Biochemical and Biophysical Research Communications **281,** 131–140 (2001)
doi:10.1006/bbrc.2001.4322, available online at http://www.idealibrary.com on IDEAL®

Cloning and Characterization of a Novel ITIM Containing Lectin-like Immunoreceptor LLIR and Its Two Transmembrane Region Deletion Variants[1]

Xin Huang, Zhenglong Yuan, Guoyou Chen, Minghui Zhang, Weiping Zhang, Yizhi Yu, and Xuetao Cao[2]

Department of Immunology, Second Military Medical University, Shanghai, 200433, People's Republic of China

Received January 8, 2001

A novel full-length cDNA was cloned from human dendritic cells (DC) by subtractive cloning and RACE. The deduced protein is a type II lectin-like membrane protein that contains an ITIM proximal to N terminal and is designated as lectin-like immunoreceptor (LLIR). The gene of LLIR is located in a region of chromosomal 12p13 and shows highest homologous with ASGPR. Two alternatively spliced transmembraneless variants of LLIR were identified by RT-PCR and named as LLIRv1 and LLIRv2. RT-PCR and immunoblotting analysis revealed that LLIR was expressed with much higher level in immature DC than in mature DC. The ITIM in LLIR was demonstrated to bind SHP-1 in HL-60 cell after the tyrosine had been phosphorylated. In addition, the mRNA expression level of LLIRv2 was raised when leukemia cells were induced to differentiate by PMA.

Key Words: **C-type lectin; immunoreceptor tyrosine-based inhibitory motif; dendritic cells; type II membrane receptor; alternative splice variant; subtractive cloning; cell differentiation.**

The indispensability of dendritic cell (DC) as antigen-presenting cell (APC) in the induction of primary immune responses is due to the high efficiency in capturing and processing antigens, the potent migratory capacity and the abundant expression of molecules necessary for T cell activation of DC (1). The functions of DC are perfectly regulated in that when DC reside in unperturbed tissues, they are in an immature stage with high antigen capturing and processing capacities, but with low T cell stimulating activity. In contrary, mature DC could present antigen with high efficiency but its antigen uptake capacity is reduced dramatically. Keyhole limpet hemocyanin (KLH) is one of potent antigens and has been used for pulsing DC in clinical trial of eliciting T cell immunity by injection with mature DC (2, 3).

The difference of cell functions is originated from the discrepancy in gene expression. Comparative studies on gene expression in different stages of DC can promote the understanding of physiological processes of DC. Subtractive cloning is a powerful method in identifying genes specially expressed in tissues and cells. In our previous study, an optimized strategy for selecting differentially expressed genes has been developed and successfully used in identifying genes differentially expressed in KLH stimulated DC (4). One of those identified gene fragments, GC13, was predicted to contain a C-type lectin domain or as carbohydrate recognition domain (CRD) at C terminal and an immunoreceptor tyrosine-based inhibitory motif (ITIM) proximal to N terminal. Biological functions of lectin-like molecules are predicted by the additional domains in the molecules, the way in which the domains link to CRD and the overall architecture of CRD (5). ITIM is a characteristic stretch in cytoplasmic tail of the members of inhibitory-receptor superfamily (IRS) (6), which can deliver inhibitory signals upon receptor cross-linking by recruiting phosphatases.

The known members of type II membrane protein have been divided into four groups: Asialoglycoprotein receptors (ASGPR) (7–9); Low affinity immunoglobulin epsilon Fc receptor (10–12); Kupffer cell receptor (13) and a number of proteins expressed on the surface of natural killer T-cell, which include NKG2 molecules (14), NKR-P1 (15), Ly-49, CD69 (16), or on the surface

Abbreviations used: APC, antigen presenting cells; ASGPR, asialoglycoprotein receptors; CRD, Ca^{2+}-dependent carbohydrate recognition domain; DC, dendritic cells; IRS, inhibitory-receptor superfamily; ITIM, immunoreceptor tyrosine-based inhibitory motif; KLH, keyhole limpet hemocyanin; NKC, NK receptor complex; ORF, open reading frame; PMA, phorbol myristic acid; PAGE, polyacrylamide gel; RACE, rapid amplification of cDNA ends.

[1] This work was supported by grants from TRAPOYT and the National Natural Science Foundation of China.

[2] To whom correspondence should be addressed. Fax: (+86-21)-6538 2502. E-mail: caoxt@public3.sta.net.cn.

0006-291X/01 $35.00

of B-cells, that include CD72, LyB-2 (17, 18). The CRD in these proteins is distantly related to other CRD; and it is yet unclear whether they are likely to bind carbohydrates (19). A number of closely related type II membrane proteins, which include NKG2 molecules, CD94, NKR-P1A/CD161 and CD69, are coded by genes localized in a region on human chromosome 12p12-p13. This region is designated as the NK gene complex (NKC). The novel gene fragment GC13 was predicted to localize in a region of chromosome 12p13. The receptors coded by NKC can mediate activation or inhibitory signals. NK cell would be activated to exhibit cytotoxicity and IFN-γ production upon cross-linking of NKR-P1A/CD161, which recognize carbohydrate ligands (20, 21). CD69 also activates NK cells to induce cytotoxic activity and costimulating cytokine production (22). NKG2 molecules have been the subjects of intense investigation over the last few years because they are specific for major histocompatibility complex (MHC) class I. CD94 is required for expression of NKG2 molecules on cell surface. The CD94 protein associates different NKG2 isoforms to form heterodimers, which function to activate or inhibit cytotoxicity of NK cell relying on the cytoplasmic domains of NKG2 isoforms. NKG2A/B contains ITIM and is inhibitory, while NKG2C lacks ITIM and is stimulatory (23). The ITIM, firstly identified in type II receptors binding the Fc part of IgG (FcγRIIB) (24, 25), is also present in growing members of receptors group that was named as IRS (15, 26–28). The negative regulation mediated by receptors of IRS is due to the activation of tyrosine phosphatases by phosphorylated ITIM (6).

Phosphorylation of the tyrosine residues within ITIM creates a docking site for the Src homology-2 domain (SH-2) of the intracellular phosphatases and results in the recruitment and activation of at least one of the SH-2 domain containing protein tyrosine phosphatases, SHP-1 and SHP-2, and/or the inositol phosphatase SHIP. The activation of these phosphatases results in dephosphorylation of signaling molecules, leading to down modulation of cell activity (29). This mechanism, combined with the pharmacological effects of pervanadate, which has been shown to induce substantial tyrosine phosphorylation of cellular substrates (30), enable the assessment of the potential ability of ITIM sequence to recruit SH-2 domain containing phosphatases.

In the present study, complete sequence of GC13 was cloned by rapid amplification of cDNA ends (RACE) and it was designated as lectin-like immunoreceptor (LLIR) based on sequence analysis of the deduced protein. The distribution of this gene in tissues and cells were analyzed by Northern blot and RT-PCR. Then two alternatively spliced variants were revealed from RT-PCR analysis and designated as LLIRv1 and LLIRv2. Furthermore, polyclonal antiserum against LLIR was prepared and used for analyzing cells distribution, biochemical characteristics. In addition, the potential of LLIR to be an inhibitory receptor was assessed by detecting its ability to recruit SHP-1. And finally, the involvement of LLIR and its variants in PMA induced leukemia cell differentiation was studied.

MATERIALS AND METHODS

Identification of differentially expressed cDNA from human dendritic cell by subtractive cloning and RACE. Human monocyte-derived DC was generated and identified by a protocol previously described by us (31). Fresh peripheral blood from normal adult was separated with lymphocyte separation media ($\rho = 1.077$, Sigma), the cells in boundary layer were collected and cultured with RPMI 1640 complete media (Gibco) in 35 mm dishes at 5×10^6 cells/ml for 2 h. Swirling the dish softly, discarding the suspending cells and the adherent cells were cultured in RPMI 1640 complete media containing rhGM-CSF (800 U/ml, Sigma) and rhIL-4 (500 U/ml, Sigma) for 5 days. The suspending cells were collected and cultured for another 1 or 2 days. The cells were human DC, which were confirmed by cytometry that more than 90% of cells were $CD1a^+$, $CD83^+$, and $HLA\text{-}DR^+$. KLH is a potent antigen, and was used to stimulate human DC. The antigen pulsed DC were obtained by incubating normal DC in KLH (10 μg/ml, Sigma) for another 24 h.

The strategy used for subtractive cloning has been described elsewhere (4). Briefly, mRNA of human monocyte-derived DC (0.5 μg) was prepared according to the instruction of PolyATtract System 1000 (Promega) and reverse-transcribed with oligo-$dT_{(12-18)}$ by the ordinary method and used as the template for DRIVER preparation. PCR amplification produced the DRIVER and biotin-21-dUTP (Clontech) was incorporated simultaneously. TESTER mRNA (0.5 μg) was prepared from KLH stimulated monocyte derived human dendritic cells and reverse-transcribed with 5′ CAP primer (5′ TAC GGC TGC GAG AAG ACG ACA GAA GGG) and 3′ anchoring primer (5′ TAC GGC TGC GAG AAG ACG ACA GAA T_{30}MN). Half of the TESTER cDNA was mixed with 100 μl of DRIVER and then hybridized at 65°C for 12 h in 6× SSC and 0.5% SDS. The hybridization mixture was passed through Sephacryl S-400 (Pharmacia) and cleared with Streptavidin Paramagnetic particles (Promega). The supernatant (containing the un-hybrid TESTER cDNA) was amplified and used to repeat subtractive hybridization if required. After two or three rounds of hybridization, DNA was resolved on agarose gel and recovered for cloning, sequencing and analyzing.

Double-strand plasmid DNA was sequenced on an ABI 377 sequencer (Applied Biosystems) using BigDye terminator technology. One of the novel gene fragments, GC13, was predicted to contain an ORF but the 5′ flanking sequence was not complete.

Double-strand cDNA with an adapter was prepared for RACE. Adapter was obtained by annealing the forward oligonucleotide 5′ CTC GAG CCC TAT AGT GAG TCG TT and backward oligonucleotide 5′ pAAC GAC TCA CTA TAG GGC TCG AG. The first strand cDNA synthesis is primed using oligo(dT) and random hexamer together and the other steps according to the instructions of SUPERSCRIPT Choice System (Gibco). The 5′ RACE primer (5′ GCT GTG TTG ATG CCT GAG GAC TTG) was used and the amplification was performed by touchdown PCR. PCR condition is 94°C for 30 s, then 94°C for 5 s and 72°C for 3 min for 5 cycles, and then 94°C for 5 s and 70°C for 3 min for 5 cycles, and finally 94°C for 5 s and 68°C for 3 min repeated 25 cycles. The amplified fragment was cloned into T-vector (Promega) for sequencing and analysis. A fragment with length of 240 bp was cloned and revealed to be 5′ flanking sequence of GC13. The full-length sequence has a length of 1305 bp.

Characteristics prediction, designation and chromosomal localization of LLIR. Sequences were analyzed with PCGENE, ANTHEPROT software and ExPASy molecular biology server. Comparisons with the GenBank databases were using the BLAST algorithm. The novel gene

was designated lectin-like immunoreceptor (LLIR) according to its predicted characteristics. Searching corresponding chromosomal sequence in the GenBank databases has allowed chromosomal localization.

Distribution analysis of LLIR by Northern blot and RT-PCR. The probe was prepared from a DNA fragment template with a length of 1220 bp from LLIR residues 20-1240 by random primer labeling. Briefly, in 25 μl reaction volume, 25 ng of template DNA, 50 μCi [α-^{32}P]dCTP (Amersham), 0.5 nmole each dGTP, dATP, dCTP and 30 μg/ml random primer (9 mer) were mixed in and denatured, then 2 units of Exo$^-$ Klenow fragment (Takara) was added and the reaction incubated at 37°C for 10 min. Passing through Sepharose G-25 (Pharmacia) column to remove unincorporated [α-^{32}P]dCTP purified the reaction product. Distribution was firstly analyzed by using Human MTN blots (Clontech) which includes mRNA of heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte. The hybridization was performed followed the manuals. Briefly, membranes were prehybridized in 5 ml of ExpressHyb solution with continuous shaking at 68°C for 30 min, then the ExpressHyb solution was replaced by 5 ml of fresh solution containing radiolabeled DNA probe and incubated with continuous shaking at 68°C for 1 h, the blots were washed in wash solution with a total time of 2 h and exposed to X-ray film (Kodak) at −70°C for 1 day.

Cultured cells or cell lines used for mRNA expression analysis were listed here: monocyte derived dendritic cells (primary culture), KLH stimulated dendritic cells (10 μg/ml KLH, stimulated for 24 h), HL-60 (acute promyelocytic leukemia), NB4 (promyelocytic leukemia), U-937 (histiocytic lymphoma), K-562 (chronic myelogenous leukemia), Jurkat (acute T cell leukemia), HuT 78 (lymphoma; cutaneous T lymphocyte), Raji and Ramos (Burkitt's lymphoma; B lymphocyte), Daudi (Burkitt's lymphoma; B lymphoblast). Total RNA was extracted with TRIZOL reagent (Gibco) as following description, 1 ml TRIZOL was used for 1×10^7 cells, after cells were homogenized with vigorous agitation, 200 μl of chloroform were added and stay at room temperature for 3 min, then centrifuged by 12,000*g* at 4°C for 15 min, the supernatants were collected and precipitated with equal volume of iso-propanol. The total RNA was reverse-transcribed by oligo-dT primering as routine. RT-PCR with the primer 5′ TCG GAA ATC ACT TAT GCT (sense primer) and 5′ ATG TCG CTG ACC TTC TG (antisense primer) was performed using Exo$^-$ *Taq* polymerase (Takara). Cycle conditions were 95°C for 10 s, 53°C for 30 s and 72°C for 30 s for 35 cycles. Synthesis of cDNA was controlled by amplifying β-actin (sense primer, 5′ GCA TCC TCA CCC TGA AGT AC; antisense primer, 5′ TTC TCC TTA ATG TCA CGC AC). Cycle conditions for β-actin were 95°C for 10 s, 57.5°C for 30 s and 72°C for 30 s for 25 cycles. The amplification products were resolved on 1.5% agarose gel.

Identification of the alternatively spliced variants of LLIR. Two variant fragments were amplified from HL-60 cells by PCR. The amplified fragments were resolved on agarose gel, recovered separately and cloned into plasmid for sequencing. Then two alternatively spliced variants of LLIR were identified and named as LLIRv1 and LLIRv2.

Preparation of polyclonal antiserum. LLIRv2 was expressed in prokaryotic expression vector pLCM182, an expression vector constructed by us (32). The vector fragment was prepared as following: 2 μg of pLCM182 was digested with *Eco*RI (New England Biolabs, NEB) and blunted with T4 DNA polymerase (NEB), this DNA fragment was retrieved on agarose gel and digested with *Bam*HI (NEB), the reaction product was precipitated and dissolved for cloning. The LLIR and LLIRv2 fragments were amplified by PCR under the condition of 95°C for 10 s, 55°C for 30 s, 72°C for 60 s and repeated for 30 cycles. PCR primers, sense primer 5′ TAT ATG ACT TCG GAA ATC ACT TAT G and antisense primer 5′ CGG GAT CCT CAT AAG TGG ATC TTC ATC ATC, would introduce blunt and *Bam*HI sites at the 5′ and 3′ ends, the start codon and *Bam*HI site are underlined. The PCR products were digested with *Bam*HI and cloned into expression vector. After the recombinants been selected, the expression levels were evaluated by hot induction. Scaling up bacterial culture to produce adequate amount of LLIRv2 protein for further using. Bacterial were suspended in ice-cold *E. coli* lysis buffer (50 mM Tris–Cl, pH 8.0, 10 mM EDTA, 100 mM NaCl) at 1 g/10 ml, then sonicated with 200 W at 50% pulse for 10 min. Lysis was complete when the cloudy cell suspension became translucent. Most of the expressed LLIRv2 protein was distributed into inclusion bodies, so the pellet was collected by centrifuging the sonicated reaction at 12,000*g* for 15 min at 4°C and washed with STE (20 mM Tris–Cl, pH 8.0, 2 mM EDTA, 500 mM NaCl) thrice then followed with 1 M urea in 100 mM Tris-Cl (pH 8.5) for three times. The inclusion bodies could be dissolved in 6 M urea and remained soluble in 1 M urea without forming of deposit. LLIRv2 protein was purified by dilution, dialysis and ion-exchange chromatography.

The purified LLIRv2 protein was used to immunize New Zealand white rabbit to produce polyclonal antibodies. About 250 μg of LLIRv2 protein mixed with equal volume of complete Freund's adjuvant was injected into multiple subcutaneous sites for priming immunization. The equal amounts of antigen were injected into multiple intramuscular sites for boosting when mixing with incomplete Freund's adjuvant 4 weeks later. Repeating the boosting twice and the rabbit blood was collected.

To remove cross-reacting antibodies, the extract of transforming host containing control plasmid pLCM182 was used. Purification was performed as following. The bacteria were resuspended in ice-cold 100 mM NaCl at 2 ml per gram, then 4 volume of acetone (−20°C) was added, mixed vigorously and stored on ice for 1 h. The suspension was centrifuged at 10,000*g* for 10 min at 4°C, the pellet was resuspended completely with same volume of acetone (−20°C) as above and was stored on ice for 10 min. The pellet was collected after recentrifugation and placed in a chemical hood to allow the acetone evaporation. When the powder was completely dry, it was added to the antiserum to a final concentration of 5% and the mixture was stored on ice for 15 min. The powder was removed by centrifugation at 10,000*g* for 10 min at 4°C and the supernatant was collected and store at −20°C as purified antiserum.

The titration of this purified antiserum was detected by Western blot analysis with the whole lysate of bacteria, which express LLIRv2. Whole lysate of the bacteria expressing LLIRv2 was separated on SDS–PAGE and the amount of expressed LLIRv2 was adjusted to about 0.5 μg in each lane, then transferred to nitrocellulose membranes (Schleicher & Schuell) and blocked with 10% nonfat dry milk for 30 min at room temperature. The purified antiserum was diluted with antibody dilution buffer (5% BSA, 0.05% Tween 20, 100 mM Tris-Cl, pH 7.5, 0.9% NaCl) by 1:500, 1:1000, 1:2000 and 1:4000 to probe target protein. The membrane was incubated in diluted antiserum for 30 min at room temperature and washed with TBST (0.1% Tween 20, 100 mM Tris-Cl, pH 7.5, 0.9% NaCl) four times for 5 min each. Then the blot was incubated with horseradish peroxidase (HRP) conjugated goat anti rabbit IgG (Santa Cruz, 1:1000 dilution) for 30 min at room temperature and washed with TBST thrice for 5 min each followed with TBS (100 mM Tris–Cl, pH 7.5, 0.9% NaCl) once for 5 min. Immunoblotted proteins were visualized by Chemiluminescence Luminol Reagent (Santa Cruz).

Immunoblotting analysis of the expressions of LLIR and its variants. HL-60 cells, NB4 cells, U-937 cells, Jurkat cells, Raji cells, Reh cells were harvested for immunoblotting. Total cell lysates were prepared as following description. Cell lysis buffer (1% NP-40, 50 mM Tris-Cl, pH 7.8, 150 mM NaCl) containing PMSF (100μg/ml), Aprotinin (1 μg/ml) and Leupeptins (1μg/ml) were added to cell pellet for suspension and incubated at 37°C for 15 min. The cell debris were removed by centrifugation for 10 min at 10,000*g,* and the supernatants were used for immunoblotting. The whole lysates were separated on SDS–PAGE and transferred onto nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in TBST at room temperature for 30 min with constant agitation. The antiserum was

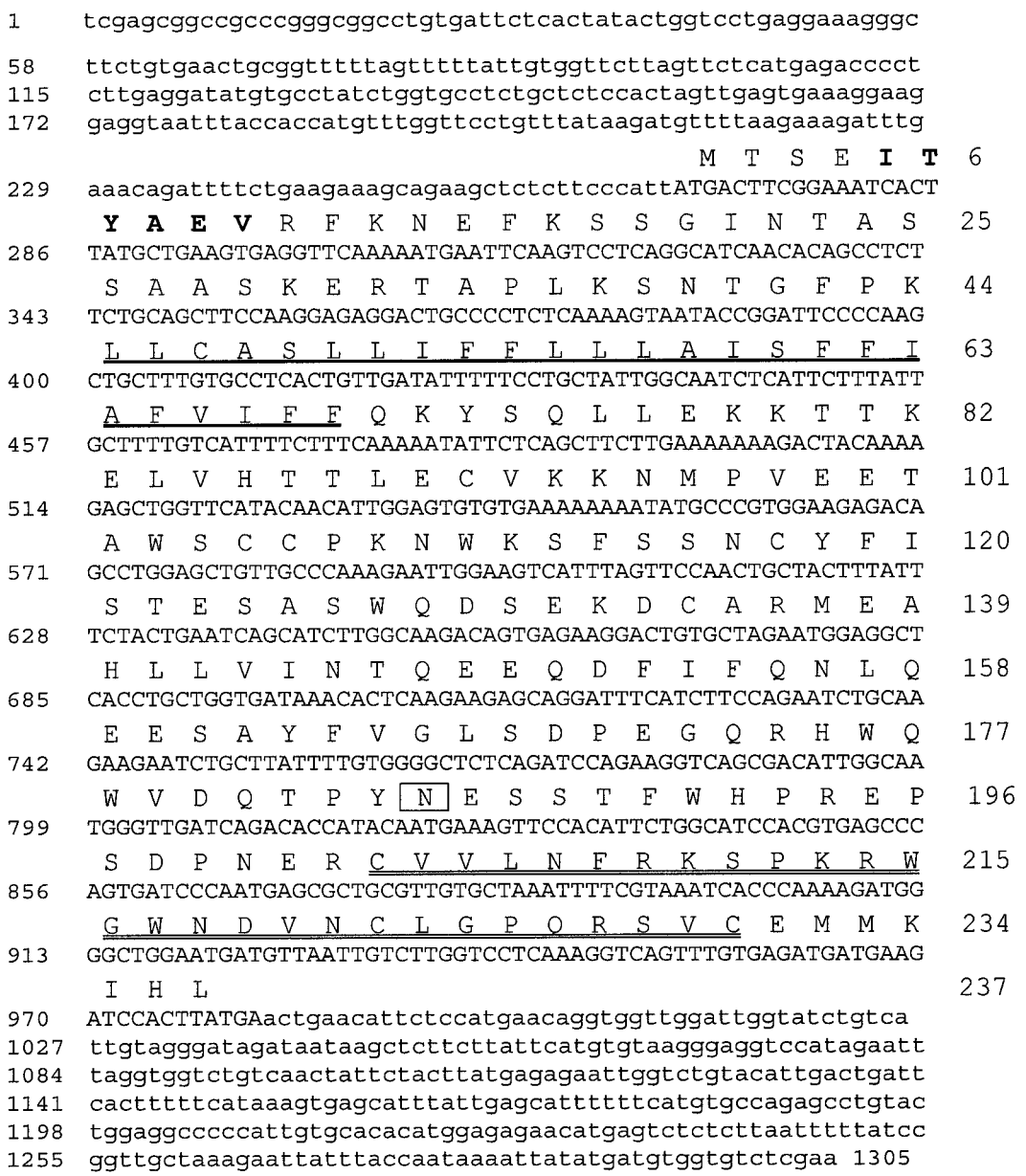

FIG. 1. Nucleotide and predicted amino acid sequences of LLIR (AF067800). The nucleotide sequence of 1305 bp contains an open reading frame from nt 269 to 982, which is shown in uppercase in contrast to the flanking part in lowercase. The derived amino acid sequence is shown above the nucleotide sequence. The C-type lectin domain signature is double underlined and the potential N-glycosylation site is boxed. The potential transmembrane region is underlined and the consensus ITIM sequence is in bold type.

diluted for 1:500 for using as primary antibody. The other steps were performed as described above.

NIH/3T3 cells transfected with LLIR and LLIR genes were used as positive control of immunoblotting. LLIR and LLIRv2 fragments for eukaryotic expression were amplified by PCR using the PCR primers: sense primer, 5′ AAC TCG AGG GCC TGT GAT TCT CAC TAT ACT G, and antisense primer, 5′ CGG GAT CCA GAG ACT CAT GTT CTC TCC ATG, which introduced *Xho*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively. The amplified fragments were cloned into eukaryotic expression vector pcDNA3.1/Myc-His(−) (Invitrogen). So the transcription was initiated by CMV promoter. Cells were transfected with plasmids using the LIPOFCTAMINE transfection reagent (Gibco) according to the manufacturer's instruction.

In vitro translation of LLIR and LLIRv2. At first, the DNAs coding sequence with proper flanking region were cloned into pcDNA3.1/Myc-His(−) which contains a promoter for T7 RNA polymerase. PCR primers used in amplifying DNA fragments for *in vitro* translation are sense primer 5′ AAG ATT TGA AAC AGA TTT TCT G and antisense primer 5′ GGA ATT CAA ATG CTC AAT AAA TGC TC. In addition, linearization and high quality of the plasmids are required for transcription, so the constructed plasmids were digested with *Eco*RI and purified by ethanol precipitation. The reaction products were used as template for transcription under the T7 RNA polymerase. The templates were mixed with 100 nmol of rNTPs, 40 units of T7 RNA polymerase (Promega) in the buffer of 80 mM Hepes-KOH (pH 7.5), 24 mM

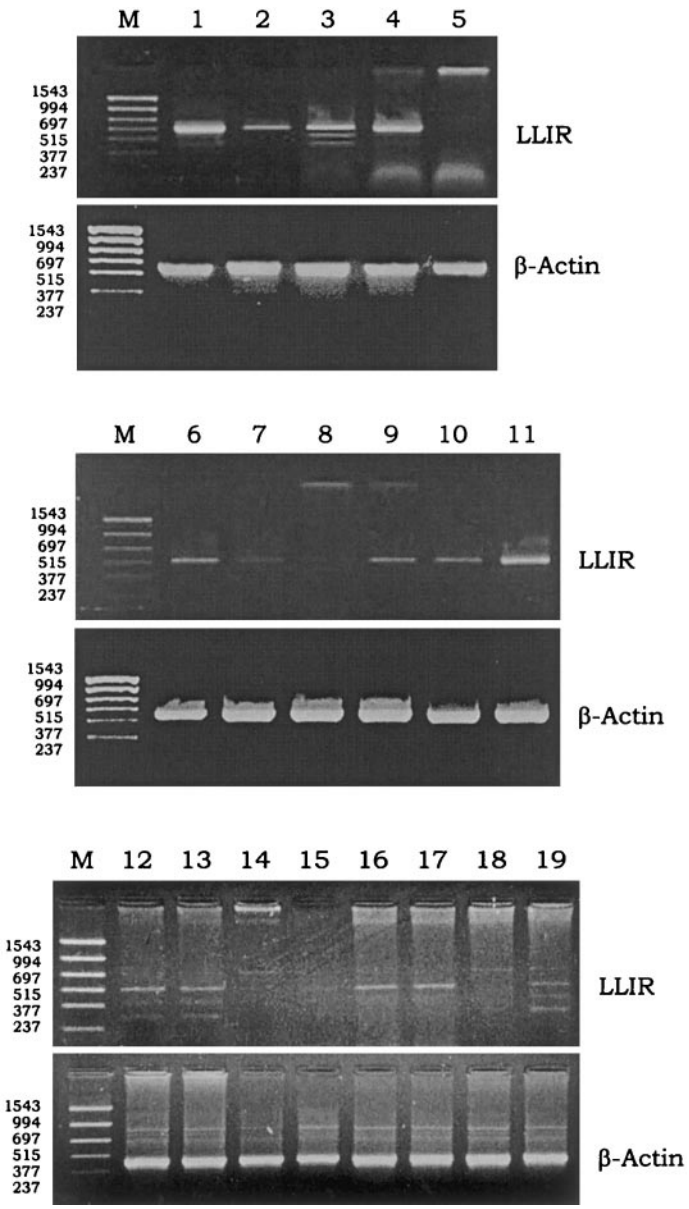

FIG. 2. RT-PCR analysis of LLIR expression in human DC, cell lines, and fetal tissues. Human cells and fetal tissues were arranged as following order: 1, DC, 2, DC-KLH, 3, HL-60, 4, NB4, 5, U937, 6, K-562, 7, Jurkat, 8, HuT78, 9, Ramos, 10, Daudi, 11, Raji, 12, fetal heart, 13, fetal brain, 14, fetal kidney, 15, fetal lung, 16, fetal liver, 17, fetal spleen, 18, fetal skeletal muscle, 19, fetal small intestine. Synthesis of cDNAs was controlled by amplifying β-actin.

$MgCl_2$, 2 mM spermidine, 40 mM DTT in the final volume of 20 μl and incubated at 37°C for 4 h. This reaction was purified by phenol:chloroform extraction, ethanol precipitation and used for *in vitro* translation. Biotin *in vitro* translation kit (Boehringer Mannheim) translated added mRNA in reticulocyte lysate into biotin-labeled proteins using a charged lysine-tRNA, labeled with biotin at the ε-amino group of lysine. The templates were mixed with 15 μl of Biotin translation mix and incubated at 30°C for 1 h. 5 μl of the products were loaded onto SDS–PAGE for detection and the proteins were transferred onto nitrocellulose membrane according to standard protocol. After blocking in 10 ml of maleic acid based blocking solution for 40 min at room temperature, the membrane was incubated in 10 ml of diluted streptavidin-POD solution for 30 min and washed with TBST for four times of 10 min each. Chemiluminescence substrate was added onto the protein-side of membrane and then blot was exposed to X-ray film for 10 min.

The products of LLIR and LLIRv2 obtained from *in vitro* translation were confirmed by immunoblotting with antiserum.

Assessment of LLIR tyrosine phosphorylation and SHP-1 binding. HL-60 cells, which is SHP-1 positive, were incubated for 10 min at 37°C in 4×10^7 cells/ml of RPMI 1640 medium alone or with 0.03% H_2O_2/100 μM sodium orthovanadate, which inhibits phosphatase activity and thus increases protein tyrosine phosphorylation. 2×10^7 HL-60 cells were then lysed at 4°C in 1 ml of RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μg aprotinin, 1 μg leupeptins, 1 mM sodium orthovanadate) for 30 min, then 10 μl of 100 mM PMSF was added and incubated for another 30 min. Cell debris were removed by centrifugation at 10,000*g* at 4°C for 10 min. The supernatants were collected as whole cell lysate. The cell lysates were precleared for 30 min at 4°C on normal rabbit serum and protein A agarose. The beads were removed by centrifugation at 1000*g* at 4°C for 5 min. The supernatants were then incubated with antiserum for 1 h at 4°C with vigorous agitation and protein A agarose were added for further binding of 4 h. Precipitates were washed 4 times with RIPA buffer, resolved by 10% SDS–PAGE, and transferred onto nitrocellulose membranes. After blocking with TBST with 5% nonfat dry milk, the membranes were incubated with anti-SHP-1 monoclonal antibody (Santa Cruz) followed by HRP-conjugated goat anti-mouse IgG. Immunoblotted proteins were visualized by chemiluminescence as described above.

Expression of LLIR and its variants in PMA-induced, differentiated leukemia cells. Promyelocytic cells, NB4 and HL-60, were used as cell model to investigate the roles of LLIR and its variants in the leukemia cells differentiation. NB4 and HL-60 cells were induced to differentiate by phorbol myristic acid (PMA, Sigma) at the concentration of 50 ng/ml for 6, 12, 24, 48 and 72 h, respectively. Expression analysis of LLIR and its variants was performed by RT-PCR and immunoblotting as described above.

RESULTS AND DISCUSSION

Identification of a Full-Length cDNA from Human Dendritic Cells That Encodes a Type II Lectin-like Immunoreceptor

One of the nucleotide fragments cloned by subtractive cloning, GC13, has a length of 1067 bp carrying an open reading frame ranging from nt 31 to 744. Because there is no stop codon in upstream region in the same reading frame, 5′ RACE was performed to amplify 5′ flanking sequence, and a 240 bp length fragment was obtained and revealed to be upstream sequence of GC13. The full length sequence is 1305 bp in length (Accession No. AF067800) and carries an open reading frame from nt 269 to 982 as shown in Fig. 1. According to consensus sequences analysis by software and server, the deduced protein carries a single CRD at the COOH terminal and belongs to type II transmembrane protein because it contains an integral transmembrane domain but without signal polypeptide. A growing group of C-type lectin molecules expressed on DC have been identified. MMR and DEC-205 are two type I lectin molecules

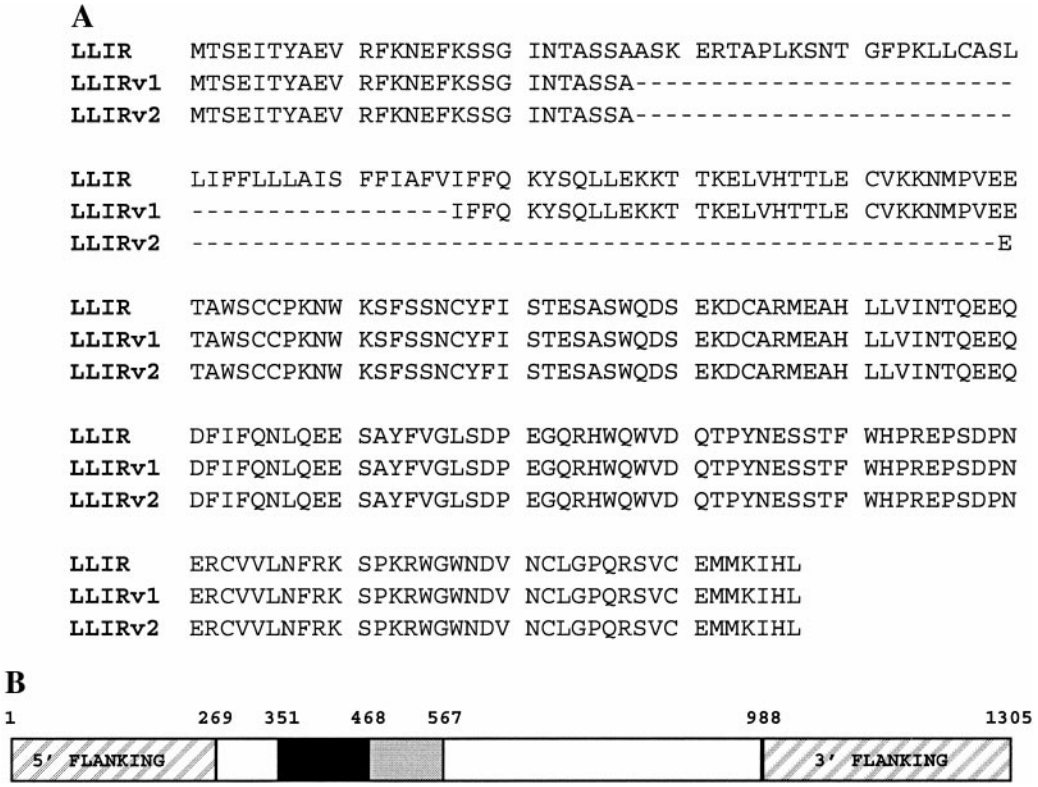

FIG. 3. Protein sequences and sketch map of LLIR and its two alternatively spliced variants (LLIRv1 and LLIRv2). (A) The comparison of three protein sequences. (B) The top numbers indicate the nt position; hatched bars represent noncoding region. In the coding region of LLIRv1, only black region is absent; and in that of LLIRv2, both of the black and dark gray regions are absent.

and expressed on DC in the case of antigen uptake and presentation. Recently, some type II lectin-like molecules were discovered, which include CLEC-1, CLEC-2, Dectin-1, Dectin-2 (33–36). Both of these molecules contain a single CRD in extracellular domain and are restrictedly expressed in DC and other myelocytic cells. There is a consensus N-glycosylation site at residue 185 and another 6 amino acid which has been predicted to be intracellular and matches ITIM (6, 37, 38). This novel gene was designated as lectin-like immunoreceptor (LLIR). Most of the alignments searched by BLAST algorithm belong to ASGPRs and the similarity always locates in extracellular part. No alignment has been found, that contains homologous intracellular sequence. LLIR is considered to be highly homologous to the group of macrophage/hepatic lectins in extracellular structure, and this result indicates the extracellular part of LLIR may recognize carbohydrate chains of antigens or ligands.

A corresponding chromosomal sequence (AC006511) of LLIR has been found in GenBank database, however uncompleted. It has been indicated that the chromosomal localization of LLIR is 12p13. Such a chromosomal localization is very close to that of NK cell receptors cluster 12p12-p13 (39, 40), in which genes of some type II lectin-like NK cell receptors locate.

LLIR Was Expressed with High Abundance in Human Dendritic Cells, Myeloid Cells, and B Cells According to RT-PCR Analysis

According to the RT-PCR amplification results shown in Fig. 2, the expression level of LLIR in KLH stimulated DC was higher than that in normal DC. In hematopoietic cell lines, mRNA of LLIR expressed with relatively high levels in promyelocytic cell line (NB4), myeloid cell line (HL-60), B lymphocytes lines (Raji, Daudi, Ramos), but with relatively low levels in chronic myelogenous leukemia cell line K-562, T lymphocyte line Jurkat. LLIR was not expressed in T lymphocyte line (HuT78) and histiocytic lymphoma cell line (U937). There were detectable mRNA expressions in fetal heart, brain, liver, spleen and small intestine, but not in fetal kidney, lung and skeleton muscle. No signals of LLIR could be detected by Northern blot analysis in human tissues and but because the signals of β-Actin was adequate, the results suggested LLIR might be in low abundance in normal tissues (data not shown). Our data suggest that LLIR be expressed in

immature DC, promyelocytic cells and B cells with high abundance and in normal tissues with low abundance.

Identification of Two Alternatively Spliced Transmembraneless Variants of LLIR

The PCR of HL-60 cells also gave two other shorter products. Sequencing of the two fragments revealed that they are two alternatively spliced variants of LLIR. The two variants were designated as LLIRv1 and LLIRv2. Comparison of the three transcripts with the uncompleted chromosomal sequence showed that the absent sequence in LLIRv1 represents one integral exon. The deletion part in LLIRv2 may be composed of two or three exons. As shown in Fig. 3, in the coding region of LLIRv1, nucleotide from 351 to 468 is absent, and in LLIRv2, nucleotide from 351 to 567 is absent. Because the transmembrane region is from nt 401 to 475, both of the two variants have no transmembrane region. In addition, the CRD is intact in both of the two variants.

LLIR Protein Was Expressed in Dendritic Cells, Myeloid Cells, and B Cells

To investigate the biological characteristics of LLIR, we need the purified LLIR protein and antibody against LLIR, so LLIR was expressed first; however, the expression level of LLIR was too low to satisfy further study, although either direct prokaryotic expression system or GST fusion expression system was used. The reason for low expression level of LLIR may be the existence of transmembrane domain and the effect of hydrophobicity on the translation of protein in bacteria. The variant of LLIR, LLIRv2, in which the transmembrane region is absent, showed much higher expression level in both direct prokaryotic expression system and GST fusion expression system (data not shown). The amount of LLIRv2 produced by direct prokaryotic expression could reach 40% of whole cell lysate. After cell disrupted, the expressed LLIRv2 was mainly stayed in inclusion bodies. The purification of LLIRv2 was performed by dilution, dialysis and ion-exchange chromatography. The purity of LLIRv2 protein was evaluated to be about 90% by silver staining. Immunization of rabbits with LLIRv2 protein was successful. The purified polyclonal antiserum could react with 0.5 μg of LLIRv2 protein at the titer of 1:4000 and not cross-react with bacterial protein. Because LLIRv2 is the shortest one among the three transcripts, the antiserum against LLIRv2 can be used to detect all three proteins. According to the results of PCR analysis, LLIR is the dominant transcript among the three transcripts in HL-60 cells, and be transcribed with different levels in cells and tissues. But, how about the distribution of the corresponding proteins? Are they translated in the same way as transcription or not? And are they glycosylated as the conserved Asn indicated? Cell lines including HL-60, NB4, U937, Jurkat, Raji and Reh have been selected for Western blot analysis. As shown in Fig. 4A, specific binding of anti-LLIRv2 polyclonal antiserum was observed in the lysates of HL-60, NB4, Raji, Reh, but not in U-937 and Jurkat. This result has some discrepancy with the observation in RT-PCR analysis. From RT-PCR analysis, Jurkat cells expressed LLIR in relatively low abundance. But no specific binding could be observed in Jurkat cells by immunoblotting analysis. This result is

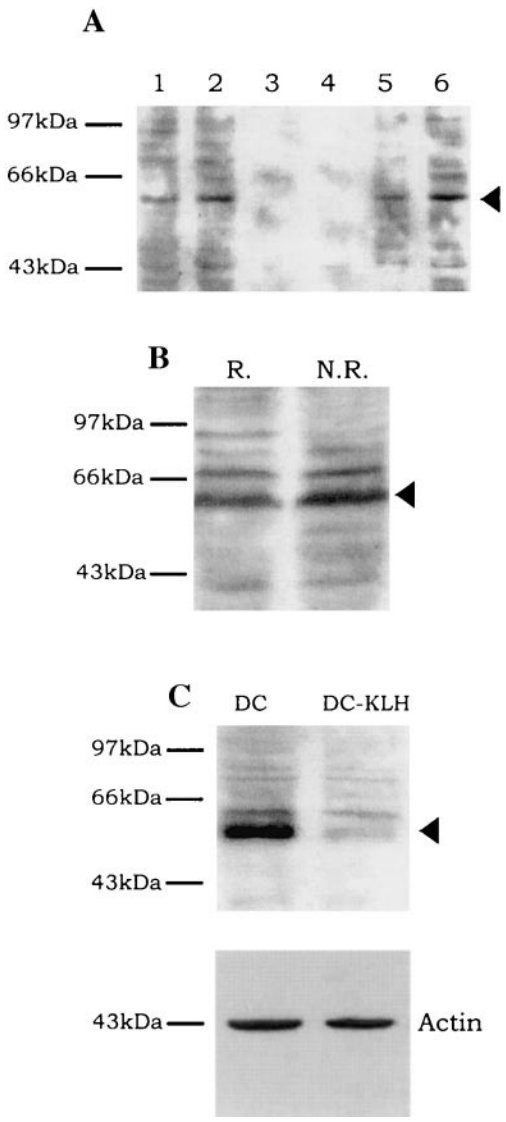

FIG. 4. Immunoblotting analysis of LLIR in human leukemia cell lines and DC. (A) The expression of LLIR in several hematopoietic cell lines. Lane 1, HL-60, lane2, NB4, lane 3, U937, lane 4, Jurkat, lane 5, Raji, lane 6, Reh. (B) The whole cell lysates of Raji cells were prepared under reducing or nonreducing conditions. (C) The whole cell lysates from DC and DC treated with KLH were probed; the concentration of each sample was determined with BCA protein assay reagents and controlled by actin detection.

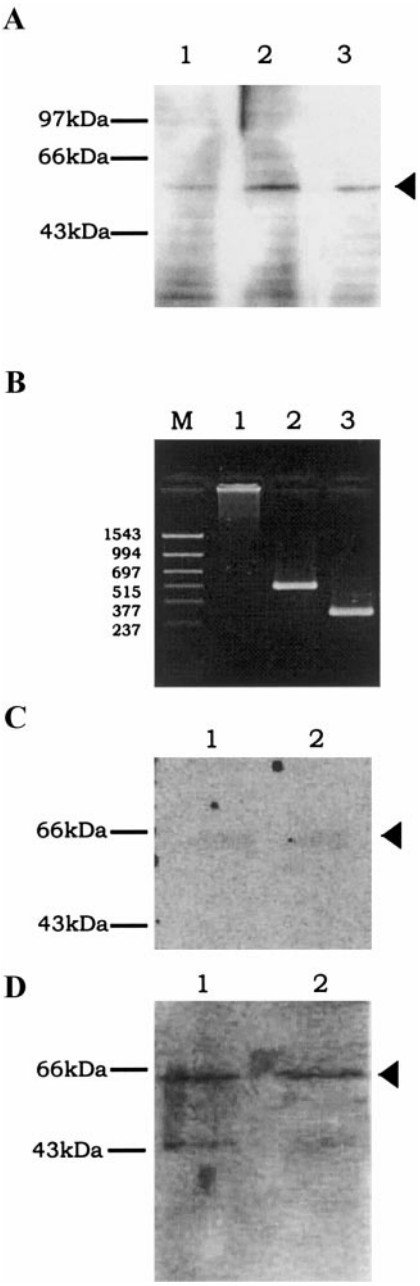

FIG. 5. Determination of LLLR and LLIRv2 by posttransfection expression and *in vitro* translation. (A and B) The detection of LLIR and LLIRv2 in transfected cells by immunoblotting and RT-PCR. In A and B: lane 1, NIH/3T3; lane 2, NIH/3T3-LLIR; lane 3, NIH/3T3-LLIRv2. (C) Streptavidin detection of translation products. (D) Immunoblotting analysis of translation products. In C and D: lane 1, product translated from LLIR gene; lane 2, product translated from LLIRv2 gene.

accordant with the implication from RT-PCR analysis, in that LLIR is likely to be absent in T lymphocytes. The specific binding band revealed the molecular weight of LLIR protein was about 60kDa, and the comparison of cell lysates under reducing condition or nonreducing condition indicated LLIR was a monomer (Fig. 4B). The expression of LLIR in DC and KLH stimulated DC was also detected by immunoblotting and the result certified that the expression of LLIR would decreased sharply upon KLH stimulation, in agreement with the result from RT-PCR analysis (Fig. 4C).

The Mature LLIR Protein and Mature LLIRv2 Protein Exhibit Equal Size

There was one specific binding band that was supposed to represent LLIR in HL-60 cells, so the question along with is where are the corresponding proteins of the two splice variants. To explore this problem, LLIR and LLIRv2 gene were subcloned into an eukaryotic expression vector and transfected into mouse fibroblast NIH/3T3 cells. After a period of positive selection, the cells were harvested for immunoblotting detection. The results were explainable but not conclusive because of the cross-reactivity of NIH/3T3 (Fig. 5A). The cross-reactivity may be due to the mouse corespondent of LLIR or members in the same or close family because it is not expressed at the level of transcription (Fig. 5B). Finally, *in vitro* translation has been employed and the products were probed with streptavidin and antiserum either (Figs. 5C and 5D). These results, together with that obtained from NIH/3T3 transfection, confirmed that LLIR and LLIRv2 displayed equal size on one-dimensional SDS–PAGE electrophoresis.

The ITIM in LLIR Could Recruit SHP-1 upon Pervanadate Treatment

HL-60 cells have been shown to express LLIR. To verify the association between LLIR and SHP-1, LLIR was immunoprecipitated with polyclonal antiserum from untreated or pervanadate-treated HL-60 cells. The immunoprecipitated proteins were subjected to anti-SHP-1 immunoblotting (Fig. 6) and suggested the

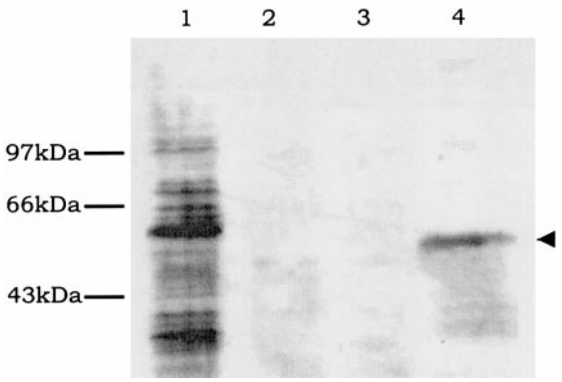

FIG. 6. Recruitment of SHP-1 in HL-60 cells upon pervanadate treatment. Lane 1, whole cell lysate of HL-60; lanes 2–4, immunoprecipitates from cell lysates with polyclonal antiserum against LLIR; lane 2, HL-60 cells; lane 3, U937 cells treated with pervanadate; lane 4, HL-60 cells treated with pervanadate.

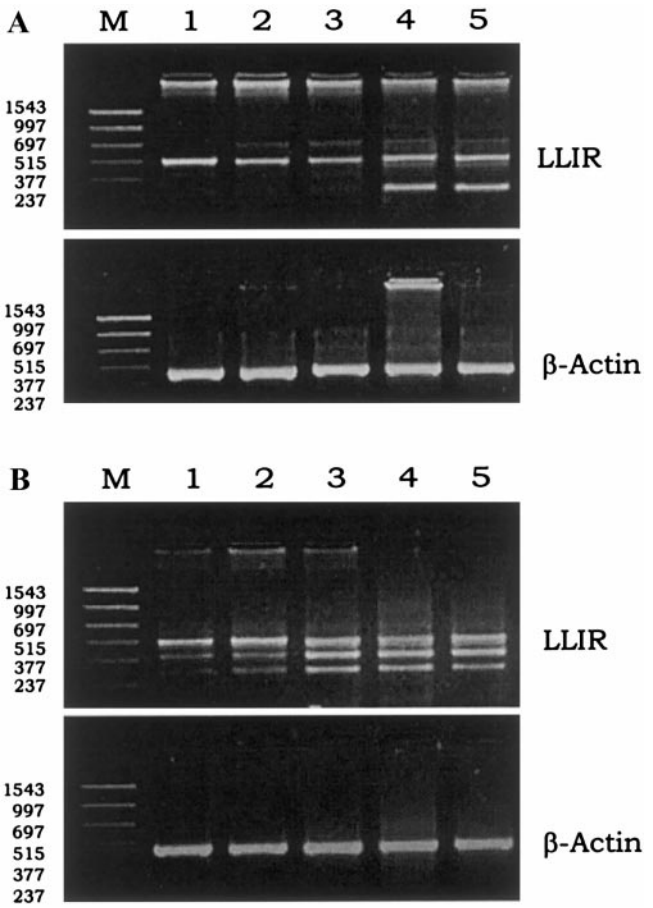

FIG. 7. Expression analysis of LLIR and its variants in differentiation of leukemia cells by RT-PCR. (A) The results of RT-PCR analysis of NB4 cells; lane 1, NB4 cells, no treatment; lanes 2–5, NB4 cells treated with PMA for 6, 12, 24, and 48 h, respectively. Chart B represents the results of RT-PCR analysis of HL-60 cells; lane 1, HL-60 cells, no treatment; lanes 2–5, HL-60 cells treated with PMA for 6, 24, 48, and 72 h, respectively.

ITIM in LLIR had the potential to recruit SHP-1 or relative phosphotyrosine phosphatase.

Expression of LLIRv2 Could Be Upregulated during PMA-Induced Leukemia Cell Differentiation

HL-60, NB4 cells were induced to differentiate by PMA at 50 ng/ml. Flow cytometry revealed HL-60 cells expressed high level of CD14, CD40, CD54 and CD86 after treatment with PMA for 24 h (data not shown). Equal amounts of cells were collected at 6, 12, 24, 48 and 72 h for immunoblotting and RT-PCR analysis. It was proposed to observe whether the expression level of LLIR would be changed during differentiation. No significant changes were observed from immunoblotting analysis (data not shown), but the results may be due to the equal size of LLIR protein and LLIRv2 protein displayed in electrophoresis. The results of RT-PCR analysis were shown in Fig. 7. The expression of LLIR was declined, but the expression of its two transmembrane-less variants, especially the LLIRv2, was improved 24 h later after PMA treatment. This data indicated that LLIR and its variants might be associated with differentiation of leukemia cells. Intracellular SHP-1 in HL-60 cells would increase upon PMA stimulation, and the increased PTPs seemed to inhibit PMA-induced differentiation (41, 42). Because LLIRv2 contains an ITIM motif, it may associate with the increase of SHP-1 in PMA-induced myelocytic cell differentiation. On the other hand, HL-60 cell would differentiate into macrophage upon PMA stimulation (43, 44), suggesting LLIRv1 and LLIRv2 are associated with functions of macrophage, such as the ability of migrating, antigen capturing and processing. Combined with the result of distribution analysis that LLIR was expressed with high abundant in DC, myeloid cells and B cells, we postulate that LLIR and its two variants may play roles in functions of APC, especially migrating, antigen capturing and processing.

REFERENCES

1. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
2. Dhodapkar, M. V., Krasovsky, J., Steinman, R. M., and Bhardwaj, N. (2000) Mature dendritic cells boost functionally superior CD8+ T-cell in humans without foreign helper epitopes. *J. Clin. Invest.* **105,** R9–R14.
3. Dhodapkar, M. V., Steinman, R. M., Sapp, M., Desai, H., Fossella, C., Krasovsky, J., Donahoe, S. M., Dunbar, P. R., Cerundolo, V., Nixon, D. F., and Bhardwaj, N. (1999) Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J. Clin. Invest.* **104,** 173–180.
4. Zhao, Z., Huang, X., Li, N., Zhu, X., Chen, S., and Cao, X. (1999) Direct cloning of cell differential expression genes with full-length by a new strategy based on the multiple rounds of "long distance" polymerase chain reaction and magnetic beads mediated subtraction. *J. Biotech.* **73,** 35–41.
5. Drickamer, K., and Taylor, M. E. (1993) Biology of animal lectins. *Annu. Rev. Cell. Biol.* **9,** 237–264.
6. Long, E. O. (1999) Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol.* **17,** 875–904.
7. Spiess, M., and Lodish, H. F. (1985) Sequence of a second human asialoglycoprotein receptor: Conservation of two receptor genes during evolution. *Proc. Natl. Acad. Sci. USA* **82,** 6465–6469.
8. Spiess, M., Schwartz, A. L., and Lodish, H. F. (1985) Sequence of human asialoglycoprotein receptor cDNA: an internal signal sequence for membrane insertion. *J. Biol. Chem.* **260,** 1979–1982.
9. Spiess, M. (1990) The asialoglycoprotein receptor: A model for endocytic transport receptors. *Biochemistry.* **29,** 10009–10018.
10. Gordon, J., Flores-Romo, L., Cairns, J. A., Millsum, M. J., Lane, P. J., Johnson, G. D., and MacLennan, I. C. (1989) CD23: A multi-functional receptor/lymphokine? *Immunol. Today* **10,** 153–157.
11. Bettler, B., Maier, R., Ruegg, D., and Hofstetter, H. (1989) Binding site for IgE of the human lymphocyte low-affinity Fc epsilon receptor (Fc epsilon RII/CD23) is confined to the domain homologous with animal lectins. *Proc. Natl. Acad. Sci. USA* **86,** 7118–7122.
12. Flores-Romo, L., Johnson, G. D., Ghaderi, A. A., Stanworth, D. R., Veronesi, A., and Gordon, J. (1990) Functional implication

for the topographical relationship between MHC class II and the low-affinity IgE receptor: occupancy of CD23 prevents B lymphocytes from stimulating allogeneic mixed lymphocyte responses. *Eur. J. Immunol.* **20,** 2465–2469.

13. Hoyle, G. W., and Hill, R. L. (1988) Molecular cloning and sequencing of a cDNA for a carbohydrate binding receptor unique to rat Kupffer cells. *J. Biol. Chem.* **263,** 7487–7492.
14. Lazetic, S., Chang, C., Houchins, J. P., Lanier, L. L., and Phillips, J. H. (1996) Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol.* **157,** 4741–4745.
15. Lanier, L. L. (1998) NK cell receptors. *Annu. Rev. Immunol.* **16,** 359–393.
16. Hamann, J., Fiebig, H., and Strauss, M. (1993) Expression cloning of the early activation antigen CD69, a type II integral membrane protein with a C-type lectin domain. *J. Immunol.* **150,** 4920–4927.
17. Van de Velde, H., von Hoegen, I., Luo, W., Parnes, J. R., and Thielemans, K. (1991) The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature* **351,** 662–665.
18. Von Hoegen, I., Hsieh, C. L., Scharting, R., Francke, U., and Parnes, J. R. (1991) Identity of human Lyb-2 and CD72 and localization of the gene to chromosome 9. *Eur. J. Immunol.* **21,** 1425–1431.
19. Drickamer, K. (1993) Ca2+-dependent carbohydrate-recognition domains in animal proteins. *Curr. Opin. Struct. Biol.* **3,** 393–400.
20. Arase, N., Arase, H., Park, S. Y., Ohno, H., Ra, C., and Saito, T. (1997) Association with FcRgamma is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1+ T cells. *J. Exp. Med.* **186,** 1957–1963.
21. Pospisil, M., Vannucci, L., Horvath, O., Fiserova, A., Krausova, K., Bezouska, K., and Mosca, F. (2000) Cancer immunomodulation by carbohydrate ligands for the lymphocyte receptor NKR-P1. *Int. J. Oncol.* **16,** 267–276.
22. Zingoni, A., Palmieri, G., Morrone, S., Carretero, M., Lopez-Botel, M., Piccoli, M., Frati, L., and Santoni, A. (2000) CD69-triggered ERK activation and functions are negatively regulated by CD94/NKG2-A inhibitory receptor. *Eur. J. Immunol.* **30,** 644–651.
23. Houchins, J. P., Lanier, L. L., Niemi, E. C., Phillips, J. H., and Ryan, J. C. (1997) Natural killer cell cytolytic activity is inhibited by NKG2-A and activated by NKG2-C. *J. Immunol.* **158,** 3603–3609.
24. Daeron, M., Latour, S., Malbec, O., Espinosa, E., Pina, P., Pasmans, S., and Fridman, W. H. (1995) The same tyrosine-based inhibition motif, in the intracytoplasmic domain of FcγRIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity.* **3,** 635–646.
25. Coggeshall, K. M. (1998) Inhibitory signaling by B cell FcγRIIB. *Curr. Opin. Immunol.* **10,** 306–312.
26. Burshtyn, D. N., Scharenberg, A. M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T., Kinet, J. P., and Long, E. O. (1996) Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* **4,** 77–85.
27. Vivier, E., and Daeron, M. (1997) Immunoreceptor tyrosine-based inhibition motifs. *Immunol. Today* **18,** 286–291.
28. Cambier, J. C. (1997) Inhibitory receptors abound? *Proc. Natl. Acad. Sci. USA* **94,** 5993–5995.
29. Bolland, S., and Ravetch, J. V. (1999) Inhibitory pathways triggered by ITIM-containing receptors. *Adv. Immunol.* **72,** 149–177.
30. O'Shea, J. J., McVicar, D. W., Bailey, T. L., Burns, C., and Smyth, M. J. (1992) Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* **89,** 10306–10310.
31. Cao, X., Zhang W., Wan, T., He, L., Chen, T., Yuan, Z., Ma, S., Yu, Y., and Chen, G. (2000) Molecular cloning and characterization of a novel CXC chemokine macrophage inflammatory protein-2 gamma chemoattractant for human neutrophils and dendritic cells. *J. Immunol.* **165,** 2588–2595.
32. Zhao, Z., Huang, X., Chen, S., and Cao, X. (2000) Construction of an integrative plasmid pLCM182 for heterogeneous gene cloning, sequencing, expression and its application in cytokine expression. *Chin. J. Microbiol. Immunol.* **20,** 136–139.
33. Ariizumi, K., Shen, G. L., Shikano, S., Ritter, R., Zukas, P., Edelbaum, D., Morita, A., and Takashima, A. (2000) Cloning of a second dendritic cell-associated C-type lectin (Dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem.* **275,** 11957–11063.
34. Ariizumi, K., Shen, G. L., Shikano, S., Xu, S., Ritter, I., Kumamoto, T., Edelbaum, D., Morita, A., Bergstresser, P. R., and Takashima, A. (2000) Identification of a novel, dendritic cell-associated molecule, Dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* **275,** 20157–20167.
35. Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R. L., O'Callaghan, C. A., Dunbar, R., Ogg, G. S., Cerundolo, V., and Rolink, A. (1998) Human myelonomocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* **160,** 3096–3100.
36. Colonna, M., Samaridis, J., and Angman, L. (2000) Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. *Eur. J. Immunol.* **30,** 697–704.
37. Unkeless, J. C., and Jin, J. (1997) Inhibitory receptors, ITIM sequences and phosphatases. *Curr. Opin. Immunol.* **9,** 338–343.
38. Vely, F., and Viver, E. (1997) Conservation of structural features reveals the existence of a large family of inhibitory cell surface receptors and noninhibitory/activatory counterparts. *J. Immunol.* **159,** 2075–2077.
39. Renedo, M., Arce, I., Rodriguez, A., Carretero, M., Lanier, L. L., Lopez-Botet, M., and Fernandez-Ruiz, E. (1997) The human natural killer gene complex is located on chromosome 12p12–13. *Immunogenetics* **46,** 307–311.
40. Brown, M. G., Scalzo, A. A., Matsumoto, K., and Yokoyama, W. M. (1997) The natural killer gene complex: a genetic basis for understanding natural killer cell function and innate immunity. *Immunol. Rev.* **155,** 53–65.
41. Zhao, Z., Shen, S. H., and Fischer, E. H. (1994) Phorbol ester-induced expression, phosphorylation, and translocation of protein-tyrosine-phosphatase 1C in HL-60 cells. *Proc. Natl. Acad. Sci. USA* **91,** 5007–5011.
42. Li, R. Y., Gaits, F., Ragab, A., Ragab-Thomas, J. M., and Chap, H. (1995) Tyrosine phosphorylation of an SH2-containing protein tyrosine phosphatase is coupled to platelet thrombin receptor via a pertussis toxin-sensitive heterotrimeric G-protein. *EMBO J.* **14,** 2519–2526.
43. Koeffler, H. P., Bar-Eli, M., and Territo, M. C. (1981) Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation. *Cancer Res.* **41,** 919–926.
44. Territo, M. C., and Koeffler, H. P. (1981) Induction by phorbol esters of macrophage differentiation in human leukaemia cell lines does not require cell division. *Br. J. Haematol.* **47,** 479–483.