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CD38-Mediated Signaling Events in Murine Pro-B Cells Expressing Human CD38 With or Without Its Cytoplasmic Domain

Akira Kitanaka,*† Toshio Suzuki,* Chikako Ito,* Hikari Nishigaki,* Elaine Coustan-Smith,* Terukazu Tanaka,‡ Yoshitsugu Kubota,‡ and Dario Campana2*‡

To elucidate the signaling mechanism of CD38 (a transmembrane molecule highly expressed in immature hematopoietic cells), we transfected Ba/F3 murine pro-B cells with a cDNA encoding human CD38. CD38 ligation with anti-CD38 Abs caused rapid, transient, dose-dependent tyrosine phosphorylation of several proteins, including the tyrosine kinase TEC and the adaptor molecule CBL, and association of tyrosine-phosphorylated proteins with phosphatidylinositol 3-kinase p85. Exposure to anti-CD38 Abs or their F(ab')2 and Fab also induced tight aggregation of CD38-transfected Ba/F3 cells, which appeared to be Ca2+ and Mg2+-independent and did not involve LFA-1. Aggregation was abrogated by addition of the tyrosine kinase inhibitor herbimycin A and was delayed by the phosphatidylinositol 3-kinase inhibitor wortmannin, suggesting a link between biochemical events and cellular effects induced by CD38. Cell aggregation was accompanied by a decrease in cell recovery. After 3 days of culture on bone marrow-derived stroma, the mean (±SD) cell recovery in the presence of anti-CD38 (T16) was 10.5 ± 9.2% (n = 7) of that in parallel cultures with an isotype-matched nonreactive Ab. Finally, CD38 ligation in Ba/F3 cells expressing a mutant human CD38 lacking the cytoplasmic domain induced tyrosine phosphorylation with intensity and kinetics similar to those seen with the entire protein. It also induced cell aggregation and decreased cell recovery. We conclude that CD38 triggers remarkably similar signaling pathways in human and murine immature B cells. This signaling is independent of the CD38 cytoplasmic domain, suggesting the existence of accessory transmembrane molecules associated with CD38.

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D38 is a 45-kDa transmembrane protein that is highly expressed by human immature lympho-hemopoietic cells, activated lymphocytes, and plasma cells (1–4). The extracellular portion of CD38 has enzymatic activity and catalyzes the conversion of nicotinamide adenine dinucleotide (NAD) and NAD phosphate to the Ca2+-mobilizing compounds cyclic adenosine diphosphate ribose (ADPR) and nicotin acid ADP, respectively (5–9). In human immature B lymphoid cells, ligation of CD38 with specific mAbs induces the tyrosine phosphorylation of the transmembrane protein CD19 (10), the tyrosine kinases SYK and TEC (11, 12), and the adaptor molecule CBL (13) as well as association of phosphatidylinositol 3-kinase (PI 3-K) with CD19 (10) and CBL (13). These biochemical events are accompanied by pronounced growth arrest and apoptosis in cells cocultured with stromal feeder layers or stroma-derived cytokines (14).

The relation between the enzymatic activity and the signaling properties of CD38 is unclear. Based on the crystal structure of Aplysia ADPR cyclase, an enzyme homologous to CD38, Prasad et al. proposed a model of CD38 as a dimer that encloses a cavity supporting the ectoenzymatic activity (15). These authors speculated that conformational changes associated with the enzymatic activity could be transmitted to the cytoplasmic domain of CD38, resulting in protein tyrosine kinase activity. The molecular mechanisms linking CD38 ligation with its effects in immature B lymphoid cells are also unclear. In mature B and T lymphocytes CD38 signaling depends on the integrity of the B and T cell Ag receptors, respectively (16–18), which are not expressed on the surface of immature B cells.

In the present study we used Ba/F3 murine pro-B cells transfected with a cDNA encoding human CD38 to further clarify the signaling mechanism mediated by CD38 in immature lymphoid cells. We found that ligation of human CD38 ectopically expressed in murine pro-B cells results in signaling events that recapitulate those seen in their human counterparts. In addition, ligation of CD38 in the murine transfecteds caused marked homotypic aggregation followed by cell death. Identical results were obtained in cells transfected with a CDNA encoding a mutant CD38 molecule lacking the cytoplasmic domain, demonstrating that the latter is not required for CD38-mediated signaling in immature lymphoid cells.

Materials and Methods

Cells and Abs

The IL-3-dependent murine pro-B cell line Ba/F3 (19) and the human B lymphoblastic leukemic cell lines RS4;11 (20) and OP-1 (21) were available in our institution. Ba/F3 was cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with IL-3 (25 U/ml; from CHO cells expressing the murine IL-3 gene), 10% FCS (BioWhittaker), L-glutamine, and antibiotics. For RS4;11 and OP-1 we used an identical tissue culture medium without IL-3. To prepare bone marrow-derived stromal layers, we collected mononucleolated cells from donor-derived normal marrow. The cells were separated on a density gradient (Lymphoprep, Nyegaard, Oslo,

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Norway) and washed three times in RPMI 1640. Stromal layers were prepared in flat-bottom 96-well plates (Costar, Cambridge, MA) and fed with RPMI 1640, 10% FCS, and 10^{-6} M hydrocortisone (Sigma, St. Louis, MO), as previously described (14, 22–26).

Anti-CD38 mAbs comprised T16 (IgG1; Immunotech, Westbrook, ME), THB7 (IgG1; American Type Culture Collection, Manassas, VA), and IB4 (IgG2a) and IB6 (IgG2b; gifts from Dr. F. Malavasi, University of Ancona, Ancona, Italy). F(ab’{	extsubscript{2}}), of IB4 (from Dr. Malavasi), and Fab and F(ab’{	extsubscript{2}}), of THB7 (prepared in our laboratory) were used in some experiments. The purity of the latter reagents was verified by SDS-PAGE and was corroborated by the lack of staining seen in CD38{	extsuperscript{+}} RS4:11 cells when a goat anti-mouse Ig Fc antisera was used as a secondary Ab. Rat anti-mouse CD71 (C2F2; Phar-Mingen, San Diego, CA), and mouse control mouse Ig control (IgG1; Becton Dickinson, San Jose, CA) were also used. mAb to phosphotyrosine (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY), Polyclonal anti-PI 3-K p85 was purchased from Transduction Laboratories (Lexington, KY). Polyclonal goat antisera to TEC and rabbit antiserum to CBL, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum to TEC was previously described (27).

DNA constructs and electroporation conditions

The CD38 expression vector was constructed by excising the human CD38 cDNA fragment from pCDM:CD38 (a gift from Dr. D. G. Jackson, Oxford, U.K.) with XbaI and inserting it into an XbaI-cleave pEF-BOS mammalian expression vector (a gift from Dr. S. Nagata, Osaka, Japan). Human CD38 with the cytoplasmic tail deleted (CD38 Δ1–20) was constructed by subcloning the XbaI fragment of human CD38 cDNA into pBluescript II SK{	extsuperscript{+}} (Stratagene, La Jolla, CA). The pBluescript-CD38 contained a unique EcoRV site at nucleotide 241 of the CD38 cDNA and a SpeI site in the multiple cloning site of the vector. Thus, a SpeI–EcoRV fragment was generated by PCR amplification (25 rounds) using 5'-GGGACTAGTATGAGGCCCCAATCTGTTG6'-3' as a 5' primer, 5'-TCTCGGGAAGCGCCTTGGTG3'-3' as a 3' primer, and pBluescript-CD38 as a template. The PCR-generated fragment was then used to replace the SpeI site, which was followed by the CD38-coding sequence including ATG as an initiation codon, three nucleotides encoding for one amino acid (Arg) of the cytoplasmic domain, and the entire transmembrane and extracellular domains through the EcoRV site. The fragment was digested with SpeI and EcoRV, and then used to replace the SpeI–EcoRV portion of pBluescript-CD38. The resulting plasmid was digested with SpeI and XbaI and the resulting cDNA fragment was inserted into pEF-BOS. The introduced mutation was confirmed by DNA sequence analysis.

Each expression plasmid (32 μg) was electroporated into 5 × 10^6 Ba/F3 cells with 3.2 μg of a second plasmid (pStNeoB) using a gene pulsar apparatus (Bio-Rad, Richmond, CA) set at 960 F and 290 V. Cells were filtered, and used at 2–10^4 cells/ml. Abs were then added to the cleared lysates, washed in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween-20 for 2 h and then with primary Abs for 1 h. After washing in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween-20, the filters were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse Ig or donkey anti-rabbit Ig (Amersham, Arlington Heights, IL), and IB4 (IgG2a) and IB6 (IgG2b; gifts from Dr. F. Malavasi, University of Ancona, Ancona, Italy). F(ab’{	extsubscript{2}}), of IB4 (from Dr. Malavasi), and Fab and F(ab’{	extsubscript{2}}), of THB7 (prepared in our laboratory) were used in some experiments. The purity of the latter reagents was verified by SDS-PAGE and was corroborated by the lack of staining seen in CD38{	extsuperscript{+}} RS4:11 cells when a goat anti-mouse Ig Fc antisera was used as a secondary Ab. Rat anti-mouse CD71 (C2F2; Phar-Mingen, San Diego, CA), and mouse control mouse Ig control (IgG1; Becton Dickinson, San Jose, CA) were also used. mAb to phosphotyrosine (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY), Polyclonal anti-PI 3-K p85 was purchased from Transduction Laboratories (Lexington, KY). Polyclonal goat antisera to TEC and rabbit antiserum to CBL, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum to TEC was previously described (27).

Immunoprecipitation, SDS-PAGE, and Western blotting

Immunoprecipitation was performed essentially as previously described (10–13). Briefly, after exposure to anti-CD38 Ab or control Ig (5–10 μg/ml), cells were lysed in 1 ml of ice-cold lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 5 mM aprotinin, 1 mM PMSF, 1 mM EDTA, and 1 mM NaVO_4) and centrifuged at 20,000 × g for 20 min at 4°C. Supernatants were preincubated by 1 h of protein A-Sepharose treatment (20 μl of a 50% slurry). Abs were then added to the cleared lysates, which were incubated at 4°C for 1–2 h. The immune complexes were collected by using protein A-Sepharose.

For SDS-PAGE, cell lysates and immunoprecipitates were resuspended in sample buffer (10% (v/v) glycerol, 5% 2-ME, 3% (w/v) SDS, 65 mM Tris-HCl (pH 6.8), and 0.002% (w/v) bromophenol blue) and separated on a 7.5% acrylamide gel (10 –13). After transfer, nitrocellulose filters were incubated first in 5% nonfat dry milk in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween-20 for 2 h and then with primary Abs for 1 h. After washing in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween-20, the filters were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse Ig or donkey anti-rabbit Ig (Amersham, Arlington Heights, IL).

**FIGURE 1.** Expression of human CD38 in transfected murine Ba/F3 cells. Cells were incubated with anti-CD38 (solid lines) or isotype-matched Ig (broken lines), both conjugated to FITC, and the intensity of fluorescence was analyzed by flow cytometry. Clones derived from single Ba/F3 cells transfected with human CD38 (Ba/F3-CD38) or human CD38 lacking the cytoplasmic portion (Ba/F3-CD38 Δ1–20) expressed high levels of CD38, in contrast to Ba/F3 cells transfected with vector only (Ba/F3-Mock), which were negative. Expression of CD38 on the human immature B cell line RS4:11 is shown for comparison.

The filters were then washed, incubated with enhanced chemiluminescence detection reagents (Amersham), and exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY). For reprobing, the filters were stripped and then reblocked, washed, and reprobed. All experiments were repeated at least three times.

**Cell culture studies**

Before each experiment, we removed the media from cultured stromal cells and washed the adherent cells with RPMI 1640 to remove hydrocortisone fully. Ba/F3 cells transfected with cDNA encoding the entire CD38 molecule, with cDNA encoding the mutant lacking the cytoplasmic tail (CD38 Δ1–20), or with the vector only were resuspended in fresh tissue culture medium with IL-3 (see above). Two hundred microliters of each cell suspension (5 × 10^5 cells/ml) were then seeded onto marrow stromal cells. In parallel experiments, transfected Ba/F3 cells were placed in the empty wells of a 96-well flat-bottom microtiter plate. For culture experiments, anti-CD38 Abs and nonreactive control Ig were dialyzed in PBS, sterile-filtered, and used at 2–10 μg/ml. β-NAD and hyaluronic acid (from human umbilical cord) were purchased from Sigma and used at the concentrations indicated in Results. All cell cultures were incubated at 37°C in 5% CO_2 with 90% humidity. At the termination of cultures, plates were observed with an inverted microscope. In some experiments cells were pretreated with the tyrosine kinase inhibitor herbimycin A, the PI 3-K inhibitor wortmannin, or their diluent DMSO (all from Sigma) for 1 h before addition of Abs, at the concentrations indicated in Results.

**Cell counting and assessment of cell death**

After culture, cells were harvested by vigorous pipetting, suspended in PBS, and passed through a 19-gauge needle to disrupt clumps. Viable cells in culture were enumerated by flow cytometry, as previously described (14, 22–26). To detect phosphatidylserine residues expressed on the cell surface (a marker of apoptosis) (28), we labeled cells with FITC-conjugated annexin V (Trevenig, Gaithersburg, MD), following the manufacturer’s instructions. In these experiments, cell membrane permeabilization was revealed by labeling cells with 5 μg/ml of propidium iodide (Trevenig) for 15 min at 20°C.

**Results**

**Signaling effects of human CD38 ligation in Ba/F3 cells**

Clonal Ba/F3 cells expressing high levels of human CD38 were obtained after transfection and single cell sorting. In the selected clones, levels of cell surface CD38 expression were similar or higher than those measured on human immature B cells (Fig. 1).

After culture, cells were harvested by vigorous pipetting, suspended in PBS, and passed through a 19-gauge needle to disrupt clumps. Viable cells in culture were enumerated by flow cytometry, as previously described (14, 22–26). To detect phosphatidylserine residues expressed on the cell surface (a marker of apoptosis) (28), we labeled cells with FITC-conjugated annexin V (Trevenig, Gaithersburg, MD), following the manufacturer’s instructions. In these experiments, cell membrane permeabilization was revealed by labeling cells with 5 μg/ml of propidium iodide (Trevenig) for 15 min at 20°C.
By contrast, mock-transfected Ba/F3 cells did not react with anti-human CD38 Abs.

To test whether CD38 ligation in transfected Ba/F3 cells caused tyrosine phosphorylation of cellular proteins, we engaged CD38 with an anti-CD38 Ab (T16) and performed anti-phosphotyrosine Western blots on cell lysates using the anti-phosphotyrosine Ab 4G10. Tyrosine phosphorylation of several proteins was detectable after 5 min of incubation with anti-CD38; the intensity of tyrosine phosphorylation was proportional to the amount of anti-CD38 used (Fig. 2). Tyrosine phosphorylation was also induced when T16 was replaced with another anti-CD38 Ab, THB7 (not shown). Time-course experiments showed that CD38-induced tyrosine phosphorylation was rapid and transient (Fig. 3). Tyrosine phosphorylation was detected as early as 1 min after exposure of cells to the anti-CD38 Ab and gradually decreased thereafter. This kinetic profile of tyrosine phosphorylation was similar to that induced by CD38 ligation in human immature B cells (11). Moreover, the pattern of tyrosine-phosphorylated bands was remarkably similar (Fig. 4).

To determine whether some of the molecules known to be activated during CD38-mediated signaling in human immature B cells were also activated in Ba/F3 cells transfected with human CD38, we tested tyrosine phosphorylation of the tyrosine kinase TEC, the adaptor molecule CBL, and the regulatory chain of PI 3-K (p85) (12, 13). For this purpose, we immunoprecipitated these proteins by using specific Abs before and after exposure of Ba/F3 to anti-CD38 and tested their tyrosine phosphorylation in Western blots probed with anti-phosphotyrosine Ab. A marked increase in tyrosine phosphorylation of TEC (Fig. 5) and CBL (not shown) proteins was observed after CD38 ligation, while no substantial changes in the levels of PI 3-K p85 tyrosine phosphorylation were observed. In these experiments, however, several tyrosine-phosphorylated proteins coprecipitated with PI 3-K p85 in cells exposed to anti-CD38 (Fig. 6), suggesting that CD38 ligation induces association of p85 with other intracellular proteins.

**FIGURE 2.** CD38 ligation in Ba/F3 cells expressing human CD38 triggers tyrosine phosphorylation. Ba/F3 cells transfected with human CD38 were exposed for 5 min to the different concentrations of anti-CD38 Ab (T16) indicated. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with the anti-phosphotyrosine Ab 4G10. Molecular mass markers (in kDa) are indicated.

**FIGURE 3.** CD38-mediated tyrosine kinase activity in Ba/F3 cells transfected with human CD38 is rapid and transient. Ba/F3 cells transfected with human CD38 were exposed for the time periods indicated to anti-CD38 Ab (T16). Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with the anti-phosphotyrosine Ab 4G10. Molecular mass markers (in kDa) are indicated.

**Cellular effects of human CD38 ligation in Ba/F3 cells**

Ligation of human CD38 with T16 (IgG1), THB7 (IgG1), IB4 (IgG2a), and IB6 (IgG2b) induced marked homotypic aggregation in cultures of Ba/F3 cells. Aggregation became distinguishable after 2 h of exposure to the Ab and was maximal after 24 h (Fig. 7). To determine whether Fc receptor engagement was required for...

**FIGURE 4.** Ligation of CD38 induces a similar pattern of tyrosine phosphorylation in human and murine pro-B cells. RS4:11 human immature B cells and Ba/F3 cells transfected with human CD38 were exposed for 5 min to anti-CD38 Ab (T16). Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with the anti-phosphotyrosine Ab 4G10. Molecular mass markers (in kDa) are indicated.
CD38-mediated cell aggregation, we performed experiments with anti-CD38 reagents lacking the Fc portion. Fab (ab’), of THB7 and IB4 induced aggregation identical with that seen with whole Abs (Fig. 7), thus excluding Fc-mediated signaling. Notably, Fab of THB7 also induced cell aggregation, suggesting that receptor cross-linking was not required. By contrast, anti-LFA-1, anti-CD71 (both reactive with Ba/F3 cells), and control Ig did not cause cell aggregation.

Cell aggregation was not noticeably affected by the addition of EDTA (1 or 5 mM) or EGTA (1 or 5 mM) to the cultures, suggesting that it occurred independently of Ca\(^{2+}\) and Mg\(^{2+}\). Likewise, aggregation was not inhibited by adding to the cultures an Ab to LFA-1 90 min before exposure to anti-CD38, indicating that the LFA-1/ICAM-1 interaction was not involved.

Ligation of CD38 in stroma-supported cultures of human immature lymphoid cells inhibits cell proliferation and induces cell death (14). To determine whether a similar effect would occur in the transfectants, we cultured these cells on bone marrow-derived stroma in the presence of anti-CD38 Ab (T16). In seven experiments, the mean cell recovery after 3 days of culture in the presence of anti-CD38 Ab (T16) was 10.5 ± 9.2% (±SD) of the mean cell recovery in parallel cultures containing an isotype-matched nonreactive Ab. Cell recovery was also suppressed by another anti-CD38 Ab (THB7), although less markedly (37.5 ± 7.6% of control cultures in three experiments). The inhibitory effects of CD38 ligation on cell recovery could also be observed when anti-CD38 Ab (THB7) was used in an F(ab’)\(_2\) form. In three experiments, cell recovery was 42.5 ± 6.2% of the recovery in parallel control cultures, an inhibitory effect similar to that observed with the whole THB7 molecule.

Decreased cell recovery in cultures containing anti-CD38 was at least in part caused by cell death. After 24 h of CD38 ligation there was a reduction in cell size accompanied by exposure of phosphatidylserine residues on the cell membrane followed by surface membrane permeabilization, compatible with apoptosis (Fig. 8).

β-NAD, hyaluronic acid, and CD31 can bind to CD38 and have been proposed as candidate natural ligands of CD38 (15, 29, 30). We therefore investigated whether addition of these molecules to cultures of Ba/F3 cells expressing human CD38 could mimic the cellular effects of anti-CD38 Abs. In repeated experiments β-NAD (5–50 μM) and hyaluronic acid (10–100 μg/ml) did not cause detectable cell aggregation of Ba/F3-CD38 cells. OP-1 cells, which express high levels of surface CD31 (H. Nishigaki and D. Campana, unpublished observation), also failed to elicit cell aggregation when added (as intact cells or as cell homogenates) to Ba/F3-CD38 cultures. 

**Relationship between biochemical and cellular effects triggered by CD38 ligation**

To determine whether the homotypic aggregation observed after CD38 ligation was dependent on the increased tyrosine kinase activity, we added anti-CD38 to cultures of CD38-transfected Ba/F3 cells exposed to herbimycin A, a tyrosine kinase inhibitor. At 20 μM, herbimycin A completely abrogated cell aggregation (Fig. 9). In parallel experiments, herbimycin A was replaced with wortmannin (100–500 nM), a PI 3-K inhibitor. In these cultures, CD38-mediated aggregation was delayed; definitive cell aggregates began to appear after 5 h or more of exposure to CD38, but after only 2 h in cultures without wortmannin (Fig. 9). However, after 24 h, cultures with and without wortmannin were indistinguishable. These results indicate that cell aggregation depends on the induction of tyrosine phosphorylation and suggest the involvement of PI 3-K in this signaling pathway.

In contrast to our previous observations with human immature B cells (13), no relation between CD38-mediated biochemical events and growth suppression could be detected. Wortmannin at 100 nM

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**FIGURE 5.** Ligation of CD38 in Ba/F3 cells induces tyrosine phosphorylation of the tyrosine kinase TEC. Ba/F3 cells transfected with human CD38 were incubated with anti-CD38 mAb T16 and isotype-matched nonreactive Ig for 5 min. Proteins immunoprecipitated with anti-TEC rabbit Ab were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti-phosphotyrosine Ab 4G10 (pTyr; upper panel), then stripped and reprobed with anti-TEC rabbit Ab (lower panel). Molecular mass markers (in kDa) are indicated. The position of Tec is indicated by an arrow.

**FIGURE 6.** CD38 ligation induces association of PI 3-K p85 with tyrosine-phosphorylated proteins. After incubation with or without anti-CD38 (T16) for 5 min, Ba/F3 cells expressing human CD38 were lysed, and immunoprecipitates were made using anti-PI 3-K p85 Ab. After blotting, the filter was first probed with anti-phosphotyrosine Ab 4G10 (upper panels), then stripped and reprobed with anti-PI 3-K p85 Ab (lower panels). CD38 ligation induced PI 3-K p85 association with tyrosine-phosphorylated proteins. Arrows point to the predicted migration position of p85. Molecular mass markers (in kDa) are indicated.
did not rescue cells from CD38-mediated suppression of cell growth, while prolonged incubations with higher concentrations of wortmannin or herbimycin A severely affected the survival of Ba/F3 cells.

The cytoplasmic portion of CD38 is not required for signaling in lymphoid cells

Human CD38 has a short cytoplasmic domain that has no tyrosine residues and no known motifs (1). To test whether this domain is required for the cellular and signaling events triggered by CD38 ligation in lymphoid cells, we prepared a construct encoding a truncated form of CD38 lacking all but the first amino acid proximal to the transmembrane portion, and expressed this construct in Ba/F3 cells.

In cells expressing the mutant CD38 (Fig. 1), ligation of CD38 induced tyrosine phosphorylation with intensity and kinetics similar to those seen in cells expressing the entire protein (Fig. 10). Consistent with these findings, ligation of CD38 in these cells induced vigorous cell aggregation that was maximal after 24 h of incubation with the Ab (not shown). Moreover, cell growth was inhibited when Ba/F3 cells expressing the CD38 cytoplasmic mutant were exposed to anti-CD38 Ab. In two separate experiments, cell recoveries after 3 days of culture on stroma in the presence of anti-CD38 (T16) were 5.6 and 15.6% of cell recovery in parallel cultures containing an isotype-matched control Ab. Taken together, these results indicate that the cytoplasmic domain of CD38 is not essential for signal transduction in immature lymphoid cells.

Discussion

To elucidate the molecular mechanism underlying CD38-mediated signaling, we generated murine pro-B cells expressing human CD38. CD38 ligation in these cells caused rapid and transient tyrosine phosphorylation of several cellular proteins, including TEC and CBL, and the association of PI 3-K with other tyrosine-phosphorylated proteins. Overall, the pattern and kinetics of tyrosine phosphorylation triggered by CD38 ligation in murine cells were remarkably similar to those observed in human immature B cells, implying the usage of identical signaling components.

The precise changes induced by Ab ligation in the expression, lateral association, or conformation of the CD38 molecule that result in signal transduction are still unclear. It is unlikely that changes in the CD38 enzymatic activity participate in this effect, as NAD hydrolysis and production of cyclic ADPR are not affected by binding of anti-CD38 to the surface of immature B cells (14). One possibility is that Ab binding induces CD38 internalization,

FIGURE 7. Ligation of CD38 induces aggregation of Ba/F3 cells. Ba/F3 cells transfected with human CD38 (bottom panels) or vector only (top panels) were exposed to isotype-matched nonreactive Ig (left panels), anti-CD38 Ab (THB7; center panels), or THB7 F(ab’)_2, for 24 h.

FIGURE 8. Ligation of CD38 induces cell death in Ba/F3 cells. Ba/F3 cells expressing human CD38 were incubated with anti-CD38 Ab (T16; bottom panels) or with isotype-matched nonreactive Ig (top panels) for 40 h. Left panels show density plots of the cells’ light scatter (FSC, forward scatter; SSC, side scatter), while right panels illustrate the intensity of labeling with annexin V FITC (x-axes) and propidium iodide (PI, y-axes). Exposure to CD38 induced a reduction in FSC (a measurement of cell size) and increases in annexin V labeling (indicating exposure of phosphatidylserine residues on the cell membrane) and PI labeling (due to cell membrane permeabilization).
which, in turn, could trigger signaling pathways, perhaps via protein ribosylation (31) or mobilization of Ca\(^{2+}\) from intracellular stores (9, 32). Indeed, capping and internalization of CD38 following exposure to anti-CD38 Abs have been documented (33), although in immature B cells this response is clearly less prominent than that of other transmembrane molecules, such as CD19, under similar circumstances (10).

The hypothetical structure of CD38, as predicted from the crystalline structure of the homologous molecule ADPR cyclase, comprises a dimer capable of hinge motion (15). Prasad et al. postulated that either enzymatic activity or ligand binding could cause conformational changes in the dimer. In turn, these could juxtapose tyrosine kinases bound to the cytoplasmic portion of CD38, triggering the tyrosine kinase cascade. We found that CD38-mediated signaling in Ba/F3 did not require the cytoplasmic portion of CD38, a result that agrees with the observations of Inoue et al. in myeloid cells (34). Taken together, these data suggest a more complex model, in which an as yet unknown transmembrane molecule (lineage specific or common to all hematopoietic lineages) is linked to the extracellular domain of CD38 and transduces conformational changes in CD38. However, we have not been successful to date in identifying tyrosine-phosphorylated proteins associated with CD38 in Ba/F3 cells.

The biochemical events triggered by CD38 ligation in Ba/F3 cells are accompanied by vigorous homotypic aggregation followed by cell death. Notably, aggregation was suppressed by inhibitors of tyrosine kinase and PI 3-K, implying a cause-effect relationship between biochemical and cellular events mediated by CD38. It is still not known whether aggregation was caused by de novo expression of surface adhesion molecules or by other mechanisms, but it appeared to be independent of Ca\(^{2+}\) and Mg\(^{2+}\) flux, and did not involve LFA-1/ICAM-1 interaction. Homotypic aggregation could also be triggered by F(ab')\(^2\), and Fab of the CD38 Ab, indicating that it is not dependent on Fc receptor cross-linking. In contrast, signaling triggered by anti-CD38 Abs in myeloid leukemic cells requires simultaneous binding of anti-CD38 Abs to Fc receptors (34).

In summary, CD38 is a surface receptor that can mediate biochemical and cellular signals in human and murine immature B cells independently from its cytoplasmic domain. The results of this and other studies (14, 16, 35–37) imply the existence of natural ligands for CD38 that can induce events similar to those triggered by ligation of CD38 with mAbs. Hyaluronic acid and CD31 have been shown to bind CD38 and may well represent natural CD38 counter-receptors (29, 30). Likewise, \(\beta\)-NAD hydrolysis could provoke hinge motion in the CD38 dimer resulting in signal transduction. However, we failed to detect signals in Ba/F3 cells expressing human CD38 after exposure to \(\beta\)-NAD, hyaluronate, or CD31. Thus, these molecules cannot account for the full range of cellular and biochemical effects mediated by CD38, suggesting the existence of other molecules capable of triggering CD38 signaling. In this respect, it is relevant that reducing agents can cause self-aggregation and internalization of the CD38 molecule (38), suggesting alternative means of triggering CD38 signaling that warrant further exploration.
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