protein bound in vitro by the Btk SH3 domain was repeatedly found to decrease after BCR stimulation (Fig. 5 *a*). To ensure that the amount of p120^{cbl} protein present in the cell lysate did not decrease after BCR stimulation, we immunoblotted the whole cell lysates, before incubation with the fusion protein, with anti-c-*cbl* antibody (results not shown) and found no detectable difference in the available p120^{cbl} protein at the various time points after BCR stimulation. The decrease in p120^{cbl} binding to SH3-GST after BCR stimulation may result from a change in the structure of the protein or from a change in the proteins associated with p120^{cbl} in vivo. Alternatively, the phosphorylation of p120^{cbl} observed after BCR stimulation (Fig. 5 *b*) may directly affect the ability of the SH3 domain to bind. The NH_2-terminal Grb2 SH3 domain showed a similar decrease in its binding of p120^{cbl} after activation of T cells (19). We cannot discount the possibility that other proteins present in the cell lysate are mediating the interaction between GST-SH3 and p120^{cbl}.

The protooncogene c-*cbl* is expressed in a large number of hematopoietic lineages; however, in vivo the oncogenic form, v-*cbl*, is mainly transforming in early B cell lineages and myeloid cells (27). The expression of Btk is limited to B cell and myeloid cell lineages and the presence of a functional Btk protein in early B cell lineages is known to be essential for the continued differentiation of these cells. The association between p120^{cbl} and the Btk SH3 domain in vitro may have implications for the signaling pathways in mature B cells and also perhaps those in early B cell lineages and myeloid lineages.

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