MOLECULAR AND CELLULAR BIOLOGY, Apr. 1990, p. 1789–1792
0270-7306/90/041789-04$02.00/0

Vol. 10, No. 4

Specific Expression of Human c-*fgr* in Natural Immunity Effector Cells

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Received 14 August 1989/Accepted 20 November 1989

The c-*fgr* gene product was shown by immune complex protein kinase assay with specific antibodies to be a 58-kilodalton protein ($p58^{c\text{-}fgr}$) with tyrosine-specific autophosphorylating activity. On examination of peripheral blood cells by immunoblotting with anti-c-*fgr* antibodies, $p58^{c\text{-}fgr}$ was found only in the fractions of monocytes, granulocytes, and natural killer cells. On the other hand, histochemical studies of hybridization demonstrated accumulation of c-*fgr* transcripts on most monocytes and large lymphocytes. In hematopoietic cell lines, $p58^{c\text{-}fgr}$ was detected in differentiated granulocytic cells as well as in differentiated monocytic cells of HL-60-cell origin. These data suggest a specific role for $p58^{c\text{-}fgr}$ in natural immunity effector cells.

The c-*fgr* gene is the cellular counterpart of the v-*fgr* gene, an oncogene of Gardner-Rasheed feline sarcoma virus (17) that induces fibrosarcomas in cats. Although the v-*fgr* gene has an actin gene-like sequence in the 5′ region upstream of the sequence for the kinase domain (16), nucleotide sequence analyses of both genomic (18) and complementary (12) DNA clones have revealed that the actin gene-like sequence is not present in the c-*fgr* gene. The c-*fgr* gene apparently encodes a non-receptor-type protein-tyrosine kinase with a calculated molecular weight of 59,478 whose structure is very similar to that of the c-*src* protein (22), except for the amino acid sequence at the amino terminus (77 residues). This unique sequence may be important for the function of the c-*fgr* gene product.

The c-*fgr* gene has been reported to be expressed only in hematopoietic tissues or cells, especially in B lymphocytes infected with Epstein-Barr virus (6), in the fetal liver (12), and in peripheral blood mononuclear cells (14).

For an examination of the normal function of the human c-*fgr* gene product, antibodies against the synthetic peptide corresponding to the N-terminal unique region (Ala-48 to Asp-67) of the predicted amino acid sequence of the c-*fgr* protein were raised. After purification by affinity chromatography, the antibodies (anti-c-*fgr*) were checked for the ability to immunoprecipitate the c-*fgr* protein specifically from extracts of F8 cells (human c-*fgr* cDNA transfectants of NIH 3T3 origin). The antibodies immunoprecipitated a 58-kilodalton protein, and an in vitro immune complex kinase assay showed phosphorylation of a 58-kilodalton protein (Fig. 1a, lane 2). Because this band was not seen when the antibodies were preincubated with the cognate peptide (Fig. 1a, lane 3) and because the 58-kilodalton protein could not be immunoprecipitated from lysates of NIH 3T3 cells (Fig. 1a, lane 1), the protein was judged to be the c-*fgr* gene product, $p58^{c\text{-}fgr}$. Phosphoamino acid analysis showed that autophosphorylation was tyrosine specific (Fig. 1b), verifying that the c-*fgr* gene product was a protein-tyrosine kinase.

Previously, we reported that c-*fgr* mRNA was detected in the fetal liver but not in the adult liver, suggesting its specific expression in hematopoietic cells (12). In an attempt to clarify which types of hematopoietic cells express $p58^{c\text{-}fgr}$, we examined the expression of this protein in normal peripheral blood cells and tonsillar B lymphocytes with the anti-c-*fgr* antibodies. Peripheral blood cells obtained from normal healthy volunteers were separated into six fractions: erythrocytes, platelets, granulocytes, monocytes, T lymphocytes (CD3-positive cells), and natural killer (NK) cells (CD3-negative, CD16-positive cells). Erythrocytes and granulocytes were obtained by centrifugation of whole blood in Ficoll-Hypaque solution. The purity of the granulocyte fraction was 98% and was measured by examining the morphology of the granulocytes under a light microscope. Monocytes were purified as described previously (11). Platelets were obtained by low-speed centrifugation of mononuclear cells. T lymphocytes and NK cells were purified by passing mononuclear cells through a nylon wool column and centrifuging them in Percoll gradients (20, 23). For further purification of NK cells, cells of the low-density fractions were treated with monoclonal antibodies (anti-CD3 and

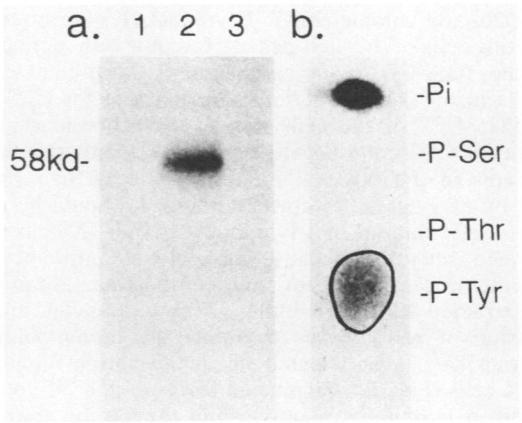

FIG. 1. Protein kinase assay and phosphoamino acid analysis. (a) In vitro protein kinase assay. Lane 1, NIH 3T3 cells; lanes 2 and 3, F8 cells (cDNA transfectants of NIH 3T3-cell origin). The assay was performed as described previously (2) by immunoprecipitating cell lysates with anti-c-*fgr* antibodies (lanes 1 and 2) or anti-c-*fgr* antibodies that had previously been blocked by treatment with an excess amount of the cognate peptide (lane 3). (b) Phosphoamino acid analysis of $p58^{c\text{-}fgr}$. The positive band in lane 2 of panel a was excised, eluted, hydrolyzed with HCl, and subjected to electrophoresis as described previously (2). Pi indicates the location of free $^{32}P_i$. The positive spot is outlined. kd, Kilodaltons.

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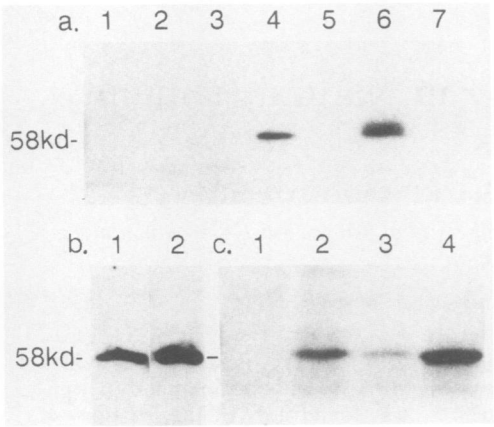

FIG. 2. Detection of p58$^{c\text{-}fgr}$ in fractionated peripheral blood cells. (a) Immunoblotting analysis with the anti-c-*fgr* antibodies. Lane 1, erythrocytes; lane 2, platelets; lane 3, granulocytes; lane 4, monocytes; lane 5, T lymphocytes; lane 6, NK cells; lane 7, tonsillar B lymphocytes. Proteins (10 μg) were applied to each lane. After electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel, the proteins were transferred onto Immobilon polyvinylidene difluoride (Millipore Corp.). The filters were probed with anti-c-*fgr* antibodies and then with ^{125}I-labeled anti-rabbit immunoglobulin antibodies (Amersham Corp.). (b) Immunoblotting analysis of peripheral blood leukocytes after DFP (3) treatment. Lane 1, total peripheral mononuclear cells (a mixture of monocytes and lymphocytes) purified by centrifugation of whole blood in Ficoll solution; lane 2, peripheral granulocytes purified as described for panel a. Membranes were extracted with TNE buffer plus trasylol (without DFP) after treatment with 2 mM DFP for 5 min at 0°C in phosphate-buffered saline, and 20 μg of protein was applied to each lane. (c) Immunoblotting analysis of HL-60 cells. Lane 1, HL-60 cells; lane 2, HL-60 cells treated with TPA at 20 ng/ml for 48 h; lane 3, HL-60 cells treated with 1.25% dimethyl sulfoxide for 5 days; lane 4, HL-60 cells treated with 1 μM retinoic acid for 5 days. Membranes were extracted with TNE buffer plus trasylol, and 20 μg of protein was applied to each lane. The autoradiographic exposure time was 20 h for each panel. kd, Kilodaltons.

anti-CD20) and complements. T lymphocytes were purified by treating cells of the high-density fraction with monoclonal antibodies (anti-CD16) and complements. More than 99% of the cells in the T-cell fraction were positive for CD3, and more than 88% of the cells in the NK-cell fraction were positive for CD16 and negative for CD3. Contamination of monocytes in the NK-cell fraction was less than 1%, as judged by nonspecific esterase staining. It should be noted that some populations of T lymphocytes and NK cells might have been activated because monoclonal antibodies and complements were used for their purification. Membranes were extracted with TNE buffer (24) plus trasylol, and the expression of p58$^{c\text{-}fgr}$ was examined by immunoblotting. Bands of p58$^{c\text{-}fgr}$ were detected only in the lanes of monocytes and NK cells (Fig. 2a). Significant levels of p58$^{c\text{-}fgr}$ could be detected in granulocytes only when they were pretreated with diisopropylfluorophosphate (DFP) (Fig. 2b), a potent proteinase inhibitor that can permeate membranes of living cells (3). The level of p58$^{c\text{-}fgr}$ in granulocytes was 2.7 times higher than that in total mononuclear cells (a mixture of monocytes and lymphocytes), as shown by analysis with a densitometer. This suggested that there is more p58$^{c\text{-}fgr}$ in monocytes and in NK cells than in granulocytes because the first two constitute only 25% of total peripheral mononuclear cells. In myeloid cell lines, p58$^{c\text{-}fgr}$ could be detected in differentiated granulocytic cells after treatment of HL-60 cells (7) with dimethyl sulfoxide (8) or retinoic acid (5) (Fig. 2c). These granulocytic cells consisted of myelocytes, metamyelocytes, banded neutrophils, and a small percentage of segmented cells (2% in dimethyl sulfoxide-treated cells and 8% in retinoic acid-treated cells). p58$^{c\text{-}fgr}$ could also be induced in differentiated monocytic cells after treatment of HL-60 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is consistent with the data obtained from the analysis of peripheral blood cells. Increased expression of p58$^{c\text{-}fgr}$ upon differentiation of HL-60 cells to either granulocytes or monocytes was also observed by Notario et al. (18a).

Cell type-specific expression of the c-*fgr* gene was also examined by in situ hybridization analysis. Peripheral blood leukocytes were sedimented onto poly-L-lysine-coated slides and hybridized in vitro with a c-*fgr*-specific probe (a mixture of *Eco*RI-*Bst*EII and *Sma*I-*Eco*RI fragments of human c-*fgr* cDNA) (12). Positive signals were detected on about half of the monocytes and 10 to 20% of the lymphocytes, whereas only background signals were observed with control vector DNA (Fig. 3). The number of grains per granulocyte was at the background level, conflicting with a recent report (15) which showed that the c-*fgr* mRNA level was highest in granulocytes. However, the fact that peripheral granulocytes have a low RNA-to-DNA ratio (about 0.08) (4) could explain this discrepancy because the method of in situ hybridization is different from S1 protection assays in that gene expression is examined at a single-cell level in the former. In fact, our Northern (RNA) hybridization data with a human c-*fgr* probe (corresponding to exons 1 and 2 of the c-*fgr* gene) suggested that the copy number of c-*fgr* mRNA per peripheral granulocyte was about one-fifth of that per c-*fgr*-positive mononuclear cell (data not shown). Here it should be noted that neutrophils have very limited protein synthesis after segmentation of their nuclei (13). Therefore, p58$^{c\text{-}fgr}$ detected in peripheral granulocytes might have been synthesized before segmentation of nuclei and might have remained stable thereafter.

The numbers of grains on monocytes and lymphocytes were counted (Fig. 4). With the c-*fgr*-specific probe, two peaks (0 to 5 and 16 to 20 grains per cell) were obtained with both monocytes and lymphocytes. On the other hand, with the control probe, a single peak of 0 to 5 grains per cell was obtained with both types of cells. Statistically significant signals (more than 16 grains per cell) were observed on 41% of the monocytes ($\chi^2 = 51.6$; $P < 0.01$) and 16% of the lymphocytes ($\chi^2 = 17.4$; $P < 0.01$).

Lymphocytes that gave positive signals were significantly larger than those that gave no signals (negative) (Fig. 3). The mean diameter ± standard deviation of positive lymphocytes was 10.0 ± 0.46 mm; that of negative lymphocytes was 8.6 ± 0.77 mm ($t = 4.95$; $P < 0.05$). The lymphocytes giving positive signals were also characterized by high cytoplasmic-to-nuclear mass ratios and kidney-shaped nuclei (Fig. 3c). These features suggested that the c-*fgr*-positive lymphocytes were large granular lymphocytes (23), third-population (non-T, non-B) lymphocytes which account for 15 to 20% of peripheral mononuclear cells. Azurophilic granules could not be seen clearly after in situ hybridization, probably because of distortion of the cells during the experiment. The data were consistent with those of the immunoblotting experiment, which showed the presence of p58$^{c\text{-}fgr}$ in NK cells, because more than 90% of large granular lymphocytes had been reported to have the surface markers of NK cells (19).

Our major conclusion from the present study is that

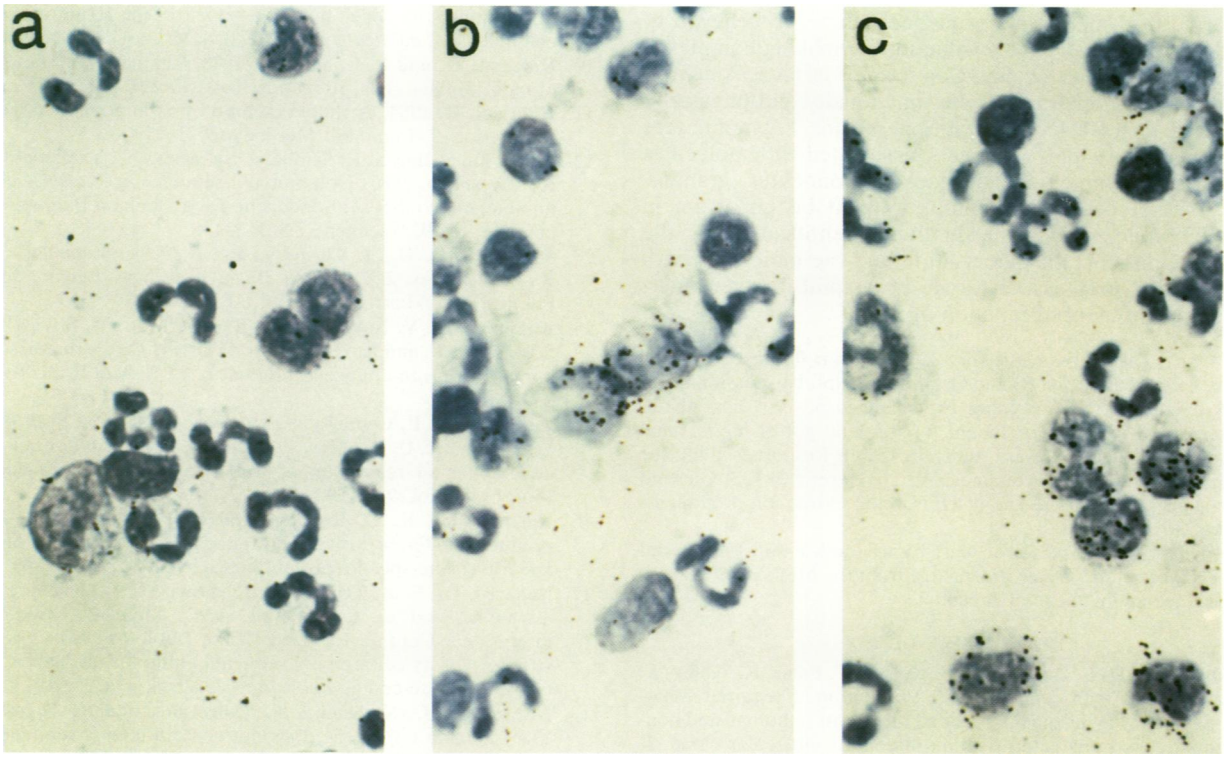

FIG. 3. In situ hybridization of peripheral blood leukocytes with human c-*fgr*-specific probe. Peripheral blood leukocytes were hybridized in vitro with control vector DNA (a) or a human c-*fgr*-specific probe (b and c) as described previously (1). Note that positive signals are found on monocytes (b and c) and large lymphocytes with high cytoplasmic-to-nuclear mass ratios (c). Magnification, ×1,000 in all panels.

$p58^{c\text{-}fgr}$ is specifically expressed in peripheral monocytes, granulocytes, and NK cells. NK cells are defined as CD3-negative, CD16-positive granular lymphocytes which mediate cytolytic reactions that do not require expression of class I or class II major histocompatibility complex molecules on target cells (10). One of the major roles of macrophages and granulocytes is cytotoxicity not subject to major histocompatibility complex restriction or immunological memory. The fact that $p58^{c\text{-}fgr}$ could be detected only in these cells suggests a specific role for c-*fgr* in natural immunity effector cells. In addition, our preliminary data showed that $p58^{c\text{-}fgr}$ could be induced in TPA-treated U937 cells but not in gamma interferon-treated cells. It is known that cell adherence and tumor cytotoxicity are induced in cells of the former type but not in those of the latter type (9). Therefore, it is suggested that $p58^{c\text{-}fgr}$ plays roles in cell adherence or cytotoxic reactions, especially against tumor cells.

Because *src*-related tyrosine kinases do not possess extra-

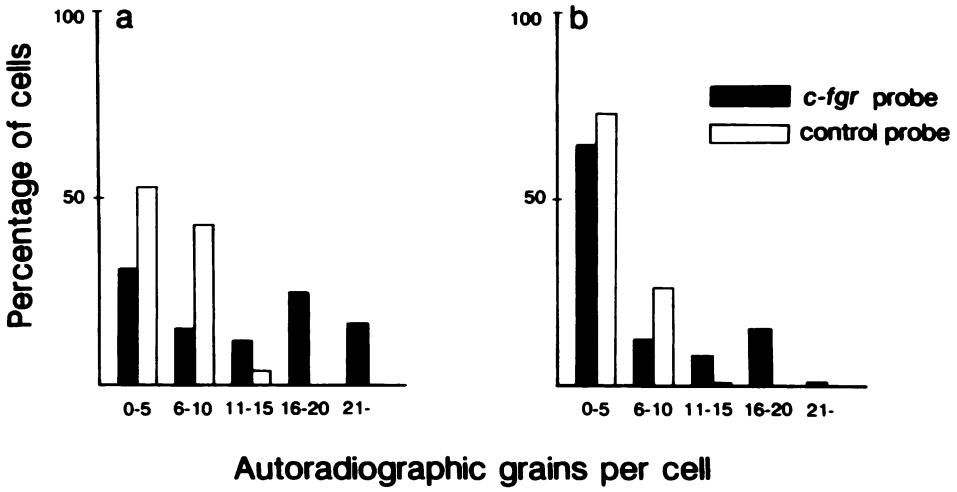

FIG. 4. Distribution of positive signals on mononuclear cells. The number of grains on each cell was counted under a light microscope, and the results are summarized as histograms. (a) Monocytes. (b) Lymphocytes.

cellular or transmembrane sequences, they are believed to play their roles in signal transduction by making complexes with other molecules localizing on the cell surface. Recently, $pp58^{lck}$ was demonstrated to be functionally and physically associated with CD4-CD8 in normal murine T lymphocytes (21, 24). Similarly, $p58^{c\text{-}fgr}$ may be associated with molecules which are present on the surfaces of monocytes, granulocytes, or NK cells. This complex might be important in transmembrane signal transduction. Identification of the target proteins for phosphorylation and the molecules with which $p58^{c\text{-}fgr}$ may associate will be helpful for the understanding of the normal function of $p58^{c\text{-}fgr}$

We thank Takuya Katagiri for his advice on fluorescence-activated cell sorter analysis, Kazuo Oshimi for helpful discussions, and Yoshiko Yamamuro for typing the manuscript. We also thank Nichirei Co., Ltd., Tokyo, Japan, for the kind gift of monoclonal antibodies and the Japanese Cancer Research Resources Bank for providing us with various cell lines. We are grateful to J. S. Gutkind for his suggestions on the use of DFP in the extraction of granulocyte proteins.

This work was supported in part by a Grant-in-Aid for Special Project Research in Cancer Bioscience from the Ministry of Education, Science and Culture of Japan.

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