The Protein Product of the Proto-oncogene c-cbl Forms a Complex With Phosphatidylinositol 3-Kinase p85 and CD19 in Anti-IgM-Stimulated Human B-Lymphoma Cells

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Multiple signal transduction cascades, consisting of multiple interacting proteins, are activated following stimulation through most cell surface receptors, including the immunoglobulin receptor of B lymphocytes. In this report, we investigated the multimolecular complexes formed following anti-Ig stimulation of a human B-lymphoma cell line, resulting in activation of phosphatidylinositol 3-kinase (PI3K). PI3K is a lipid kinase that consists of an 85-kD regulatory subunit, bound to a 110-kD catalytic subunit. CD19 is a 95-kD B-cell surface marker that contains a consensus binding motif for PI3Kp85 in the cytoplasmic domain and recruits PI3K activity in activated B cells. The protein product of the c-cbl proto-oncogene is a 120-kD protein that is expressed in early B-lineage cells and in myeloid cells and is phosphorylated on tyrosine following receptor-mediated signaling in T and B lymphocytes. We demonstrate here that phosphorylated c-cbl complexes with CD18 and with PI3Kp85 via its C-terminal SH2 domain, and that both c-cbl and CD19 are associated with active PI3K in anti-Ig-stimulated cells. Although we cannot differentiate between a three-component, c-cbl/CD19/PI3Kp85 complex and individual two-component complexes, these studies suggest that c-cbl may function as a docking protein, possibly linking distinct signal transduction pathways.

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TIMULATION THROUGH the immunoglobulin (Ig) receptor on B lymphocytes activates many different signaling pathways. Initial studies elucidated a role for phospholipase C-γ (PLC-γ)-mediated cleavage of membrane phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate, resulting in the activation of protein kinase C (PKC) and the release of intracellular Ca++, respectively. This pathway was also shown to be dependent on the activation of tyrosine kinases. Several investigators demonstrated a potential role for classical G proteins while more recent evidence has clearly shown that a pathway including p21ras is also activated. One pathway that has recently become an area of intense investigation is that involving the lipid kinase, phosphatidylinositol 3-kinase (PI3K) (reviewed in Kapeller). This enzyme is comprised of an 85-kD regulatory subunit bound to a 110-kD catalytic subunit, and its primary function is to catalyze the phosphorylation of inositol lipids on the D-3 hydroxyl position. Activation of PI3K has been shown to occur following ligation of many growth factor receptors containing intrinsic tyrosine kinase activity, as well as receptors associated with src-like tyrosine kinases, such as the antigen receptors on T and B lymphocytes and costimulatory receptors CD28 and CD19. There is increasing evidence that "crosstalk" occurs among these different pathways largely through protein-protein interactions.

CD19 is a 95-kD protein expressed on B lymphocytes from the pre-B stage until just before terminal differentiation into plasma cells (reviewed in Fearon). It is usually found in a complex with several other B-cell surface proteins including CD21, CD81, and Leu13. Furthermore, cross-linking CD19 simultaneously with surface IgM has been shown to augment Ig-mediated responses, including activation of PI3K. Stimulation of B cells through the Ig receptor results in tyrosine phosphorylation of CD19 and is thought to recruit PI3K to the complex via its binding to CD19. CD19 has also been shown to form complexes with the src-like kinases lyn and fyn. Thus, CD19 appears to play a role in bringing other signaling molecules into close proximity with the Ig receptor to amplify or diversify the response.

Many of the multimolecular complexes formed in activated cells occur via binding of src homology 2 (SH2) domains to phosphorylated tyrosine residues, or binding of src homology 3 (SH3) domains to proline-rich regions in target proteins. Both lyn and PI3Kp85 bind to the tyrosine phosphorylated cytoplasmic region of CD19. Another protein that has been found to associate with PI3Kp85 in Jurkat T cells and with btk in B cells, is the proto-oncogene c-cbl. The 120-kD c-cbl protein is expressed in lymphoid and myeloid cells, and has been shown to be phosphorylated on tyrosine after activation of Tp and B lymphocytes. The viral oncogene v-cbl is formed by the truncation of more than 50% of the COOH terminus of c-cbl, thus producing a protein that localizes to the nucleus, binds DNA and becomes transforming in early B-lineage and myeloid cells. There is no known function of c-cbl; however, it contains 17 proline-rich regions for potential SH3 binding and multiple tyrosine residues, some of which contain SH2-binding motifs. Thus, it may be an important docking molecule in signal transduction cascades.

We have been investigating anti-Ig-mediated growth inhibition of human B-lymphoma cells, and have previously demonstrated that tyrosine phosphorylation and activation of PI3K are both critical to this response. In this report, we examine molecular complexes involved in PI3K activation following anti-Ig stimulation. We demonstrate that c-cbl,
PI3Kp85, and CD19 are all phosphorylated on tyrosine following activation. In addition, we show that c-bcl forms a complex with CD19 and with the C-terminal SH2 domain of p85. Thus, both c-bcl and CD19 are involved in recruiting active PI3K to the Ig receptor complex.

MATERIALS AND METHODS

Cell lines and reagents. The RL cell line was grown from the ascites of a patient with diffuse large cell lymphoma. It is an IgM-, IgD-, B-cell line that has been shown by Southern blot analysis to be Epstein-Barr virus (EBV) genome negative and has been described previously. The cells are passed twice weekly in RPMI 1640 (Bio-Whittaker, Walkersville, MD), containing 2 mmol/L L-glutamine, 1,000 U/mL penicillin, and 100 μg/mL streptomycin, and 10% fetal calf serum (FCS), all obtained from GIBCO (Grand Island, NY), with no other growth factors added. Normal rabbit IgG (Rγγ) and rabbit anti-human IgM (RαIgM) conjugated to acrylamide beads were obtained from Irvine Scientific (Santa Ana, CA). Rabbit anti-cbl antiserum was obtained from Santa Cruz (Santa Cruz, CA). Anti-PI3K p85 monoclonal antibody (MoAb) and polyclonal rabbit antiserum, and the glutathione S-transferase (GST) fusion proteins of the N-terminal and C-terminal SH2 domains of PI3K p85 were all purchased from UBI (Lake Placid, NY). Anti-CD19 antibodies were purchased from Immunotech, Inc (CD19A, Westbrook, ME), Accurate Chemical and Scientific Corp (CD19B, Westbrook, NY), and Cappel Organon Teknika (CD19C, Durham, NC).

Immunoprecipitation and Western blotting. RL cells were incubated with Rγγ or RaIgM beads for 5 to 10 minutes, pelleted, and lysed in buffer containing 20 mmol/L Tris, 2 mmol/L EDTA, 1% NP40, 137 mmol/L NaCl, and 10% glycerol, with aprotinin (2 μg/mL), PMSF (100 μmol/L), leupeptin (5 μg/mL), and Na2VO4 (1 mmol/L) added immediately before lysing. Lysates were incubated on ice for 15 to 30 minutes, and cleared by spinning 12,000g in a microcentrifuge for 30 minutes at 4°C. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce Chemical Co, Rockford, IL), and 50 μg/lane were used for straight immunoblotting. For immunoprecipitations, 200 μg to 500 μg (approximately 2 to 5 × 106 cell equivalents, respectively) of lysate were added to eppendorf tubes containing Protein A Sepharose (Pharmacia LKB Biotech, Piscataway, NJ) and 10 μL of μL of appropriate anti serum. Tubes were rotated 2 hours to overnight, and pellets were washed three times in lysis buffer, and one time in 10 mmol/L Tris containing 0.1% NP40. For precipitations using the GST-SH2 domain-agarose conjugates, 500 μg of lysate were first precleared with protein A sepharose, rotated at 4°C with 5 μL of the conjugate, plus protein A sepharose as a carrier, and washed three times in dH2O. Electrophoresis sample buffer was added to the washed pellets, samples were boiled for 10 minutes, and run on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini-gels (Novex, Encinitas, CA). Gels were transferred to Hybond-ECL nitrocellulose (Amersham Corp, Arlington Heights, IL), and blocked for 1 hour in Tris-buffered saline containing 0.1% Tween-20 (TBST), 5% powdered milk, and 5% normal goat serum (TBST/MB/NGS). Primary antibodies were added, and blots were incubated for 2 hours at room temperature. After washing in TBST, horse-radish peroxidase-linked secondary antibodies were added and the blots were washed in TBST/MB/NGS. Blots were then washed in TBST and developed using enhanced chemiluminescent (ECL) kits from Amersham.

p13 kinase assays. Assays for PI3K activity were performed as previously described. Immunoprecipitates were prepared from control or anti-Ig-stimulated RL cells exactly as described above. They were washed three times in lysis buffer, and three times in 10 mmol/L Tris, pH 7.4. A total of 10 μL of sonicated PI substrate (Avanti Polar Lipids, Inc, Alabaster, AL) were added to each sample and they were incubated for 10 minutes on ice. Forty microliters of kinase buffer containing 30 mmol/L Hepes pH 7.4, 3.0 mmol/L MgCl2, 50 μmol/L ATP, and 200 μmol/L adenosine were added, followed by 10 μCi γ-32P ATP [3,000 Ci/mmol] (DuPont Co, NEN, Boston, MA). Samples were incubated for 20 minutes at room temperature, and the reaction was ended with 100 μL 1 mol/L HCl. Phospholipids were extracted with 200 μL chloroform/methanol (1:1). Equal volume aliquots from the bottom chloroform layer were spotted onto thin layer chromatography plates (MCB reagents, Merck & Co, Inc, Gibbstown, NJ), and developed in chloroform/methanol/dH2O/ammonium hydroxide (45:35:7.5:2.5). Plates were dried briefly and placed with X-ray film (Eastman Kodak, Rochester, NY) for 1 to 7 days at -70°C.

RESULTS AND DISCUSSION

We previously demonstrated that anti-Ig stimulation of the RL human B lymphoma cell line resulted in tyrosine phosphorylation of multiple proteins, one of which was the 85-kD subunit of PI3K. Furthermore, we showed that both tyrosine phosphorylation and activation of PI3K were critical to the ensuing growth inhibition of anti-Ig-treated cells. In this report, we add to those findings by examining the complex of proteins that are involved in PI3K activation following anti-Ig stimulation. The cytoplasmic region of CD19 contains a consensus YXXM motif, which on activation, becomes phosphorylated and binds to the p85 SH2 domains, thus recruiting active PI3K to the Ig receptor complex.

We next examined the ability of these proteins to associate with each other in activated cells. When lysates from control or anti-Ig-treated RL cells were immunoprecipitated with anti-p85 and p85 for anti-Ig-induced tyrosine phosphorylation (Fig 1). RL cells were stimulated with control or anti-Ig conjugated to acrylamide beads for 10 minutes at 37°C. Lysates were immunoprecipitated with anti-p85 and anti-p85 (Fig 1A), or three different anti-CD19 antibodies (Fig 1C), run on 10% SDS-PAGE mini-gels and transferred to nitrocellulose. Blots were probed with anti-PTyr and clearly identified phosphorylated bands of the correct molecular weights for c-bcl (120 kD), PI3Kp85 (85 kD), and CD19 (95 kD) in lysates from the anti-Ig, but not control, treated cells. The anti-cbl and anti-p85 blots were then reprobed with the immunoprecipitating antibody, demonstrating that equal amounts of protein were evaluated. These data demonstrate that all three proteins are major substrates of tyrosine phosphorylation following anti-Ig treatment of RL cells.
amounts of immunoprecipitated protein. In Fig 2C, we demonstrate that CD19 and PI3Kp85 also associate in anti-Ig-activated RL cells. Finally, immunoprecipitates were made using three different anti-CD19 antibodies, anti-PI3Kp85, anti-Class II major histocompatibility complex (MHC), or mouse IgGl, and analyzed by Western blotting (Fig 2D). The blots were probed with anti-cbl, the 120-kD c-cbl product was identified in the anti-p85 lane, and in each anti-CD19 immunoprecipitate, but not with the mouse IgGl or anti-Class II MHC antibodies. As had been noted by Donavan et al, we also observed an unidentified 60 kD protein with the anti-cbl antisera. None of the anti-CD19 antibodies was able to immunoblot, so the reverse of this experiment could not be performed. Meisner et al have demonstrated that c-cbl forms separate complexes with PI3Kp85 and the adaptor protein Grb2. These data suggest that c-cbl forms a link between the ras signaling pathway and the PI3K pathway in activated T cells. In B cells, ras has been shown to interact with the Ig receptor and to be activated following Ig ligation, and coligation of CD19 with surface Ig has been shown to augment membrane translocation of a multimeric Shc/Grb2 complex. Furthermore, Weng et al demonstrated that signaling through CD19 in human B-cell precursors results in complexes between vav, p85, and CD19, suggesting activation of both the ras/MapK pathway and the PI3K pathway. It will be interesting to determine if c-cbl is a member of the Grb2/Sos/Shc complex involved in regulation of ras in activated B cells. Our data showing c-cbl complexed with CD19 and with PI3Kp85 combine with the above-mentioned studies to demonstrate that c-cbl may act

Fig 1. Anti-Ig stimulation of RL cells leads to phosphorylation on tyrosine of c-cbl, PI3Kp85, and CD19. RL cells were incubated with control beads (CBI) or anti-IgM beads (μB) for 10 minutes at 37°C. Lysates (500 μg) were immunoprecipitated with anti-cbl, anti-p85, or various anti-CD19 antibodies with protein-A sepharose overnight at 4°C and run on 10% SDS mini-gels. Immunoblots were prepared and probed with anti-PTyr, anti-cbl, or anti-p85 as indicated. These results are representative of at least three separate experiments.

Fig 2. Anti-Ig stimulation of RL cells results in multimolecular complexes containing CD19, c-cbl, and PI3Kp85. Lysates and immunoprecipitates were prepared from control bead (CBI) or anti-IgM bead (μB)-treated RL cells as described above. The antibodies used to perform the immunoprecipitations (IP) are indicated above the figure, and the blotting antibodies (WB) are shown below. In panel C, anti-CD19C was used in the immunoprecipitation. These results are representative of at least three separate experiments.
as a docking or bridging protein among multiple signal transduction pathways. It may function in a three-way complex with CD19 and p85 to bring other molecules to the complex, perhaps as regulators of CD19-mediated PI3K activation. Alternatively, it may form separate complexes with p85, thus linking PI3K activation with another pathway, such as the ras/MapK pathway. Whether it has some other function, perhaps as a transcription factor, remains to be elucidated.

The c-cbl protein has multiple sites available for binding to other proteins via their SH2 or SH3 domains. It has been shown to bind to the SH3 domains of several proteins, including btk, Nck, p55, PLCγ, and others. In addition, it has been shown to complex with both the SH2 and SH3 domains of Grb2 in T cells. Furthermore, it contains two YXXM motifs as putative binding sites for the PI3Kp85 SH2 domains. Therefore, we investigated the ability of GST-fusion proteins containing the SH2 domains of PI3Kp85 to bind to c-cbl in anti-Ig-activated RL cells (Fig 3). Lysates from control or anti-Ig-treated cells were incubated with a GST-agarose control or the GST-fusion protein containing the C-terminal SH2 domain of PI3Kp85 bound to agarose (I). The associated complexes were resolved on SDS-PAGE gels and transferred to nitrocellulose. Blots were probed with anti-PTyr (Fig 3, panels A and B, left), or anti-cbl (panels A and B, right). It is clear that multiple phosphorylated proteins bound to the p85 C-terminal SH2 domain, but not to the GST-agarose control, and one of these was identified as p120 c-cbl. Current studies are underway.

Fig 3. The protein product of the c-cbl proto-oncogene binds to the C-terminal SH2 domain of PI3Kp85. Lysates of control (CB) or anti-Ig-treated (μB) RL cells were prepared as described above. Lysates were precleared with protein-A sepharose, and incubated overnight with GST-agarose (II) or the GST-SH2-agarose conjugate (I) plus protein-A sepharose as a carrier. Precipitates were washed three times with PBS, boiled with SDS sample buffer and run on 10% SDS-PAGE mini-gels. Blots were probed with anti-PTyr (panels A and B, left) or with anti-cbl (panels A and B, right). These results are representative of three separate experiments.

Fig 4. Immunocomplexes of c-cbl and CD19 obtained from anti-lg-stimulated RL cells contain increased PI3K activity. Lysates of control or anti-lg-treated RL cells were prepared as described above. Immunoprecipitates made by overnight incubation with the indicated antibodies were subjected to PI3K assays as described in Materials and Methods. PI-3P is the product of phosphorylated PI and is indicated on the figure. These results are representative of three separate experiments.
to identify the other associated phosphoproteins, including the 77-kd and 95-kd bands. Similar experiments were done using the N-terminal SH2 domain GST-fusion protein; however, the results were difficult to interpret due to an extremely high background with the rabbit anti-cbl antisera. Our data demonstrate that c-cbl binds to the C-terminal SH2 domain of PI3Kp85 in activated RL cells, but do not rule out the possibility that binding via the p85 N-terminal SH2 domain, or the p85 SH3 domain, could also occur.

We next performed PI3K assays to determine if immunocomplexes containing c-cbl and CD19 exhibited increased PI3K activity in anti-Ig-activated RL cells (Fig. 4). Lysates from control or anti-Ig-treated RL cells were immunoprecipitated with anti-PTyr, anti-cbl, anti-CD19B or anti-CD19C, and PI3K assays were performed as previously described. 24 It is clear that both anti-cbl and anti-CD19 were able to bring down significant levels of PI3K activity in the anti-Ig-treated, but not the control bead-treated cells, demonstrating that both c-cbl and CD19 are involved in recruiting active PI3K to the Ig receptor complex. Current data demonstrate that subunits of PI3K are members of protein families and many different functions have been described for the products of various family members, including activation of PKC, 20 activation of S6 ribosomal kinase, 21 activation of the protein kinase encoded by the Ak receptor oncogene, and protein trafficking and sorting. 22 Taken together, our data show that anti-Ig stimulation of human B-lymphoma cells results in tyrosine phosphorylation of multiple proteins including c-cbl, CD19, and PI3Kp85, formation of multimolecular complexes containing c-cbl and CD19 in association with PI3Kp85, and recruitment of active PI3K to the complex. All of these events appear to be critical for receptor-mediated growth inhibition, and we are currently investigating the functional effect of Ig receptor/CD19 coligation on PI3K activation and growth arrest, and the role that c-cbl plays in this response.

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