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.

This is a US government work. There are no restrictions on its use.
PI3Kp85, and CD19 are all phosphorylated on tyrosine following activation. In addition, we show that c-cbl forms a complex with CD19 and with the C-terminal SH2 domain of p85. Thus, both c-cbl 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, 1000 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 (RμμGm) conjugated to acrylamide beads were obtained from Irvine Scientific (Santa Ana, CA). Rabbit anti-c-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 (Pierce Chemical Company, Rockford, IL), and 50 μg/lane were used for straight immunoblotting. For immunoprecipitations, 200 μg to 500 μg (approximately 2 to 5 × 10⁷ cell equivalents, respectively) of lysate were added to eppendorf tubes containing Protein A Sepharose (Pharmacia LKB, Biotech, Piscataway, NJ) and 10 μL of mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a control. Calcein and 500 μg of lysate were first preclarified 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 diH₂O. Lysates were incubated with anti-c-cbl (Fig 1A), anti-p85 (Fig 1B), 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-cbl (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.

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 are 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 YYXM motif, which on activation, becomes phosphorylated and binds to the p85 SH2 domains, thus recruiting active PI3K to the Ig receptor complex. CD19 has also been found to be associated with the src-like kinases lyn and fyn. Similarly, the p120 protein product of the protooncogene c-cbl has been shown to be complexed with the tyrosine kinase btk in activated B cells, and with PI3Kp85 in Jurkat T cells. We were interested in determining if c-cbl and CD19 were also involved in PI3K activation in the RL cells. Because tyrosine phosphorylation is important in many of the intermolecular complexes formed, we first examined c-cbl, CD19, 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-cbl (Fig 1A), anti-p85 (Fig 1B), 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-cbl (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.
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 (FBI) 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 (FBI)-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.
The protein product of the c-abl proto-oncogene binds to the C-terminal SH2 domain of P13Kp85. 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 (I) or the GST-SH2-agarose conjugate (II) 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.

The c-abl 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^Grb2, 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-abl 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 (Fig 3, 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-abl. Current studies are underway.
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 antiserum. 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. 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 PKCζ, activation of S6 ribosomal kinase, activation of the protein kinase encoded by the Akt proto-oncogene, and protein trafficking and sorting. 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.

ACKNOWLEDGMENT

We thank Dr Chou-Chi Li for careful reading of the manuscript.

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M Beckwith, G Jorgensen and DL Longo