Tyrosine Phosphorylation of the c-cbl Proto-oncogene Product Mediated by Cell Surface Antigen CD38 in HL-60 Cells*

(Received for publication, September 1, 1995, and in revised form, November 9, 1995)

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The human cell surface antigen CD38 is a 46-kDa type II transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long Cys-rich C-terminal extracellular one. We demonstrated previously that the extracellular domain of CD38 has NAD$^+$ glycohydrolase (NADase) activity and that the ecto-form NADase activity induced in HL-60 cells during cell differentiation by retinoic acid is due to CD38. In the present study, we investigated the intracellular signaling mediated by CD38 in retinoic acid-differentiated HL-60 cells with an anti-CD38 monoclonal antibody. The addition of anti-CD38 monoclonal antibody to the cells induced rapid tyrosine phosphorylation of the cellular proteins with molecular weights of 120,000, 87,000, and 77,000. An increase in tyrosine kinase activity in the anti-phosphotyrosine immunoprecipitates of the cells was also observed after the addition of anti-CD38 monoclonal antibody. Moreover, one of the prominent tyrosine-phosphorylated proteins stimulated by the anti-CD38 monoclonal antibody was identified as the c-cbl proto-oncogene product, p120$cbl$. These results indicated that tyrosine phosphorylation of cellular proteins, including p120$cbl$, is possibly involved in transmembrane signaling mediated by CD38.

The human cell surface antigen CD38, originally termed T10 (1), is a 46-kDa type II glycoprotein with a single transmembrane domain. However, CD38 exhibits no significant homology with other known cell surface molecules (2, 3). Cytological studies revealed that CD38 is predominantly produced on the cell surface in both the early and late stages of T and B cell maturation but not in intermediate ones (1, 4, 5). We recently demonstrated that the extracellular domain of CD38 exhibits NAD$^+$ glycohydrolase (NADase) activity and that the ecto-form NADase activity induced by RA in HL-60 cells is due to CD38 (6). Moreover, it has been reported that CD38 catalyzes not only the hydrolysis of NAD, but also the formation and hydrolysis of cyclic ADP-ribose (7-9), which is a novel candidate that mediates Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores (see Refs. 10 and 11 for reviews). Besides these enzyme activities, CD38 has the ability to bind hyaluronate, which is a large glycosaminoglycan existing in the extracellular matrix and on the cell surface (12). Recent studies revealed that stimulation of CD38 with anti-CD38 mAbs induces various cell responses including DNA synthesis by human thymocytes in the presence of accessory cells (13), the proliferation of mouse B cells in the presence of IL-4 (14), and the rescue of germinal center cell apoptosis (15). Moreover, anti-CD38 mAb inhibited lymphocyte adhesion to endothelial cells (16) and suppressed the growth of immature B lymphoid cells in the bone marrow microenvironment (17). Regardless of these observations, little is known concerning the intracellular signaling mediated by CD38.

In hematopoietic cells, the stimulation of T and B cell antigen receptors activates multiple protein kinases, resulting in the phosphorylation of numerous intracellular substrate proteins. Extensive research on these protein kinases and their substrates has revealed that protein-tyrosine phosphorylation plays a crucial role in transmembrane signaling via hematopoietic receptors (18-20). In the present study, we examined the possibility that the tyrosine phosphorylation of cellular proteins might be involved in the CD38-mediated signaling pathway. We found that stimulation of RA-differentiated HL-60 cells with anti-CD38 mAbs induces rapid tyrosine phosphorylation of cellular proteins. One of the prominent phosphorylated proteins was identified as the c-cbl proto-oncogene product, p120$cbl$.

**EXPERIMENTAL PROCEDURES**

Cdl Culture—HL-60 cells were cultured and caused to differentiate by various inducers as described previously (21). Mouse hybridoma HB136 cells, which produce an anti-CD38 mAb (HB-7, subclass IgG1), were obtained from the American Type Culture Collection and cultured in serum-free Cosmid-001 (Cosmo Bio Co., Ltd., Tokyo, Japan) containing insulin and transferrin.

Materials—The anti-CD38 mAb, HB-7, was purified from the culture medium of HB136 cells by means of column chromatography on protein A-Sepharose (Seikagaku-Kogyo, Tokyo, Japan). Another anti-CD38 mAb, T16, and the subclass-matched control IgG1 were purchased from Cosmo Bio (IOB6) and ICN Biochemicals, Inc. (64-335), respectively. The anti-PY mAb PY-20 and rabbit anti-PY pAb were obtained from Leinco Technologies Inc. (Bailwin, MO) and Chemicon International Inc. (Temecula, CA), respectively. An anti-p120$cbl$ pAb was purchased from Santa Cruz Biotechnology (SC-170). Sepharose 4B and protein G-Sepharose 4-FF were from Pharmacia Biotech. (Uppsala, Sweden). The tyrosine kinase substrate, Raytide, and P-81 ion exchange chromatography paper were purchased from Oncogene Science Inc. (Uniondale, NY) and Whatman (Maidstone, United Kingdom), respectively. [32P]ATP and [32P]-protein A were from DuPont NEN. All other reagents were of analytical grade from commercial sources.

Stimulation of HL-60 Cells with Anti-CD38 mAbs and Analysis of Tyrosine Phosphorylated Proteins—HL-60 cells which had been cultured with or without various inducers were washed three times with ice-cold phosphate-buffered saline and then resuspended in serum-free RPMI 1640 containing 10 mM Na-Hepes (pH 7.4) at a cell density of 3 × 10$^7$ cells/mL. After preincubation at 37°C for 5 min, the cells (1.2 × 10$^7$ cells) were incubated at 37°C for 5 min with or without various inducers and anti-CD38 mAbs. The cell lysates were subjected to SDS-PAGE followed by autoradiography.
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RESULTS

Stimulation of Protein Tyrosine Phosphorylation by Anti-CD38 mAbs in RA-differentiated HL-60 Cells—We first investigated the possibility that the tyrosine phosphorylation of cellular proteins might be induced on stimulation with anti-CD38 Abs. HL-60 cells that had been cultured with RA to produce CD38 were stimulated with an anti-CD38 mAb, T16, and then a lysate was prepared from the cells. Tyrosine-phosphorylated proteins in the cell lysate, after being immunoprecipitated with PY20, were separated by SDS-PAGE and visualized as described above.

However, the subclass-matched control mAb (IgG1) had no effect on the tyrosine phosphorylation (lane 2). The specificity of the action of HB-7 was confirmed by its inability to stimulate the tyrosine phosphorylation in undifferentiated or dibutyryl cAMP-differentiated HL-60 cells (Fig. 1B), in which CD38 is not expressed (6). These results clearly indicated that the anti-CD38 mAb stimulates the tyrosine phosphorylation of cellular proteins through CD38 produced on the surface of HL-60 cells.

Fig. 2A shows the time course of protein tyrosine phosphorylation after the addition of HB-7 to RA-differentiated HL-60 cells. All the tyrosine phosphorylation of p120, p87, and p77 occurred within 1 min, the maximal levels being reached at 1-2 min, followed by gradual decreases in the phosphorylation levels (Fig. 2B). The overall pattern of tyrosine phosphorylation induced by HB-7 was not altered by further cross-linking of the membrane-bound anti-CD38 mAb with an anti-mouse IgG antibody (data not shown). This may imply that the aggregation of CD38 molecules is not essentially required for the CD38-induced tyrosine phosphorylation in cells.

Stimulation by Anti-CD38 mAb of Tyrosine Kinase Activity in a Cell Lysate Immunoprecipitated with Anti-PY pAb—It has been generally observed in tyrosine kinase-induced signaling pathways that the activation of tyrosine kinases induces the autophosphorylation of tyrosine residues and/or association with tyrosine-phosphorylated proteins. Thus, we next measured the tyrosine kinase activity in the lysate of HL-60 cells that had been stimulated with HB-7. Tyrosine-phosphorylated proteins and/or proteins associated with them in the cell lysate were immunoprecipitated with PY20, and the in vitro tyrosine kinase assay was carried out with Raytide as the substrate (22). Fig. 3 shows the time course of the tyrosine kinase activity in the PY20-immunoprecipitated fraction after the addition of HB-7 to RA-differentiated HL-60 cells. HB-7 rapidly stimu-
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The relationship between this p120c-cbl and p120 previously identified as the tyrosine-phosphorylated protein in the same RA-differentiated HL-60 cells was further investigated as follows. A cell lysate obtained from HB-7-stimulated HL-60 cells was first treated with the anti-p120c-cbl antibody to deplete p120c-cbl from the lysate. The treated lysate, after being immunoprecipitated with PY20, was then separated by SDS-PAGE and then subjected to immunoblotting with the anti-p120c-cbl pAb or anti-PY pAb (Fig. 4B). As expected, the depletion of p120c-cbl from the cell lysate was almost completely achieved (lanes 1 and 2). When the cell lysate was analyzed by immunoblotting with the anti-PY pAb, a marked decrease in the immunoreactivity of p120 to the pAb was observed without significant changes in any other tyrosine-phosphorylated proteins (lanes 3 and 4). These results indicated that the major fraction of the tyrosine-phosphorylated p120 observed on the stimulation of CD38 represents the phosphorylated p120c-cbl in the HL-60 cells.

DISCUSSION

We demonstrated that stimulation with anti-CD38 mAbs induces tyrosine phosphorylation of cellular proteins in HL-60 cells caused to differentiate into granulocytes by RA. The action of the anti-CD38 mAb, HB-7, was specifically observed in CD38-producing HL-60 cells; there was no stimulatory effect of the mAb on undifferentiated or dibutyryl cAMP-differentiated HL-60 cells, in which CD38 is not produced. Protein tyrosine phosphorylation induced by anti-CD38 mAb has been also reported in mouse B cells (27). However, none of the phosphorylated proteins was analyzed in the B cells, although some of the anti-CD38-immunoreacted proteins appeared to be similar to those observed in the RA-differentiated HL-60 cells in terms of molecular weight.

In the present study, we found that one of the major tyrosine-phosphorylated proteins stimulated by anti-CD38 mAb is the c-cbl proto-oncogene product with M<sub>s</sub> of 120,000, p120<sup>c-cbl</sup>. The cbl gene was initially identified as a transforming component of Cas NS-1 retrovirus that induces early B-lineage lymphoma (28). The c-cbl proto-oncogene is mainly expressed in hematopoietic cells (29). Recent studies revealed that p120<sup>c-cbl</sup> is tyrosine-phosphorylated in response to T cell receptor, Fc receptor, and epidermal growth factor receptor activation (23–26). It was also shown that tyrosine-phosphorylated p120<sup>c-cbl</sup> has an ability to bind the SH2 domains of Fyn, Lck, and Blk protein-tyrosine kinases, GTPase-activating protein and phospholipase Cγ, and that p120<sup>c-cbl</sup> also binds to the SH3 domain of Nck, Lyn, and the N-terminal SH3 domain of Grb-2 (23, 30). Moreover, the conversion of c-cbl to a transforming gene involves tyrosine phosphorylation of its protein products (31). Thus,
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**Fig. 4. Tyrosine phosphorylation of the cbl proto-oncogene product upon stimulation of CD38.** A, a cell lysate was prepared from RA-differentiated HL-60 cells which had been incubated with the control IgG1 (C) or anti-CD38 mAb HB-7 (H) at 37 °C for 2 min and then p120<sup>cybl</sup>-immunoprecipitation in the cell lysate was immunoprecipitated with its pAb. The precipitated proteins, after being separated by SDS-PAGE, were subjected to immunoblot analysis with the anti-PY pAb (lanes 1 and 2) or anti-p120<sup>cybl</sup>-pAb (lanes 3 and 4), as described under “Experimental Procedures.” The position of p120<sup>cybl</sup> is indicated by the arrow. B, a cell lysate prepared from HB-7-stimulated HL-60 cells was first subjected to three rounds of immunoprecipitation with anti-CD38 mAb HB-7 (lanes 2 and 4). The lysates, after being immunoprecipitated with PY20, were separated by SDS-PAGE and then analyzed by immunoblotting with the anti-p120<sup>cybl</sup>-pAb (lanes 1 and 2) or anti-PY pAb (lanes 3 and 4).

p120<sup>cybl</sup> appears to play an important role in the signal transduction in hematopoietic cells.

In the case of T cell receptor or Fc receptor stimulation (23, 24), p120<sup>cybl</sup> might be tyrosine-phosphorylated via intracellular protein-tyrosine kinases which bind to a common cytoplasmic motif, termed ITAM (immune tyrosine-based activation motif, which has been referred to as ARAM or TAM), in those receptors (19, 32). Such a sequence motif is not found in the cytoplasmic domain of CD38 (2); however, we can’t 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 not determined what kind of protein-tyrosine kinase(s) is activated upon stimulation of CD38 in RA-differentiated HL-60 cells. In this regard, an interesting finding has been reported that B cells from X-linked immunodeficient mice, which have a defect in Btk, contain an unidentified motif that is capable of interacting with a cellular tyrosine kinase(s). We have not determined what kind of protein-tyrosine kinase(s) is activated upon stimulation of CD38 in RA-differentiated HL-60 cells. In this regard, an interesting finding has been reported that B cells from X-linked immunodeficient mice, which have a defect in Btk, contain an unidentified motif that is capable of interacting with a cellular tyrosine kinase(s). We have not determined what kind of protein-tyrosine kinase(s) is activated upon stimulation of CD38 in RA-treated HL-60 cells would provide useful information for understanding the transmembrane signal mediation by CD38.

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