ACCELERATED PUBLICATION

Cloning of a new type II cytokine receptor activating signal transducer and activator of transcription (STAT)1, STAT2 and STAT3

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In the present paper, we report the identification of a new gene encoding a transmembrane protein of 520 amino acids, showing 22% amino acid identity with the extracellular domain of the interleukin (IL)-20 receptor. This gene, termed likely interleukin or cytokine receptor-2 (*LICR2*), is located on chromosome 1, at 25 kb from the *IL22R* (IL-22 receptor) gene, and is constitutively expressed in most tissues. A chimaeric receptor, consisting of the extracellular domain of the IL-10 receptorα chain and the intracellular domain of LICR2, activated signal transducer and activator of transcription (STAT)1, STAT2, STAT3 and STAT5 upon IL-10 stimulation, in a Janus kinase 1-dependent manner. In contrast, none of the IL-10-related cytokines described so far could activate LICR2-transfected cells, suggesting that LICR2 is a signalling receptor for a new cytokine of the IL-10 family.

Key words: cytokine receptor, Janus kinase (JAK), signal transduction, signal transducer and activator of transcription (STAT).

INTRODUCTION

Type II cytokine receptors include receptors for type I and II interferons (IFNs), and for interleukin (IL)-10-related cytokines [1]. These transmembrane proteins are almost exclusively related by their extracellular part, which consists of tandem fibronectin type III domains, whereas the cytoplasmic domain is associated with a tyrosine kinase of the Janus kinase (JAK) family. The type II cytokine receptor (R) family consists of 11 members, including two pairs of two receptor subunits for both type I (IFNα, IFNβ, IFNω and IFNτ) and type II (IFNγ) interferons, and IL-10Rα, IL-10Rβ, IL-20Rα, IL-20Rβ, IL-22R, IL-22 binding protein (IL-22BP) and tissue factor (TF) [1–4]. Four of the corresponding genes, *IFNAR1*, *IFNAR2*, *IL10R2* and *IFNGR2* are clustered on human chromosome 21; *IL22BP*, *IFNGR1* and *IL20R1* genes map to chromosome 6; *TF* and *IL22R* genes are located on chromosome 1, *IL20R2* is on chromosome 3 and *IL10R1* is on chromosome 11.

The IL-10-related cytokine family consists of six members, including IL-10, for which biological activities and receptor usage have been extensively described [5]. More recently, a series of new cytokines, structurally related to IL-10, have emerged. This family includes melanoma-differentiation-associated gene-7 ('mda-7' or IL-24), IL-19, IL-20, IL-10-related T-cell-derived inducible factor (IL-TIF or IL-22) and IL-26 (or AK155) [6–8]. Although none of these IL-10-related cytokines mimics IL-10 activities, they are likely to be involved in inflammatory processes as well. IL-24, IL-19 and IL-20 form a subfamily, based on conserved amino acid sequences, and on the use of shared receptor complexes [9,10]. Functional studies have stressed the potential tumour-suppressing activity of IL-24 [11], while IL-20 and IL-19 seem to be involved in skin abnormalities [2,12]. IL-22 binds to a complex formed by IL-22R and IL-10Rβ [3], and was shown to up-regulate acute-phase-reactant production by liver and pancreatic cells [13,14]. Finally, for IL-26 (AK155), originally described as encoded by a gene induced upon T-cell transformation by herpesvirus saimiri [15], functional data are still lacking to determine its biological activities and receptor usage.

In order to complete the picture of the cytokine receptor II family, we screened genomic DNA databases for similarity with the extracellular domains of type II cytokine receptors. In the present paper, we describe a new member of this family and show that the cytoplasmic domain of this new receptor is able to activate signal transducer and activator of transcription (STAT) factors and that this activation is JAK1-dependent.

EXPERIMENTAL

DNA sequence similarity searches

A search for similarity was performed with the protein sequence of IL-22BP using the TBLASTN software to screen the human genome databases. Regions presenting a putative similarity were analysed further by the NIX analysis program (http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/) developed by the U.K. Human Genome Mapping Project Resource Centre. A phylogenetic tree was generated by multiple alignment of the extracellular domains of the class II cytokine receptors using the ClustalX Multiple Sequence Alignment program [16] available from http://www-igbmc.u-strasbg.fr./BioInfo/ClustalX/Top.html

Abbreviations used: ERK, extracellular-signal-regulated kinase; FCS, foetal calf serum; IFN, interferon; IL, interleukin; IL-22BP, IL-22 binding protein; IL-10Rα (etc.), IL-10 receptor α (etc.); JAK, Janus kinase; LICR2, likely interleukin or cytokine receptor-2; MAPK, mitogen-activated protein kinase; ORF, open reading frame; RT, reverse transcriptase; STAT, signal transducer and activator of transcription; TF, tissue factor; TK, thymidine kinase.

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The nucleotide sequence data for the two genes encoding the two LICR2 isoforms have been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AJ534330 and AJ534331.

Cell cultures, transfections and cytokines

H4IIE rat hepatoma cells and HT-29 human intestinal epithelial cells were grown in Iscove–Dulbecco's medium supplemented with 10 % (v/v) foetal calf serum (FCS), 0.55 mM L-arginine, 0.24 mM L-asparagine and 1.25 mM L-glutamine. 2C4, U4C (generously provided by Dr Ian Kerr, Cancer Research UK, London Research Institute, London, U.K.) and γ2A (generously provided by Dr George Stark, Cleveland Clinic Research Institute, Cleveland, OH, U.S.A.) fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % FCS and with 400 μg/ml geneticin (Invitrogen). Recombinant human IL-22 and IL-26 were produced in *Escherichia coli* as described in [13,15]. Recombinant human IL-10 was purchased from Peprotech Inc. (Rocky Hill, NJ). Recombinant IL-19, IL-20 and IL-24 were produced by transient transfection of HEK-293 cells [9].

Likely interleukin or cytokine receptor-2 (LICR2) and chimaeric IL-10R–LICR2 expression constructs

Total RNA was isolated from various organs using the TRIzol® (Roche) method according to the manufacturer's recommendations. Reverse transcription was performed on 5 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 5 ng of total RNA was amplified for 33 cycles by PCR with specific primers for LICR2 as follows: sense 5′-GGGAACCAAGGAGCTGCTATG-3′ and antisense 5′-TGGCACTGAGGCAGTGGTGTT-3′. These primers are located on exons 3 and 5 of *LICR2* respectively. The annealing temperature was 56 °C. The PCR products were analysed in ethidium-bromide-stained 1 % (w/v) agarose gels. Alternatively, another PCR was performed with primers located on exons 5 and 7 of *LICR2* as follows: sense 5′-ACCTGCTTCTTGCTGGAGGTC-3′ and antisense 5′-AAGCTGACGCCATCTTCTGTG-3′. The full-length open reading frame (ORF) was amplified by reverse transcriptase (RT)-PCR in two steps. The 5′ region was amplified from colon oligo(dT)-primed cDNA using sense primer 5′-AAGGCCATGGCGGGGCCCGA-3′ and antisense primer 5′-CAGAAGGTCAGTGTCTGAAG-3′, located on exons 1 and 7 respectively, generating the expected fragment of 1424 nucleotides. The 3′ region of the ORF was amplified from a HepG2 cell line using oligonucleotide primers: sense 5′-ACCTGCTTCTTGCTGGAGGTC-3′ and antisense 5′-CATCAGATTCGGTGGGATGTC-3′, which amplified a fragment of 951 nucleotides from exons 5 to 7 (overlapping with the 5′ fragment). Both fragments were cloned and digested at the *Xho*I site located at position 916 from the start codon and present on both amplified fragments. The full-length ORF was next reconstituted by ligation of the two fragments into a pCEP4 plasmid.

The construct encoding a chimaeric protein with the extracellular part of IL-10R and the intracellular part of LICR2 was generated as follows. The extracellular part of mouse IL-10Rα was amplified using a mutated sense primer that introduced a *Nco*I site 5′-GCTCCATGGGACGATGCCGCTGTG-3′ and a mutated antisense primer with an *Ssp*I site 5′-GTGAAATATTGCTCCGTCGT-3′. Human LICR2 transmembrane and intracellular domains were amplified from the pCEP4–LICR2 construct using a mutated sense primer that introduced an *Ssp*I site 5′-GAAGAATATTGGGCTTTCCTGGTGCTG-3′ and an antisense primer located on pCEP4 plasmid. After amplification, both PCR products were digested and cloned into pEF/myc/cyto plasmid (Invitrogen, Groningen, The Netherlands) under the control of the elongation factor-1 promoter. The clones obtained were sequenced with an automated fluorescence-based system (Applied Biosystems 310) using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA).

Western blots and luciferase assay

Functional STAT activation was assessed by measuring luciferase production by cells transfected with the pGRR5 construct (provided by Dr P. Brennan, University of Wales College of Medicine, Cardiff, Wales, U.K.). This construct contains five copies of the STAT-binding site of the *FcγRI* gene inserted upstream from a luciferase gene controlled by the thymidine kinase (TK) promoter. As an internal control, we used the pRL–TK vector (Promega, Madison, WI) containing the *Renilla* luciferase gene under the control of the TK promoter.

A sample of 10^6 HT-29 cells were transiently transfected by electroporation (250 V, 1200 μF and 192 Ω) with 15 μg of chimaeric IL-10R–LICR2 construct, 15 μg of pGRR5 and 1 μg of pRL–TK. Cells were then seeded in 12-well plates (Nunc, Rochester, NY) at 8×10^5 cells/well. After 5 h, cells were stimulated for 2 h with or without IL-10 or IL-22. Luciferase assays were performed using the dual luciferase reporter assay kit (Promega, Madison, WI).

Transient transfections of 2C4, U4C, and γ2A fibrosarcoma cells [17,18] were carried out as described in [19]. Briefly, cells were seeded in 24-well plates at 2×10^5 cells/well 1 day before transfection. Transfection was carried out using the LIPOFECTAMINE™ method (Invitrogen, Groningen, The Netherlands), according to the manufacturer's recommendations, with 500 ng of IL-10R–LICR2 chimaeric cDNA, 40 ng of pRK5-JAK1, pRK5-JAK2, 100 ng of pGRR5, and 100 ng of pRL–TK. At 5 h after transfection, cells were stimulated with control medium or human IL-10 (10 ng/ml) for 4 h. Luciferase assays were performed using the dual luciferase reporter assay kit.

IL-10R–LICR2 chimaeric receptor cDNA was transiently transfected by electroporation (250 V, 1200 μF and 192 Ω) in H4IIE cells. Transfected cells were seeded in six-well plates for 5 h, before stimulation for 15 min with or without IL-10 or IL-22. Cells were then lysed in 500 μl of Laemmli buffer (Bio-Rad) and boiled for 3 min before loading on pre-cast Novex (Carlsbad, CA) SDS/polyacrylamide gels (8 or 14 %) and electrophoretic transfer to nitrocellulose membranes (Hybond C; Amersham Biosciences). Membranes were blocked in 5 % (w/v) non-fat dry milk, washed, and probed using antibodies specific for phosphorylated STAT2-Y-689 (Upstate Biotechnology, Lake Placid, NY), STAT1-Y701, STAT3-Y705, STAT5-Y694 and extracellular-signal-regulated kinase (ERK)1/2 (Cell Signaling, Beverly, MA). Blots were reprobed with anti-(β-actin) antibodies (Sigma, Bornem, Belgium) as a control. A SuperSignal West Pico detection kit (Pierce, Rockford, IL) was used for detection.

RESULTS

Characterization of a new gene of the class II cytokine receptor family

In order to identify putative new members of the class II cytokine receptor family, which includes receptors for IFNs and IL-10, we screened human genome databases with the IL-22BP protein sequence. One short region of homology was found, at about 25 kb from the *IL22R* gene, showing 38 % amino acid identity with residues 68 to 113 from IL-22BP, suggesting the presence of a new member of this gene family. This genome sequence was further analysed using the NIX analysis program. This software predicted seven exons stretching over approx. 30 kb. The genomic organization is shown in Figure 1 and the intron/exon junctions

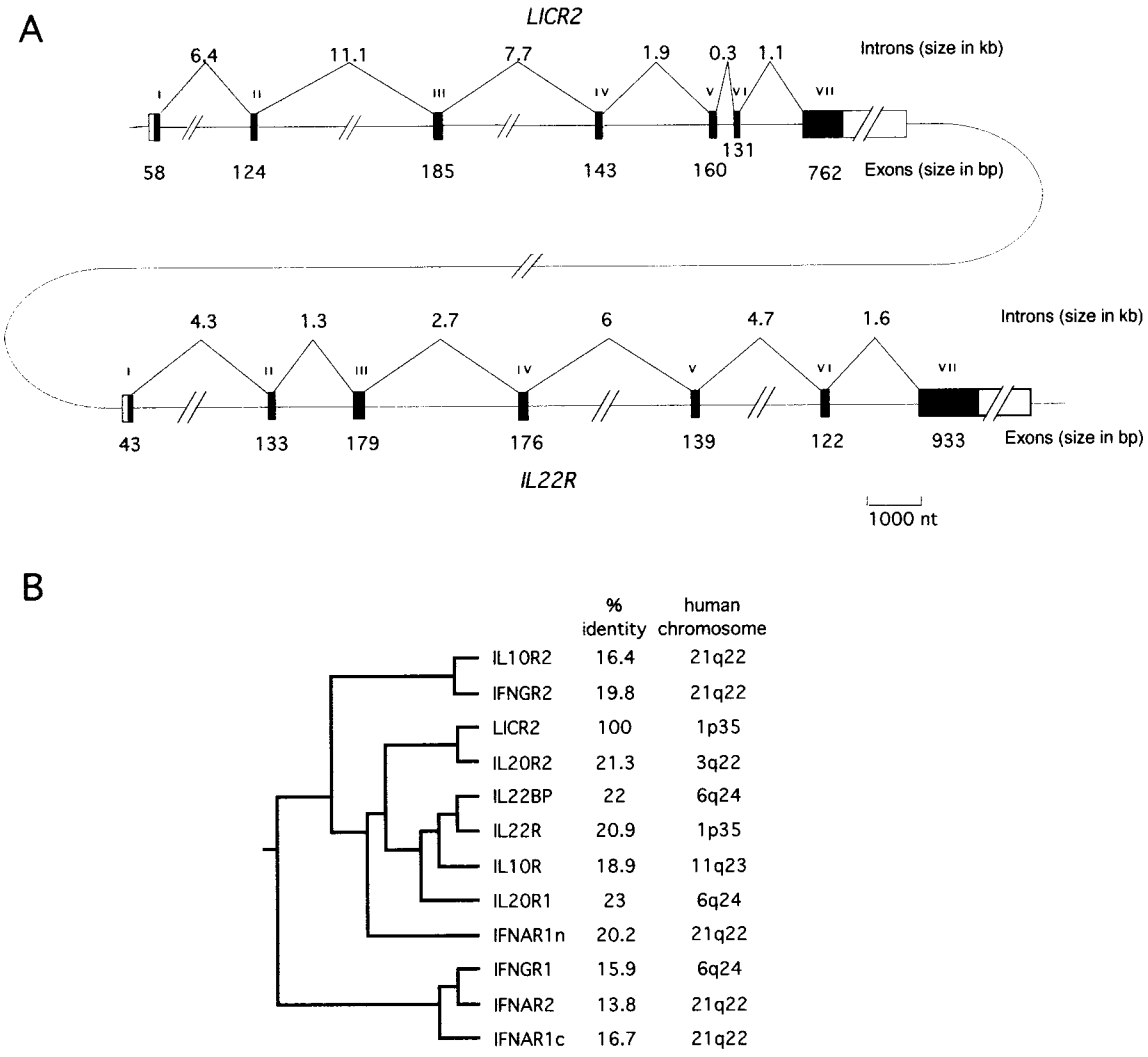

Figure 1 Genomic organization and phylogenetic analysis of the *LICR2* gene

(**A**) Exons are shown as boxes, and their size is indicated in bp for the coding sequences. White boxes correspond to non-coding regions and black boxes to coding regions. The introns are represented by lines and their length is indicated in kb. The splicing patterns of *LICR2* and *IL-22R* are shown above the respective genes. (**B**) A phylogenetic tree was generated by multiple alignment of the extracellular domains of the class II cytokine receptors using the ClustalX Multiple Sequence Alignment program. The percentage of amino acid identity with the extracellular domain of LICR2, and the chromosomal location is indicated for each receptor.

were confirmed by a series of RT-PCRs using oligonucleotides located in the predicted exons. The gene, which was provisionally designated *LICR2* (for likely cytokine or interleukin receptor-2), is located together with *IL22R* on chromosome 1, in the same orientation as *IL22R* (Figure 1A).

A cDNA fragment of 1600 nucleotides, including a 1560 bp ORF, was amplified by RT-PCR from human colon and hepatoma mRNA. The predicted LICR2 protein contains 520 amino acids (EMBL/GenBank® accession number AJ534330), including a stretch of hydrophobic residues at the N-terminus, compatible with a signal peptide, and an internal hydrophobic segment, encoded by exon 6, and which might correspond to the transmembrane domain. The extracellular domain, encoded by exons 2 to 5, showed between 18 and 23 % amino acid identity with IL-22BP and with the extracellular domain of IL-20R, IL-20Rβ, IL-22R, IL-10Rα (Figure 1B).

The expression of the *LICR2* gene was analysed by RT-PCR with oligonucleotides located in exons 3 and 5. As shown in Figure 2A, *LICR2* expression is ubiquitous, with the strongest expression in breast, colon, liver and testis. Interestingly, a second PCR performed with oligonucleotides located in exons 5 and 7 detected a second smaller band that was expressed at lower level in some tissue samples such as liver (Figure 2B). This PCR product was sequenced, and turned out to correspond to an alternative splicing product that lacked exon 6 sequences. Translation of this variant resulted in a shorter protein of 244 amino acids without any transmembrane and cytoplasmic domains (EMBL/GenBank® accession number AJ534331).

LICR2 cytoplasmic domain activates STAT factors

To address whether LICR2 is capable of transmitting a signal upon activation, we constructed a chimaeric receptor consisting of the extracellular part of the IL-10Rα chain joined to the transmembrane and intracellular parts of LICR2. Upon IL-10 stimulation, this chimaeric receptor should dimerize with

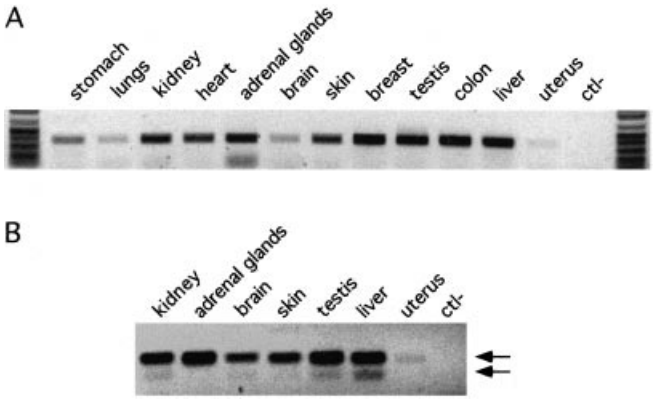

Figure 2 Tissue distribution of LICR2 mRNA

Total RNA was isolated from various normal organs. RT-PCR amplification was performed with oligonucleotides specific for exons 3 and 7 (**A**) or for exons 5 and 7 (**B**) of the *LICR2* gene. The two amplified LICR2 cDNAs are indicated by arrows in (**B**).

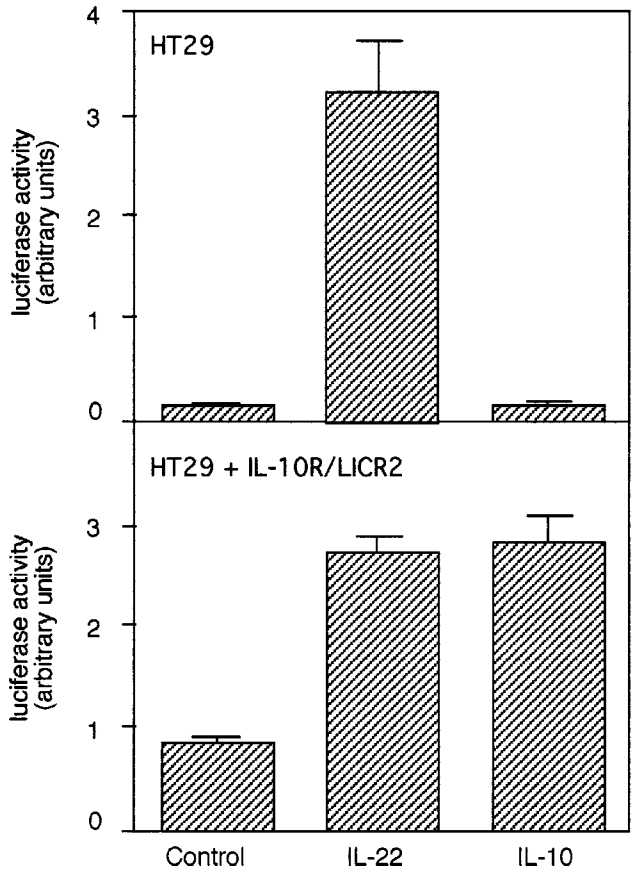

Figure 3 Transcriptional activation of a STAT-responsive promoter by the cytoplasmic domain of LICR2

HT-29 cells were transiently electroporated with the pGRR5-luc reporter plasmid alone (upper panel) or together with the cDNA encoding the chimaeric IL-10R–LICR2 (lower panel). After 5 h, cells were stimulated with IL-22 (100 ng/ml) or IL-10 (10 ng/ml). The luciferase assay was performed after 2 h of stimulation. These results are representative of two independent experiments.

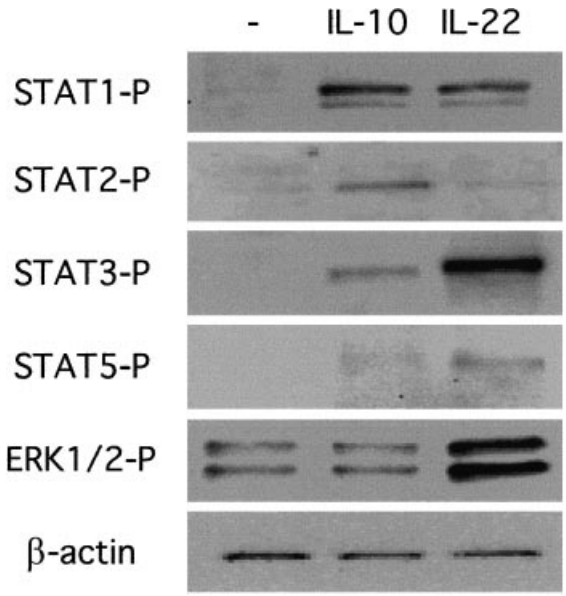

Figure 4 The cytoplasmic domain of LICR2 induces STAT1, STAT2 and STAT3 phosphorylation

H4IIE cells were transiently electroporated with the cDNA encoding the chimaeric IL-10R–LICR2 receptor, together with pGRR5-luc reporter plasmid. Transfected cells were seeded in six wells of a six-well plate. At 5 h later, cells were stimulated with IL-22 (300 ng/ml) or with IL-10 (10 ng/ml) for 15 min or were left unstimulated. Total lysates were analysed by Western blotting with antibodies directed against phosphorylated STAT1, STAT2, STAT3, STAT5 and ERK1/2. The membranes were then reprobed with an anti-(β-actin) antibody.

IL-10Rβ. As the short cytoplasmic domain of IL-10Rβ recruits the Tyk2 tyrosine kinase, but does not directly activate STAT transcription factors, this chimaeric construct can be used to assess the ability of the cytoplasmic domain of LICR2 to activate STAT factors. HT-29 cells, which express endogenously IL-10Rβ, but not IL-10Rα, were electroporated with the cDNA for the chimaeric receptor and with pGRR5, a luciferase reporter construct regulated by five copies of a STAT-binding sequence recognized at least by STAT1, STAT3 and STAT5. As shown in Figure 3, IL-10 induced a significant increase of luciferase activity in cells transfected with the chimaeric receptor. Similar results were obtained after transfection of the chimaeric construct in HEK-293 kidney cells and in H4IIE rat hepatoma cells (results not shown). To characterize further which STAT factors are activated by the LICR2 cytoplasmic domain, we performed a Western blot using H4IIE cells transiently transfected with the IL-10R–LICR2 chimaeric receptor. Cells were stimulated with IL-10 or with IL-22 as a positive control. As shown in Figure 4, activation of the chimaeric receptor resulted in phosphorylation of STAT1, STAT2 and STAT3. A weak, but reproducible, phosphorylation of STAT5 was also observed. No phosphorylation of ERK1/2 was observed upon IL-10 stimulation, in contrast with IL-22 [19].

STAT activation by LICR2 is JAK1-dependent

As JAKs are known to be responsible for STAT phosphorylation in response to cytokines, we next investigated which JAK is activated by the cytoplasmic domain of LICR2, using 2C4 fibroblasts and their JAK-deficient variants [17,18]. These cells, which do not express endogenous IL-10Rα [20], were transfected

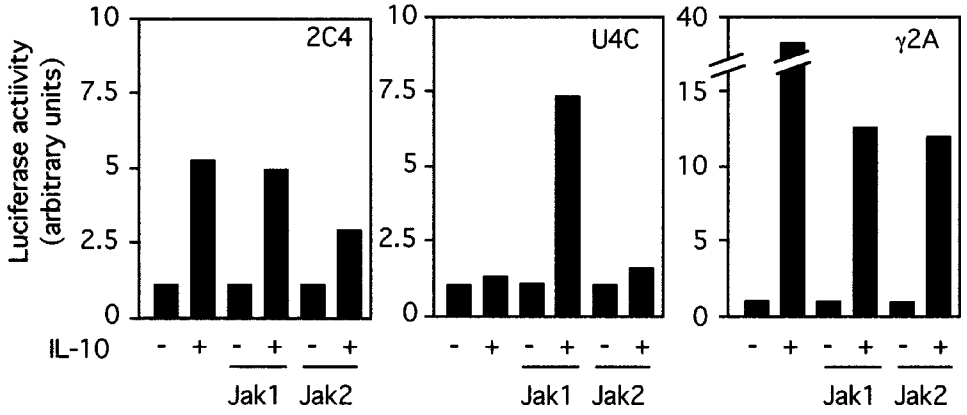

Figure 5 JAK1, but not JAK2, is required for STAT activation by the LICR2 cytoplasmic domain

Parental 2C4, JAK1-deficient U4C or JAK2-deficient γ2A cells were transfected with a vector coding for the IL-10R–LICR2 chimaeric receptor, together with pGRR5-luc and, when mentioned, with a vector coding for JAK1 or JAK2. Cells were stimulated with IL-10 (10 ng/ml) or with control medium for 4 h before a luciferase assay was performed. These results are the means for duplicate cultures and are representative of two independent experiments.

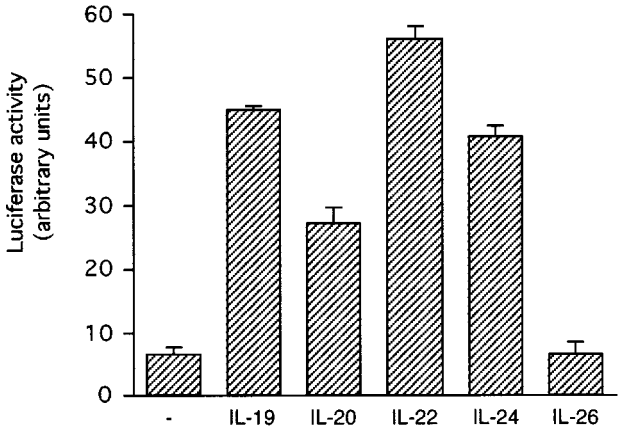

Figure 6 LICR2 transfection does not confer IL-26-responsiveness

HT-29 cells were electroporated with vectors encoding IL-20Rα, IL-20Rβ, LICR2 and with the pGRR5-luc reporter plasmid. After 5 h, cells were stimulated with IL-19, IL-20, IL-24 (10% of transfected HEK-293 cell supernatant), IL-22 (100 ng/ml) or IL-26 (10 ng/ml). The luciferase assay was performed after 2 h of stimulation. These results are representative of four independent experiments.

with the IL-10R–LICR2 chimaeric cDNA, together with pGRR5-luc reporter plasmid. As shown in Figure 5, IL-10 failed to up-regulate luciferase activity in JAK1-deficient cells unless these cells were transfected with JAK1 cDNA, indicating that STAT activation induced by LICR2 is JAK1-dependent. In contrast, parental cells or JAK2-deficient cells were both able to respond to IL-10 upon IL-10R–LICR2 transfection.

STAT activation by IL-10 homologues through LICR2

In order to assess if LICR2 could be a functional receptor for IL-26, an orphan cytokine of the IL-10 family, we transfected HT-29 cells, which endogenously express IL-22R and IL-10Rβ, with expression constructs for IL-20Rα, IL-20Rβ, LICR2 and with the pGGR5-luc reporter plasmid. IL-19, IL-20, IL-22 and IL-24, all induced luciferase activity in transfected cells, confirming that these receptors were functionally expressed. In contrast, IL-26 stimulation did not lead to STAT activation (Figure 6), indicating that LICR2 cannot associate with IL-10Rβ, IL-22R, IL-20Rα nor IL-20Rβ to form a functional IL-26 receptor.

DISCUSSION

In the present paper, we describe a novel protein that displays the typical genomic organization and structural features of type II cytokine receptors. It shares significant similarity with known members of this receptor family, most notably IL-20R and IL-22BP, and its gene is found in close physical proximity to *IL22R* on human chromosome 1. The gene encoding the mouse orthologue is located on mouse chromosome 4, also at 25 kb from the mouse IL-22R (results not shown).

We have assessed the signalling capacity of LICR2 by fusing its intracellular domain to the ligand-binding domain of IL-10Rα, which forms a heterodimer with IL-10Rβ upon IL-10 stimulation. IL-10Rβ is known to be associated with Tyk2 tyrosine kinase, but studies on IL-10 and IL-22 signal transduction have shown that its short cytoplasmic domain does not recruit any STAT transcription factor or any adaptor protein for the mitogen-activated protein kinase (MAPK) pathway ([21]; D. Lejeune and J.-C. Renauld, unpublished work). Our results demonstrate that the IL-10R–LICR2 chimaeric molecule was able to transduce a signal, and caused phosphorylation of STAT1, STAT2, STAT3 and STAT5, but not of the ERK MAPK. This STAT activation was JAK1-dependent, pointing to JAK1 as the tyrosine kinase associated with LICR2. Activation of STAT1, STAT3 and STAT5 by cytokines is a common feature and has been associated with a wide range of biological activities, including cell-growth regulation and differentiation [22]. In contrast, STAT2 activation has, at present, been restricted to type I IFNs [23,24], and our results suggest that LICR2 might be also involved in anti-viral responses.

Because LICR2 is mostly related to IL-20Rα and IL-22BP, it might be a receptor for any known member of the IL-10-related cytokine family. IL-26 (AK155) was an interesting candidate as its receptor has not been characterized at the present time. However, transfection of LICR2 failed to confer responsiveness to IL-26, even in cells expressing most other members of the IL-10R family (IL-10Rβ, IL-20Rα, IL-20Rβ and IL-22R). Similar

negative results were obtained by co-transfecting LICR2 and the different IFN receptor chains (results not shown). In addition, polyclonal antibodies generated against the extracellular domain of LICR2 failed to inhibit IL-26 activity on human epithelial cells (results not shown). Taken together, these results strongly suggest that LICR2 is not responsible for IL-26 biological activities. We have also investigated the ability of all IL-10-related cytokines, including IFNs, to activate STAT-3 in LICR2-transfected HEK293 cells, and failed to notice any activation (results not shown). Altogether, these observations suggest that LICR2 binds to a new cytokine, or acts through dimerization with another cytokine receptor chain. The identification of this putative ligand or receptor chain will be a critical step to the further understanding of the biological functions of LICR2.

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