The Role of the DAP12 Signal in Mouse Myeloid Differentiation[1]

Naoko Aoki,[2]* Shoji Kimura,† Yumi Takiyama,* Yoshiaki Atsuta,* Asami Abe,* Keisuke Sato,* and Makoto Katagiri*

DAP12 is a recently cloned, immunoreceptor tyrosine-based activation motif-bearing transmembrane adapter molecule that is associated with the NK-activating receptors. Previous reports showed that the DAP12 message could be detected not only in NK cells but also in granulocytes, monocytes, dendritic cells, and macrophages. In this study we found a significant level of DAP12 protein expression in macrophage-related cell lines and organs. Additionally, we observed increased expression of DAP12 after LPS-induced differentiation of M1 cells into macrophages. To examine the role of DAP12 in the myeloid cell lineage, we established M1 FLAG-DAP12 transfectants (FDAP-M1) and demonstrated the marked morphological changes in FDAP-M1 cells caused by signaling through DAP12. Cell surface phenotypic analysis showed up-regulation of macrophage markers CD11b, 2.4G2, and adhesion molecule B7-2. Additionally, after stimulation through DAP12, phosphorylated FLAG -DAP12 could be immunoprecipitated using anti-phosphotyrosine mAbs. Collectively, these findings indicate that direct DAP12 signaling has an important role in macrophage differentiation. ***The Journal of Immunology*****, 2000, 165: 3790–3796.**

Terminal differentiation of the myeloid cell precursors into macrophages is accomplished through a series of complicated molecular steps during the process of hemopoiesis (1–3). However, the mechanisms of the signaling cascades during differentiation are still not totally understood.

Recently, a novel immunoreceptor tyrosine-based activation motif (ITAM)[3]-bearing transmembrane adapter molecule called DAP12 was identified and cloned by Lanier et al. (4). DAP12 was expressed on the cell surface of NK cells and associated noncovalently with the killer cell-activating receptors (KARs) (4–7). Although the expression of KARs is restricted to NK and T cell subsets (reviewed in Ref. 8), DAP12 transcription is distributed among a wide variety of cell types, including peripheral blood granulocytes, monocytes, and dendritic cells (4, 6, 9).

Independently in our laboratory, during the course of exploring the molecular mechanism underlying the growth requirement of a CTLL-2 derived subline (named CTL(−)) which has no need of exogenous IL-2 for its growth, we have identified a novel ITAM-containing molecule. The DNA sequence of the gene was same as that of DAP12. The transcript was easily detected in macrophage cell lines in addition to CTL(−), but not in cell lines with T or B cell characteristics (our unpublished observations). Even though reports on the DAP12 molecule of NK cells are accumulating (10–12), little is known about the effects of DAP12 on macrophage lineage.

In this report we first demonstrated the precise expression pattern of the DAP12 protein on various cell lines and tissues in the presence or the absence of stimulation. Secondly, to explore the function of DAP12 in macrophage differentiation, we used mouse myeloid leukemic M1 cells, which can be induced to differentiate into macrophages by treatment with various agents, including LPS, phorbol ester, and various cytokines (13, 14). We established an M1 FLAG-DAP12 transfectant and investigated the importance of signal transduction via the DAP12 molecule for M1 cell differentiation into a macrophage.

Materials and Methods

Mice, cells, and culture conditions

BALB/c mice were bred in our facility and used at 6–10 wk of age. 1029 and 8072, myeloid cell lines of C3H/He origin, were made available by Dr. N. Tada (Tokai University, Isehara, Japan). P388D1 and J774-1, macrophage cell lines derived from DBA/2 and BALB/c, respectively, were provided by Dr. K. Tomonari (Fukui Medical School, Fukui, Japan). The M1 cell line was obtained from Riken Gene Bank (Wakou, Japan). CTLL-2 was obtained from the American Type Culture Collection (Manassas, VA) and has been maintained with exogenous IL-2. The CTL(−) line was established from CTLL-2 by gradual omission of exogenous IL-2 from the culture medium, and it has been maintained under IL-2-free conditions. Cells were cultured in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS and 5×10^{-5} M 2-ME.

Antibodies

Rabbit anti-mouse DAP12 polyclonal Ab was generated by immunizing a rabbit (Japanese White) with the glutathione-*S*-transferase-mouse DAP12 cytoplasmic domain fusion protein (DAP12 CY). The fusion protein was produced as described previously (15). Anti-CD11b mAb (M1/70) and anti-CD11c mAb (N418) were purchased from Chemicon (Temecula, CA). Anti-B7-2 mAb (GL-1), anti-CD16/CD32 (2.4G2), and anti-CD14 mAb (rmC5-3) were purchased from PharMingen (San Diego, CA). Anti-MHC class II mAb K24.64 culture supernatant (16) was prepared in our laboratory.

*Department of Pathology and †School of Nursing, Asahikawa Medical College, Asahikawa, Japan

Received for publication March 14, 2000. Accepted for publication July 14, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant-in-Aid 09470060 from the Ministry of Education, Science, Sports, and Culture, Japan.

[2] Address correspondence and reprint requests to Dr. Naoko Aoki, Department of Pathology, Asahikawa Medical College, Higashi 2-1-1, Midorigaoka, Asahikawa 078-8510, Japan. E-mail address: aoq@asahikawa-med.ac.jp

[3] Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; FDAP, FLAG-DAP12; KAR, killer cell-activating receptor; DAP12 CY, DAP12 cytoplasmic domain fusion protein; PY, phosphotyrosine.

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00

Immunoprecipitation, electrophoresis, and blotting

Cells were lysed in lysis buffer (0.5% Triton X-100, 50 mM Tris (pH 7.2), 140 mM NaCl, and 10 mM EDTA) containing the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). Lysates were clarified by centrifugation and immunoprecipitated with Ab bound to rProtein A-Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1–2 h at 4°C. The resulting immunocomplexes were washed and run on 4–12% NuPage bis-Tris SDS-PAGE gels (Novex, San Diego, CA) under reducing conditions. Proteins were then blotted onto Immobilon-P (Millipore, Bedford, MA), blocked in 5% skim milk or 3% BSA, and probed with rabbit anti-DAP12 Ab or anti-FLAG mAb (M2; Sigma, St. Louis, MO) followed by donkey anti-rabbit IgG-HRP (Amersham Pharmacia Biotech) or sheep anti-mouse IgG-HRP (Amersham Pharmacia Biotech). The ECL system (Amersham Pharmacia Biotech) was used for detection. Tyrosine phosphorylation was examined using the following lysis buffer: 1% digitonin, 0.1% Triton X-100, 50 mM Tris (pH 7.2), 140 mM NaCl, 10 mM EDTA with protease inhibitor mixture, sodium fluoride, and sodium orthovanadate. Immunoblots were probed with anti-phosphotyrosine (anti-PY) mAb (4G10; Upstate Biotechnology, Lake Placid, NY) followed by anti-mouse IgG-HRP.

DAP12 expression vectors

The cDNA encoding DAP12 was originally cloned as an ITAM-containing molecule by PCR subtraction between CTL(−) and CTLL-2 cell lines. The CTL(−) line was established as a subline of CTLL-2, showing IL-2-independent growth as described above.

A cDNA containing the CD8 leader segment, followed by the FLAG peptide epitope (DYKDDDDK) and joined to the extracellular, transmembrane, and cytoplasmic regions of mouse DAP12 was constructed using the above cDNA as a template and the PCR overlap extension method (17). The primers used were: 5′-GCGAATTCCGCGTCATG GCCTTACCAGTGA-3′ (5′CD8), 5′-CCTCTAGAGGGGACAGAAAT GGTACAATGT-3′ (3′DAP12), and overlapping primers, 5′-GTCATCG TCGTCCTTGTAGTCCGGCCTGGCGGCGTGGAG-3′, 5′-CTCCACG CCGCCAGGCCGGACTACAAGGACGACGATGAC-3′, 5′-TACAA GGACGACGATGACAAGCAGAGTGACACTTTCCCA-3′, and 5′-TG GGAAAGTGTCACTCTGCTTGTCATCGTCGTCCTTGTA-3′. The PCR fragment was first cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), then the sequence was confirmed and subcloned into the pME18S neo expression vector. Similarly, the DAP12 cytoplasmic fragment was cloned in-frame into the pGEX-KG vector (15). pME18S neo and pGEX-KG were provided by Dr. K. Maruyama (Tokyo University, Tokyo, Japan) and Dr. J. E. Dixon (Purdue University, West Lafayette, IN), respectively.

Transfection

M1 cells were stably transfected by electroporation (Gene Pulsor, Bio-Rad, Hercules, CA). To obtain the FLAG-DAP12 (FDAP) M1 transfectant, M1 cells were transfected with pME18S neo-FLAG-DAP12. pME18S neo-B2 M7 (18) was used as the control G418-resistant clone. In either case, a pulse was delivered to 800 μl of suspension containing 1×10^7 cells and 30 μg of plasmid DNA. After 48 h, cells were subjected to selection in growth medium containing 0.5 mg/ml of geneticin (G418). Resistant clones were isolated and selected by limiting dilution.

Assays for differentiation

M1 cells were cultured with 10 μg/ml of LPS from *Escherichia coli* serotype 0111:B4 for 3 days. In the case of CSF, cells were cultured with 50 ng/ml of GM-CSF, M-CSF, or G-CSF (R&D Systems, Minneapolis, MN) for 8 days.

Assays for differentiation by DAP12 signaling

To immobilize Abs, the SonicSeal Slide Wells (Nalge Nunc International, Naperville, IL) were incubated with anti-FLAG mAb (M2; 20 μg/ml in PBS; Sigma) or isotype control of mouse IgG1 (20 μg/ml in PBS; Chemicon) overnight at 4°C and washed with culture medium twice. M1 or FDAP-M1 cells were incubated with or without LPS (10 μg/ml; Sigma) overnight (10–14 h) and washed with culture medium before being transferred to Ab-coated SonicSeal Slide Wells. Cells were cultured on the SonicSeal Slide Well coated with anti-FLAG mAb (M2) or mouse IgG1 isotype control for 4 days. Cells were stained with hematoxylin-eosin or were used for flow cytometry analysis and Western blotting.

Flow cytometry

Cells (1×10^6) were incubated with saturating amounts of primary mAbs for 30 min in staining buffer (PBS, 1% FCS, and 0.1% sodium azide) at 4°C. As a second Ab, FITC-donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), FITC-rabbit anti-mouse IgG (Dako, Glostrup, Denmark), FITC-goat anti-hamster IgG (Jackson ImmunoResearch Laboratories), or avidin-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used. Dead cells were gated out by using 2 μg/ml of propidium iodide at the last step of staining.

Results

Protein expression pattern of DAP12 on tissues and cell lines with or without stimulation

To gain a more precise knowledge of the expression pattern of DAP12 protein, we performed Western blotting analysis on a variety of cell lines and organs (Fig. 1). DAP12 protein was strongly expressed in macrophage cell lines such as P388D1 and J774-1 as well as CTL(−), an autonomous growing CTLL-2 subline from which we first cloned DAP12 gene by PCR subtraction analysis. In agreement with the finding of DAP12 expression in macrophage cell lines, DAP12 protein was found in lung, liver, thymus, lymph node, spleen, and peritoneal macrophages, but not in brain, kidney, or testis. DAP12 in each organ was formed by dual bands, but the amount of the predominant band differs from one organ to another. For example, in the lung it is the upper band, while in the thymus it is the lower one (Fig. 1*B*). This may be due to modification of DAP12 at different activation states. Compared with other organs, the m.w. of DAP12 protein in bone marrow is slightly smaller. This finding suggests a difference in glycosylation or another modification of DAP12 between bone marrow and other organs.

M1 is a murine myeloblastic leukemic cell line capable of differentiating into macrophages upon stimulation by LPS or cytokines such as IL-6 (13, 14). We studied the mode of expression of DAP12 during macrophage differentiation. As shown in Fig. 2*A*, DAP12 protein was not detectable in uninduced M1 cells, but the expression level was noticeably increased following induction of differentiation by LPS. A similar result was obtained when murine bone marrow cells were cultured with LPS (Fig. 2*B*). Although

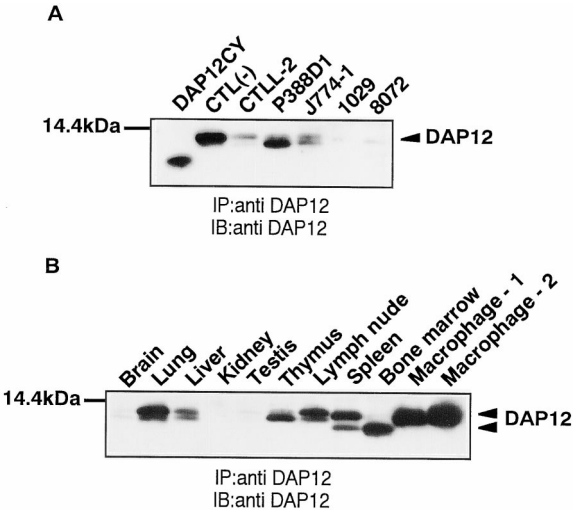

FIGURE 1. Expression of DAP12 in different cell lines and organs. *A*, Lysates prepared from 1×10^7 cells were immunoprecipitated with affinity-purified rabbit anti-mouse DAP12 polyclonal Abs. Samples were analyzed by Western blot using anti-DAP12 Ab. GST-DAP12 CY (0.1 μg) was used as a positive control. CTL(−) is the IL-2-independent cell line derived from CTLL-2. CTL(−) and CTLL-2 are T cell lines. P388D1 and J774-1 are macrophage cell lines.1029 and 8072 are cell lines of myeloid origin. *B*, One milligram of cell lysate (500 μg of cell lysate for macrophage) was prepared from each BALB/c organ. Macrophages were collected from the abdominal cavity. Each cell lysate was immunoprecipitated with anti-DAP12 and analyzed by Western blot using anti-DAP12 Ab.

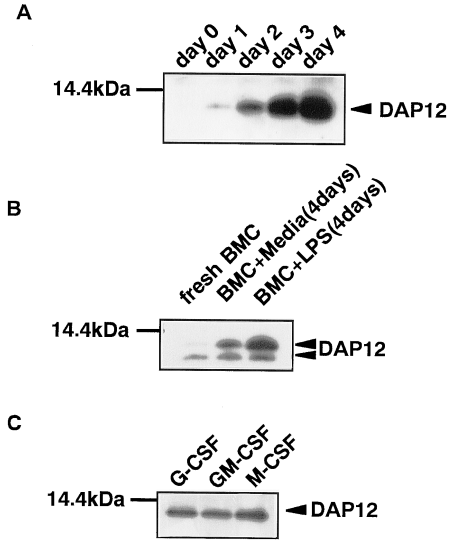

FIGURE 2. Expression pattern of DAP12 in M1 cells or bone marrow cells following treatment with various stimuli. *A,* Cells from a murine leukemic cell line, M1, were stimulated with LPS (10 μg/ml) for 4 days. Lysates prepared from 1×10^6 cells were immunoprecipitated with anti-DAP12 and analyzed by Western blot using anti-DAP12 Ab. *B,* Bone marrow cells prepared from BALB/c mice were cultured in the presence or the absence of LPS (10 μg/ml) for 4 days. Lysates prepared from 5×10^6 cells were immunoprecipitated with anti-DAP12 Ab and analyzed by Western blot using anti-DAP12 Ab. *C,* M1 cells were stimulated with 50 ng/ml of G-CSF, GM-CSF, and M-CSF for 8 days. Lysates prepared from 2×10^6 cells were immunoprecipitated with anti-DAP12 Ab and analyzed by Western blot using anti-DAP12 Ab.

murine bone marrow cells have only the smaller m.w. DAP12 without simulation, the macrophage-type DAP12 was induced following stimulation by LPS. DAP12 protein can be also detected in M1 cells by treatment with G-CSF, GM-CSF, or M-CSF (Fig. 2*C*).

Establishment of a FDAP-M1 transfectant and cell surface expression of FLAG-DAP12 with LPS stimulation

To examine the role of DAP12 in macrophages, several FDAP-M1 clones were established as described (Fig. 3*A*). As shown in Fig. 3*B*, uninduced FDAP-M1 cells revealed massive FLAG-DAP12 protein expression. Consistent with Fig. 2*A*, LPS-induced FDAP-M1 cells express a large amount of endogenous DAP12 as well as FLAG-DAP12. It has been reported previously that DAP12 alone cannot be expressed on the cell surface, but it requires an associate molecule, such as the NK cell-activating receptor, for cell surface expression (7). Although uninduced FDAP-M1 cells had massive amounts of FLAG-DAP12 protein in their cytoplasm, they were not transported to the cell surface (Fig. 3*C*). After LPS induction, FLAG-DAP12 became expressed at the cell surface with the aid of newly induced, possible associate molecule.

Morphological change and cell surface phenotypic change in FDAP-M1 cells due to cross-linking of DAP12

Next, we investigated whether stimulation through DAP12 affects differentiation of M1 cells into macrophages. As noted previously in Fig. 3*C*, FLAG-DAP12 protein alone could not be expressed on cell surface; therefore, we pretreated FDAP-M1 cells with LPS

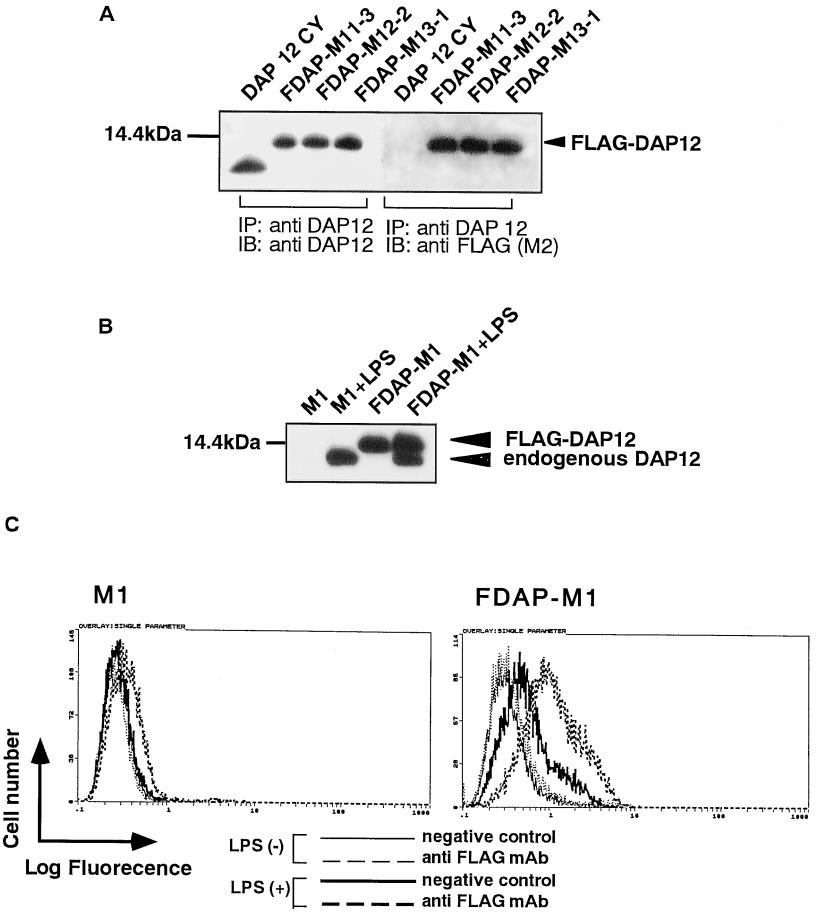

FIGURE 3. Establishment of M1 cell lines that express FLAG-DAP12 (FDAP-M1 cells) and their surface expression of DAP12 following LPS stimulation. *A,* Cell lysates prepared from 5×10^6 cells of each FDAP-M1 clone were immunoprecipitated with anti-DAP12 Ab and analyzed by Western blot using anti-DAP12 Ab or anti-FLAG mAb (M2). DAP12 CY (0.1 μg) was used as a control. *B,* M1 cells or FDAP-M1 cells were cultured in the presence or the absence of LPS (10 μg/ml) for 3 days. Lysates prepared from 5×10^6 cells were immunoprecipitated with anti-DAP12 Ab and analyzed by Western blot using anti-DAP12. *C,* M1 cells or FDAP-M1 cells were cultured in the presence or the absence of LPS (10 μg/ml) for 3 days. Cells were collected and blocked with 50% goat serum to avoid nonspecific binding and were stained with biotin-conjugated anti-FLAG mAb (M2) followed by avidin-FITC. Samples were analyzed by flow cytometry.

overnight before stimulation by immobilized anti-FLAG mAb to induce expression of the associate molecule of DAP12 on the cell surface. After stimulation with anti-FLAG mAb, hematoxylin-eosin staining of FDAP-M1 transfectants on the SonicSeal Slide Well showed dramatic changes to a macrophage-like morphology (Fig. 4*B*), similar to human monocytes stimulated with M-CSF (19). Nonspecific esterase staining was positive on these macrophage-like cells (data not shown). Compared with LPS-supported macrophage (Fig. 4*F*), cells that had undergone differentiation by stimulation through DAP12 showed marked spreading out of adherent cells, with elongation and spindle shape. No morphological change was observed in pME18S neo-B2M7-transfected M1 cells with LPS pretreatment plus anti-FLAG mAb (data not shown). Interestingly, stimulation via DAP12 produced a large number of giant cells that had abundant cytoplasm, and multiple nuclei were scattered on the slide (Fig. 4*E*). Even smaller cells appeared to have two or more nuclei; thus, a notable number of mirror-image cells and giant cells were detectable (Fig. 4*E*, *upper left panel*). Although LPS-supported macrophage showed a few giant cells, the rate of giant cell formation was low.

Cell surface phenotypic analysis of the macrophage-like cells generated by stimulation through DAP12 revealed up-regulation of class II, CD11b (Mac-1), CD16/CD32 (2.4G2), CD86 (B7-2), and CD11c (Fig. 5). No expression of CD14 was detected. In addition to morphological changes, cell surface analysis indicated that stimulation via DAP12 induced terminal differentiation of FDAP-M1 transfectants into a macrophage phenotype.

Involvement of phosphorylated DAP12 in the initiation of M1 differentiation

In an attempt to confirm DAP12 signaling, we performed anti-PY (phosphotyrosine) blotting during FLAG-DAP12 cross-linking using anti-FLAG mAb (M2). As shown in Fig. 6, FLAG-DAP12 is phosphorylated in FDAP-M1 cells constitutionally (Fig. 6*B*, *lanes 1–4*, *bands b1* and *b2*). Interestingly, an additional 17-kDa phosphorylated molecule (Fig. 6*B*, *lane 3*, *bands a1* and *a2*) was coimmunoprecipitated only when FDAP-M1 cells had been stimulated with anti-FLAG mAb. Immunoprecipitation by anti-PY mAb (4G10) showed a significant amount of FLAG-DAP12 after stimulation with anti-FLAG mAb (M2; Fig. 6*C*, *lane 3*, *bands b1* and *b2*). In contrast, overnight pretreatment with LPS or stimulation with control mouse IgG1 did not give rise to these bands. Coated anti-FLAG mAb without cross-linked secondary Abs brought about the same result (data not shown). These observation suggests that signal transduction via DAP12 plays a important role in our system of M1 terminal differentiation.

Discussion

DAP12 is a novel ITAM-bearing transmembrane adaptor molecule associated with KARs (4) and its role in KAR signaling has been relatively well characterized (4, 10, 11). Interestingly, DAP12 is expressed not only in NK cells, but also in other types of cells, such as macrophages (4) (Fig. 1). To our knowledge, the functional

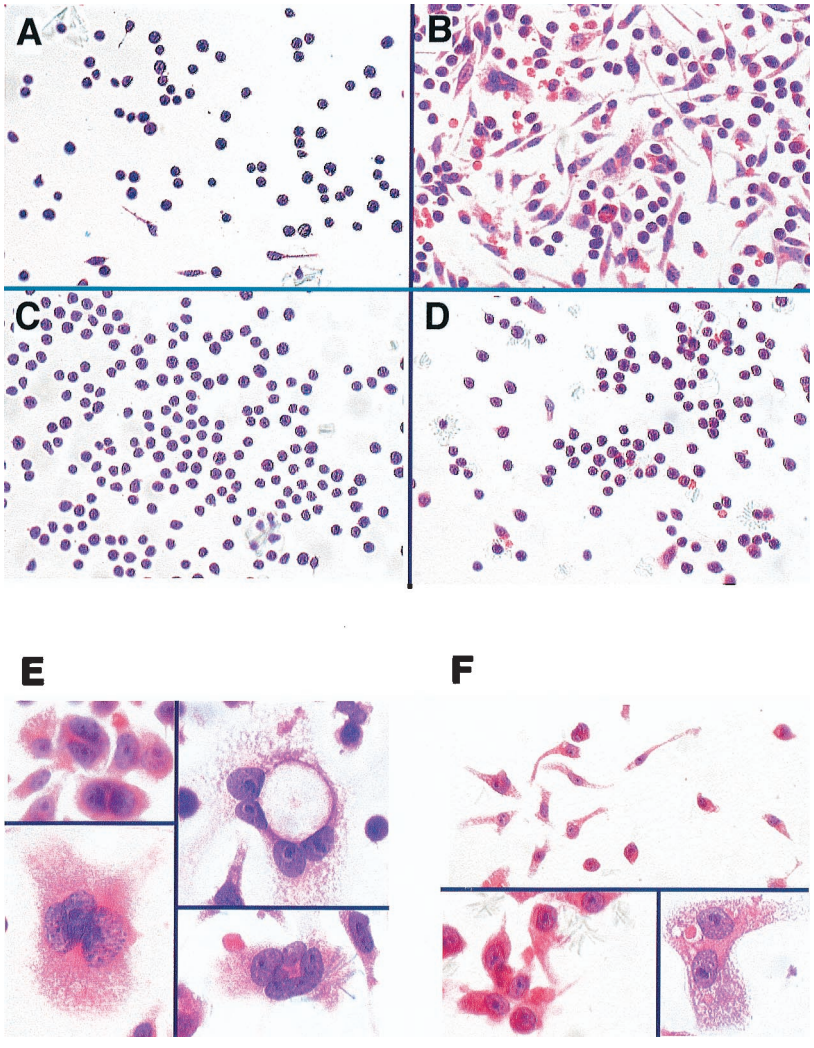

FIGURE 4. Morphological change in FDAP-M1 cells incubated with immobilized anti-FLAG mAb. M1 or FDAP-M1 cells were incubated overnight (10–14 h) with or without LPS (10 μg/ml). Cells were cultured on SonicSeal Slide Wells coated with anti-FLAG Ab or control mouse IgG1 for 4 days. Cells were stained with hematoxylin-eosin and observed under the microscope. *A*, FDAP-M1 with LPS pretreatment plus mouse IgG1. *B*, FDAP-M1 with LPS pretreatment plus anti-FLAG mAb. *C*, M1 with LPS pretreatment plus anti-FLAG mAb. *D*, FDAP-M1 without LPS pretreatment plus anti-FLAG mAb. *E*, Giant cell formation was found under the same culture conditions as those in *B*. *F*, FDAP-M1 cells were cultured with LPS (10 μg/ml) for 3 days. Magnification: *A–D* and *upper part* of *F*, $\times$100; *E* and *lower part* of *F*, $\times$400.

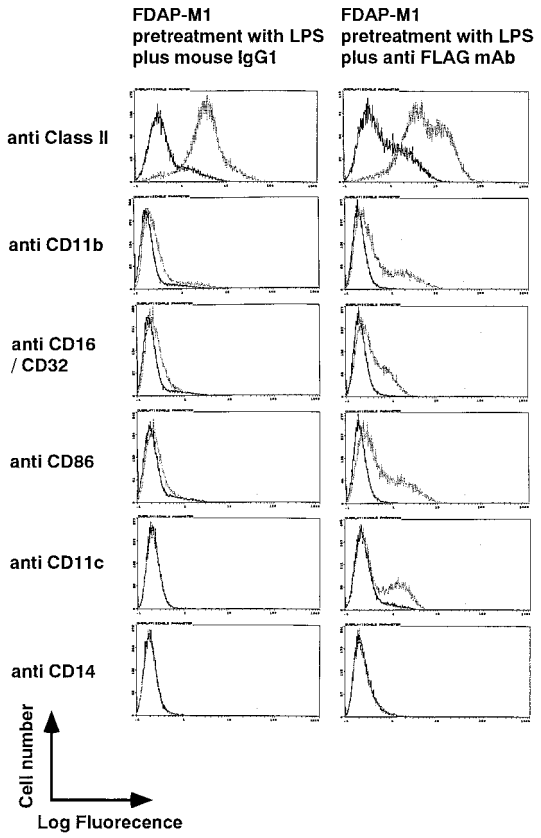

FIGURE 5. Cell surface phenotypic analysis of the macrophage-like cells supported by stimulation via DAP12. FDAP-M1 cells were incubated overnight with LPS (10 μg/ml), and cells were cultured on anti-FLAG Ab or control mouse IgG1-coated culture dishes for 4 days. Cells were collected and stained with the indicated mAb followed by FITC-conjugated second Ab (dotted line). The negative control was stained with isotype-matched Ig of each mAb (solid line). Samples were analyzed by flow cytometry.

role of DAP12 has never been scrutinized in these cells despite its abundance in macrophages.

In this report we demonstrated that DAP12 protein was detected in many organs and cell lines that are involved in the immune response (Fig. 1). In view of the significant amount of DAP12 expression in peritoneal macrophages, the DAP12 protein should also be expressed in alveolar macrophages of the lung and Kupffer cells in the liver. Although microglial cells share properties with macrophages (20), we could not detect DAP12 protein in the brain. Considering one case of DAP12 expression in a neural cell line (6), the expression of DAP12 on macrophage-like cells possibly depends on their activation state or differentiation stage. Moreover, we found that DAP12 in bone marrow has a lower m.w. than that in other organs. Even though there was an increase in the macrophage type of DAP12 following LPS stimulation in bone marrow, the amount of the smaller type of DAP12 was almost unchanged. One possible explanation is that these two types of DAP12 have different roles in regulating the immune response or differentiation.

One of the remarkable morphological changes after DAP12 stimulation is a distinct aptitude for cell fusion in differentiated M1 cells (Fig. 4, *B* and *E*). Previous studies demonstrated that monocyte/macrophages differentiated into multinucleated giant cells after induction by various cytokines, bacteria, and their combinations, including IL-4, M-CSF, GM-CSF, and Calmette-Guérin bacillus (21–23). Although, the precise mechanism is still unknown, it seems that up-regulation of adhesion molecule by stimulation of DAP12 plays an important role in giant cell formation. Lemaire et al. (21) have shown that endogenous IL-6 increases multinucleated giant cell formation in rat alveolar macrophages following stimulation by GM-CSF. M1 cells can also be induced to differentiate into macrophages by IL-6 alone (13, 24), hinting at the possibility that DAP12 signaling affects endogenous transcripts of IL-6 during differentiation. In our system although transcripts of IL-6 were up-regulated during pretreatment with LPS, no remarkable increase in IL-6 transcripts was detected after stimulation with anti-FLAG mAb (data not shown). However, the possibility, that signaling via DAP12 influenced the IL-6/Janus kinase/Stat cascade, which is known as one of the major pathways in M1 cell differentiation (24, 25), still remains.

In FDAP-M1 cells we observed DAP12 expression on the cell surface only after LPS stimulation (Fig. 3*C*), and we presumed that the association of DAP12 with newly induced proteins enabled the complex to be transferred to the cell surface. DAP12 has a negatively charged aspartic acid in its transmembrane region, which has been thought to bind with a positively charged lysine or arginine residue in the transmembrane region of KARs (4). Paired Ig-like receptors and Ig-like transcripts families, preferentially expressed in mouse and human myeloid cells, also have a positively charged arginine residue (26, 27). Nevertheless, it has been reported that molecules of Ig-like transcript/paired Ig-like receptor families associate with the Fc receptor γ-chain (26, 28), but not with DAP12, although they share structural homology (28, 29). Recently, a novel C-type lectin, myeloid DAP12-associating lectin-1, was cloned by Bakker et al. (29) as a DAP12-associating receptor in myeloid cells. However, we could not detect myeloid DAP12-associating lectin-1 transcripts in cDNA from LPS-stimulated M1 cells (data not shown), suggesting the presence of other DAP12-associating receptors in M1 cells. While we were preparing this manuscript, other associate molecules of DAP12 in myeloid cells, signal regulatory protein-β1 and triggering receptor-1 expressed on myeloid cells, were reported (30, 31). We have not examined the expression of signal regulatory protein-β1 or triggering receptor-1 expressed on myeloid cells in M1 cells, but they would be possible candidates for the associate molecule of DAP12 in M1 cells.

Finally, we investigated the phosphorylation of DAP12 and its possible associate proteins after DAP12 cross-linking. Several bands, two corresponding to DAP12 (Fig. 6, *bands b1* and *b2*) and two with slower mobility (17 kDa), were observed (Fig. 6*B*, *bands a1* and *a2*). As those slowly migrating two bands were barely detected by anti-FLAG mAb (M2; Fig. 6, *A* and *C*), although they were immunoprecipitated with the same anti-FLAG mAb (Fig. 6, *A* and *B*), these 17-kDa molecules may be DAP12-associated phosphorylated proteins but not DAP12 itself. Additionally, as shown in Fig. 6*C*, DAP12 was detected in the immunoprecipitate by anti-PY mAb only when FDAP-M1 cells had been stimulated with anti-FLAG mAb, although constitutional DAP12 phosphorylation was seen in Fig. 6*B*. These observations suggest that anti-PY mAb (4G10) could not interact with PY residues of DAP12 strongly enough to bring down the molecule, and it is, in fact, a coimmunoprecipitate of other phosphorylated associate molecules such as the 17-kDa phosphorylated band. Alternatively, these two 17-kDa bands may indeed be the phosphorylated DAP12 described in other reports (4, 32). In any event these phosphorylated bands were seen only after DAP12 cross-linking. Thus, it is conceivable that signaling through DAP12 has an important role in M1 differentiation for macrophages. A recent report by McVicar et al. (10)

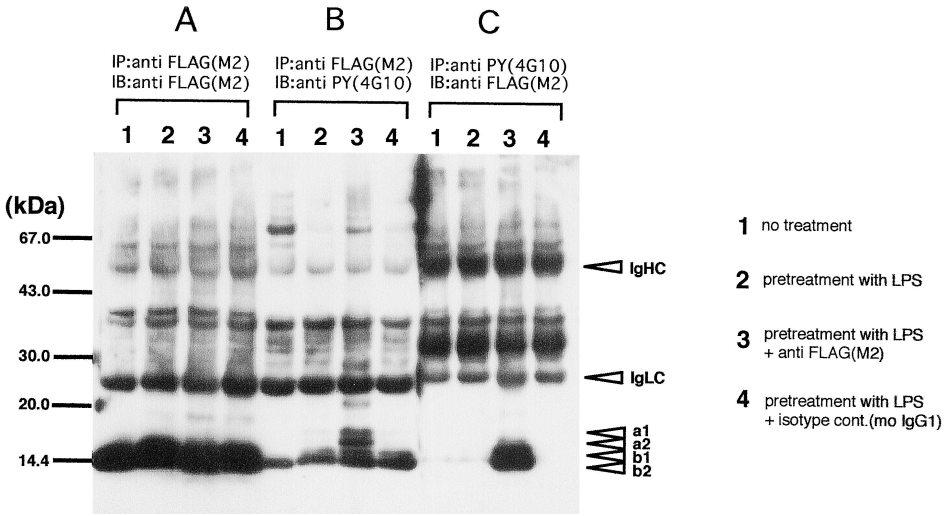

FIGURE 6. Immunoprecipitation of FLAG-DAP12 after stimulation with anti-FLAG mAb. FDAP-M1 cells were incubated overnight with LPS (10 μg/ml), and cells were stimulated by a soluble form of anti-FLAG mAb (M2; 20 μg/ml) or control mouse IgG1 (mo IgG1; 20 μg/ml) for 5 min. Anti-mouse IgG (5 μg/ml) was used for the cross-linking. FDAP-M1 cells without LPS pretreatment were used as a control. Cell lysates were immunoprecipitated with anti-FLAG mAb or anti-PY mAb (4G10) and were analyzed by Western blot using anti-FLAG mAb or anti-PY mAb.

clearly demonstrated the preferential use of Syk tyrosine kinase following Ly49D cross-linking. It is expected that Syk tyrosine kinase would be recruited to DAP12 tyrosine residues in our system of M1 differentiation.

In conclusion, this is the first report of the possible role of DAP12 in terminal differentiation of the M1 leukemic cell line. Additional experiments are necessary to determine the unknown receptor on M1 cells and the precise signaling pathway via DAP12 during differentiation.

Acknowledgments

We thank S. Kato (Asahikawa Medical College Hospital, Asahikawa, Japan) for supplying the protocol for cell staining, and Dr. Y. Katagiri (National Children's Medical Research Center, Tokyo, Japan) for helpful suggestions.

References

1. Lord, K. A., A. Abdollahi, B. Hoffman-Liebermann, and D. A. Liebermann. 1993. Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation. *Mol. Cell. Biol. 13:841.*
2. Krishnaraju, K., B. Hoffman, and D. A. Liebermann. 1998. The zinc finger transcription factor Egr-1 activates macrophage differentiation in M1 myeloblastic leukemia cells. *Blood 92:1957.*
3. Nicholson, S. E., R. Starr, U. Novak, D. J. Hilton, and J. E. Layton. 1996. Tyrosine residues in the granulocyte colony-stimulating factor (G-CSF) receptor mediate G-CSF-induced differentiation of murine myeloid leukemic (M1) cells. *J. Biol. Chem. 271:26947.*
4. Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature 391:703.*
5. Lanier, L. L., B. C. Corliss, J. Wu, and J. H. Phillips. 1998. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity 8:693.*
6. Tomasello, E., L. Olcese, F. Vely, C. Geourgeon, M. Blery, A. Moqrich, D. Gautheret, M. Djabali, M. G. Mattei, and E. Vivier. 1998. Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. *J. Biol. Chem. 273:34115.*
7. Smith, K. M., J. Wu, A. B. Bakker, J. H. Phillips, and L. L. Lanier. 1998. Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J. Immunol. 161:7.*
8. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359.*
9. Cantoni, C., C. Bottino, M. Vitale, A. Pessino, R. Augugliaro, A. Malaspina, S. Parolini, L. Moretta, A. Moretta, and R. Biassoni. 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J. Exp. Med. 189:787.*
10. McVicar, D. W., L. S. Taylor, P. Gosselin, J. Willette-Brown, A. I. Mikhael, R. L. Geahlen, M. C. Nakamura, P. Linnemeyer, W. E. Seaman, S. K. Anderson, et al. 1998. DAP12-mediated signal transduction in natural killer cells: a dominant role for the Syk protein-tyrosine kinase. *J. Biol. Chem. 273:32934.*
11. Gosselin, P., L. H., Mason, J. Willette-Brown, J. R. Ortaldo, D. W. McVicar, and S. K. Anderson. 1999. Induction of DAP12 phosphorylation, calcium mobilization, and cytokine secretion by Ly49H. *J. Leukocyte Biol. 66:165.*
12. Mason, L. H., J. Willette-Brown, A. T. Mason, D. McVicar, and J. R. Ortaldo. 2000. Interaction of Ly-49D$^+$ NK cells with H-2Dd target cells leads to Dap-12 phosphorylation and IFN-γ secretion. *J. Immunol. 164:603.*
13. Gearing, D. P. 1989.Comparison of factors which induce differentiation of the murine myeloid leukaemic cell line M1. *Hamatol. Bluttransfus. 32:208.*
14. Yamashita, U., Y. Tanaka, and F. Shirakawa. 1993. Suppressive effect of interleukin-4 on the differentiation of M1 and HL60 myeloid leukemic cells. *J. Leukocyte Biol. 54:133.*
15. Guan, K. L., and J. E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. *Anal. Biochem. 192:262.*
16. Koch, N., G. J. Hammerling, N. Tada, S. Kimura, and U. Hammerling. 1982. Cross-blocking studies with monoclonal antibodies against I-A molecules of haplotypes b, d and k. *Eur. J. Immunol. 12:909.*
17. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene 77:51.*
18. Kimura, S., M. Okamoto, and M. Katagiri. 1997. The importance of the membrane proximal region of the mouse mammary tumor virus encoded superantigen. In *T Cell Activation in Viral Superantigens*. K. Tomonari, ed. CRC Press, New York, p. 65.
19. Young, D. A., L. D. Lowe, and S. C. Clark. 1990. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture: analysis of macrophage antibody-dependent cellular cytotoxicity. *J. Immunol. 145:607.*
20. Matyszak, M. K., S. Denis-Donini, S. Citterio, R. Longhi, F. Granucci, and P. Ricciardi-Castagnoli. 1999. Microglia induce myelin basic protein-specific T cell anergy or T cell activation, according to their state of activation. *Eur. J. Immunol. 29:3063.*
21. Lemaire, I., H. Yang, V. Lafont, J. Dornand, T. Commes, and M. F. Cantin. 1996. Differential effects of macrophage- and granulocyte-macrophage colony-stimulating factors on cytokine gene expression during rat alveolar macrophage differentiation into multinucleated giant cells (MGC): role for IL-6 in type 2 MGC formation. *J. Immunol. 157:5118.*
22. Dugast, C., A. Gaudin, and L. Toujas. 1997. Generation of multinucleated giant cells by culture of monocyte-derived macrophages with IL-4. *J. Leukocyte Biol. 61:517.*
23. Gasser, A., and J. Most. 1999. Generation of multinucleated giant cells in vitro

by culture of human monocytes with *Mycobacterium bovis* BCG in combination with cytokine containing supernatants. *Infect. Immun. 67:395.*

24. Piekorz, R., B. Schlierf, R. Burger, and G. M. Hocke. 1998. Reconstitution of IL6-inducible differentiation of a myeloid leukemia cell line by activated Stat factors. *Biochem. Biophys. Res. Commun. 250:436.*
25. Nakajima, K., Y. Yamanaka, K. Nakae, H. Kojima, M. Ichiba, N. Kiuchi, T. Kitaoka, T. Fukada, M. Hibi, and T. Hirano. 1996. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. *EMBO J. 15:3651.*
26. Kubagawa, H., C. C. Chen, L. H. Ho, T. Shimada, L. Gartland, C. Mashburn, T. Uehara, J. V. Ravetch, and M. D. Cooper. 1999. Biochemical nature and cellular distribution of the paired immunoglobulin-like receptors, PIR-A and PIR-B. *J. Exp. Med. 189:309.*
27. Samaridis, J., and M. Colonna. 1997. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol. 27:660.*
28. Nakajima, H., J. Samaridis, L. Angman, and M. Colonna. 1999. Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor γ-chain. *J. Immunol. 162:5.*
29. Bakker, A. B., E. Baker, G. R. Sutherland, J. H. Phillips, and L. L. Lanier. 1999. Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. *Proc. Natl. Acad. Sci. USA 96:9792.*
30. Dietrich, J., M. Cella, M. Seiffert, H. J. Buhring, and M. Colonna. 2000. Signal-regulatory protein β1 is a DAP12-associated activating receptor expressed in myeloid cells. *J. Immunol. 164:9.*
31. Bouchon, A., J. Dietrich, and M. Colonna. 2000. Inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol. 164:4991.*
32. Mason, L. H., J. Willette-Brown, S. K. Anderson, P. Gosselin, E. W. Shores, P. E. Love, J. R. Ortaldo, and D.W. McVicar. 1998. Characterization of an associated 16-kDa tyrosine phosphoprotein required for Ly-49D signal transduction. *J. Immunol. 160:4148.*