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Molecular cloning of a novel CC chemokine, interleukin-11 receptor α-locus chemokine (ILC), which is located on chromosome 9p13 and a potential homologue of a CC chemokine encoded by molluscum contagiosum virus

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Abstract Molluscum contagiosum virus (MCV) encodes a CC chemokine MC148R which is likely to have been acquired from the host. By a homology search employing MC148R as a probe, we have identified a novel CC chemokine whose gene exists next to the IL-11 receptor α (IL-11Rα) gene in both humans and mice. Thus, this chemokine maps to chromosome 9p13 in humans where IL-11Rα has been assigned. We term this novel chemokine IL-11Rα-locus chemokine (ILC). ILC has the highest homology to MC148R among the known human CC chemokines. Furthermore, ILC is strongly and selectively expressed in the skin where infection of MCV also takes place. Thus, ILC is likely to be the original chemokine of MC148R.

Key words: Chemokine; Molluscum contagiosum virus; Interleukin 11 receptor

1. Introduction

Chemokines are small (~8 kDa), structurally related, mostly basic, heparin binding chemotactic cytokines that play important roles in the inflammatory and immunological responses primarily by inducing emigration of leukocytes and lymphocytes [1–3]. In humans, over 40 members have been identified. Based on the arrangement of the first two of the four conserved cysteine residues, the chemokines are divided into two major subfamilies, the CXC and CC ones. Furthermore, the molecules now representing the C and CX3C subfamilies have been identified [2,3]. Leukocytes respond to chemokines through seven-transmembrane G protein-coupled receptors [4]. HIV and SIV exploit certain chemokine receptors as coreceptors essential for their entry into target cells after binding to CD4 [5]. Furthermore, certain viruses such as herpesviruses encode chemokines and/or chemokine receptors which are considered to have been acquired from the host and to facilitate their in vivo growth by modulating host immune responses [6].

Molluscum contagiosum virus (MCV) is a poxvirus which infects young children and sexually active adults to causes benign proliferative lesions of the skin (3–5-mm papules) that last for months to years before spontaneous regression. MCV infection typically elicits only a weak immune response and almost no inflammatory reaction around the hyperplastic, virus-filled epidermal lesions [7]. There are at least two types of MCV, MCV-1 and MCV-2, based on restriction endonuclease cleavage patterns of viral DNA [8]. Analysis of the complete genome sequence of MCV-1 has revealed a 104-amino acid open reading frame (MC148R) that is structurally related to the CC subfamily of chemokines [9]. The chemokine homologues from MCV-1 and MCV-2 were expressed and were found to have no chemotactic activity on monocytes but to block the response of monocytes to macrophage inflammatory protein (MIP)-1α [10]. Consistent with the absence of chemoattractant activity and the role as a cellular chemokine antagonist, the chemokine homologues of MCV have a deletion in the amino-terminal activation domain [11]. MC148R is considered to have been derived from a CC chemokine of the host but the host homologue of MC148R has not been identified. Here we report a novel CC chemokine, interleukin-11 receptor α-locus chemokine (ILC), which is a potential homologue of MC148R.

2. Materials and methods

2.1. Identification of mouse and human ILC

Using the amino acid sequence of the MCV-1 CC chemokine homologue MC148R1 [9] as a probe, we searched the public database of expressed sequence tags (EST) with the BLAST program and identified two murine ESTs potentially encoding a novel CC chemokine (W10954 from mouse embryo day 19.5; AA024054 from mouse placenta). These clones were obtained and sequenced. The determined cDNA sequence encoding a novel mouse CC chemokine (mILC) has been deposited in the DDBJ/GenBank/EBI Data Bank (accession number AB013398). Using the mouse cDNA as a probe, we next identified the mouse genomic clone containing this novel chemokine gene (GenBank accession number X94161). By analyzing the genomic sequence, we found that the chemokine gene is located in the very

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close vicinity of the IL-11 receptor α (IL-11Rα) gene in a tail-to-tail orientation. Taking advantage of this information, we first identified the human genomic sequence containing the IL-11Rα gene (GenBank accession number U32323) and analyzed the adjacent sequence with the gene-predicting program GenScan. We identified exons 2 and 3 of a CC chemokine-like gene located close to the human IL-11Rα gene. We then determined the full-length cDNA sequence by the method of rapid amplification of cDNA ends (RACE) [12]. In brief, two-step polymerase chain reaction (PCR) was carried out using human thymus Marathon-Ready cDNA (Clontech) as template. The first step PCR was carried out with the first gene-specific primers (5′-RACE primer, 5′-TGGGCAGAGTCCCATGGAGCTTTCT-3′; 3′-RACE primer, 5′-TTCCTACTGCCACCCAGCACTGCCT-3′) and AP1 primer which is complementary to the adapter ligated at both ends of cDNA. Then, the second step PCR was carried out using the second gene-specific primers (5′-RACE primer, 5′-CACTGTGACAGGC-TGGGGTTCTGGG-3′; 3′-RACE primer, 5′-CCAGGTGGAACTG-CAGGAGGCTGAC-3′) and AP2 primer which is also complementary to the adapter ligated at both ends of cDNA. The amplification products were cloned into the pGEM-T Easy vector (Promega) by T-A ligation and sequenced on both strands. The full-length cDNA sequence of human ILC has been deposited in the DDBJ/GenBank/EBI Data Bank (accession number AB010445).

2.2. Expression analysis

Total RNA samples were prepared from various tissues of normal BALB/c mice and normal human skin tissues by using Trizol reagent (Gibco-BRL). RNA samples were further purified by using RNeasy (Qiagen). Human neonatal epidermal keratinocytes were purchased from Clonetics (Iwaki Glass, Japan). Total RNA samples were prepared from keratinocytes that were treated without or with lipopolysaccharide from *Escherichia coli* (Sigma), IL-1α, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-4 (all purchased from PeproTech), singly or in combination, for 24 h. Multi-tissue Northern blot filters were purchased from Clontech. Northern blot analysis was carried out as described previously [13]. RT-PCR was carried out as follows. Total RNA samples (290 ng each) were reverse transcribed by using 6 pmol of oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Gibco-BRL). The resulting first-strand DNA (15 ng of total RNA equivalent) and original total RNA (15 ng) were subjected to PCR amplification in a final volume of 20 μl containing 10 pmol of each primer and 1 unit of Ex-Taq polymerase (Takara, Japan). The primers used were: +5′-CCATTTTCCTTAGCATCCCA-3′ and −5′-GGACCTTTCTCTACCCAGGC-3′ for human ILC and +GCC-AAGGTCATCCATGACAACTTTGG-3′ and −5′-GCCTGCTTC-zACCACCTTCTTGATGTC-3′ for glyceraldehyde 3-phosphate dehydrogenase (G3PDH). Amplification conditions were 94°C for 5 min, 32 cycles (for ILC) or 27 cycles (for G3PDH) of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and finally 72°C for 5 min. Amplification products (6 μl each) were separated by electrophoresis on 2% agarose and stained with ethidium bromide.

2.3. Production of recombinant human ILC

Recombinant human ILC was produced as a fusion protein with the Flag tag as described previously [14]. In brief, the open reading frame of human ILC fused with the coding sequence of the Flag tag was subcloned into pDREF-Hyg [15] to generate pDREF-ILC-Flag (see Fig. 4A). 293/EBNA-1 cells (Invitrogen) were transfected with pDREF-ILC-Flag using Lipofectamine (Gibco-BRL). After 3–4 days of culture, ILC-Flag secreted in the culture supernatant was purified by using anti-FLAG M2 affinity gel (Kodak) and reverse phase high performance liquid chromatography (HPLC) essentially as described previously [14]. For immunoblot analysis, samples were electrophoresed on SDS-PAGE (15–25% gradient) and electrophoretically transferred onto a filter. The filter was incubated with mouse monoclonal anti-Flag and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG in succession. The HRP enzymatic activity was detected by chemiluminescence using ECL kit (Amersham).

2.4. Functional assays

L1.2 murine pre-B cells stably expressing CCR1, CCR2b, CCR3, CCR4, CCR5, CCR6, CCR7, CCR10, XCR1, and CX3CR1 were described previously [16]. Chemotaxis and calcium flux assays were carried out as described previously [14].

3. Results

3.1. Identification of a mouse novel CC chemokine

MCV, which is a human poxvirus causing benign proliferative lesions of the skin [7], encodes a 104-amino acid open reading frame (MC148R) that is structurally related to the CC subfamily of chemokines [9–11]. It is probable that MCV acquired the gene from the human host and extensively remodeled it during the viral evolution to employ it to evade host immune responses. Therefore, we wished to identify its mammalian homologue and to understand the structural and functional relationship between the original host chemokine and its MCV version. Using the amino acid sequence of MC148R1 [9] as a probe, we searched the public EST database for homologous sequences. We found mouse ESTs, W10954 (from mouse embryo day 19.5) and AA024054 (from mouse placenta) that potentially encoded a novel CC chemokine. These clones were obtained and sequenced. The cDNA sequence thus determined contains an open reading frame that has an in-frame stop codon in the upstream and encodes a polypeptide of 120 amino acids (indicated as mILC in Fig. 1B). The amino-terminal region is highly hydrophobic and likely to be a signal peptide with a potential cleavage site between Ala-25 and Leu-26. The putative mature protein, which is a CC chemokine with a distant homology to other murine CC chemokines, consists of 95 amino acids with a calculated molecular weight of 10 937 and p*I* of 11.94. We searched the GenBank using the cDNA as a probe and found that the gene was located just in the downstream of the mouse IL-11Rα gene [17] in a tail-to-tail orientation. We call this novel chemokine murine ILC (mILC) for the reason described below.

3.2. Tissue expression of mILC

By Northern blot hybridization, we examined the expression of mILC in various organs of normal mice. As shown in Fig. 2A, a strong signal of about 1 kb was observed only in the skin. We also observed weak signals in the brain and testis using a commercial multiple tissue blot filter (data not shown). These tissues also contained transcripts of larger sizes that might be due to alternative splicing or differential polyadenylation signals. Thus, mILC appeared to be constitutively and selectively expressed in the normal skin at high levels.

3.3. Isolation of the human cDNA

There were no human EST sequences homologous to mILC. Since the mouse gene was found to be located very close to the IL-11Rα gene with a space of only a few nucleotides, we speculated that this might be also true for the human counterpart. Therefore, we searched a chemokine gene in the human genomic sequence (GenBank, U32323) that contained the IL-11Rα gene [18,19]. We indeed found exons potentially encoding a novel CC chemokine in a tail-to-tail arrangement with the IL-11Rα gene with a space only a few nucleotides (Fig. 3). Based on the exon sequences, we generated specific primers and carried out 5′- and 3′-RACE [12] using a human thymus cDNA library. As shown in Fig. 1A, the full-length cDNA thus determined contains an open reading frame of 112 amino acids. The amino-terminal region is estimated to be a signal peptide with the probable cleavage site between Ala-24 and Phe-25. The mature protein is a CC chemokine with 88 amino acids with a calculated molecular weight of 10 150 and

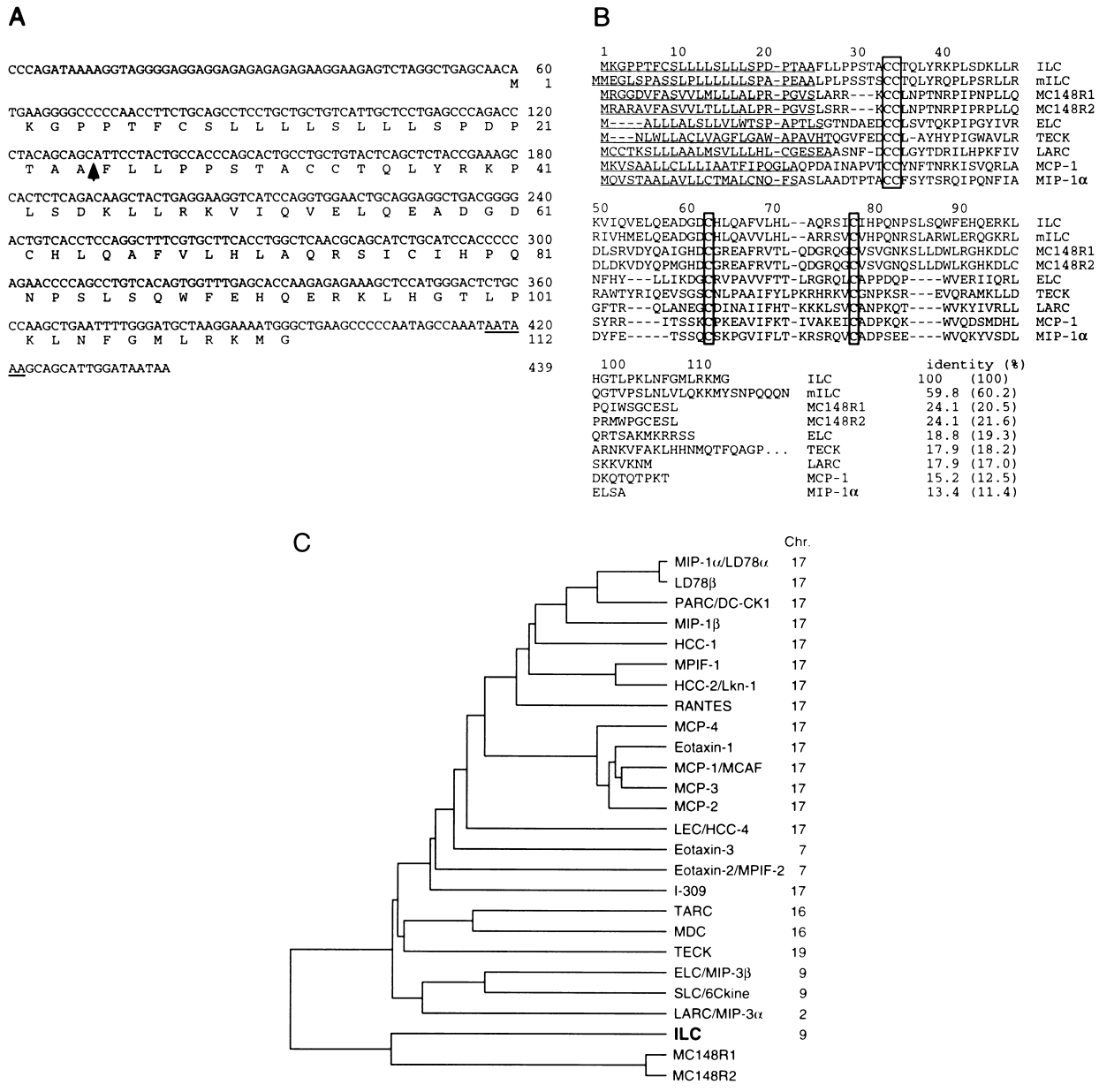

Fig. 1. A: The nucleotide and deduced amino acid sequences of human ILC. The signal peptide cleavage site is indicated by an arrow. The polyadenylation signal is underlined. B: Amino acid alignment. The signal peptide sequences are underlined. Four conserved cysteine residues are boxed. The % amino acid identity between human ILC and each indicated chemokine is shown in the right. Percent identity between mature proteins is shown in parentheses. MC148R1 and MC148R2 are the CC chemokine homologues encoded by MCV subtypes 1 and 2, respectively [10,11]. ELC, EBI1-ligand chemokine; TECK, thymus-expressed chemokine; LARC, liver and activation-regulated chemokine; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α. C: Phylogenetic tree of the human CC chemokines with MCV chemokines (MC148R1 and MC148R2). Evolutionary distance was estimated using the prrp and phylp programs [21]. The chromosome on which each chemokine has been mapped is indicated on the right.

p*I* of 10.33. Since both human and mouse genes for this novel chemokine are located in close vicinity to the IL-11Rα gene, we have termed this novel chemokine interleukin-11 receptor α-locus chemokine (ILC) (mILC for the murine counterpart). Its systemic nomenclature and gene symbol are CCL27 (mCCL27 for the murine counterpart) and *SCYA27*, respectively. The human IL-11Rα gene has been mapped to chromosome 9p13 [18,19].

3.4. Amino acid alignment

The human and mouse ILC amino acid sequences are aligned with other chemokines in Fig. 1B. The human and mouse proteins show the highest homology among the known CC chemokines with 60.2% identity (mature proteins). The amino acid sequence of human ILC is only remotely related to other human CC chemokines with identities below 20%. In fact, the human ILC sequence has the highest homology to MC148R1 and MC148R2 from MCV subtypes 1 and 2, respectively [10,11]. Notably, these four chemokines (ILC, mILC, MC148R1 and MC148R2) also share two extra regions consisting of three amino acids (V/M-E/D-L/Y) and four amino acids (S-L-S/A/L-Q/R/D) that are not seen in other chemokines. The phylogenetic tree of the human CC chemokines with MC148R1 and MC148R2 also supports that

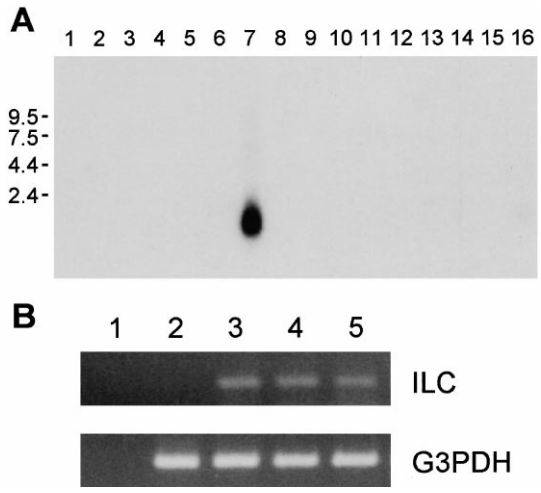

Fig. 2. Expression analysis. A: Northern blot analysis of mILC in normal mouse tissues. 1, uterus; 2, ovary; 3, bone marrow; 4, lymph node; 5, thymus; 6, spleen; 7, skin; 8, skeletal muscle; 9, colon; 10, small intestine; 11, stomach; 12, kidney; 13, liver; 14, lung; 15, heart; 16, brain. The size markers are shown on the left. B: RT-PCR analysis of human ILC expression in the skin. 1, no templates; 2, PHA-activated PBMC; 3–5, normal human skin tissues.

ILC and MC148R were diverged from a common ancestor (Fig. 1C).

3.5. Tissue expression of ILC

By Northern blot hybridization using a commercially available filter (Clontech), the expression of ILC in various human organs was examined. A weak signal of about 0.8 kb was detected in the thymus and placenta (data not shown). We also examined the expression of ILC by PCR using multiple tissue cDNA panels (Clontech). ILC expression was detected in thymus, placenta, testis and ovary (data not shown). Since these commercial samples did not contain skin samples, we separately examined the expression of ILC in normal human skin tissues by RT-PCR. As shown in Fig. 2B, ILC transcripts were consistently detected in normal human skin tissues. However, human neonatal epidermal keratinocytes cultured and treated without or with LPS, IL-1α, IFN-γ, TNF-α and IL-4 alone or in combination for 24 h did not express ILC at levels detectable by RT-PCR (data not shown).

3.6. Production of recombinant ILC and functional analysis

293/EBNA-1 cells were transfected with an expression vector for human ILC fused with a Flag tag [14] and culture supernatants were analyzed by immunoblot using anti-Flag. As shown in Fig. 4B, ILC-Flag was secreted in the culture supernatant. We therefore purified ILC-Flag from the culture supernatant by anti-Flag affinity gel and HPLC. ILC-Flag was eluted as a single peak from HPLC and migrated as a single band of 12 kDa on SDS-PAGE (not shown). The amino acid sequencing analysis revealed that the amino-terminus of the mature secreted protein was ^{25}F-L-L-P-P-S as expected.

We examined the biological activity of recombinant ILC-Flag using murine L1.2 cells stably transfected with CCR1, CCR2b, CCR3, CCR4, CCR5, CCR6, CCR7, CCR10, XCR1, and CX3CR1 [16]. ILC-Flag failed to induce any chemotactic or calcium flux responses in these panel of chemokine receptor-expressing L1.2 cells even though the chemokines used as positive controls for the respective receptors induced vigorous responses (data not shown). Furthermore, ILC-Flag at a 10-fold excess did not show any inhibitory effect on the activities of the chemokines acting on these receptors in calcium flux assays. ILC-Flag also failed to induce any significant migration of peripheral blood mononuclear cells in a transendothelial migration assay [14].

4. Discussion

We have identified a novel CC chemokine ILC whose gene exists in close conjunction with the IL-11Rα gene in a tail-to-tail orientation with a space of a few nucleotides in both human and mouse (Fig. 3). Importantly, the human IL-11Rα gene has been mapped to chromosome 9p13 [18,19] where two CC chemokines, secondary lymphoid-tissue chemokine (SLC) and EBI1-ligand chemokine (ELC), are also assigned [3]. Indeed, we have evidence that these three CC chemokines exist within 200 kb. Thus, ILC, SLC and ELC exist in a minicluster of chemokine genes at chromosome 9p13. SLC and ELC share CCR7 and have overlapping biological functions [3]. This suggests that SLC and ELC have diverged from a common ancestor. ILC may have derived from the same ancestor but may have diverged further to act on a distinct receptor.

ILC is likely to be the homologue of the MCV encoded chemokine MC148R since (1) ILC has the highest homology

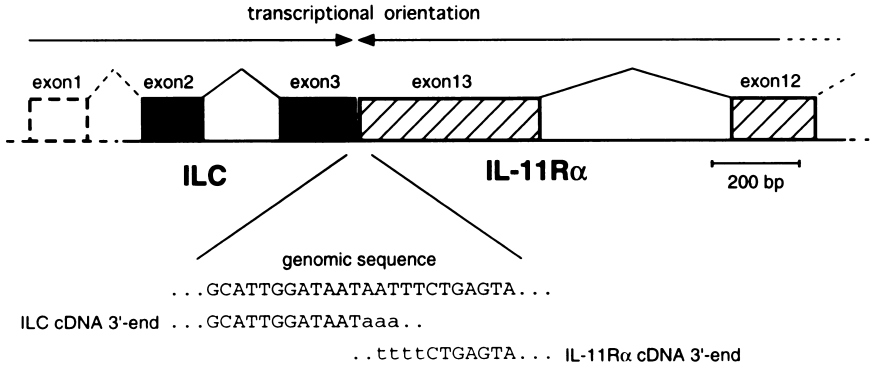

Fig. 3. The physical arrangement of the human ILC and IL-11 receptor α genes. The arrows indicate transcriptional orientation. The closed boxes indicate the exons of the ILC gene. The hatched boxes indicate the exons of the IL-11Rα gene. The genomic sequence, the 3′-end of ILC cDNA and the 3′-end of IL-11Rα cDNA are aligned in the bottom. The small letters, a or t, at the 3′ ends of the cDNAs indicate the poly(A) sequences.

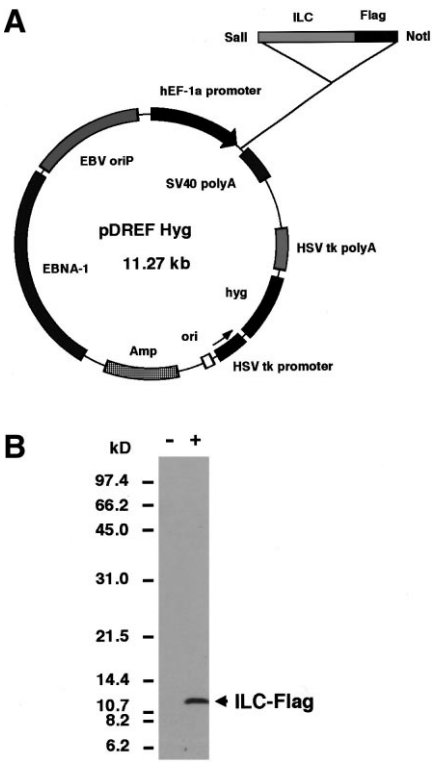

Fig. 4. Production of recombinant ILC. A: The physical map of the expression vector pDREF-ILC-Flag. The open reading frame encoding ILC fused to Flag tag [14] was inserted under the control of the EF-1α promoter as indicated. B: Detection of ILC-Flag by immunoblotting. Culture supernatants from 293/EBNA-1 cells transfected with pDREF-Hyg (−) or pDREF-ILC-Flag (+) were electrophoresed on SDS-polyacrylamide gel (15–25% gradient) and transferred to a filter. ILC-Flag was detected by immunoblotting. Size markers are shown on the left.

to MC148R, (2) ILC shares with MC148R two regions of a few extra amino acids not seen in other chemokines, (3) ILC and MC148R have the closest relationship in the phylogenetic tree, and (4) ILC is strongly expressed in the skin where infection and replication of MCV also take place. Thus, ILC may be applicable to antagonize the MCV chemokine for therapeutic purposes.

ILC failed to induce any responses in a panel of L1.2 cells expressing various cloned chemokine receptors. At present, therefore, we do not know the biological activity of ILC. Its strong and selective expression in the skin may indicate that some migratory cells unique to the skin are the targets of ILC. We did not detect expression of ILC in normal epidermal keratinocytes without or with stimulation by various cytokines. Thus, the tissue cells expressing ILC also remain to be identified.

During the final stage of this work, Hromas et al. [20] reported a novel murine CC chemokine termed ALP for its amino-terminal peptide sequence. ALP is identical to mILC. ALP was shown to be expressed in the heart, liver and testis. The skin was not examined. In transwell chemotaxis assays, ALP did not show any chemotactic activity for bone marrow granulocytes or monocytes, T cells from the thymus, or B cells or T cells from the spleen [20].

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