The murine c-fgr gene product associated with Ly6C and p70 integral membrane protein is expressed in cells of a monocyte/macrophage lineage

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ABSTRACT The c-fgr gene is a member of the Src family of protooncogene tyrosine kinases. A monoclonal antibody (2H2) that recognizes the specific region of the N-terminal domain of the murine c-fgr gene product (Fgr) has been established. With an immune complex kinase assay in a monocytic leukemia cell line, 2H2 monoclonal antibody was shown to precipitate a 59-kDa protein that corresponds in molecular mass to murine Fgr. Fgr was expressed highly in lymph nodes, slightly in spleen and peripheral blood leukocytes, and barely in the thymus and was not detected in bone marrow. In the presence of a mild detergent, Fgr was coimmunoprecipitated with a 70-kDa protein (p70) or with p70 plus several other molecules that were expressed on the cell-surface membrane of macrophage tumor cell lines PU5-1.8 and J774.1, respectively. By contrast, Fgr was not coimmunoprecipitated with a low-affinity receptor for the Fc portion of IgG that is associated with human Fgr. The molecule was also coimmunoprecipitated with the Ly6C molecule from a macrophage cell line (J774.1) that showed protein-tyrosine kinase activity. Peptide mapping revealed that this kinase activity was derived from Fgr. The similarity of relationship between this intramembrane p70 and/or Ly6C and cytoplasmic Fgr to relationships previously reported between T-cell antigen receptor complex, including CD4 and CD8 coreceptors, and Lck or Fyn in T cells and between surface IgM and Lyn or Blk in B cells, suggests that the Fgr and p70 or Ly6C are, indeed, associated with each other and in the murine system may be responsible for recognition of extracellular substances (either cellular or noncellular) and for signal transduction in cells of monocyte/macrophage lineage.

Phosphorylation of cellular proteins on critical tyrosine residues is essential to control activation and/or proliferation of normal cells and tumor cell transformation (1–3). Tyrosine kinases may be divided into two groups. One consists of integral membrane proteins with an extracellular domain, transmembrane region, and an intracellular tyrosine kinase domain. This group contains growth factor receptors—e.g., epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin receptor. Binding of growth factor to the extracellular domain triggers the intrinsic tyrosine kinase activity.

Another group of tyrosine kinases includes nonreceptor proteins with only cytoplasmic kinase domains. These link to cellular membranes by posttranslational myristoylation at the N termini. The membrane-associated protein-tyrosine kinases belong to the Src family of tyrosine kinases: encoded by c-src, c-fgr, c-yes, lyn, lck, hck, lyn, blk, and yrk (4–11). These Src family kinases possess N-terminal myristoyla-

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Abbreviations: MBP, maltose-binding protein; LN, lymph node; mAb, monoclonal antibody; SH2 and SH3, Src-homology regions 2 and 3, respectively; FcγRII, low-affinity receptor for Fe portion of IgG; PtdIns, phosphatidylinositol.

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the p70 or Ly6C, Fgr may function as a mediator of transduction pathways that lead to activation and/or proliferation of the cells involved.

**MATERIALS AND METHODS**

**Mice.** Female (C57BL/6 × C3H)F1 mice were purchased from Shizuku Animal Center (Hamamatsu, Japan).

**Cell Lines and mAbs.** Murine tumor cell lines used were as follows: P5U-1.8 and J774A.1, macrophage-like cell lines. mAbs used include MAR18.5 (mouse IgG2a, anti-rat κ) (29), PK136 (mouse IgG2b, anti-natural killer 1.1) (30), 34-2-11 (mouse IgG2a, anti-Ly6C) (31), MOL171 (mouse IgG1, anti-p50/52) (32), 2.4G2 (rat IgG2a, Anti-Fcγ II, III) (33), and 14B2C11 (hamster IgG, antimouse CD3ε) (34).

**Production of Fusion Proteins.** RNA was isolated under standard conditions (35). cDNA was synthesized from total cellular RNA templates by using random primer and Moloney murine leukemia virus reverse transcriptase. RNA (5 μg) was dissolved in 10 μl of distilled H2O heated to 95°C for 5 min and added to a reaction mixture containing 0.3 μg of primer, 10 mM each deoxynucleoside triphosphate, 10 mM dithiothreitol, 20 units of RNasin (Promega), and 200 units of reverse transcriptase (GIBCO/BRL). DNA transcripts were used as templates in a PCR that contained 10 pmol of 5’ primer (5’-CTCTCTAGAGGCGATCGTAGG-3’) and 3’ primer (5’-CTCAAGCTTTGATGCCCAGTCAGG-3’), including XbaI, HindIII restriction cleavage sites, respectively. The 5’ primer (5’-CTCTCTAGACCGGCACCATGTC-3’) and 3’ primer (5’-CTCAAGCTTTGATGCCCAGTCAGG-3’) were used for a fusion protein containing only the kinase domain of Fgr, and 5’ primer (5’-CTCTCTAGAGGCGATCGTAGG-3’) and 3’ primer (5’-CTCAAGCTTTGATGCCCAGTCAGG-3’) were used for a fusion protein containing only the N-terminal specific domain of Fgr. PCR amplification was done with program temp control system PC-700 (ASTEC, Fukuoka, Japan) with 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After PCR, amplified products were digested by XbaI and HindIII and separated by electrophoresis on a 1% low-melting-temperature agarose gel (FMC). The expected band was eluted and subcloned into pMAL-c2 bacterial expression vector (NEB). A maltose-binding protein (MBP)-Fgr fusion protein was expressed in Escherichia coli strain TB1, induced by 0.3 mM isopropyl β-D-thiogalactoside, and purified by using amylose resin-affinity chromatography according to the manufacturer’s procedure.

**Immunizations and Screening of mAb.** MBP–Fgr fusion protein dissolved in phosphate-buffered saline (PBS) (0.6 mg/ml) was emulsified in complete Freund’s adjuvant, and 0.5 ml was injected i.p. twice into each of several mice. Three weeks later, the mice were killed, and their spleens were dissected out and used as the source for antibody-producing cells. Fusions of spleen cells were done with myeloma cell line P3U1 by standard techniques (36). Culture supernatant was screened with ELISA. In brief, the fusion protein dissolved in bicarbonate buffer (pH 8.0) was coated on a 96-well plate at room temperature for 2 hr and blocked with PBS/1% bovine serum albumin. Culture supernatants from each well were treated at room temperature, and the wells were washed with PBS/0.05% Triton X-100. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1 μg/ml) was treated at room temperature for 1 hr, washed, and mixed with 0-phenyldiamine. The absorption rate for the contents of each well was measured at a 492-nm wavelength. The cells in positive wells were diluted and seeded for cloning. The subclasses of established mAbs were determined by the use of a mouse mAb isotyping kit (Amersham).

**Immune Complex Kinase Assay.** Cells (1 × 10⁹) were solubilized with 1 ml of lysis buffer containing 1% Nonidet P-40, 10 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and aprotinin at 2 μg/ml; rotated at 4°C for 30 min; and centrifuged at 21,800 × g for 20 min at 4°C. The supernatant was preclarified with 50 μl of normal mouse serum and mixed with 100 μl of protein A–Sepharose 4B or protein G–agarose overnight at 4°C and centrifuged at 21,800 × g for 5 min at 4°C. The supernatant was treated with 10 μl of ascites containing mAb for 2 hr at 4°C and then with 20 μl of protein A–Sepharose 4B or protein G–agarose overnight at 4°C. After being washed with lysis buffer, the beads were equilibrated with kinase buffer containing 40 mM Heps (pH 7.4), 10 mM MgCl₂, and 3 mM MnCl₂. The beads were incubated in 20 μl of kinase buffer containing 370 kBg of [γ-⁳²P]ATP (Dupont/NEN) at 30°C for 10 min. The kinase reaction was stopped by adding 20 μl of sample buffer twice concentrated. The beads were then boiled for 5 min and applied to SDS/9% PAGE. The gel was dried, exposed for 2 hr, and developed.

**Immunoprecipitation.** Cells (5 × 10⁷) were washed twice with 4°C PBS and iodine-labeled by the lactoperoxidase method (37). After washing with PBS, cells were solubilized with PBS containing 1% Brij 96, 1 mM phenylmethylsulfonyl fluoride, aprotinin at 2 μg/ml; and 1 mM sodium orthovanadate and centrifuged at 21,800 × g for 5 min at 4°C. The supernatant was preclarified and treated with 10 μl of mAbs followed by protein A–Sepharose 4B at 4°C overnight. After being washed, the beads were boiled with sample buffer for 5 min, and applied to SDS/9% PAGE. The gel was dried, exposed for 5 days, and developed.

**Peptide Mapping.** After immune complex kinase assay, the gels containing phosphorylated protein were cut out and homogenized with 50 mM NH₄HCO₃ (100 μl). The homogenates were incompletely digested with 100 units of V8 protease (Sigma) at 25°C for 12 hr, boiled with sample buffer for 5 min, and then subjected to SDS/16% PAGE. The gel was dried, exposed for 2 or 14 hr, and developed.

**Surface Marker Analysis.** Flow cytometry analysis was performed on a FACScan (Becton Dickinson) with the indirect immunofluorescent method. The cell-bound antibodies were revealed by fluorescein isothiocyanate-conjugated antimouse or anti-rat immunoglobulin (Cappel) after incubation with the mAbs. Dead cells were excluded by propidium iodide staining (Sigma).

**RESULTS**

**Establishment of a mAb Against Murine c-Fgr Product.** We first attempted to generate mAb against murine Fgr. Spleen cells from (B6 × C3H)F1 mice immunized with fusion proteins of whole Fgr plus MBP (Fig. 1A) were fused with myeloma cell line P3U1. Hybridoma supernatants were screened and selected for specific antibodies that reacted with Fgr, MBP, and/ or unique antigens (Fig. 1B).

**Fig. 1.** Fgr–MBP fusion proteins used for immunization and ELISA. The domains of the fusion proteins are illustrated. (A) Fusion protein containing the full length of Fgr. (B) Fusion protein containing only the N-terminal unique domain of Fgr. (C) Fusion protein containing only the kinase domain of Fgr. unique, N-terminal specific nonhomologous domain.
with the immunogens and fusion proteins of the unique N-terminal region of Fgr plus MBP (Fig. 1B) but did not react with the fusion proteins of the homologous kinase domain of Fgr plus MBP (Fig. 1C). Finally, we could establish a hybridoma, 2H2. In ELISA, the mAb from hybridoma 2H2 was shown to bind specifically to whole Fgr–MBP fusion protein and Fgr–MBP fusion protein that contains only the nonhomologous N-terminal domain but not to the other kind of Fgr–MBP fusion protein that contained only the homologous kinase domain (data not shown). Thus, 2H2 mAb apparently binds to a specific Fgr region. The isotype of the 2H2 mAb was shown to be IgG1 κ.

Expression of Murine c-fgr Gene Product in Various Hematopoietic and Lymphoid Tissues. To determine tissue distribution and molecular mass of the antigen recognized by 2H2 mAb, cell lysates from hematopoietic and lymphoid tissues were immunoprecipitated with the putative anti-Fgr mAb (2H2) coupled to protein A–Sepharose. Thereafter, the immune complexes were characterized by using an in vitro kinase reaction because Fgr can undergo autophosphorylation. The immune complexes phosphorylated in vitro were then analyzed on SDS/9% PAGE under reducing conditions. Fig. 2 shows that the 2H2 mAb precipitates a phosphorylated protein band of slightly higher molecular mass than the 59 kDa deduced from murine c-Fgr CDNA. The higher molecular mass appears attributable to the phosphorylation. Among various murine hematopoietic and lymphoid tissues, lymph node (LN) cells expressed very high levels of Fgr, spleen cells expressed relatively high levels, and thymus and peripheral blood leukocytes expressed very low levels. With this procedure Fgr was not detected in bone marrow cells. These results are compatible with those of Yi and Willman (26) that c-fgr gene product is expressed only in mature macrophages. No phosphorylated protein band of 59 kDa was detected in lysates eluted from protein A–Sepharose coupled with a control anti-rat κ chain mAb, MAR18.5 (Fig. 3 and data not shown).

To confirm that tyrosine residues but not serine/threonine residues of Fgr molecules were autophosphorylated, after immune complex kinase assay the gel was treated with alkaline solution (1 M KOH). Phosphotyrosine is relatively resistant to alkaline treatment, but phosphoserine/threonine is sensitive. Because the band did not disappear after alkaline treatment, we could conclude that the phosphorylated amino acids of Fgr are tyrosine residues and are not serine/threonine residues (data not shown).

Fine Specificity of 2H2 mAb Against Murine c-fgr Gene Product. To further analyze the antigen specificity recognized by 2H2 mAb, sequential immunoprecipitation analysis was performed by using 2H2, anti-Lck mAb (MOL171), and a negative control mAb (MAR18.5). Precleared lysates of LN cells with 2H2 mAb or MOL171 mAb were further immunoprecipitated with 2H2 or MOL171 mAb followed by in vitro kinase assay. After preclearance with 2H2 mAb, the immune complexes formed with MOL171 mAb were autophosphorylated at the same level as those after preclearance with MAR18.5 mAb. By contrast, no autophosphorylated band was detectable in immune complexes formed with 2H2 mAb (Fig. 3). Preclearing with the MOL171 mAb did not affect precipitation of the 59-kDa band by the 2H2 mAb, but no autophosphorylated band was seen in the immune complexes formed with MOL171 mAb. These findings show in another way that 2H2 mAb recognizes neither the SH2 and SH3 domains nor the kinase domains shared by Fgr and Lck.

**Immunoblot Analysis.** Lysates of LN cells or thymocytes precleared with MAR18.5, 2H2, or MOL171 mAb were separated on SDS/9% PAGE, and immunoblot analysis was performed by using MOL171 mAb. The lysate precleared with 2H2 mAb contained Lck proteins at the same level as that precleared with MAR18.5 mAb, but the lysate precleared...
with MOL171 mAb did not contain Lck protein (data not shown). These findings permit us to conclude that 2H2 mAb recognizes specifically a unique and nonhomologous N-terminal domain of the murine Fgr molecule.

The Cell-Surface Molecules Associated with Murine Fgr. Some members of Src family of kinases are functionally and physically associated with the internal domains of cell-surface molecules in T cells and B cells (12, 14, 15). On the other hand, the cell-surface molecules associated with the murine Fgr have not yet been identified. Because strong detergents such as Nonidet P-40 or Triton X-100 quickly dissociate the complexes of Src family kinases and surface-membrane molecules (e.g., Fyn and T-cell receptor), we used a relatively mild detergent, Brij 96, to obtain molecules associated with intracellular Fgr. Cell-surface proteins of PU5-1.8 or J774.1 cells were radioiodinated with lactoperoxidase. These cells were then solubilized with 1% Brij 96 lysis buffer and immunoprecipitated with 2H2 mAb. Fig. 4 shows that a 70-kDa protein immunoprecipitated with the Fgr molecule is present in the lysate from PU5-1.8 cells. Similarly, an 80-kDa plus several high-molecular-mass proteins other than the 70-kDa protein were found in the lysates from J774.1 cells. Thus, Fgr is apparently associated with a variety of molecules that are integrated in the cell-surface membrane. Alternatively, these molecules may be derived from the same group (represented by p70) and have been differently modified after translation.

Ly6C Physically Associated with Intracellular Fgr Molecule. We then determined which molecules are associated with Fgr as described above. Several phosphatidylinositol (PtdIns)-linked cell molecules have been reported (17, 19) to be physically associated with some members of the Src family. In addition, cells of macrophage lineage have been shown to express the Ly6C molecule as a PtdIns-linked cell-surface molecule (38). On the other hand, recently it has been reported that human Fgr is associated physically with a receptor for theFc of IgG (FcγRII) on neutrophils. Thus a murine macrophage cell line, J774.1, was chosen for further analysis because flow cytometric analysis revealed that this cell line expressed both Ly6C and FcγRII molecules (Fig. 5A). Cell lysates from J774.1 were immunoprecipitated with anti-Ly6C (mAb 34-2-11) or anti-FcγRII (mAb 2.4G2) and analyzed by using an in vitro kinase assay (Fig. 5B). The immunoprecipitates with anti-Ly6C mAb showed kinase activities at the same molecular-mass band as obtained with the 2H2 mAb. By contrast, the immunoprecipitates with anti-FcγRII mAb showed no kinase activity.

To ascertain whether the kinase activity exhibited by the immunoprecipitates with anti-Ly6C was derived from the physically associated kinase molecule Fgr, the phosphorylated proteins were incompletely digested with V8 protease. Fig. 5C shows a one-dimensional peptide mapping pattern that compares the protein bands phosphorylated from 2H2 mAb immunoprecipitates with the anti-Ly6C immunoprecipitates. The two immunoprecipitates showed an identical pattern, although the exposure times differed. Almost the same results were obtained when LN cells were analyzed (data not shown). These findings demonstrate that the murine Fgr molecule is associated with the Ly6C molecule but is apparently not associated with the FcγRII molecule (28).

**DISCUSSION**

Some members of the Src kinase family are involved in regulation of activation and differentiation of lymphoid cells and cells of the brain. Recently, tissue-specific expression and developmental regulation of the human fgr protoonco-
gene products in monocytes and macrophages have been reported. However, little has been known concerning murine c-fgr gene products. By immunizing mice with bacterially expressed fusion proteins of MBP and murine Fgr, we have established the hybridoma cell line 2H2, which produces a mAb specific for the c-fgr protooncoenzyme product. Using 2H2 mAb, we semiquantitatively analyzed the expression of Fgr
in lymphoid tissues and identified the cell-surface molecules associated with murine Fgr.

Fgr was expressed at highest levels in LN. The Fgr expression in spleen was also relatively high, as compared with expression in other tissues but was lower than that seen in LN. This finding is consistent with data from immunohistochemical analysis in situ because the proportion of Fgr-expressing cells in LN was higher than that in the spleen (S.H., K.I., K.O., R.A.G., K. Kajino, K. Takami, and M. Katoh, unpublished work). Fgr was not seen in other tissues studied (e.g., thymus and bone marrow).

Cell-surface receptors or adhesion molecules represent cellular machinery essential to cell-to-cell interactions. Human Fgr is physically and functionally associated with FcγRII and may be involved in FcγRII-mediated signal-transduction pathways. In immunoprecipitates with 2H2 mAb of membrane lysates from macrophage-like cell lines in the presence of a mild detergent (Brij 96), we detected surface molecules (e.g., 70-kDa) associated with the Fgr molecule. These molecules were slightly larger than murine FcγRII (40–70 kDa). Furthermore, the immunoprecipitates with anti-FcγRII mAb had no kinase activity. These findings suggest that murine Fgr may not be associated with FcγRII, in contrast with human Fgr. When comparing sequences of murine c-Fgr sequences with human c-Fgr, impressive differences were noted in residues 12–62 of the N-terminal domain (26). This nonhomologous N-terminal domain of the Src family kinases is presumably one that mediates substrate specificity and determines the associated molecules and subcellular location of each kinase. Thus, the divergent N-terminal sequence of the N-terminal domain between murine and human Fgr suggests that these differences may be consequential to the kinase function and may reflect evolutionary divergence of functions of cells of macrophage lineages between these two species. It is not surprising that the cell-surface molecules associated with the murine Fgr molecule differ from those associated with the human Fgr molecule. We consider that the molecules represented by p70 correspond to FcγRII in the human case. To determine the relationship between p70 and FcγRII, further precise analyses need to be done. Several PtdIns-linked cell molecules are physically associated with some members of the Src family—e.g., in T cells: Fyn and/or Lck with Thy-1 or Ly6A/E (38). In addition, certain lineages among macrophages express the Ly6C molecule as a PtdIns-linked cell-surface molecule. We show here that the Ly6C molecule expressed on macrophage cell lines is associated with molecules that contain tyrosine kinase activity. Moreover, this kinase activity is derived from Fgr. Some PtdIns-linked molecules appear to transduce intracellular signaling, which results in increased intracellular Ca2+ influx. Thus, Fgr molecules are probably involved in the Ly6C-mediated intracellular signaling in cells of the monocyte/macrophage lineage. To finally ascertain the function of Fgr, further studies are needed to characterize molecules other than Ly6C that are associated with Fgr. Here we have described a hybridoma cell, 2H2, that secretes a mAb directed specifically against the murine c-fgr gene product. This 2H2 mAb should facilitate further studies on the regulation of expression and function of Fgr but also may be useful in dissecting the roles played by this kinase in proliferation, differentiation, functional activation, and cell-to-cell interaction of cells in monocyte/macrophage lineage.

We thank Drs. S. Kobayashi (Hokkaido University) and Y. Koga (Kyushu University) for providing valuable cell lines and mAbs. We thank Ms. Michiyoko Konishi for her technical and secretarial assistance and Ms. Tazim Verjee for manuscript preparation and editorial assistance. This work was supported, in part, by Grant-in-Aid for Scientific Research (B, C), Grant-in-Aid for Cancer Research, The Ministry of Education, Science and Culture, Japan, and The National Institute of Health Cellular Engineering Grant AG05628-09.