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Ⅱ．過去の研究歴

昭和62年 福建医学院（中華人民共和国）卒業，福建医学院附属協和医院勤務後，平成4年6月1日より，大阪歯科大学博士課程専攻生として，内科学講座において膠原免疫学の研究を開始する。

Ⅲ．過去の研究実績

1. 黄建勇他5名．自己免疫発症における細胞活能の解析．厚生省特定疾患"免疫異常の発症機構調査研究班"平成6年度研究報告書 16-18, 1995.

2. 黄建勇他5名．NK細胞におけるCD2, FcγR架橋刺激によるPI-3キナーゼの活性化と細胞内顆粒放出能との関係．厚生省特定疾患 平成7年度研究報告書 31-34. 1996

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

1. 第25回 日本臨床免疫学会．CD2架橋刺激によるNK細胞の活性化シグナルと細胞傷害能
2. 第27回 日本免疫学会．CD2架橋刺激によるNK細胞活性化シグナル（1）
3. International Symposium on Recent Advances of Human Tumor Immunology and Immunotherapy. Involvement of protein tyrosine kinase, p72^{Syk} and PI-3kinase in —

(2) 学会誌等に発表した論文　　無 ・ (有)　　（雑誌名・論文名）

Journal of Immunology, Vol 159: 1200-1207, 1997.
"Involvement of protein Tyrosine kinase p72^{Syk} and phosphatidylinositol 3-kinase in CD2-mediated granular exocytosis in the natural killer cell line, NK3.3"

Ⅴ．今後の研究計画及び希望

来年度より，米国カリフォルニア州サンディエゴ，La Jolla Institute for Allergy & Immunology" の Dr. Howard M. Grey のもとで，研究留学に入る予定です。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 4,000字以上で記載して下さい。別紙可）

私は，養子免疫療法の改良にあたり，NK 細胞の活性化メカニズムを CD2 分子を介したシグナル伝達の面より解析いたしました．A) CD 2 分子架橋刺激による細胞内顆粒放出能についての検討： CD2 抗体濃度依存性に細胞内顆粒放出の増強を認めた．この効果は，抗 CD16 抗体にても同様に認められたが，コントロール IgG や，抗 LFA-1, CD28 抗体では認められなかった．また，チロシンキナーゼ阻害剤である Herbimycin と PI 3 キナーゼ阻害剤である Wortmannin は，この効果を完全に抑制した．B) CD2 架橋刺激による，NK 細胞内蛋白のチロシンリン酸化：CD2,および CD16 架橋刺激により，数種のチロシンリン酸化蛋白が出現した． さらに，免疫沈降 Syk のチロシンリン酸化と in vitro kinase assay による検討により，CD2,および CD16 架橋刺激により Syk の活性化が起こっていることを確認した． C) CD2 架橋刺激による PI 3ーキナーゼの活性化：PI 3-キナーゼは，p85 subunit の SH2 domain を介して細胞内チロシンリン酸化蛋白に結合し活性増強を受けることが知られている．そこで，CD2 架橋刺激後に，チロシンリン酸化蛋白を免疫沈降し，これに結合する PI 3-キナーゼを immuno blott 法により検出したところ，CD2,および CD16 架橋刺激により，チロシンリン酸化蛋白に結合した PI 3-キナーゼが増加していた．さらにこの免疫沈降物の PI 3-キナーゼ活性も増強していた．D) 細胞内アダプター蛋白 Shc のチロシンリン酸化と PI 3-キナーゼとの結合： Shc は，CD2 および CD16 架橋刺激により著明にチロシンリン酸化を受けていた．しかも，チロシンリン酸化 Shc に結合した PI 3-キナーゼ活性も有意に増強していた．以上の結果より，NK 活性化レセプターである CD2 分子は，チロシンキナーゼ Syk と PI 3-キナーゼの活性化を介して，細胞内顆粒放出を引き起こしていることが明らかとなった．以上の結果を Journal Immunology vol:159, 1200-1207, 1997 に発表いたしました．

Ⅶ. 指導教官の意見

黄　建勇　君は，約４年半のあいだ勤勉に研究に取り組み，彼の優れた資質により，立派な成果を残しました．これも一重に日中医学協会の御援助のお陰と感謝致しております．また，研究室内での人間関係も良く，スムーズな研究室の運営に貢献致しました．来年度より，米国カリフォルニア州サンデイエゴの La Jolla Institute for Allergy and Immunology, Dr. Howard M Grey のもとで研究を続けることが決まっております．今後も，彼のさらなる飛躍を期待いたしております．

左から2人目

Involvement of Protein Tyrosine Kinase p72^{syk} and Phosphatidylinositol 3-Kinase in CD2-Mediated Granular Exocytosis in the Natural Killer Cell Line, NK3.3[1]

Hisanori Umehara,[2]* Jian-Yong Huang,* Takeshi Kono,† Fazal H. Tabassam,* Toshiro Okazaki,* Eda T. Bloom,‡ and Naochika Domae*

The granular exocytosis pathway is one mechanism by which NK cells and CTLs induce cytolysis of target cells. Triggering through adhesion molecules such as CD2 and LFA-1 as well as FcγRIII (CD16) can invoke this pathway. CD2 is a cell surface glycoprotein present on CTLs and NK cells that plays an important role in both cellular adhesion and signal transduction. Here we report that cross-linking of CD2 as well as CD16 by immobilized Abs enhances granular exocytosis in an NK cell line, NK3.3. Herbimycin, a protein tyrosine kinase (PTK) inhibitor, or wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI 3-K), inhibited completely or almost completely CD2- or CD16-mediated granular exocytosis, suggesting the involvement of protein tyrosine kinases and PI 3-K in both CD2- and CD16-mediated granular exocytosis. We also observed that cross-linking of CD2 as well as CD16 enhances p72^{syk} tyrosine kinase activity, and this enhancement correlated well with the increased tyrosine phosphorylation of several cellular proteins, including the adapter protein Shc. Furthermore, we have observed that cross-linking of CD2 as well as CD16 enhances the PI 3-K activity associated with the tyrosine-phosphorylated cellular proteins and Shc. These results provide insight into the signaling pathways by which triggering of CD2 and CD16 on NK cells leads to cytolysis of target cells. *The Journal of Immunology,* **1997, 159: 1200–1207.**

NK cells express a low affinity FcγRIII[3] (CD16) and are believed to mediate immunity against viruses and surveillance against neoplastic transformation. The granular exocytosis pathway, using lytic mediators, such as perforin, and several proteases (granzymes) residing within cytoplasmic granules, is one mechanism by which NK cells or CTLs mediate cytotoxicity (1). This mechanism induces both necrotic and apoptotic death of target cells (2, 3) and can be triggered by CD16 as well as adhesion molecules on NK cells (4, 5). We previously reported that the β_2 integrin, LFA-1 (CD11a/CD18), on NK cells was tyrosine phosphorylated during the generation of LAK cells, and that cross-linking of LFA-1 on LAK cells enhanced the production of inositol 1,4,5-triphosphate and induced a Ca^{2+}-dependent increase in granular exocytosis (6). Kanner et al. reported that co-cross-linking of LFA-1 with CD3 in T cells increased tyrosine phosphorylation of phospholipase C-γ (PLC-γ) and mobilization of intracellular calcium (7). Signaling through other receptors on NK cells, such as CD2 and CD16, is also reported to induce tyrosine phosphorylation of PLC-γ, resulting in Ca^{2+} influx and cytolytic activity (8–10). Although the protein tyrosine kinase (PTK) and PLC-γ pathways, leading to phosphatidylinositol (PtdIns) breakdown and the mobilization of intracellular calcium, are important in signaling through adhesion molecules, other signaling molecules, such as GTP binding proteins and phosphatidylinositol 3-kinase (PI 3-K), may be involved in granular exocytosis by NK cells (11–13).

It is well established that receptor-type tyrosine kinases, such as platelet-derived growth factor and epidermal growth factor receptors, mediate activation of PI 3-K as well as that of PLC-γ (14). Several lines of evidence demonstrate that stimulation of TCR and B cell Ag receptors (BCR), which lack tyrosine kinase domains but are functionally coupled to PTKs such as p56^{lck} (*Lck*), p59^{fyn} (*Fyn*), and p53/56^{lyn} (*Lyn*), can also mediate PI 3-K activation (15–17). PI 3-K consists of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit that phosphorylates PtdIns at the 3-D position of the inositol ring (18, 19). The products of PI 3-K are not substrates for PLC-γ and, therefore, must participate in cellular signaling through a novel mechanism. CD2 is a glycoprotein adhesion molecule present on CTLs and NK cells and reported to be crucial for the activation of T cells or NK cells via *Lck* and *Fyn* (4, 20, 21), which mediate PI 3-K activation (14–17). We, therefore, hypothesized that signaling through adhesion molecules on NK cells, such as CD2, involves PI 3-K.

Here we report that cross-linking of CD2 by immobilized Ab enhances exocytosis of cytoplasmic granules, and that both herbimycin, a tyrosine kinase inhibitor, and wortmannin, a potent and selective inhibitor of PI 3-K, prevent CD2-mediated granule exocytosis in an NK cell line, NK3.3, suggesting the involvement of

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[3] Abbreviations used in this paper: FcγR (FcγRIII), low affinity Fc receptor for immunoglobulin G; PLC, phospholipase C; PTK, protein tyrosine kinase; PtdIns, phosphatidylinositol; PI 3-K, phosphatidylinositol 3-kinase; BCR, B cell antigen receptor; *Lck*, p56^{lck}; *Fyn*, p59^{fyn}; *Lyn*, p53/56^{lyn}; p85, p85 subunit of PI 3-K; *Syk*, p72^{syk}; BLTE, N^{α}-benzyloxycarbonyl-L-lysine thiobenzyl ester; MBP, myelin basic protein; PSL, photostimulated luminescence.

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PTKs and PI 3-K in CD2-mediated granular exocytosis. We further observed that cross-linking of CD2 activates the PTK $p72^{syk}$ (*Syk*), enhances tyrosine phosphorylation of cellular proteins including an adapter protein Shc, and enhances PI 3-K activity associated with tyrosine-phosphorylated proteins and Shc.

Materials and Methods

Cell and cell culture

The human NK cell line, NK3.3, was provided by Dr. Jacki Kornbluth (University of Arkansas, Little Rock, AR) (22) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Upstate Biotechnology, Inc., Lake Placid, NY), 2 mM L-glutamine, penicillin, and 2 nM rIL-2 (Shionogi Co., Ltd., Osaka, Japan). NK3.3 cells expressed CD2, LFA-1α (CD11a), LFA-1β (CD18), CD45, ICAM-1 (CD54), and FcγRIII (CD16), but not CD3 or CD28 (data not shown), characteristics similar to those of NK cell populations present in peripheral blood.

Abs and reagents

Hybridomas producing Abs against CD11a (LFA-1α: TS1/22; IgG1), CD18 (LFA-1β: TS1/18; IgG1), and CD2 (TS2/18; IgG1) were purchased from the American Type Culture Collection (Rockville, MD), and mAbs were purified as previously described (6). Monoclonal anti-phosphotyrosine Ab (4G10) and polyclonal Abs against the p85 subunit of PI 3-K and Shc were obtained from Upstate Biotechnology. Control IgG1 (MOPC-31c), N^{α}-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLTE), 5,5′-bithiobis(2-nitro-benzoic acid), and myelin basic protein (MBP) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-FcγRIII (CD16: B-E16; IgG1) mAb was purchased from Serotec (Oxford, U.K.). Anti-human CD28 mAb (TN228; IgG1) was provided by Dr. M. Azuma (Juntendo University, Tokyo, Japan). Anti-CD45 mAb (UCHL1; IgG2a) was purchased from Dako (Glostrup, Denmark). Anti-$p72^{syk}$ (*Syk*) mAb and wortmannin were obtained from Wako Pure Chemical Industries (Osaka, Japan), and rabbit anti-human *Syk* Ab, useful for Western blot analysis, was provided by Dr. T. Kurosaki (Lederle Laboratory, New York, NY). Rabbit anti-mouse IgG mAb was purchased from Cappel (Durham, NC). The ECL immunodetection system and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG mAb were obtained from Amersham International (Amersham, Aylesbury, U.K.). Polybeads (polystyrene microspheres, 2.5% solid latex; diameter = 6 μm; no. 07312) were purchased from Polysciences, Inc. (Warrington, PA).

Cross-linking of surface Ags and measurement of BLTE esterase activity

Flat-bottom microtiter plate wells were coated with 20 μg/ml rabbit anti-mouse IgG at 4°C overnight. Wells were then washed and incubated with control IgG1 (MOPC 31c) or Abs against LFA-1α, LFA-1β, CD2, CD28, CD45 (20 μg/ml), and CD16 (1/100 dilution) at 37°C for 1 h, followed by several PBS washes. NK3.3 cells (2×10^5) were added in 200 μl of phenol red-free RPMI 1640 (IBL, Fujioka, Japan) containing 2% FCS and incubated at 37°C for 4 h, and 100-μl aliquots were harvested from each well for BLTE esterase assay. In some experiments, cells were incubated in the presence of herbimycin or wortmannin in DMSO at 37°C for 2 h and 20 min before the assay, respectively. The final concentration of DMSO did not exceed 0.1%. The BLTE esterase assay was adapted to 200 μl for use in 96-well plates (6). Optical density was read at 414 nm on an ELISA microplate reader (Iwaki, Osaka, Japan), and the percentage of BLTE esterase activity was calculated as: (experimental BLTE esterase release − spontaneous BLTE esterase release)/(maximum BLTE esterase release − spontaneous BLTE esterase release) × 100.

Cell stimulation, solubilization, and immunoprecipitation

Polybeads were prepared as described previously (23), by coating with 20 μg/ml rabbit anti-mouse IgG, followed by storage with the Ab, and extensive washing before use. NK3.3 cells (5×10^6) were washed and resuspended in 100 μl of RPMI 1640 medium and incubated with Abs (20 μg/ml or 1/100 dilution for anti-CD16) for 30 min on ice. After washing, cells were resuspended in 100 μl of RPMI 1640 containing 2% FCS and stimulated with rabbit anti-mouse IgG-bound polystyrene beads at a ratio of 1:2 at 37°C for 3 min in polypropylene round-bottom tubes in a final volume of 200 μl/tube. The reaction was terminated by the addition of 800 μl of ice-cold RPMI 1640 and centrifuged for 2 min. Cells were solubilized with lysis buffer containing 50 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 300 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate by gentle rocking at 4°C for 30 min. Insoluble material was removed by centrifugation, and the supernatants were subjected to SDS-PAGE for Western blotting or to immunoprecipitation with protein G beads precoated with the anti-phosphotyrosine mAb (4G10) or anti-Shc Ab.

Electrophoresis, Western blotting, and immunoblotting

Cell lysates and immunoprecipitated proteins were eluted by boiling in SDS-containing sample buffer and fractionated by SDS-PAGE (8%) (24). Proteins were electrophoretically transferred to polyvinylidene difluoride (Immobilon-P) membranes (Sigma Chemical Co.). Membranes were blocked using 10% Blockace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl overnight. Membranes were incubated for 3 h with anti-phosphotyrosine mAb, 4G10 (1 μg/ml) or anti-p85 subunit, and anti-Shc Ab (1/1000), as indicated, in PBS containing 0.05% Tween-20 and 10% Blockace. Peroxidase-conjugated secondary Abs (Amersham) were used at a 1/1000 dilution, and immunoreactive bands were visualized using ECL (Amersham).

In vitro kinase assay

The in vitro kinase reaction was essentially as described by Minami et al. (25). Briefly, anti-*Syk* immunoprecipitates were washed once with kinase buffer (25 mM HEPES (pH 7.4), 0.1% Nonidet P-40, 10 mM $MgCl_2$, 3 mM $MnCl_2$, and 1 mM orthovanadate) and resuspended in 30 μl of the kinase buffer containing 2 μg of MBP as the substrate. Reactions were initiated by the addition of 3.75 μM [γ-^{32}P]ATP (10 μCi of [γ-^{32}P]ATP/sample, 5000 Ci/mmol; Amersham), incubated for 5 min at 25°C, and terminated by the addition of an equal volume of 2× Laemmli buffer. Samples were subjected to SDS-PAGE (11% gels) under reducing conditions and were electrophoretically transferred to polyvinylidene difluoride membrane filters, which were treated with 1 N KOH for 2 h at 55°C, fixed, and subjected to autoradiography. Radioactivity within bands was also estimated using a Bio-Imaging analyzer (BAS 2000, Fuji Photo Film Co. Ltd., Tokyo, Japan), corrected for background, and expressed as photostimulated luminescence (PSL) arbitrary units.

Measurement of PI 3-K enzymatic activity

The PI 3-K activity of anti-phosphotyrosine or anti-Shc immunoprecipitates was measured by the method of Whitman et al. (26). Briefly, immunoprecipitates were washed twice with lysis buffer, followed by once with PBS, once with 0.5 M LiCl/0.1 M Tris-HCl, pH 7.5, once with distilled water, and once with 0.1 M NaCl/1 mM EDTA/20 mM Tris-HCl, pH 7.5, and then assayed for PI 3-K activity. All wash solutions contained 0.2 mM sodium orthovanadate. To evaluate PI 3-K activity, PtdIns (Sigma Chemical Co.) and [γ-^{32}P]ATP (10 μCi/sample, Amersham) were added to the immunoprecipitates at room temperature for 10 min. PtdIns was suspended in 10 mM HEPES/1 mM EGTA, pH 7.5, sonicated before use, and added to the immunoprecipitates at a final concentration of 0.2 mg/ml. [γ-^{32}P]ATP was added in a solution of 50 mM ATP, 5 mM $MgCl_2$, and 1 mM HEPES. The lipid-containing organic phase was resolved on oxalate-coated TLC plates (Silica Gel 60, MCB reagents, Merck, Rahway, NJ), developed in chloroform/methanol/water/ammonium hydroxide (43/38/7/5), and lipid species were visualized by autoradiography. Radioactivity within spots of PtdIns-3′-monophosphate was estimated with a Bio-Imaging analyzer and expressed as PSL arbitrary units.

Results

CD2-mediated granular exocytosis in NK3.3 cells and effects of herbimycin and wortmannin on BLTE secretion

It has been reported that various surface receptors, such as CD2 and CD16, can participate in target cell lysis via the granular exocytosis pathway (4). Therefore, we first examined whether cross-linking of CD2 induced granular exocytosis in the NK cell line, NK3.3. Cross-linking of CD2 on NK3.3 cells enhanced BLTE esterase secretion in an Ab-dose dependent manner (Fig. 1*A*). Although CD2 lacks an intrinsic tyrosine kinase domain, it has been reported to be functionally and physically associated with *src* family tyrosine kinases, *Fyn* and *Lck* (27, 28). Therefore, we examined the effect of a specific tyrosine kinase inhibitor, herbimycin, on CD2-mediated BLTE esterase release. Herbimycin suppressed CD2-mediated granular exocytosis in a dose-dependent manner (data not shown), and treatment of cells with 10 μM herbimycin significantly reduced CD2- as well as CD16-mediated granular

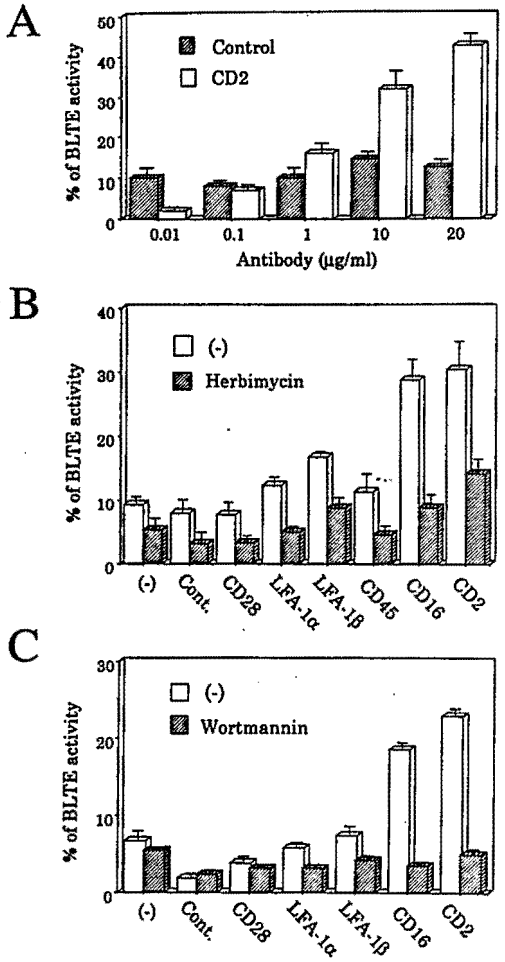

FIGURE 1. Induction of BLTE esterase release by cross-linking of CD2 and effects of herbimycin and wortmannin on BLTE secretion. Cross-linking of CD2 enhances BLTE esterase secretion that depends on the dose of Ab used. NK3.3 cells (5×10^5) were stimulated with immobilized CD2 mAb or control IgG1 at the indicated concentration at 37°C for 4 h. BLTE esterase activity was measured and expressed as described in *Materials and Methods* (*A*). NK3.3 cells were incubated in the absence or the presence of 10 μM herbimycin for 2 h, then stimulated with the indicated Abs (1/100 for CD16 mAb or 20 μg/ml for the others) for 4 h, and supernatants were assayed for BLTE esterase activity. Herbimycin inhibited CD2- as well as CD16-mediated BLTE secretion (*B*). NK3.3 cells were incubated in the absence or the presence of 10^{-7} M wortmannin for 20 min, then stimulated with the indicated Abs (1/100 for CD16 mAb or 20 μg/ml for the others) for 4 h, and supernatants were assayed for BLTE esterase activity. Wortmannin inhibited CD2- as well as CD16-mediated BLTE secretion (*C*). These data are representative of more than three independent experiments.

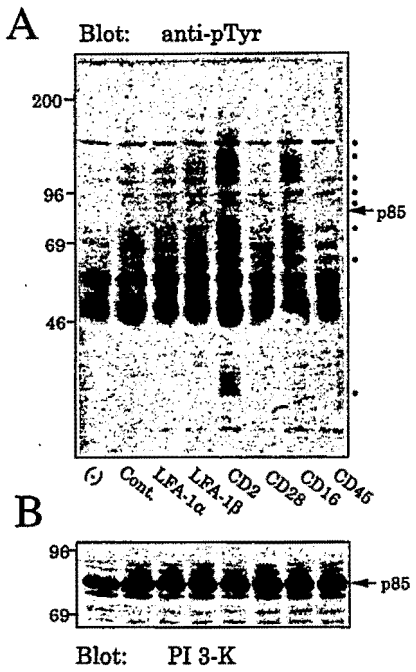

FIGURE 2. Induction of protein tyrosine phosphorylation by cross-linking of CD2 in NK3.3 cells. NK3.3 cells were treated with the indicated Abs and stimulated using polybeads coupled to rabbit anti-mouse IgG Ab at 37°C for 3 min. Detergent-soluble proteins were fractionated by SDS-PAGE (8% polyacrylamide gel) and were transferred to an Immobilon-P membrane. The membrane was immunoblotted with anti-phosphotyrosine mAb (*A*) and then stripped and immunoblotted with Ab against the p85 subunit of PI 3-K (*B*). Open circles indicate tyrosine-phosphorylated proteins, and the arrow indicates p85. The migration positions of m.w. markers are indicated. Data are representative of five independent experiments.

exocytosis (Fig. 1*B*). Previously, we reported that cross-linking of LFA-1 had no effect on BLTE release in fresh NK cells, while it increased BLTE secretion in LAK cells that had been produced by IL-2 stimulation of NK cells, suggesting a functional change in LFA-1 during IL-2 activation (6). A slight or moderate increase in BLTE secretion following cross-linking of LFA-1 was also observed in NK3.3 cells (Fig. 1, *B* and *C*), a finding consistent with our previous results, since NK3.3 cells are continuously cultured with IL-2.

Since PI 3-K is reported to be involved in various biologic functions such as membrane ruffling (29), endocytosis (30), and histamine secretion (31), the PI 3-K pathway as well as the PLC-γ pathway, may be crucial for cytoskeletal rearrangement and granular exocytosis at the site of effector-target cell conjugation. Therefore, we examined the effect of a specific inhibitor of PI 3-K, wortmannin, on CD2-mediated BLTE esterase release. The data demonstrated that wortmannin inhibited CD2-mediated granular exocytosis in a concentration-dependent manner (data not shown) and that treatment of cells with 10^{-7} M wortmannin also essentially abrogated the CD2- as well as CD16-mediated granular exocytosis (Fig. 1*C*). These results suggest that PTKs and PI 3-K are both involved in CD2- and CD16-induced granular exocytosis.

Cross-linking of CD2 induces tyrosine phosphorylation of cellular proteins in NK3.3

To ascertain whether stimulation through CD2 activates PTKs in NK3.3 cells, CD2 was cross-linked, and tyrosine-phosphorylated proteins were analyzed by Western blotting using anti-phosphotyrosine Ab. The data (Fig. 2*A*) demonstrated that cross-linking of CD2 as well as CD16 triggered tyrosine phosphorylation of several cellular proteins migrating with apparent molecular mass of 160, 140, 120, 105, 90, 72, and 56 kDa, suggesting that these receptors may use similar signaling pathways. In contrast, cross-linking of LFA-1α, LFA-1β, or CD45 or of CD28 as a negative control or use of an isotype-matched control IgG1 induced no detectable increase in tyrosine phosphorylation of cellular proteins in NK3.3 cells. The same membrane was stripped and immunoblotted with Ab against the p85 subunit of PI 3-K. Although considerable amounts of p85 were detected in lysates (Fig. 2*B*), tyrosine phosphorylation of a band corresponding to p85 was not observed in any of the NK3.3 cell lysates (Fig. 2*A*, arrow).

Cross-linking of CD2 activates Syk *in NK3.3*

It has been reported that CD2-mediated signals depend in part on the CD3 ζ-chain, a signal-transducing subunit of the TCR/CD3 complex, and that CD16 on NK cells is functionally equivalent to the TCR on T cells for coupling CD2 to its signaling pathway (10, 32, 33). At least four PTKs have been shown to be implicated in the TCR/CD3-mediated signal pathway: two kinases of the *src* family, *Lck* and *Fyn*, and two of the *Syk* family, ZAP-70 and *Syk*. The recruitment of the *Syk* family tyrosine kinases, ZAP-70 and *Syk*, to the TCR/CD3/ζ complex is probably a key step in the CD3-mediated signal transduction, most likely by an interaction through SH2 domains (20). Therefore, we examined whether cross-linking of CD2 activates *Syk* in NK3.3 cells. Cells were stimulated by cross-linking of surface receptors for 3 min, and detergent-soluble proteins were immunoprecipitated with anti-*Syk* mAb, resolved by SDS-PAGE, and immunoblotted with antiphosphotyrosine mAb (Fig. 3*A*). The same membrane was stripped and immunoblotted with anti-*Syk* polyclonal Ab (Fig. 3*B*). The results indicated that tyrosine phosphorylation of a band corresponding to *Syk* was induced by cross-linking of CD2 and CD16 (Fig. 3*A*). We then examined *Syk* kinase activity using an in vitro kinase assay. Figure 3*C* revealed that cross-linking of CD2 markedly increased *Syk* kinase activity using MBP as substrate (1768 PSL U/mm^2), and cross-linking of CD16 moderately increased *Syk* activity (1280 PSL U/mm^2) compared with engagement of other surface receptors or treatment with control IgG1 (991, 923, 638, and 765 PSL U/mm^2 for LFA-1β, CD28, CD45, and control IgG1, respectively). The less marked *Syk* activation as a result of CD16 cross-linking compared with CD2 cross-linking may be due to the differential surface expression of CD16 and CD2 on NK3.3. FACS analysis revealed that the intensity of CD16 expression on NK3.3 cells is substantially lower than that of CD2 and LFA-1 (data not shown). Therefore, CD16 cross-linking may not be sufficient to activate *Syk* in NK3.3 cells.

Cross-linking of CD2 increases the association between PI 3-K and tyrosine-phosphorylated proteins

It has been reported that tyrosine phosphorylation of p85 is induced by platelet-derived growth factor or IL-2 stimulation and correlated with increased PI 3-K activity (34, 35). Since our Western blotting experiments did not reveal increased tyrosine phosphorylation corresponding to p85 after cross-linking of CD2 (Fig. 2*A*), we immunoprecipitated p85 and examined its tyrosine phosphorylation to verify whether cross-linking of CD2 directly induces tyrosine phosphorylation of p85. These data also revealed no tyrosine phosphorylation of p85 after cross-linking of CD2 or CD16 (data not shown). p85 is reported to contain two SH2 domains and bind to tyrosine-phosphorylated YXXM motifs (17, 36). Therefore, we examined the possible association between PI 3-K and tyrosine-phosphorylated cellular proteins following cross-linking of CD2. NK3.3 cells were stimulated by cross-linking of surface receptors for 3 min, and detergent-soluble proteins were immunoprecipitated with anti-phosphotyrosine Ab and immunoblotted with Ab against p85. The data in Figure 4*A* clearly showed that cross-linking of CD2 as well as CD16 increased the association between PI 3-K and immunoprecipitable tyrosine-phosphorylated proteins. Anti-phosphotyrosine immunoprecipitates were also examined for PI 3-K activity by in vitro PI 3-K enzymatic assay, and radioactivity of spots relevant to PtdIns-P was estimated with a Bio-Imaging analyzer. The results revealed that PI 3-K activity associated with tyrosine-phosphorylated proteins was also augmented following cross-linking of CD2 or CD16 (Fig. 4*B*).

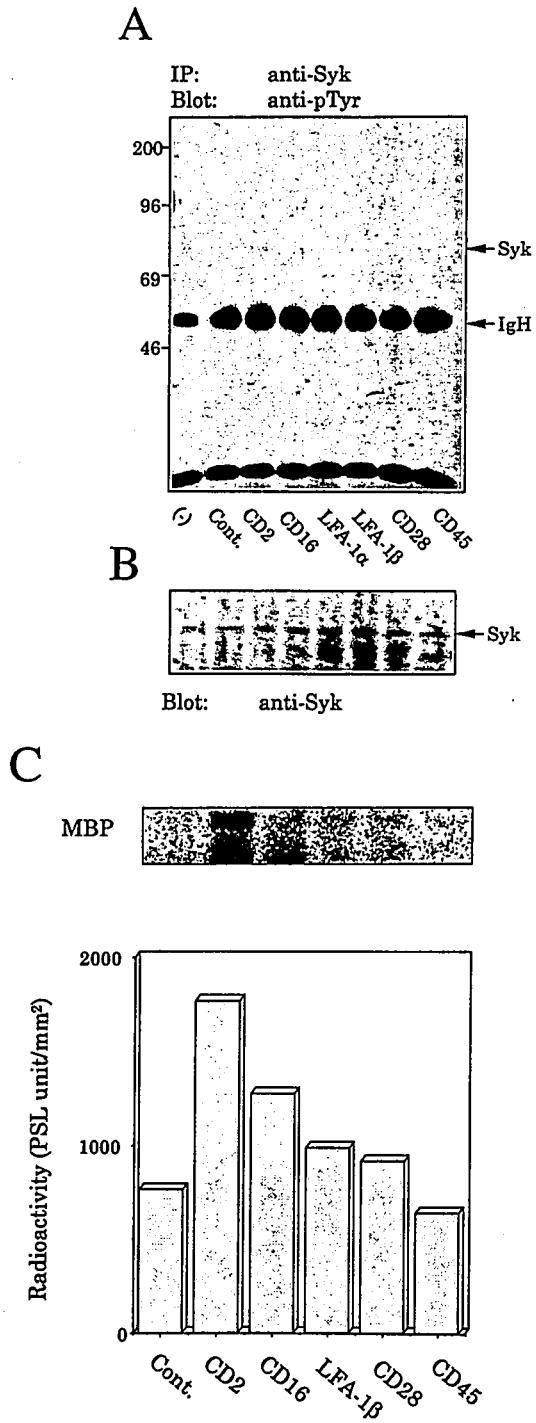

FIGURE 3. Cross-linking CD2 activates *Syk* protein kinase. NK3.3 cells (5 × 10^6/sample) were stimulated with the indicated Abs using polybeads coupled to rabbit anti-mouse IgG Ab at 37°C for 3 min, and detergent-soluble proteins were immunoprecipitated with anti-*Syk* mAb, resolved by SDS-PAGE, and immunoblotted with antiphosphotyrosine mAb (*A*). The same membrane was stripped and immunoblotted with anti-*Syk* polyclonal Ab (*B*). Arrows indicate the position of *Syk* and the heavy chain of IgG (IgH). The migration positions of m.w. markers are indicated. Anti-*Syk* immunoprecipitates were subjected to in vitro kinase assay as described in *Materials and Methods*. Tyrosine phosphorylation of MBP was monitored for evaluating *Syk* kinase activity (*top panel*), and radioactivity within bands was also estimated by a Bio-Imaging analyzer and expressed as PSL arbitrary units as described in *Materials and Methods* (*bottom*; *C*). These data are representative of three for *A* and *B*, and two for *C*.

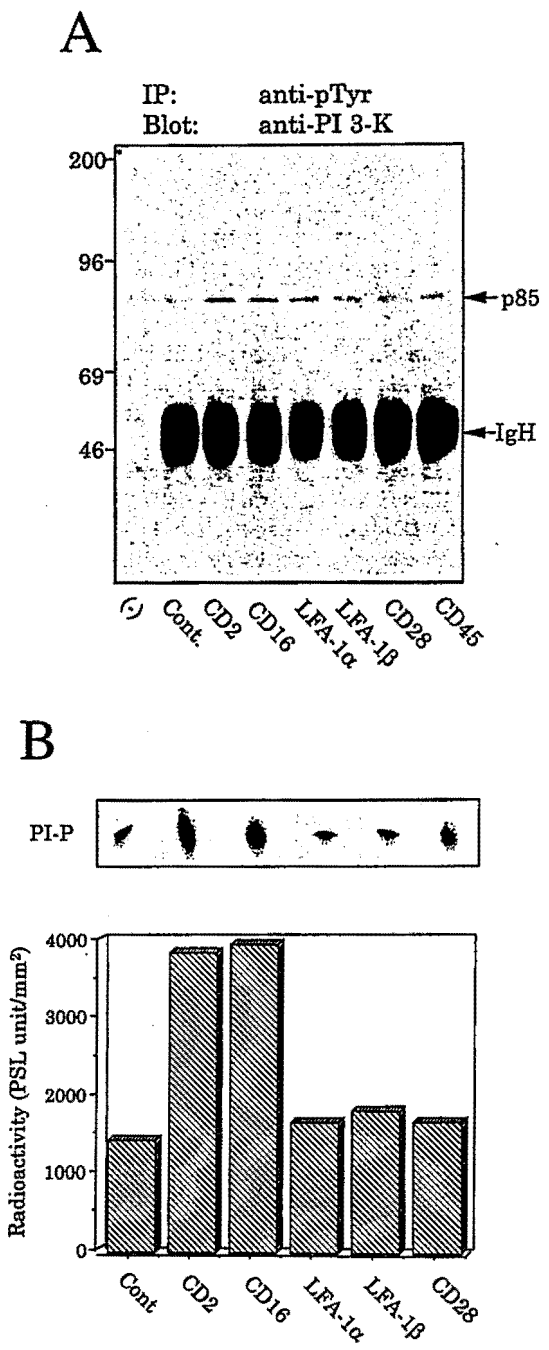

FIGURE 4. Association of PI 3-K with tyrosine-phosphorylated proteins induced by cross-linking of CD2. NK3.3 cells were treated with the indicated Abs and stimulated with polybeads coupled to the rabbit anti-mouse IgG Ab at 37°C for 3 min. Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine mAb (4G10) coupled to protein G beads. Precipitated proteins were fractionated by SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was immunoblotted with Ab against the p85 subunit of PI 3-K. Arrows indicate the position of p85, and the huge bands at M_r 50 kDa are nonspecific binding of rabbit IgG used for cross-linking of receptors. The migration positions of weight markers are indicated (*A*). Immunoprecipitates, obtained as described for *A*, were assayed for PI 3-K activity as described in *Materials and Methods*. The reaction products were subjected to TLC and visualized by autoradiography. Radioactivity within spots of PtdIns-P (PI-P; *top panel*) was estimated by a Bio-Imaging analyzer and expressed as PSL arbitrary units as described in *Materials and Methods* (*B*). These data are representative of two independent experiments.

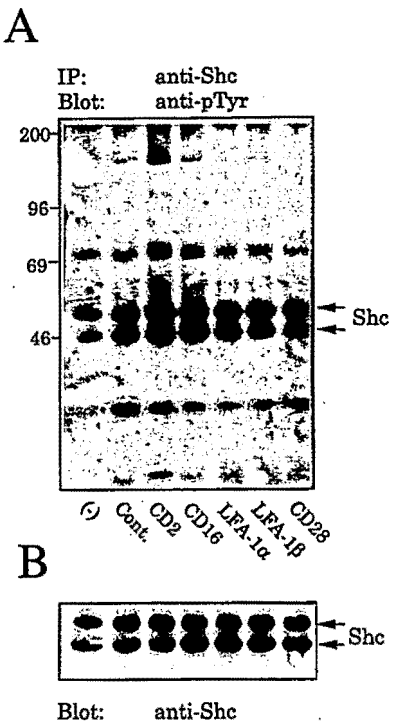

FIGURE 5. Cross-linking of CD2 enhances tyrosine phosphorylation of Shc. NK3.3 cells (5×10^6/sample) were stimulated with the indicated Abs using polybeads coupled to rabbit anti-mouse IgG Ab at 37°C for 3 min, and detergent-soluble proteins were immunoprecipitated with anti-Shc Ab, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine mAb (*A*). The same membrane was stripped and immunoblotted with anti-Shc polyclonal Ab (*B*). Arrows indicate the positions of p46 and p52 Shc proteins, and the migration positions of m.w. markers are indicated.

Cross-linking of CD2 enhances the tyrosine phosphorylation of Shc and increases PI 3-K activity associated with Shc

Shc is an adapter protein that is widely expressed in all tissues and contains an SH2 domain and a collagen-like domain but no obvious catalytic domain. Shc proteins of 46 and 52 kDa encoded by a 3.4-kb mRNA are ubiquitously expressed, whereas a 66-kDa Shc is likely to be encoded by a distinct Shc transcript and is absent in some hemopoietic cells (37). Because this protein is phosphorylated on tyrosine residues in cells transformed by nonreceptor tyrosine kinases such as v-*src* and v-*fps* (37) and by cross-linking of CD3 in T cells (38) or through the stimulation of growth factors, we speculated that Shc might couple the PI 3-K and be involved in the CD2-mediated signal pathway. To evaluate the role of Shc, we determined whether Shc is tyrosine-phosphorylated by cross-linking of CD2 in NK cells. Cells were stimulated by cross-linking of surface receptors for 3 min, and Shc were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine mAb (Fig. 5*A*). The same membrane was stripped and immunoblotted with anti-Shc Ab (Fig. 5*B*). The results indicated that tyrosine phosphorylation of 46- and 52-kDa Shc proteins was enhanced by cross-linking of CD2 as well as CD16 (Fig. 5*A*). Specifically, CD2-mediated tyrosine phosphorylation of Shc was observed in a CD2 Ab concentration-dependent manner, increased within 1 min, and peaked at 3 min (data not shown). To ascertain the association between Shc and PI 3-K, anti-Shc immunoprecipitates were also examined for PI 3-K activity by in vitro PI 3-K enzymatic assay, and radioactivity of spots relevant to PtdIns-P was estimated by a Bio-Imaging analyzer. The results revealed that PI 3-K activity

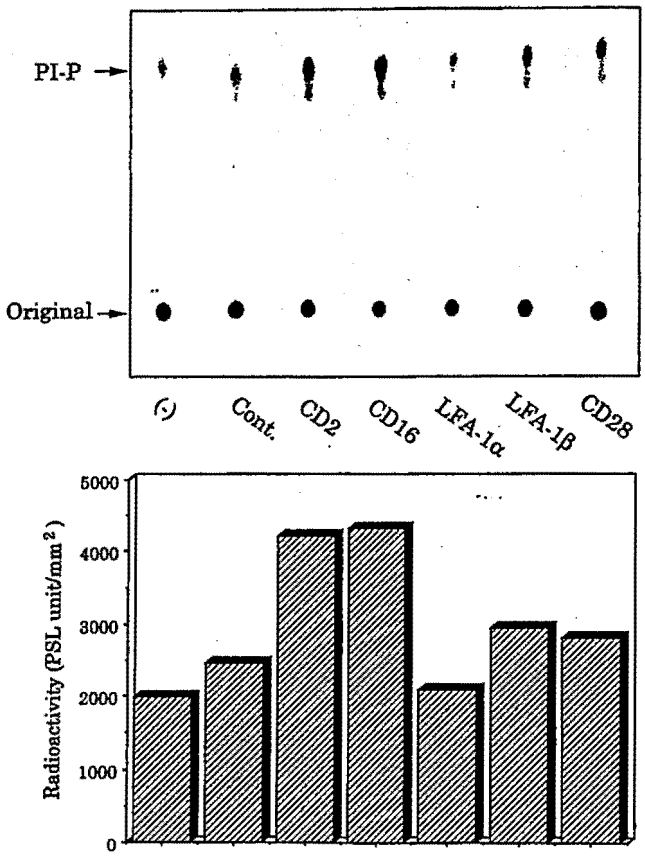

FIGURE 6. Cross-linking of CD2 increases the PI 3-K activity associated with Shc. Shc immunoprecipitates, obtained as described in Figure 5, were assayed for PI 3-K activity as described in *Materials and Methods*. The reaction products were subjected to TLC and visualized by autoradiography (*top panel*). Radioactivity within spots of PtdIns-P (PI-P) was estimated by a Bio-Imaging analyzer and expressed as PSL arbitrary units as described in *Materials and Methods* (*bottom*). These data are representative of two independent experiments.

associated with Shc precipitates was also augmented by cross-linking of CD2 as well as CD16 (Fig. 6).

Discussion

Although CD2 is believed not only to serve adhesion functions by binding its ligands CD48, CD58, and CD59, but also to generate transmembrane signals during the activation of T cells and NK cells, there have been conflicting reports about the signal transduction ability of CD2 on T cells and NK cells. The anti-CD2 mAb (IgG3) in the form of $F(ab')_2$ is reported to have no effect on cytoplasmic calcium influx and cytolytic activity in NK cells, and the trace amounts of contamination of cross-linking Ab in the form of whole IgG have been found to induce calcium influx (9, 10), suggesting that bridging and comodulation of CD2 and CD16 may be required for CD2-mediated signal transduction. In our assay, we used the isotype-matched Abs (IgG1) against CD2, CD16, LFA-1α, LFA-1β, CD28, control IgG1, and cross-linking Ab in the form of whole IgG. In contrast to the effects seen by cross-linking CD2 or CD16, we could not detect a significant increase in BLTE esterase secretion (Fig. 1), tyrosine phosphorylation of cellular proteins (Fig. 2), or Syk activation (Fig. 3) by cross-linking of LFA-1α or LFA-1β or by use of control anti-CD28 or control IgG1, suggesting that the nonspecific binding of the Fc portion of Abs to CD16 did not stimulate NK3.3 cells. Furthermore, co-cross-linking experiments with anti-CD2 and anti-CD16 Ab revealed no synergistic effects on either granular exocytosis or tyrosine phosphorylation of cellular proteins (data not shown). Therefore, we concluded that multivalent cross-linking of CD2 itself was sufficient to transduce signals in NK3.3 cells and may represent another means of triggering a similar response to that through CD16.

CD2-induced signaling events depend on the cytoplasmic domain of CD2, which is relatively large (116 amino acids); is highly conserved among humans, rats, and mice; and lacks intrinsic kinase activity (39). The remarkable capacity of CD2 to activate T cells and NK cells raises the possibility that, like CD3/TCR, CD2 interacts with cytoplasmic PTKs. Previous studies revealed that CD2 and CD16 are functionally and physically associated with the *src* family tyrosine kinases, *Fyn* and *Lck* (28, 40, 41), and induce tyrosine phosphorylation of PLC-γ (8, 27). Recently, Bell et al. clearly demonstrated that the SH3 domain of *Lck* binds to proline-rich sequences in the cytoplasmic domain of CD2 (42). It has been reported that signal transduction via CD2 in NK cells is dependent in part on the CD3/ζ-chain, a signal-transducing subunit of the TCR complex, or the γ subunit of CD16, which is homologous to the CD3/ζ-chain (10, 33, 43). The current model of T cell activation is based on a sequential interaction of *src* and *Syk* family PTKs with the TCR/CD3/ζ complex, which by itself lacks intrinsic kinase activity. *Lck* or *Fyn* is thought to be responsible for the tyrosine phosphorylation of the CD3/ζ-chain on the immunoreceptor tyrosine-based activation motif (ITAM; YXXL-based sequences interspaced by six to eight amino acids) that is present as three copies in each ζ subunit and as two copies in γ subunit of CD16, which lead to recruitment of the tyrosine kinase ZAP-70, most likely by an interaction mediated by SH2 domains (20). *Syk* is structurally homologous to ZAP-70, with two tandemly arranged SH2 domains and a C-terminal kinase domain (44), and a similar mechanism of recruitment has been proposed for *Syk*. *Syk* has been reported to be activated by various receptors, such as CD3/TCR (45–47), BCR (48), FcεRI (49), and FcγRIIA (50). In NK cells, engagement of CD16 has been reported to induce tyrosine phosphorylation of cellular proteins (51) and to increase the catalytic activity of both ZAP-70 and *Syk* (52, 53). Therefore, we examined whether cross-linking of CD2 activates *Syk* in NK cells, and we found that cross-linking of CD2 or CD16 enhances tyrosine phosphorylation of *Syk* and activates its kinase activity against MBP substrates (Fig. 3, *A* and *C*), suggesting that CD2 and CD16 use similar signaling pathways.

Involvement of PI 3-K in CD16-mediated signal transduction and granular exocytosis in NK cells has been reported (12, 13). Our results extend these analyses by demonstrating that cross-linking of CD2 as well as CD16 stimulates *Syk* kinase activity (Fig. 3, *A* and *C*) and enhances tyrosine phosphorylation of cellular proteins and an adapter protein, Shc (Figs. 2*A* and 5*A*), resulting in the increase in PI 3-K activity associated with tyrosine-phosphorylated Shc (Fig. 6). Although we could not detect tyrosine phosphorylation of p85 in NK3.3 cells, we have observed increased PI 3-K activity in phosphotyrosine immunoprecipitates following cross-linking of CD2 or CD16, as reported for CD3 stimulation of T cells (54, 55). The p85 subunit contains two SH2 domains that bind to tyrosine-phosphorylated YXXM motifs, one SH3 domain that binds to the proline-rich region of *src* family kinases, and two proline-rich regions that are docking sites for SH3 of *src* family PTKs (17, 36). Thus, PI 3-K participates in the assembly of signal transducing complexes through multiple association sites. Although Shimizu et al. have reported constitutive association of PI 3-K and CD2 in a CD2-transfected cell line (56), we found PI 3-K associated with CD2 in CD2 immunoprecipitates as well as in control immunoprecipitates (data not shown). Therefore, our data

do not support a specific association between PI 3-K and CD2 in NK3.3 cells. A possible alternative explanation for the participation of PI 3-K in the CD2-mediated signaling and granular exocytosis may be that the SH2 domains of the p85 subunit bind to tyrosine-phosphorylated receptors and/or adapter proteins. It is reported that full activation of PI 3-K occurs only when both SH2 domains of p85 are occupied by tyrosine-phosphorylated YXXM motifs, and partial activation occurs when either SH2 domain is occupied (57, 58). We speculated that Shc, an adapter protein, may associate with and regulate PI 3-K activity, because it has been reported to be phosphorylated on tyrosine residues by *Syk* as well as other PTKs (59, 60). Shc has also been reported to associate with PI 3-K or to be involved in the assembly of signaling complexes that include PI 3-K following stimulation through CD3/TCR, BCR, or cytokine receptors (37, 61–63). Therefore, we examined whether cross-linking of CD2 increases Shc tyrosine phosphorylation and mediates the association between PI 3-K and Shc. The results clearly demonstrated that cross-linking of CD2 increases tyrosine phosphorylation of Shc (Fig. 5*A*) and enhances PI 3-K activity associated with Shc (Fig. 6). We examined Grb2-associated PI 3-K activity and found that no remarkable change in PI 3-K activity was induced by cross-linking of CD2 or CD16. Furthermore, we examined the Grb2-associated molecules by binding assay using glutathione-*S*-transferase fusion proteins of Grb2 and found that Grb2 associated with Shc, but not with PI 3-K. These results indicate that neither direct association of Grb2 and PI 3-K nor a trimolecular complex of Shc, Grb2, and PI 3-K was formed in significant amounts (data not shown). Although several reports have indicated the physical association of PI 3-K with *src* family PTKs (15–17), we propose an additional mechanism for the involvement of PI 3-K in the CD2-mediated signal pathway: 1) cross-linking of CD2 as well as CD16 activates *Syk* directly or dependent upon tyrosine phosphorylation of the γ subunit of CD16 through *Lck* activation; 2) activated *Syk* induces phosphorylation of Shc on tyrosine residues; and 3) full or partial activation of PI 3-K is mediated by association with Shc via SH2 domains of p85.

In conclusion, we have clearly demonstrated that CD2-mediated granular exocytosis in NK cells is dependent on PTKs and PI 3-K activity, since herbimycin or wortmannin strongly inhibited or completely abrogated CD2-mediated granular exocytosis. Moreover, we have observed that cross-linking of CD2 increases *Syk* kinase activity and enhances tyrosine phosphorylation of cellular proteins such as Shc, resulting in increased PI 3-K activity associated with Shc. These results suggest a potential role for *Syk* and PI 3-K in the CD2-mediated signal pathway and granular exocytosis.

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理事長　中島章殿

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Ⅱ．過去の研究歴

C型肝炎ウイルス（HCV）に感染した母子例を対象として、HCVのエンベロープ領域にある超可変領域の塩基及びアミノ酸配列を解析して、その多様性及び母子間の相同性を明らかにし、感染経路の解明を試みる。

Ⅲ．過去の研究実績

上記の研究について いくつかの成果をあげた。自検例の研究によって小児のC型肝炎のほとんどがその母親から移されたと明らかな結果を得ました。そして第一作者で日本小児科学会雑誌に論文を三つ載せた。また 共同研究者としても 数個の論文を発表した。

Ⅳ．本年度の研究業績

(1) 学会、研究会等においての口頭発表（学会名・内容）

(2) 学会誌等に発表した論文　無 ・ (有)　（雑誌名・論文名）

"Homology of E2 gene in 8 child-mother pairs infected with hepatitis C virus." The Tohoku Journal of Experimental Medicine 1997, 183(3).

"Sequential change of hepatitis C virus E2 gene in two mother-child pairs." ～
（この論文は書いているところで、まだ最後に完成していない）

Ⅴ．今後の研究計画及び希望

今後もHCVについて研究を続けるが、また、G型肝炎及びまだ未知のウイルス性肝炎の病因や感染経路についての研究を進めたいです。

研究助成金を与えていただいて、大いに助かりまして、まことにありがとうございます。

今後も研究助成金を頂くチャンスを与えてほしいです。

Ⅵ. 研　究　報　告（日本語、又は英語で書いて下さい。 4,000字以上で記載して下さい。別紙可）

研究報告は別紙にて.

Ⅶ. 指導教官の意見

李柔代が貴財団の研究助成を得て行った研究結果は、今日のわが国における小児のC型肝炎の感染経路を明らかにするもので、成人の領域では国民病とされるC型肝炎の予防および治療対策を小児の立場から明らかにする上で重要な知見が提供されたものと考える。李柔代の研究に対する姿勢は真摯で、助成の効果を十分生かせたものと思う。しかし、更に研究を発展させるためには貴財団の研究助成が継続できる体制が望まれる。

目的

（1）小児におけるC型肝炎ウイルス（HCV）の感染機序を解明するため、HCVに感染した母子を対象にHCV E2領域の塩基配列を解析し、母子感染の確認に役立つか否かを検討する。

（2）HCVの変異状況と持続感染の関係を解明するため、HCVに感染した母子を対象にHCV E2領域の塩基配列を経時的に解析する。

対象

感染経路の解明については、名古屋市立大学病院小児科外来に通う小児8例とその母親8例とした。8例の性別は男児3例、女児5例で、年齢は2ヵ月から12歳であった。4例の児はHCV感染の母親から生まれ、フォローアップ中にHCVに感染した。他の4例の児はHCV抗体スクリーニングによって、HCV感染が判明し、母子共にHCV感染と診断された。児は全例輸血歴はなく、HCV水平感染のリスク因子もなかった。家族1の母子以外全例血液サンプルを同時に採取した。血液サンプルは採取後直ちに血清に分離し、－30℃に保存した。

母子感染例におけるHCVE2領域の経時的解析は2組の母子で行った。即ち、

家族1：母親は妊娠初期に流産に対する免疫治療として夫の白血球の皮下注射を受けた。治療前の母親のHCV抗体は陰性であった。治療後夫がHCVキャリアであることが判明した。母親は分娩前にHCV抗体陽性となっていた。

児は生後3カ月時HCV RNA陽性で、その後ウイルス血症が持続している。

血清サンプルは児においては、生後3、6、9、12、15、21、27、および30カ月の8時点で、母親においては、分娩後3、9及び12ヵ月の3時点で採取した。

家族2：母親は無症候性HCVキャリアであった。生後4カ月に行った児のHCV RNA検査が陽性で、その後HCV RNA陽性が続いている。血清サンプルは児においては、生後4、6、9、12、18、24及び30カ月の7時点で、母親においては、分娩後4、6、9、12及び30ヵ月の5時点で採取した。

方法

RNA抽出とRT-PCRについて

血清100μlよりAGPC(acid guanidium thiocyanate-phenol-chloroform)法でRNAを抽出し、random hexamerをprimerとして、MMLV(maloney murine leukemia virus)逆転写酵素を用いてcDNAを合成した。

HCVゲノタイプの同定は岡本らの測定方法を一部改変して行った。即ち岡本らはHCVのC領域に位置するuniversal primer (sense) と4種類のtype-specific primers (antisense) の混合液を用いてPCR反応を行い、4つのgenotypeにHCVを分類しているが、4種類のtype-specific primersを別々に用いてPCR反応を行った。

HCVのE2領域ゲノムの増幅は岡田らの方法に従って行った。1st及びnested PCRはそれぞれ1種類のsense primerと2種類のantisense primerを用いた。PCR反応はthermal cyclerを用い、1st PCRでは35サイクル、nested PCRでは30サイクル行った。denaturation温度は94℃、annealing温度は55℃、extension温度は72℃とし、反応時間はそれぞれ1分とした。

cDNAサブクリーニング法について

増幅したcDNAはアガロース泳動した後目的のバンドを切り出し、ガラスパウダーで抽出した。制限酵素EcoRIとBclIで処理した後cDNAのフラグメントをplasmid vectors (pUC119)のEcoRI/BamHIのサイトに組み込むことでクローン化した。1つの検体についてそれぞれ5つのcDNAクローンを解析した。

シークエンス解析について

塩基配列の解析はABI (Applied Biosystems)のTaq Dye Deoxy Terminator Cycle Sequencing Kitと373A蛍光DNA sequencerを用いて、プラス鎖とマイナス鎖について行った。

塩基及びアミノ酸の番号はgenotype IIでは、HCV-BK株、genotype IIIでは、J6株を基準とした。本研究では解析した全領域をF領域と定義し、更にそれを3つの部分、即ち超可変領域(HVR)-1、HVR-2、及びHVR-1と2の間の領域に分けて検討した。超可変領域-1と2の間の領域はI領域と定義した。

系統発生学的解析について

I 領域に対して系統発生学的解析を行った。この解析はソフトウエア GENETYX-MAC を用い、neighbor-joining法で行った。

結果と考察

HCVゲノタイプをtype-specific primersを用いたPCR法で検索した結果、家族１、４、６、７及び８の母子はtype II と判定され、家族２、３と５の母子は type III と判定された。一方cDNAクローンの塩基配列に最も類似した塩基配列をGenBankに登録されている塩基配列の中から検索すると、家族１、４、６、７及び８の母子のcDNA塩基配列は type II の HCV-BK 株が選択された。同様に家族２、３、と５の母子では type III の HCV-J6 株が選択された。即ち二つの異なった方法によってgenotype分類を検討したところ同じ結果であった。

Table 1は各症例それぞれ５クローン間の塩基及びアミノ酸配列の同一性を検討した結果を示す。各項の左側の値は各領域の塩基及びアミノ酸配列の最も高い変異率を示し、右側は最も高い保存率を意味する。百パーセントは解析した５クローンの cDNA の中に完全に一致したクローンが少なくとも２クローンあったことを意味する。

超可変領域-1 において クローンの種類を検討すると 図 1に示した通りである。

図 1のaは家族１－５までの超可変領域のアミノ酸配列を示す。家族３の児では単一のアミノ酸配列が見られたが、他の症例では多種類の配列が見られた。図 1のbは家族６－８までの超可変領域のアミノ酸配列を示す。家族６の母子とも単一のアミノ酸配列を示す。家族７の児と家族８の母親は以前に interferon の治療を受けていたが HCV ウイルス血症が続いていた。これらの症例では多種類の アミノ酸配列が観察された。なお、家族７の児の１０クローン中７クローンには３塩基、１アミノ酸の挿入を認め、またその母親の１１クローン中の９クローンには１塩基の欠失を認め、frame shift により生じたstop codon が多数認められた。

Table 2には児とその母親の間、児と他の児及び他の母親との間、また児と GenBankに登録されている塩基配列との間の一致率を各領域について検討した結果を示す。I 領域について相同性をみると８組中６組（７５%）（家族１、２、３、５、６と８）では児とその母親の間の塩基配列の相同性は、５クローン全部が児とほかの児、他の母親及び GenBank に登録されている最も類似性の高い塩基配列の間の相同性より高った。１組（１３%）（家族７）は一部のクローンのみ高い相同性を示した。即ち、児の１０クローン中６クローンと母親の１１クローン中の５クローンの間の相同性は児と他の児、他の母親及び GenBank からの塩基配列の間の相同性より高い値を示した。他の１組（１３%）（家族４）は母子間の塩基配列の相同性が児の５クローン中の４クローンと家族７の母親の１１クローン中の５クローンの間の相同性より低い値を示した。F 領域について同様のことをみた結果をTable 2に示した。即ち母子間の相同性を比較する場合、F 領域よりI 領域の方がいくぶん高いという成績が得られた。

I 領域の塩基配列を用いて系統発生学的関係を検討した結果を 示す。８家族で６４個、 GenBank から得た２２個の ユニークな 塩基配列を用いて分析した。家族１、２、３、４、６及び８の児のDNA塩基配列は母親のそれと同じ枝に分類されたが、 GenBank から得られた１つの塩基配列と家族５の塩基配列が同じ枝に分類され、また家族７では、 GenBank から得られた多数の塩基配列とその家族の半数のクローンは比較的類似性の高い塩基配列として分類された。

今回の検討で我々は１症例について、５クローンを解析し、HVR-1 と２の間のI領域について母子間の相同性をみると、８家族中７家族で母親とその児の間で一致率が高いことを実証できた。これはまた系統発生学的分析法によっても証明された。即ちこの研究の結果によって、I 領域についての塩基配列を解析することで、 HCV の母子感染の確認が可能と考えられた。

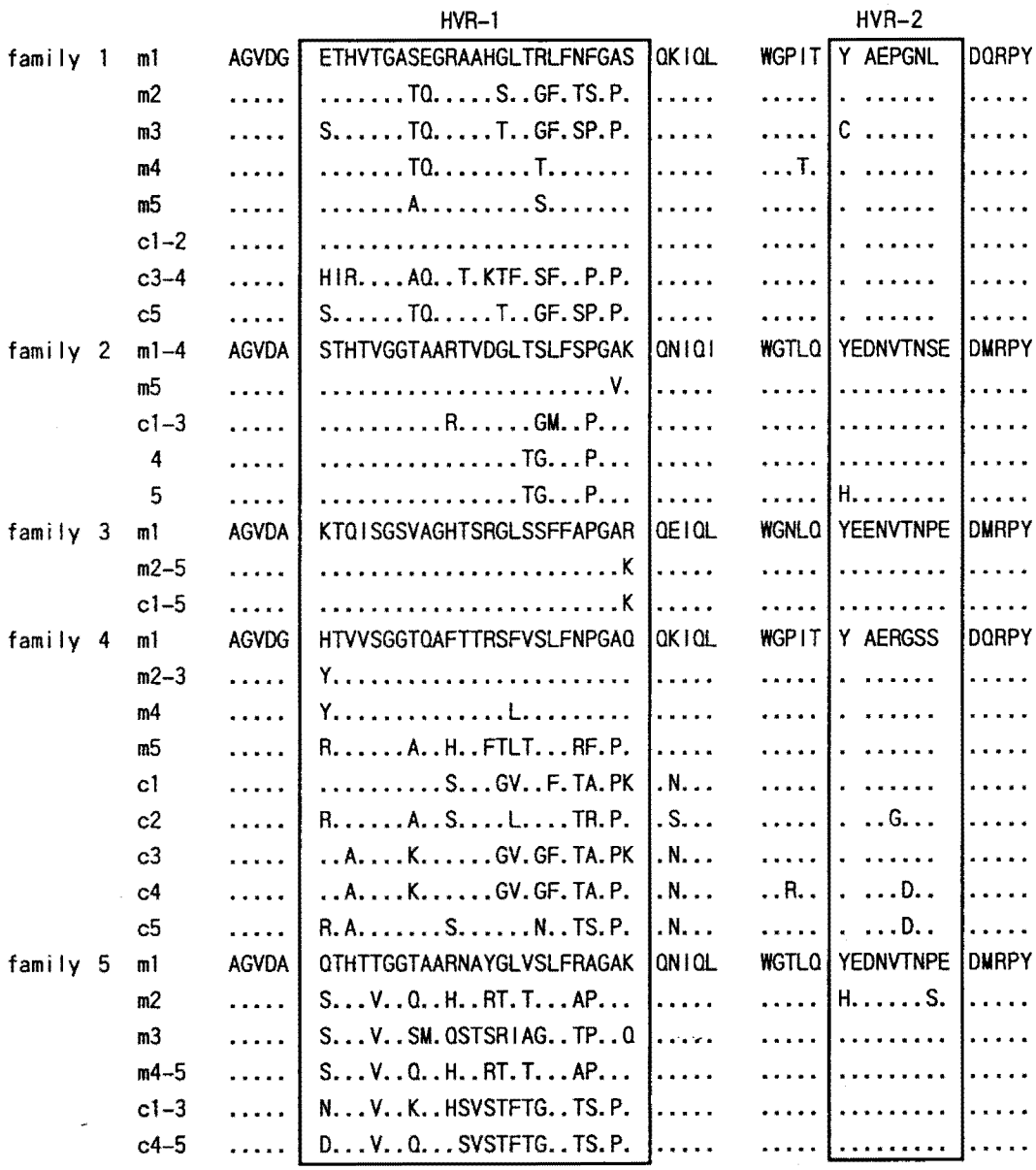


Fig. 1 a. Amino acid sequences for HCV HVR-1 and HVR-2. The consensus sequences for the predicated amino acid of the mother is shown by a single letters on the top line. A dotted line indicates identical residue to consensus. * indicates stop codon. m indicates mother. c indicates child. The regions enclosed in boxes indicate HVR-1 and HVR-2.

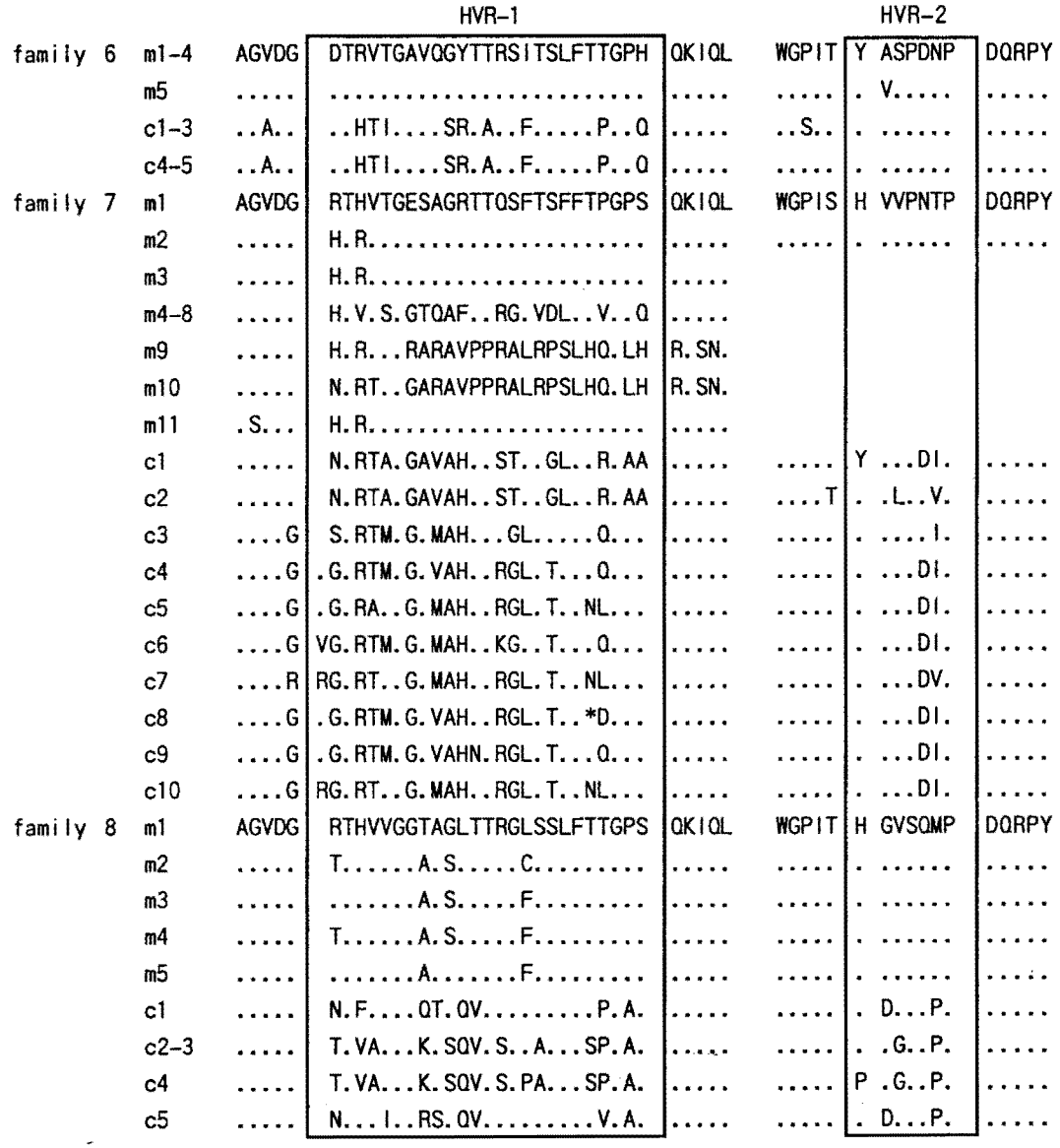
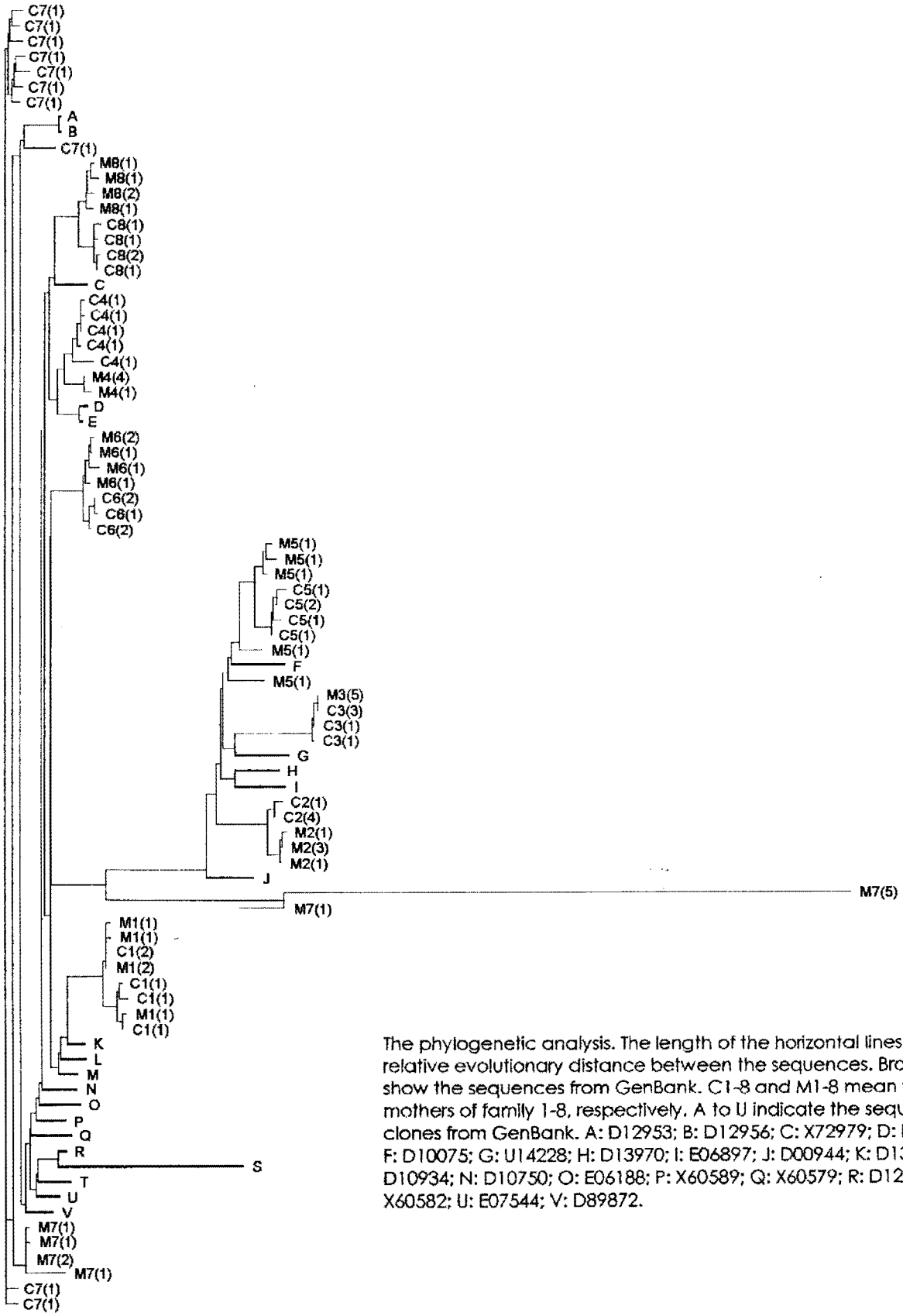


Fig. 1 b. Amino acid sequences for HCV HVR-1 and HVR-2. The consensus sequences for the predicated amino acid of the mother is shown by a single letters on the top line. A dotted line indicates identical residue to consensus. * indicates stop codon. m indicates mother. c indicates child. The regions enclosed in boxes indicate HVR-1 and HVR-2.

Fig. 2



The phylogenetic analysis. The length of the horizontal lines indicates the relative evolutionary distance between the sequences. Broad lines in trees show the sequences from GenBank. C1-8 and M1-8 mean the children and mothers of family 1-8, respectively. A to U indicate the sequences of cDNA clones from GenBank. A: D12953; B: D12956; C: X72979; D: D50481; E: D50485; F: D10075; G: U14228; H: D13970; I: E06897; J: D00944; K: D13406; L: X60581; M: D10934; N: D10750; O: E06188; P: X60589; Q: X60579; R: D12958; S: D12965; T: X60582; U: E07544; V: D89872.

Table 1 Homology search for nucleotide and amino acid sequences among the clones from 8 children and their mothers

Patient No.		Region F		HVR-1		Region I		HVR-2	
		Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)
Family 1	Child	93.8-99.8	88.1-99.4	73.3-100	48.0-100	96.4-100	93.9-100	100	100
	mother	94.7-98.7	92.6-98.9	78.7-96.0	64.0-92.0	95.9-100	95.4-100	95.2-100	85.7-100
Family 2	Child	98.1-100	94.9-100	94.7-100	88.0-100	99.0-100	96.9-100	96.3-100	88.9-100
	mother	99.4-100	98.9-100	98.7-100	96.0-100	99.0-100	98.5-100	100	100
Family 3	Child	99.4-100	99.4-100	100	100	99.0-100	100	100	100
	mother	99.1-99.8	98.3-100	97.3-100	96.0-100	100	100	100	100
Family 4	Child	96.0-98.3	91.5-96.0	80.0-98.7	60.0-96.0	95.4-99.5	95.4-98.5	90.5-100	71.4-100
	mother	94.7-99.1	92.1-98.9	72.0-100	60.0-100	99.0-100	98.5-100	100	100
Family 5	Child	97.6-99.3	96.1-99.4	94.7-100	88.0-100	97.4-100	100	96.3-100	100
	mother	87.1-98.5	86.5-98.3	60.0-100	40.0-100	88.7-98.0	90.8-100	88.9-100	77.8-100
Family 6	Child	98.9-100	98.3-100	100	100	98.5-100	98.5-100	100	100
	mother	98.5-99.4	97.2-98.9	100	100	98.0-100	95.4-100	95.2-100	85.7-100
Family 7	Child	89.8-97.6	85.9-99.4	66.7-100	42.3-100	89.7-97.4	89.2-100	81.0-100	42.9-100
	mother	85.1-100	----- a	60.0-100	----- a	89.7-100	----- a	52.4-100	-----a
Family 8	Child	94.3-99.8	90.3-100	76.0-100	52.0-100	98.0-100	98.5-100	76.2-100	57.1-100
	mother	97.9-99.6	95.5-98.9	92.0-98.7	84.0-96.0	97.4-100	93.9-100	100	100

a Data could not be obtained.

Table 2 Homologous frequencies of nucleotide and amino acid sequences between 8 child-mother pairs

Child No.	Region F				HVR-1		Region I				HVR-2	
	Nucleotide			Amino acid	Nucleotide	Amino acid	Nucleotide			Amino acid	Nucleotide	Amino acid
	Child-mother	Child-other children and mothers[a]	Child-GenBank data[b]	Child-mother	Child-mother	Child-mother	Child-mother	Child-other children and mothers[a]	Child-GenBank data[b]	Child-mother	Child-mother	Child-mother
1	93.9-99.6	87.1	86.7	88.1-100	73.3-100	48.0-100	95.9-100	88.7	92.3	92.3-100	95.2-100	85.7-100
2	96.8-97.8	88.0	86.1	92.7-95.5	92.0-94.7	76.0-84.0	95.9-97.4	89.2	85.1	90.8-95.4	96.3-100	88.9-100
3	99.1-100	84.3	82.4	98.3-100	97.3-100	96.0-100	99.0-100	85.1	84.6	100	100	100
4	92.6-95.3	97.2	92.1	86.9-91.5	70.7-84.4	48.0-76.0	92.3-95.4	96.9	94.9	87.7-92.3	95.2-100	85.7-100
5	87.5-94.4	87.8	88.4	87.1-89.3	65.3-84.0	44.0-60.0	89.2-95.4	85.1	88.7	90.8-93.9	88.9-100	77.8-100
6	93.8-94.5	86.6	85.2	90.3-92.1	80.0	64.0	95.9-98.5	90.8	92.8	93.9-98.5	95.2-100	85.7-100
7	83.4-95.3	86.7	88.8	------	54.4- 85.3	------	86.7-96.9	90.3	93.9	------	42.9-90.5	------
8	91.1-93.4	85.7	85.6	85.8-91.5	69.3-78.7	48.0-68.0	93.9-96.4	90.3	90.8	89.2-93.9	71.4-85.7	57.1-71.4

a Highest homologous frequency of nucleotide sequence between one child and the other children or mothers for Region F and I.

b Highest homologous frequency of nucleotide sequence between one child and accession cases in GenBank database for Region F and I.