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Specific association of phosphatidylinositol 3-kinase with the protooncogene product Cbl in Fcγ receptor signaling

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Abstract **A tyrosine-phosphorylated protein with a molecular mass of 115 kDa was reported to be tightly associated with the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, when the enzyme was essentially activated upon ligand engagement of Fcγ receptors (FcγR) leading to engulfment of IgG-coated erythrocytes by phagocytes [Ninomiya et al. (1994) J. Biol. Chem. 269, 22732–22737]. Here, the 115-kDa protein is identified as the product of human c-*cbl* protooncogene. Cross-linking of FcγRII on the surface of THP-1 cells triggered (a) prominent tyrosine phosphorylation of Cbl, (b) activation of PI 3-kinase that was immunoprecipitated with the anti-Cbl or the anti-phosphotyrosine antibody, and (c) specific association of Cbl with p85. Thus, Cbl functions in phagocytes as a result of its association with PI 3-kinase in response to FcγR ligation.**

Key words: Phosphatidylinositol 3-kinase; Cbl, Fcγ receptor; Tyrosine phosphorylation; Phagocytes

1. Introduction

Immune complex- or opsonized antigen-mediated cross-linking of Fc receptors for IgG (FcγR) on the surface of phagocyte activates their various biological functions such as phagocytosis, exocytosis, and superoxide anion production. Although much is known about the gene and protein structure of FcγR [1–3], the intracellular signal transduction system arising from the receptor stimulation has not been well established. Numerous studies have suggested that the system involves activation of intracellular protein tyrosine kinases such as Lyn, Fyn, Fgr, Hck, Yes and Syk, as a result of cross-linking of FcγR [4–10]. Although relatively few tyrosine phosphorylated peptides have as yet been identified, a protooncogene product, Cbl, has recently attracted attention among them [5]. Cbl is tyrosine phosphorylated upon stimulation of T-cell receptor, B-cell receptor and some cytokine receptors as well [11–17]. Some Src family tyrosine kinases are reported to be associated with Cbl in hematopoietic cells [5,12,15]. Thus, Cbl emerges as a potential player in immune recognition receptor signaling.

PI 3-kinase is a lipid kinase which catalyzes phosphorylation at the D-3 position of the inositol ring of PI, PI 4-phosphate, and PI 4,5-bisphosphate (for review, see refs. [18–20]). The activity of this enzyme in cells increases upon stimulation of a number of growth factor receptors having intrinsic tyrosine kinase activities, such as those for platelet-derived growth factor, epidermal growth factor, insulin, and colony stimulating factor-1 [21–26]. PI 3-kinase has also been activated after stimulation of a variety of the receptors lacking tyrosine kinase domains. These tyrosine kinase-lacking receptors include cytokine receptors, T-cell and B-cell antigen receptors [27–33]. Most of the latter receptors, when stimulated, are physically associated with and activate Src-type tyrosine kinases. The latest paper has reported that PI 3-kinase associates with Cbl upon activation through TCR/CD3 or immunoglobulin complex in jurkat T cells [12,13,15].

Previously, we have reported that stimulation of FcγR by the antibody cross-linking gives rise to increased PI 3-kinase activity in the anti-phosphotyrosine-precipitable fraction prepared from whole cell lysate in the monocytic U937 cell line [34]. A specific inhibitor of PI 3-kinase, wortmannin [35], completely inhibits endocytosis of IgG-coated sheep erythrocytes that has been bound to FcγR, affording evidence for an indispensable role of PI 3-kinase activation in signaling cascades arising from the receptor stimulation and leading to this eventual cellular response [34]. We have also indicated that a tyrosine-phosphorylated 115-kDa protein may communicate between FcγR-coupled Src-type tyrosine kinase and PI 3-kinase by binding to the SH2 domain of the p85 regulatory subunit of the enzyme [34]. Here, we report on identification of the 115-kDa protein as Cbl.

2. Materials and methods

2.1. Materials

Mouse monoclonal antibody IV.3 (anti-FcγRII) was from Medarex (East Annandale, NJ); $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) from Jackson ImmunoResearch (West Grove, PA); monoclonal anti-phosphotyrosine antibody (PY-20), polyclonal anti-PI 3-kinase p85 antibody and rabbit anti-Cbl antibody from Santa Cruz (California); ^{125}I-protein G from New England Nuclear; phospholipids from Avanti Polar Lipids Inc. All other reagents from commercial sources were of analytical grade.

2.2. Cells

The human monocytic cell line THP-1 obtained from the American Type Culture Collection was maintained in the RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 20 mM HEPES (pH 7.2). The cells had been cultured with 0.5 mM dibutyryl cAMP for 72 h before use.

2.3. Cross-linking of FcγRs on THP-1 cells

The cultured cells were washed twice and suspended in Krebs-Ringer-HEPES buffer (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM glucose, 0.2% bovine serum albumin, 20 mM HEPES, pH 7.4). Aliquots (10^7 cells/0.5 ml) of the cell suspension were first treated with monoclonal IV. 3 (anti-FcγRII) (1 μg/ml) for 25 min on ice and warmed to 37°C for 5 min and cross-linked with 10 μg/ml of goat anti-mouse IgG for the period indicated. The reactions were terminated with 0.5 ml of ice-cold lysis buffer consisting of 2% NP-40, 4 mM $NaVO_3$, 100 mM NaF, 40 mM HEPES (pH 7.8), 100 units/ml of aprotinin, 2 mM PMSF, 2 mM dithiothreitol, 10 mM EDTA and 0.2% bovine serum albumin.

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Abbreviations: FcγR, Fc receptor for IgG; PI, phosphatidylinositol; p85, 85-kDa subunit of PI 3-kinase; PI-3P, PI 3-phosphate

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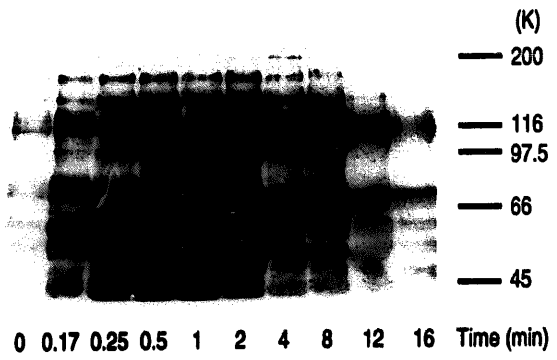

Fig. 1. Time course of protein tyrosine phosphorylation after cross-linking for FcγR. THP-1 cells treated with monoclonal antibody against FcγRII at 0°C for 25 min were incubated at 37°C for 5 min without any further addition and then with the $F(ab')_2$ fragment of goat anti-mouse IgG for the period indicated at the bottom of panel. After incubation, the cells were lysed with NP-40, and phosphotyrosine-containing proteins were immunoprecipitated with anti-phosphotyrosine antibodies (PY-20). Immunoprecipitates were then analyzed by SDS-PAGE followed by immunoblotting with polyclonal rabbit anti-phosphotyrosine antibodies. Mobility of the protein standards is indicated in kDa of molecular masses. The data are from a typical experiment representative of more than three.

2.4. Immunoprecipitation and immunoblotting

The cell lysate was precleared with preimmune IgG and protein G-Sepharose at 4°C for 1 h, and subjected to immunoprecipitation with appropriate antibodies for more than 1 h, and then with the addition of protein G-Sepharose for 1 h or a longer period of time. After repeated washings, immunoprecipitates were heated at 100°C for 3 min in 30 μl of the sample buffer consisting of 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue and 62.5 mM Tris (pH 6.8). Solubilized peptides were then separated by SDS-PAGE on 7.5% slab gel and transferred electrophoretically to a nitrocellulose membrane at 2 mA/cm^2 for 40 min. After blocking in 3% fatty acid-free bovine serum albumin, the blot was incubated with appropriate antibodies, washed, and then with ^{125}I-protein G. Following repeated washings, associated radioactivity was visualized with a Fuji BAS2000 bioimaging analyzer.

2.5. Assay of PI 3-kinase activities

The anti-phosphotyrosine (PY-20), anti-Cbl or anti-p85 immunoprecipitates were assayed for their PI 3-kinase activities. The immunoprecipitates prepared as above were washed twice with the buffer consisting of 40 mM Tris-HCl (pH 7.4), 5 mM EDTA and 1% NP-40, twice with the same buffer supplemented with 0.5 M LiCl, and three times with the buffer supplemented with 100 mM CaCl. Aliquots (2×10^6 cells eq.) of immunoprecipitates were suspended in 0.1 ml of the reaction mixture consisting of 40 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.2 mM PI, 0.2 mM phosphatidylserine, 5 mM $MgCl_2$, and 0.1 mM (2 μCi) [γ-^{32}P]ATP. The reaction was allowed to proceed at 37°C for 15 min before termination by the addition of 20 μl of 8% $HClO_4$ and 0.45 ml of chloroform/methanol (1:2). After vigorous stirring, the mixture was added with 0.15 ml of chloroform and 0.15 ml of 8% $HClO_4$ to separate the organic phase, which was washed with chloroform-saturated 0.5 M NaCl containing 1% $HClO_4$ and then evaporated to dryness. The extract was dissolved in 30 μl of chloroform/methanol (9:1) to be spotted on a silica gel plate (Silica Gel 60, Merck). The plate was developed in chloroform/methanol/28% NH_4OH/H_2O (70:100:25:15), dried, and visualized for radioactivities in the PI-3P fraction with a Fuji BAS2000 bioimaging analyzer.

3. Results and discussion

3.1. Protein tyrosine phosphorylation after FcγRII cross-linking

Cross-linking of FcγRII on THP-1 cells with monoclonal anti-FcγRII antibody and $F(ab')_2$ fragment of anti-mouse IgG induced tyrosine phosphorylation of several proteins with molecular masses of approximately 200, 160, 140, 100–130, 95, 75, 70, 62, 53, 47 and 44 kDa (Fig. 1). The phosphorylation of most of these proteins was observed within 15 s after stimulation and peaked at 1 min and then gradually decreased up to 16 min. Several lines of evidence have raised the possibility that tyrosine phosphorylation of Cbl might be involved in immune recognition systems in hematopoietic cells [11–17]. To test the tyrosine phosphorylation of Cbl upon FcγR stimulation, whole cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (PY20) and analyzed by blotting with anti-Cbl antibodies. As shown in Fig. 2, phosphorylation of Cbl migrating with a mobility corresponding to 120 kDa was observed within 15 s after stimulation and reached a maximum at 1 min and then decreased to almost the basal level at 8 min.

3.2. Association of PI 3-kinase activity with anti-Cbl antibody upon FcγRII cross-linking

In a previous study, we have sought the tyrosine phosphorylated proteins that are associated with the 85-kDa subunit of PI 3-kinase during FcγR-dependent activation of PI 3-kinase [34]. Cross-linking of FcγR resulted in association of several tyrosine-phosphorylated proteins with the lipid kinase. Among them, the 115-kDa protein bound to p85 so firmly as to be observable even after heat and acid denaturation of proteins [34]. Cbl is a possible candidate for the 115-kDa

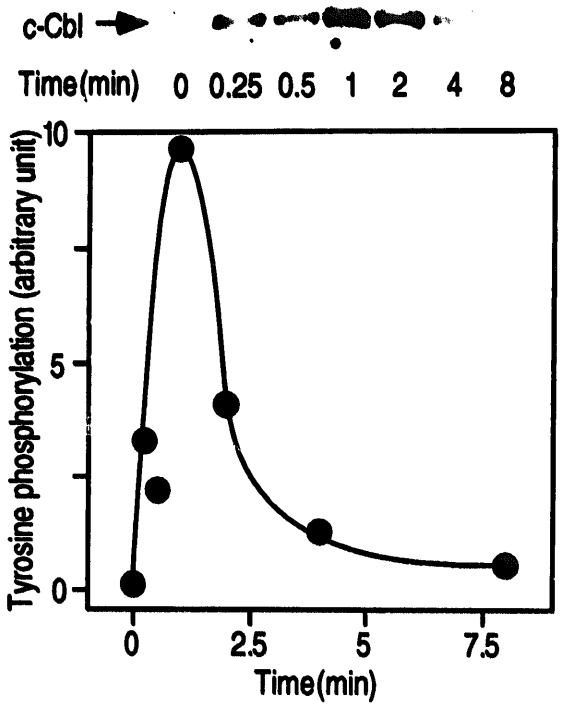

Fig. 2. Time course of tyrosine phosphorylation of Cbl after cross-linking of FcγRII. THP-1 cells treated with monoclonal antibodies against FcγRII were incubated with the $F(ab')_2$ fragment of goat anti-mouse IgG for the indicated times. The cells were lyzed, and anti-phosphotyrosine immunoprecipitates were analyzed by immunoblotting with anti-Cbl antibodies (upper panel). The phosphorylation was quantified by an image analyzer and plotted in an arbitrary unit as a function of incubation time (lower panel). The data are from a typical experiment; similar results were obtained three times.

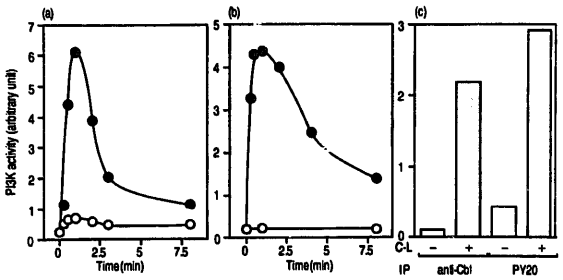

Fig. 3. Increased PI 3-kinase activity in anti-phosphotyrosine and anti-Cbl immunoprecipitates after crosslinking of FcγRII. THP-1 cells untreated (●) or treated (○) with monoclonal antibody against FcγRII were incubated with $F(ab')_2$ fragment of a goat anti-mouse IgG for the indicated times before the extraction with NP-40. PI 3-kinase activities immunoprecipitated with anti-phosphotyrosine (a) or anti-Cbl (b) were measured as described under section 2. The generation of PI-3P was quantified by an image analyzer and plotted in an arbitrary unit as a function of incubation time. (c) THP-1 cells untreated (open column; Cross-Linking −) or treated (dotted column; C-L +) with monoclonal antibodies against FcγRII were incubated with $F(ab')_2$ fragment of goat anti-mouse IgG for 1 min. PI 3-kinase activities immunoprecipitated (IP) with anti-Cbl or PY-20 were measured as above.

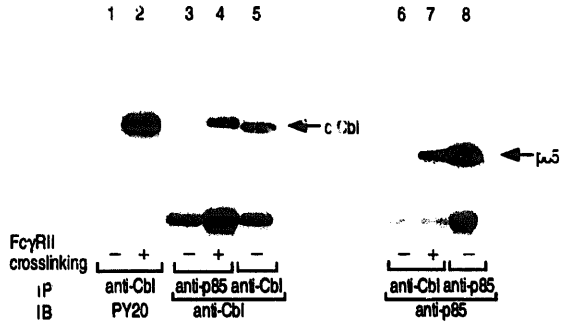

Fig. 4. Association of tyrosine-phosphorylated Cbl with the 85-kDa subunit (p85) of PI 3-kinase after crosslinking of FcγRII. THP-1 cells untreated (lanes 1, 3, 5, 6 and 8 as shown by [−]) or treated (lanes 2, 4 and 7 [+]) with monoclonal antibody against FcγRII were incubated with the $F(ab')_2$ fragment of goat anti-mouse IgG for 1 min before the extraction with 1% NP-40-containing buffer. Proteins in the extract were immunoprecipitated (IP) with anti-Cbl antibodies (lanes 1, 2, 5, 6 and 7 as shown by [anti-Cbl]) or with anti-p85 antibodies (lanes 3, 4 and 8 [anti-p85]), separated by SDS-PAGE and analyzed by immunoblotting (IB) with PY-20 (lanes 1 and 2), anti-Cbl antibodies (lanes 3–5) or anti-p85 antibodies (lanes 6–8). The data are from a typical experiment after similar results have been obtained more than three times.

protein. Crosslinking the FcγRII on THP-1 cells caused a marked increase in the PI 3-kinase activity in the anti-phosphotyrosine (Fig. 3a) and anti-Cbl (Fig. 3b) immunoprecipitates from the whole cell lysate. The maximum activation was observed at 1 min in both fractions (Fig. 3a,b). Although the amount of PI 3-kinase in Cbl immunoprecipitates represented only a small fraction (4–5%) of the total p85 pool, most fraction (roughly 85 percent) of the increments in PI 3-kinase activity of anti-phosphotyrosine immunoprecipitates upon FcγR engagement was accounted for by the enzyme activity increment in anti-Cbl immunoprecipitates in the same experiments (Fig. 3c).

3.3. *Specific association of Cbl with PI 3-kinase*

Crosslinking of FcγRII resulted in tyrosine phosphorylation of Cbl as observed in experiments in which immunoprecipitation of cell lysates with anti-Cbl was followed by immunoblotting with PY20 (Fig. 4, lanes 1 and 2) or inversely immunoprecipitation with PY20 was followed by immunoblotting with anti-Cbl, as shown in time-course data in Fig. 2. The thus phosphorylated Cbl, which migrated at a slightly lower rate than did the non-phosphorylation one upon SDS-PAGE, was mostly associated with the regulatory subunit of PI 3-kinase as detected by immunoprecipitation with anti-p85 antibody followed by immunoblotting with anti-Cbl antibody (Fig. 4, lanes 3–5). The association was again observed when two antibodies used were replaced each other for immunoprecipitation and immunoblotting (Fig. 4, lanes 6–8).

The tight association of tyrosine-phosphorylated Cbl with p85, upon FcγR stimulation, is very likely to be responsible for marked increases in PI 3-kinase activity in anti-Cbl and anti-phosphotyrosine immunoprecipitates observed under similar conditions in Fig. 3. The increase in PI 3-kinase must be essential for FcγR-mediated phagocytosis, since it was totally blocked by wortmannin, a selective inhibitor of the enzyme [34]. The initial step of signaling following ligand engagement of FcγRII could be activation of a non-receptor-type protein-tyrosine kinase, one of the substrates of which is Cbl, as actually reported for Lyn in HL-60 cells [5] and Fyn/Lck in human jurkat T-cells [15]. Cbl proteins thus phosphorylated on tyrosines should associate with SH2 domains of p85 because the direct association between Cbl and p85 is reported in vitro using a fusion protein comprising the SH2 domains of p85 in CD3-activated jurkat T cells [13]. Actually, in THP-1 cells, the binding between Cbl and p85 was almost completely blocked by phenyl phosphate which competitively inhibits the binding between tyrosine phosphorylated protein and the SH2 domain (paper in preparation). Furthermore, when anti-Cbl antibody immunoprecipitate was separated by SDS-PAGE, blotted to a membrane, and probed with GST-p85 fusion protein and anti-GST antibody, the bound GST-p85 was detectable at the position of Cbl migrated (paper in preparation). The direct binding of phosphorylated Cbl to PI 3-kinase must lead to phagocytosis as a result of activation of the lipid kinase. A possibility could not be excluded, however, for other signaling roles of Cbl, which is capable of binding to SH3 domains of intracellular proteins via its proline-rich motifs [36,37]. In fact, Grb2/Ash coprecipitated with Cbl from lysates of stimulated or unstimulated THP-1 cells (unpublished observation), in accordance with previous reports on T-cell lines [12,15]. Such adaptor proteins might mediate Ras-MAP kinase cascades via Sos, an activator of small GTP-binding proteins. These possibilities would be the subjects of further investigations.

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