Association of phosphatidylinositol 3-kinase with the proto-oncogene product Cbl upon CD38 ligation by a specific monoclonal antibody in THP-1 cells

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Received 24 June 1996; revised version received 30 September 1996

Abstract **We reported that ecto-NAD$^+$ glycohydrolase activity induced upon differentiation of HL-60 cells with retinoic acid is localized on the extracellular domain of CD38 and that CD38 ligation by a specific monoclonal antibody, HB-7, is followed by rapid tyrosine phosphorylation of cellular proteins including a proto-oncogene product, Cbl. In the present study, we investigated intracellular signaling linked to the HB-7-induced Cbl phosphorylation in dibutyryl cAMP-treated THP-1 cells. The 85-kDa regulatory subunit (p85) of phosphatidylinositol (PI) 3-kinase was immunoprecipitated with anti-Cbl antibody in a manner dependent on the tyrosine phosphorylation of Cbl. PI 3-kinase activity was also observed in the immunoprecipitated fractions containing tyrosine-phosphorylated Cbl. The phosphorylated form of Cbl, which had been separated from the CD38-stimulated cells, was capable of directly binding to a recombinant p85 fused to glutathione *S*-transferase. Thus, the direct association of tyrosine-phosphorylated Cbl with PI 3-kinase, possibly leading to the kinase activation, appeared to be involved in intracellular signaling caused by the CD38 ligation.**

Key words: CD38; Phosphatidylinositol 3-kinase; Proto-oncogene product Cbl; Tyrosine phosphorylation

1. Introduction

The human cell surface antigen CD38 is a 46-kDa type II glycoprotein with a single transmembrane domain [1]. Analysis of expression of CD38 is widely used as a phenotypic marker of differentiation or activation of human T and B lymphocytes [2]. We previously demonstrated that NADase activity induced by all-*trans* retinoic acid in human leukemic HL-60 cells is due to the extracellular domain of CD38 [3] and that the same molecule is able to bind hyaluronate [4]. CD38 displayed an amino acid sequence similar to *Aplysia* ADP-ribosyl cyclase, an enzyme which catalyzes the formation of cyclic ADP-ribose from NAD$^+$ [5]. The cyclic nucleotide has been considered a new mediator or modulator of Ca^{2+} release from intracellular stores insensitive to inositol 1,4,5-trisphosphate [6]. Indeed, CD38 catalyzes not only the hydrolysis of NAD$^+$, but also the formation and hydrolysis of cyclic ADP-ribose, although at much lower levels [7].

CD38 is considered an important regulatory molecule in the immune system, as inferred from the observation that CD38 ligation by agonistic mAbs elicits a variety of cell functions, including cell proliferation, lymphopoiesis, apoptosis, adhesion and cytokine production [2,8,9]. CD38 ligation by mAbs is believed to mimic some of the events triggered by the binding with a natural ligand. In this regard, a cell surface protein with the M_r of 120000, which is predominantly produced in endothelial cells, was recently identified as one of the CD38-binding proteins [10]. Quite recently, we found that ligation of CD38 with selected mAbs induces rapid tyrosine phosphorylation of cellular proteins in retinoic acid-treated HL-60 cells [11]. Such protein-tyrosine phosphorylation was also observed in mouse [12,13] and human [14] B cells and retinoic acid- or Bt_2cAMP-treated monocytic THP-1 cells [15]. The tyrosine-phosphorylated proteins included phospholipase C-γ, PI 3-kinase, Syk [14] and Btk [13] tyrosine kinases, and a proto-oncogene product, Cbl [11]. However, intracellular signaling associated with the CD38-dependent tyrosine phosphorylation is yet to be defined.

Cbl, one of the prominent phosphorylated proteins by CD38 ligation [11], is also known to be phosphorylated upon stimulation of T- and B-cell receptors, Fc receptors for IgG (FcγR), and some cytokine receptors as well [16–19]. The oncogene product is capable of binding to non-receptor type tyrosine kinases, receptor-tyrosine kinases, adaptor proteins, and PI 3-kinases [16–18]. Among them, a typical PI 3-kinase is a heterodimer with an 85-kDa regulatory (p85) subunit and a 110-kDa catalytic (p110) subunit (for review, see [20–22]). p85 possesses two SH2 and one SH3 domains. Proteins containing a consensus motif, *YXXM*, bind either of the two SH2 domains of the p85 subunit upon its tyrosine phosphorylation, thereby activating the catalytic function of the associated p110 subunit. The motif, *YXXM*, is found in the intracellular domain of tyrosine kinase-type receptors and in tyrosine kinase-substrate proteins, such as the insulin receptor substrate 1, polyoma middle T antigen, and Cbl. Recently, we have presented evidence suggesting that tyrosine-phosphorylated Cbl may communicate between FcγR-coupled tyrosine kinase and PI 3-kinase in Bt_2cAMP-treated THP-1 cells [19]. The association has a crucial effect upon FcγR stimulation in phagocytes [23]. Here we examined the possibility that tyrosine phosphorylation of Cbl by CD38 ligation might also result in association with PI 3-kinase.

2. Materials and methods

2.1. Materials

The anti-CD38 mAb, HB-7, was purified from the culture medium

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Abbreviations: Ab, antibody; Bt_2, dibutyryl; mAb, monoclonal antibody; NADase, NAD$^+$ glycohydrolase; PI, phosphatidylinositol; p85, 85-kDa subunit of PI 3-kinase; PI-3P, PI 3-phosphate; PY, phosphotyrosine; GST, glutathione *S*-transferase

PII S0014-5793(96)01151-9

of HB 136 cells as described previously [11]. GST-p85 fusion protein was purified from *E. coli* producing the protein. The anti-PY mAb PY-20, polyclonal anti-p85 Ab and rabbit anti-Cbl Ab were obtained from Santa Cruz; ^{125}I-labeled protein G and [γ-^{32}P]ATP from DuPont/NEN; 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 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 were cultured with 0.5 mM Bt_2cAMP for 72 h before use.

2.3. Stimulation of THP-1 cells with anti-CD38 mAb

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, and 20 mM HEPES, pH 7.4). Aliquots (10^7 cells/0.5 ml) of the cell suspension were warmed to 37°C for 5 min, and stimulated with 2 μg/ml of HB-7 at 37°C for the indicated times. 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.

2.4. Immunoprecipitation and immunoblotting

The cell lysate was precleared with preimmune IgG and protein G-Sepharose at 4°C for 1 h. The supernatant was incubated with appropriate antibodies for more than 1 h, and for a further 1 h or a longer period of time with the addition of protein G-Sepharose. After repeated washing, 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 polyvinylidene difluoride membrane at 2 mA/cm^2 for 40 min. After blocking in 3% bovine serum albumin, the blot was incubated with appropriate antibodies and then with ^{125}I-labeled protein G. Where indicated (Fig. 1D), the blot was first incubated at 4°C for 1 h with 5 μg/ml of GST-p85 in Tris-buffered saline supplemented with 3% bovine serum albumin and then probed with anti-p85 Ab and ^{125}I-labeled protein G to test the direct association of the blotted proteins with p85. Following repeated washing, associated radioactivity was located with a Fuji BAS2000 bioimaging analyzer.

2.5. Assay of PI 3-kinase activity

The materials associated with anti-phosphotyrosine (PY-20), anti-Cbl or anti-p85 were assayed for PI 3-kinase activity. The samples prepared as above were washed twice with a buffer consisting of 40 mM Tris-HCl (PH 7.4) and 5 mM EDTA supplemented with 1% NP-40, twice with the buffer containing 0.5 M LiCl, and three times with the buffer containing 100 mM NaCl. Aliquots (2×10^6 cells) 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 [γ-^{32}P]ATP (2 μCi). 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, v/v). After vigorous stirring, 0.15 ml of chloroform and 0.15 ml of 8% $HClO_4$ was added to the mixture 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, v/v). The radioactivity in the PI-3P fraction was determined with a Fuji BAS2000 bioimaging analyzer.

3. Results

Monocytic THP-1 cells were cultured with Bt_2cAMP for 3 days to produce CD38 on the cell surface [15], and the cells were then stimulated with an anti-CD38 mAb, HB-7. Lysate from the cells was subjected to immunoprecipitation with anti-Cbl Ab, and the precipitated proteins, after being separated by SDS-PAGE, were visualized by means of immunoblotting with an anti-phosphotyrosine Ab (PY-20). As shown in Fig. 1A, tyrosine phosphorylation of Cbl with a M_r of 120000 was very rapidly observed after the addition of HB-7; the phosphorylation occurred within 30 s, the maximum level being reached at 2 min, followed by a gradual decrease in the phosphorylation level. When the immunoblot analysis was performed with anti-Cbl Ab instead of PY-20, there were no marked differences in the immunoreactive 120-kDa Cbl among the various fractions, indicating that the same amounts of Cbl could be immunoprecipitated under the present conditions (Fig. 1B). The same anti-Cbl-precipitated fractions were separations by SDS-PAGE and visualized with anti-p85 Ab. As shown in Fig. 1C, the 85-kDa regulatory subunit of PI 3-kinase appeared to be present in the Cbl-containing fractions only upon the CD38 ligation by HB-7. The time course of the appearance of the immunoreactive p85 (see Fig. 1C) was similar to that observed in the tyrosine phosphorylation of Cbl (see Fig. 1A), suggesting that p85 is capable of binding only to the tyrosine-phosphorylated form of Cbl, since all the precipitated fractions contained the same amounts of 120-kDa Cbl (see Fig. 1B).

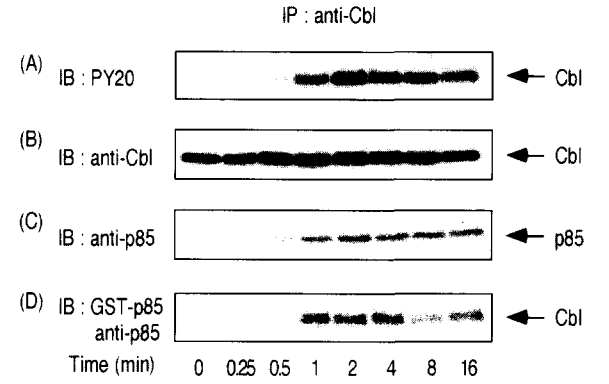

Fig. 1. Association of the 85-kDa regulatory (p85) subunit of PI 3-kinase with tyrosine-phosphorylated Cbl after stimulation of THP-1 cells with HB-7. THP-1 cells that had been cultured with Bt_2cAMP were stimulated with 10 μg/ml of HB-7 at 37°C for the indicated times. The cell lysate was immunoprecipitated (IP) with anti-Cbl Ab, separated by SDS-PAGE, and analyzed by immunoblotting (IB) with the indicated Abs (A–C) as described in Section 2.4. In D, the samples immunoprecipitated with anti-Cbl Ab were separated by SDS-PAGE and blotted to a membrane. The membrane was further incubated with a recombinant GST-p85 fusion protein followed by immunoblotting with anti-p85 Ab as described in Section 2.4. The data are from a typical experiment; similar results were obtained more than three times.

When lysate from the anti-CD38 mAb-treated cells was first immunoprecipitated with anti-p85 Ab and then subjected to immunoblotting with anti-Cbl Ab, we could not see the immunoreactive band corresponding to 120-kDa Cbl (data not shown). This might suggest that only a limited amount of Cbl could associate with the p85 of PI 3-kinase. Alternatively, the anti-p85 Ab used in the present study might be unsuitable for the immunoprecipitation of a specific form of p85 associated with tyrosine-phosphorylated Cbl, probably due to the masking of its epitopic site(s).

The existence of a catalytic subunit of PI 3-kinase, which might be associated with p85, was estimated in the anti-Cbl-precipitated fractions by measuring the kinase activity as fol-

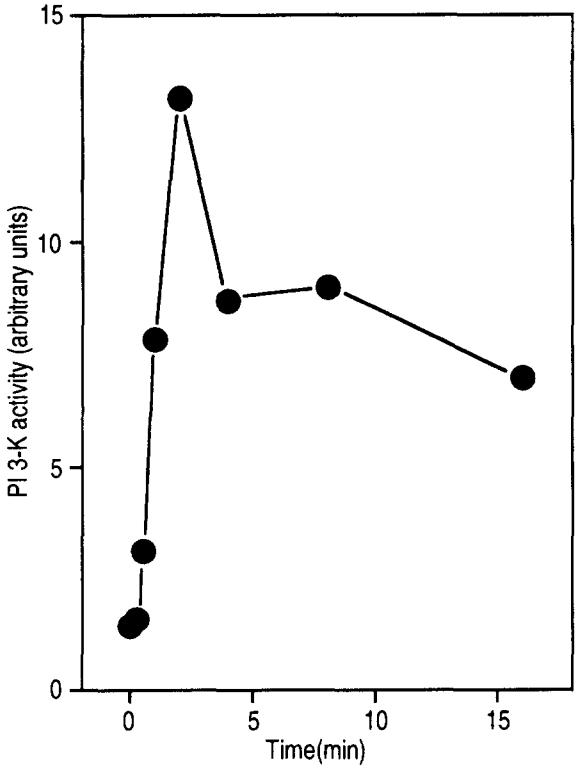

Fig. 2. Association of the catalytic activity of PI 3-kinase with tyrosine-phosphorylated Cbl after stimulation of THP-1 cells with HB-7. The Bt_2cAMP-treated THP-1 cells were stimulated with 10 mg/ml of HB-7 at 37°C for the indicated times. The cell lysate was immunoprecipitated with anti-Cbl Ab and then subjected to the assay for PI 3-kinase as described in Section 2.5. The generation of PI-3P is plotted in an arbitrary unit as a function of incubation time. The data are from a typical experiment; similar results were obtained more than three times.

lows. The precipitated fractions were incubated with PI and [γ-^{32}P]ATP, and radioactivity incorporated into PI 3-P was then separated by thin-layer chromatography. As shown in Fig. 2, there was a marked increase of PI 3-kinase activity in the immunoprecipitated fractions upon CD38 ligation by HB-7. The time course of the activity increase was again correlated with that of tyrosine-phosphorylated Cbl (see Fig. 1A).

The possible association of the phosphorylated Cbl with the p85 subunit of PI 3-kinase was further investigated with a recombinant GST-p85 fusion protein. The anti-Cbl-immunoprecipitated fractions, after being separated by SDS-PAGE, were blotted to a membrane, and the membrane was further incubated with GST-p85. When the membrane was visualized with anti-p85 Ab, the p85-fusion protein was clearly observed at the position of 120-kDa Cbl only in the fractions containing its phosphorylated form (Fig. 1D). Cbl contains several proline-rich motifs and potential tyrosine phosphorylation sites that might mediate its interaction with proteins possessing SH3 and SH2 domains, respectively [16–18]. Since the association of the two polypeptides observed in Fig. 1D appeared to be inhibited by the addition of an excess amount of phenylphosphate (data not shown), it is very likely that PI 3-kinase binds to tyrosine-phosphorylated Cbl via the SH2 domain(s) of p85.

4. Discussion

Our data in this communication clearly indicate that the tyrosine phosphorylation of a proto-oncogene product, Cbl, upon CD38 ligation by HB-7 results in direct association of Cbl with PI 3-kinase in Bt_2cAMP-treated THP-1 cells. 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. The activity of this enzyme in cells increases upon stimulation of numerous immune inflammatory stimuli such as interleukin (IL-2, IL-3, IL-4, or IL-7) and granulocyte monocyte colony stimulating factor (GMCSF) receptors, T-cell receptors, B-cell antigen receptors, and Fc receptors [21,22]. Most of these receptors possess the consensus sequence termed a 'tyrosine activation motif' (TAM), which is found in several receptor subunits that associate with cytoplasmic tyrosine kinases and when stimulated, are physically associated with and activate Src-type tyrosine kinases or Syk/Zap70 which are believed to mediate the receptor signaling [24,25]. Although such a sequence motif is not found in the cytosolic domain of CD38, we cannot totally rule out the possibility that the short cytoplasmic domain of CD38 contains an unidentified motif that is capable of interacting with a cellular tyrosine kinase(s).

We have demonstrated that CD38 ligation by selected anti-CD38 mAbs induces tyrosine phosphorylation of cellular proteins in human leukemia cell lines including HL-60 [11] and THP-1 [15] cells in their differentiated states. These cells acquire the high responsiveness to the stimulation with chemotactic receptors coupled to heterotrimeric GTP-binding proteins during the differentiation and consequently they are capable of generating O_2^- in response to the receptor agonists. We have reported an indispensable role of PI 3-kinase in the chemotactic receptor-induced O_2^- production in granulocytes [26]. Interestingly, the receptor-induced O_2^- production is dramatically enhanced by the simultaneous stimulation of CD38 by the mAbs in the same Bt_2cAMP-treated THP-1 cells (manuscript in preparation). Since the formation of intracellular PIP_3, a product of PI 3-kinase, in response to chemotactic receptor stimulation is synergistically increased by the simultaneous addition of the agents which cause tyrosine kinase-mediated activation of PI 3-kinase [27], the augmentation of O_2^- production might be dependent on the CD38-mediated activation of PI 3-kinase. Our earlier finding that the G$\beta\gamma$ subunit of heterotrimeric GTP-binding proteins greatly increased the PI 3-kinase activity associated with tyrosine-phosphorylated proteins supports the hypothesis [27]. Alternatively, since O_2^- production requires Ca^{2+} other than PI 3-kinase as we have indicated [28], CD38 might supply Ca^{2+} rather than PIP_3 because CD38 catalyzes the formation of cyclic ADP-ribose, which is a novel candidate for Ca^{2+} release from intracellular Ca^{2+} stores. We are currently analyzing this in detail in our laboratories.

During the preparation and revision of the manuscript, A. Kitanak et al. reported similar results showing that CD38-mediated tyrosine phosphorylation of Cbl is accompanied by its association with PI 3-kinase in human immature B-cell lines [29].

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