THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 32, Issue of August 6, pp. 33343–33351, 2004

Printed in U.S.A.

The T-cell Lymphokine Interleukin-26 Targets Epithelial Cells through the Interleukin-20 Receptor 1 and Interleukin-10 Receptor 2 Chains*

Received for publication, May 5, 2004, and in revised form, June 1, 2004
Published, JBC Papers in Press, June 3, 2004, DOI 10.1074/jbc.M405000200

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The cellular members of the interleukin-10 (IL-10) cytokine family share sequence homology with IL-10, whereas their sites of expression and their functions are divergent. One of these factors, AK155 or IL-26, was discovered because of its overexpression in human T lymphocytes after growth transformation by the simian rhadinovirus herpesvirus saimiri. In addition, the gene is transcribed in various types of primary and immortalized T-cells. Here we describe epithelial cells, namely colon carcinoma cells and keratinocytes, as targets of this T-cellular lymphokine. Purified recombinant IL-26 induced the rapid phosphorylation of the signal transducer and activator of transcription factors 1 and 3. As a result, secretion of IL-10 and IL-8, as well as cell surface expression of CD54 were enhanced. Moreover, we show that the IL-26 protein binds to heparin, is released from the cell surface, and can be functionally inhibited by heparin. The sensitivity to recombinant IL-26 of various cell lines strictly correlated with the expression of the long chain of the IL-20 receptor. Because blocking antibodies against either the short chain of the IL-10 receptor or the long chain of the IL-20 receptor inhibited IL-26-dependent signal transduction, and transient expression of these receptor chains induced IL-26 responsivity in non-sensitive cells, we propose that the IL-20 receptor 1 and IL-10 receptor 2 chains participate in forming the IL-26 receptor. Targeting epithelial cells, the T-cell lymphokine IL-26 is likely to play a role in local mechanisms of mucosal and cutaneous immunity.

Virus-infected or virus-transformed cells have been a valuable source for the identification of novel cytokines such as interleukin (IL)[1] 10 (IL-10), IL-17, IL-26, and IL-27 (1–6). For example, one component of the heterodimeric IL-27 was found to be strongly overexpressed by Epstein-Barr virus-transformed B cells (1). Similarly, by subtractive hybridization, we identified IL-26 (AK155) as a cytokine that is strongly overexpressed by human T-cells after transformation with the simian rhadinovirus herpesvirus saimiri (HVS) (4). Various types of primary and transformed T-cells and native peripheral blood cells were found to transcribe IL-26 at low levels (4, 7). Because of sequence similarity, IL-26 was assigned to the IL-10 family of cytokines (8–12). Similar to IL-10, IL-26 forms stable homodimers (4). Because IL-26 overexpression was one of the very few functional differences between native and HVS-transformed T-cells (4, 13, 14), a role for this cytokine in viral growth transformation seemed likely.

The IL-10 cytokine family harbors six cellular members: IL-10, IL-19, IL-20, IL-22 (IL-TIF), IL-24 (MDA-7), and IL-26 (8–12). In addition, the three interferon-like cytokines molecules IL-28A, IL-28B, and IL-29 (IFN-λ1–3) can be included into this family because of sequence homology rather than to functional similarity (15, 16). Whereas the genes for IL-19, IL-20, and IL-24 cluster near the IL-10 locus on human chromosome 1q32 (17, 18), those for IL-22 and IL-26 are found close to the IFN-γ gene on chromosome 12q15 (4, 19–21). Correspondingly, IL-10, IL-19, IL-20, and IL-24 are primarily secreted by activated macrophages, whereas T-cells are the main source for IL-22, IL-26, and IFN-γ (4, 7, 9, 22, 23). In contrast to the pleiotropic IL-10, which has predominantly immunosuppressive functions (24), the members of the IL-10 family have diverse effects: for IL-19, pro-apoptotic functions have been proposed in monocytes (25). In transgenic mice, IL-20 overexpression caused skin disorders reminiscent of psoriasis (17). IL-24 (MDA-7) showed anti-tumor and pro-apoptotic activities (26–28). The T-cellular product IL-22 mediated acute phase responses in the liver (23).

The family of type II cytokine receptor molecules (29) comprises a series of initially orphan receptor chains in addition to the prototypic heterodimeric receptor for IL-10. The IL-10 receptor (IL-10R) consists of a long transmembrane chain (IL-10R1, Ref. 30) mediating signal transduction and activation of signal transducer and activator of transcription (STAT) factors, and a short transmembrane chain which is required for signal transduction and enhances ligand binding (IL-10R2, Ref. 31). Most orphan receptors of the type II cytokine receptor family have recently been functionally assigned to members of the IL-10 cytokine family. IL-22 signals via IL-22R/IL-10R2 com-

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[1] The abbreviations used are: IL, interleukin; HVS, herpesvirus saimiri; IFN, interferon; IL-20R1, interleukin 20 receptor 1 chain; STAT, signal transducer and activator of transcription; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay.

plexes (32, 33). IL-20, IL-19, and IL-24 target IL-20R1/IL-20R2 heterodimers (17, 34). In addition, IL-20 and IL-24, but not IL-19, bind also to IL-22R/IL-20R2 complexes (34–36). The IFN-like molecules IL-28A, IL-28B, and IL-29 signal via IL-28R/IL-10R2 heterodimers (15, 16, 37). Thus, our aim was to define the role of IL-26 in this complicated functional environment of the IL-10 cytokine family members and their receptors.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human Colo-205 and SW-403 colon carcinoma, HepG2 hepatoma, HeLa cervical carcinoma, Panc-I pancreatic carcinoma, RTS3b (kindly provided by Irene Leigh, London) and SCC12F skin carcinoma (kindly provided by Lawrence Young, Birmingham), and 293T human embryonal kidney cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human Lovo and Colo-320 colon carcinoma cells, the Hodgkin's line KMH-2, the T-leukemia line Jurkat, and Chinese hamster ovary cells were kept in RPMI 1640 medium with 10% fetal bovine serum. The primary human keratinocyte line HaCaT in an early passage was kindly provided by Norbert Fusenig (Heidelberg) and cultivated as published (38). Cultures of CB-15, CB-84, and other HVS-transformed human T-cells followed published protocols (39, 40). Monocytes were purified from fresh blood using magnetic beads with antibodies against CD14 (Miltenyi Biotech, Bergisch Gladbach). Dendritic cells were differentiated from primary CD14+ monocytes (41). 293T cells were transfected using LipofectAMINE (Invitrogen, Karlsruhe) with described FLAG-tagged IL-26 expression constructs (4). For the reconstitution of the IL-26 receptor in a non-responsive cell line, Chinese hamster ovary cells were transfected in 24-well plates using LipofectAMINE 2000 (Invitrogen) and 2 μg of plasmid DNA per well. The IL-20R1 and IL-10R2 cDNAs were expressed from pcDEF3-based vectors (31, 34). pEGFP-N1 (Clontech) served as a transfection control. The transfected Chinese hamster ovary cells were treated with IL-26 or control medium at 36 h post-transfection for 30 min at room temperature and then lysed for Western blot analysis. The heparin drug Liquemin N was purchased from Roche Pharmaceuticals, biotinylated heparin from porcine intestinal mucosa was purchased from Merck (Schwalbach), chondroitin sulfate A (chondroitin-4 sulfate) from bovine trachea, chondroitin sulfate B (dermatan sulfate) from porcine intestinal mucosa, chondroitin sulfate C (chondroitin-6 sulfate) from shark cartilage, heparan sulfate from bovine kidney, and heparin from porcine intestinal mucosa were purchased from Sigma (Taufkirchen).

Cytokine Purification and Interaction Studies—The cell-free supernatant of HVS-transformed human T-cells was passed through a heparin-agarose column (1 ml, Amersham Biosciences). After washing with phosphate-buffered saline, proteins were eluted with 0.6 and 2.0 M of NaCl. The fractions were tested for IL-26 by Western blotting. Recombinant N-terminally histidine-tagged IL-26 (His-IL-26) was highly purified (at least 95%, according to Coomassie-stained SDS-polyacrylamide gels) after denaturation via nickel-chelate chromatography from bacterial lysates and stepwise renaturation, as published previously (4). For each individual preparation, the concentration-dependent biological activity was titrated on Colo-205 cells for STAT3 phosphorylation in Western blots in order to permit comparable test conditions. The purified His-IL-26 protein was also used to raise polyclonal antisera in rabbits (Charles River, Kisslegg).

To test for a direct interaction between IL-26 and heparin, polystyrene microwell surfaces (MaxiSorp plates, Nunc, Wiesbaden) were coated with IL-26 (up to 20 μg/ml) for 2 h at room temperature. Nonspecific binding was blocked with phosphate-buffered saline supplemented with 3% fetal bovine serum for 2 h at room temperature. As probe for the interaction, biotinylated heparin (up to 10 μg/ml) was added in phosphate-buffered saline for 2 h at room temperature. After washing twice with water, a streptavidin horseradish peroxidase conjugate (R&D systems, Wiesbaden; 1:200 dilution in phosphate-buffered saline) was added for 1 h at room temperature followed by two washing steps with water. Finally, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) was added, and the reaction was stopped with 5% H_2SO_4. The absorbance was read at 450 nm using a Multiskan Ascent microplate photometer (Thermo Electron, Dreieich).

Similar solid phase interaction experiments were performed to test the interaction of IL-26 or IL-20 with the extracellular domain of IL-20R1. For this purpose, 293T cells were transiently transfected with expression constructs for either the extracellular domain of IL-20R1, FLAG-tagged IL-20 (IL-20FLAG in pCEP4, Ref. 33) or GFP (pEGFP-N1, Clontech) as a control. The extracellular domain of IL-20R1 was amplified using the primers HF894 5′-GGCCCAGCCGGCCGTTCCCT-GTGTCTCTGGTGG-3′ and HF898 5′-GGATCCCGTTCTGTTTGATT-TAGTATTC-3′ after reverse transcription from HaCaT total RNA and cloned into pSeqTagHygroB (Invitrogen). Culture supernatants were harvested 48 h after transfection. Polystyrene plates were coated with 200 μl of supernatant from 293T cells expressing the extracellular domain of IL-20R1 or GFP overnight at 4 °C. The probes with either purified, recombinant His-IL-26 (0.1 or 1.0 ng), or IL-20 FLAG-containing culture supernatant, or phosphate-buffered saline alone were then incubated for 2 h at room temperature. After three washes with phosphate-buffered saline containing 0.05% Tween 20, the plates were incubated for 2 h at room temperature with mouse monoclonal antibodies against the FLAG tag (mouse anti-FLAG M2, Sigma; 1:2000 in phosphate-buffered saline containing 3% fetal calf serum) or the His tag (mouse anti-penta-His, Qiagen 1:1000 in phosphate-buffered saline containing 3% fetal calf serum), respectively. This was followed by another 3-fold washing and incubation with peroxidase-conjugated rabbit anti-mouse antibodies (Dianova, Hamburg, 1:2000 in phosphate-buffered saline containing 3% fetal calf serum) for 1 h at room temperature. The plates were washed again, and binding was detected and quantified as described.

Three chemically synthesized peptides (Biosyntan, Berlin, Germany or Coring, Gernsheim, Germany) with blocked end groups were used for circular dichroism spectroscopy studies of the cytokine-heparin interaction: IL-10AB (residues 14–58 of mature IL-10, representing the helices A and B: HFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL-LLKESLLEDFKG); IL-26AB (residues 14–57 of mature IL-26, representing the helices A and B: RGTLSQAVDALYIKAAWLKATIPE-DRIKNIRLLKKKTKKQF-MKN); IL-26B (residues 37–54 of mature IL-26, representing helix B: EDRIKNIRLLKKKTKKQF). Far-ultraviolet circular dichroism spectra of IL-10AB, IL-26AB, and IL-26B were recorded at 20 °C in 10 mM potassium phosphate, pH 7.4 in a 10-mm cell from 195 to 250 nm at 50 nm/min on a Jasco J810 circular dichroism spectropolarimeter at a peptide concentration of 10 μM. For the heparin titration experiments a 1-, 2-, and 3-fold molar excess of heparin (Roth, Karlsruhe, Germany) was added to the peptide samples. At each heparin concentration, reference spectra were measured in order to allow for a subsequent correction for the heparin signal in the CD spectra. All spectra were measured eight times and averaged for each peptide and reference sample. The ellipticity $[\Theta]_{MRW}$ was calculated after subtraction of the reference from the peptide spectra.

Reverse Transcription Polymerase Chain Reaction—The expression of type II cytokine receptor chains was tested by RT-PCR. Total cellular RNA was prepared by the acidic phenol-chloroform method. Using Superscript II reverse transcriptase (Invitrogen) and random oligonucleotide primers, cDNA was prepared. The following primers were used for PCR: HF855 5′-ACTTCAGCCTCCTAACCTC-3′ and HF856 5′-CT-ATTGCTGCTGCCACTAC-3′ (IL-10R1, 534-bp fragment), HF857 5′-T-ATTGGACCCCCTGGAATG-3′ and HF858 5′-GTAAACGCACCACAG-CAAG-3′ (IL-10R2, 411-bp fragment), HF859 5′-CTGTGTCTCTGGTG-GTTTG-3′ and HF860 5′-CGCAGTAAAGAGTGTTCGG-3′ (IL-20R1, 553-bp fragment as main product), HF861 5′-CAGACAGAATGTGTG-GAGG-3′ and HF862 5′-TTAGACAAACCACCCTGCC-3′ (IL-20R2, 449-bp fragment), HF863 5′-GATATGTCACCAAGCCGCC-3′ and HF864 5′-GTCTTTGCCAGAACCTTCC-3′ (IL-22R, 484-bp fragment), JCS1146 5′-GGGAACCAAGGAGCTGCTATG-3′ and JCS1147 5′-TGG-CACTGAGGCAGTGGTGTT-3′ (IL-28R, 370-bp fragment), HF147 5′-GCAGGGGGGAGCCAAAAGGG-3′ and HF148 5′-TGCCAGCCCCAG-CGTCAAAG-3′ (glyceraldehyde phosphate dehydrogenase, GAPDH, 570-bp fragment).

Immunoprecipitation, Western Blotting, and Flow Cytometry—For immunoprecipitation, up to 10 ml of culture supernatant were used. Anti-IL-26 serum (100 μl) was first preincubated with 50 μl of protein G-agarose (Roche Applied Science). After washing the beads in phosphate-buffered saline, the culture supernatants were added to the coated beads and agitated overnight at 4 °C. After 3-fold washing, the beads were heat-denatured and loaded on polyacrylamide gels. For Western blotting, proteins from denaturing polyacrylamide gels were transferred onto polyvinylidine difluoride membranes (Millipore, Eschborn) by semidry electrotransfer. STAT factor signaling was investigated with the following rabbit antibodies: STAT1 (9172), pSTAT1 (against phosphorylated tyrosine residue 701 of STAT1, 9171), STAT3 (9132), pSTAT3 (against phosphorylated tyrosine residue 705 or STAT3, 9131, all from Cell Signaling Technology, Frankfurt). In cultures, the following antibodies were used in order to block IL-26-mediated signaling: polyclonal rabbit antisera against recombinant His-IL-26 (Charles River), affinity-purified polyclonal goat antibody against IL-26 (AF1375, R&D Systems), murine monoclonal anti-IL-10R1 (3F9,

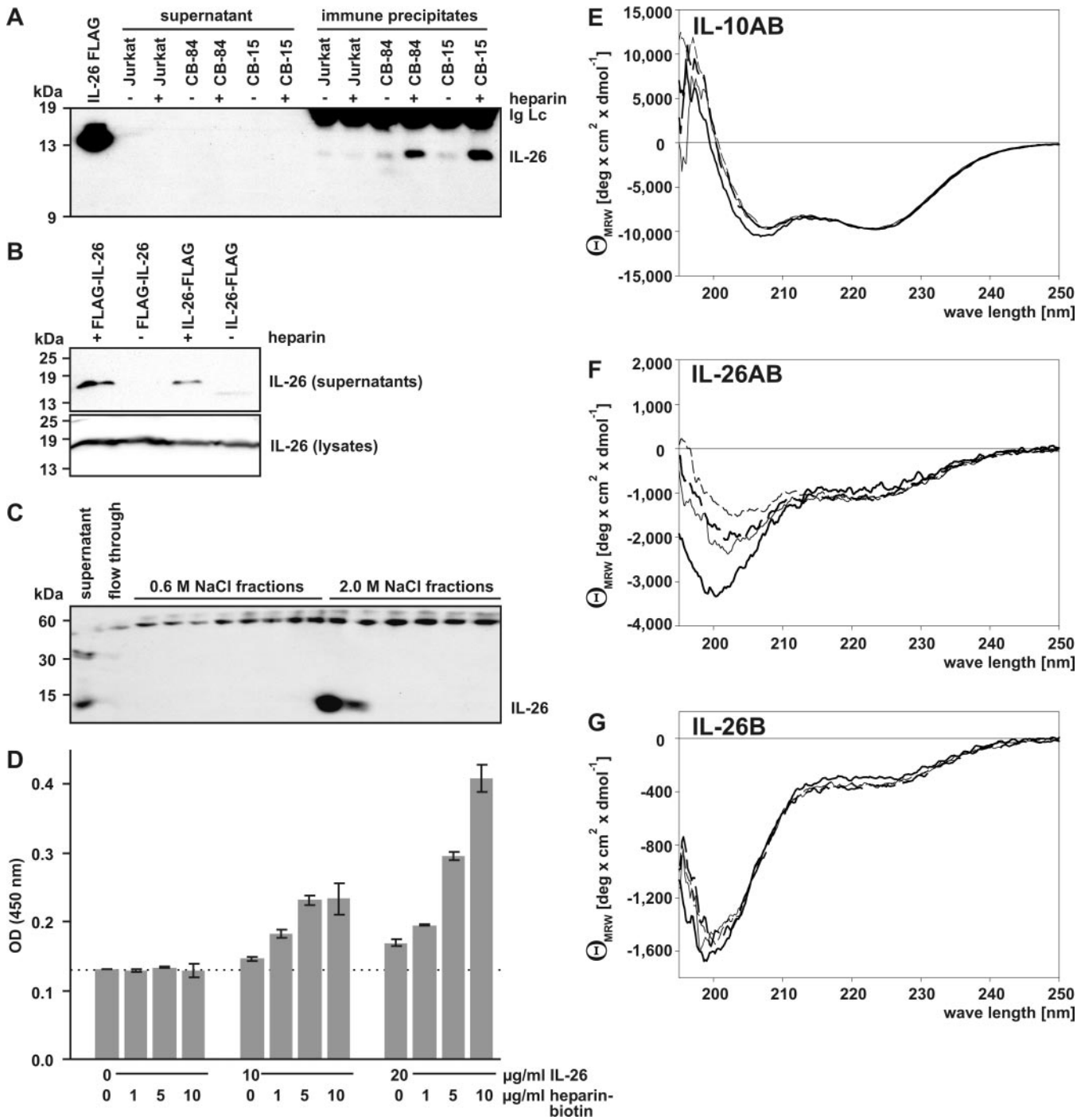

FIG. 1. **Binding of IL-26 to cell surfaces and heparin.** *A*, IL-26 release by heparin into the culture supernatant. HVS-transformed T-cells are the only cell type found to produce easily detectable amounts of IL-26. The influence of heparin (20 units/ml) on IL-26 amounts in the supernatant of HVS-transformed T-cells (CB-15) was investigated. Supernatants (20 μl) and immunoprecipitates from 10 ml of supernatant were analyzed by Western blot with a rabbit anti-IL-26 antiserum. After immunoprecipitation from supernatants, strong IL-26 signals were only detectable in the presence of heparin. The strong signals on top seem to result from large amounts of immunoglobulin light chains (*Ig Lc*). Lysates of transiently transfected IL-26 FLAG-expressing 293T cells were used as a positive control. *B*, IL-26 release into the culture supernatant after transient transfection of 293T cells in the presence of heparin. 293T cells were transiently transfected with N- or C-terminally FLAG-tagged IL-26 expression constructs (4). Whereas all the cell lysates had similar amounts of IL-26, the supernatants contained detectable quantities of IL-26 only in presence of heparin (20 units/ml), as shown by Western blot with a rabbit anti-IL-26 antiserum. *C*, IL-26 enriched by heparin affinity chromatography. Supernatant (50 ml) of the HVS-transformed human T-cell line CB-15 was loaded onto a 1-ml heparin agarose column. The first high salt fractions were enriched for IL-26, as shown by Western blot using a rabbit anti-IL-26 antiserum. *D*, direct binding of heparin to recombinant IL-26. The direct binding of heparin to purified, recombinant IL-26 was analyzed in a sandwich test on polystyrene microwells. The well surface was coated with 0, 1, or 2 μg of purified His-IL-26/well in 100 μl. After blockade of unspecific reactions with 3% fetal bovine serum in phosphate-buffered saline, biotinylated heparin (0–10 μg/ml) was added. Absorption signals ($OD_{450\ nm}$) were detected after using a streptavidin horseradish peroxidase conjugate and a 3,3′,5,5′-tetramethylbenzidine substrate. The *dotted line* represents the background absorption observed under these conditions. *E–G*, circular dichroism spectra of IL-10 and IL-26 peptides with increasing amounts of heparin. Peptides (10 μM) representing helices A and B of IL-10 (IL-10AB, residues 14–58, *panel E*) or helices A and B of IL-26 (residues 14–57, IL-26AB, *panel F*), or only helix B of IL-26 (residues 37–54, IL-26B, *panel G*) were analyzed at pH 7.4 and in the presence of increasing amounts of heparin. The far ultraviolet circular dichroism spectra are shown as *thick solid* (peptide alone), *thin dashed* (equimolar heparin), *thick dashed* (2-fold excess), and *thin solid* lines (3-fold excess), respectively. Most pronounced heparin-dependent changes were observed for the spectra of peptide IL-26AB (*panel F*), indicating that both helices A and B are contributing to the pronounced heparin affinity of IL-26.

DNAX Research Institute, Palo Alto), and murine monoclonal anti-IL-10R2 (MAB874, R&D Systems). To produce polyclonal murine anti-IL-20R1 antibodies, P815 mastocytoma cells were transfected with the human IL-20R1 cDNA cloned into the pEF-BOS plasmid before injection into DBA/2 mice. After rejection of the tumors, the sera of these mice had high titers of neutralizing anti-hIL-20R1 antibodies and were used at a 1:500 dilution. IL-8 and IL-10 production was measured using Duoset ELISA antibody sets (R&D Systems). Flow cytometry was performed with antibodies against CD50 (ICAM-3, clone HP2/19), CD54 (ICAM-1, clone 84H10), and CD102 (ICAM-2, clone BT-1; all from Beckman Coulter).

RESULTS

Cell Surface Association of IL-26—In contrast to eukaryotic expression systems for other related cytokines, only a small fraction of IL-26 was detectable in secreted form, whereas most of the intrinsically expressed or recombinant IL-26 was found to be cell-associated (4). Therefore, we speculated that this association could be caused by the attachment of IL-26 to cell surface proteoglycans. In the presence of low heparin concentrations (20 units/ml) in the culture medium of HVS-transformed T-cells, the yields of IL-26 immunoprecipitations using rabbit antisera against the recombinant His-tagged IL-26 protein were strongly enhanced (Fig. 1*A*). Moreover, the amounts of IL-26 in the culture supernatants of transfected 293T cells were strongly augmented when heparin was added to the medium (Fig. 1*B*). Correspondingly, native IL-26 from supernatants of HVS-transformed T-cells was efficiently enriched on a heparin column. Whereas 0.6 M salt did not influence the retention of the cytokine on the column, IL-26 was eluted in the first fractions in 2 M sodium chloride (Fig. 1*C*). This strongly suggests that IL-26 is attached to the cell surface through binding to proteoglycans.

The direct interaction of IL-26 with heparin was further investigated by solid phase binding experiments on polystyrene surfaces in which the amounts of both IL-26 and biotin-conjugated heparin correlated with signal strength (Fig. 1*D*). To further confirm this interaction, we used circular dichroism spectroscopy to monitor heparin-dependent structural changes in IL-10- and IL-26-derived peptides. The choice of the IL-26 peptides investigated was guided by the published sequence alignment of the IL-10 cytokine family (9) and by the length and location of the secondary structure elements deduced from the IL-10 crystal structure. One peptide (IL-26B) corresponds to helix B of the IL-10 family cytokines and contains an IL-26-specific cluster of basic amino acids, which are good candidates for interacting with the negatively charged groups of heparin. Another peptide (IL-26AB) additionally includes the N-terminally adjacent residues corresponding to helix A of mature IL-10 family cytokines. In helix A, IL-26 exhibits an unusual amino acid composition compared with its homologs (9), rendering a further structural characterization relevant. The structure and heparin-binding properties of a peptide spanning helices A and B of IL-10 (IL-10AB) was tested as a reference.

In the absence of heparin, IL-10AB adopts a helical structure as evidenced by the two pronounced minima at 208 and 222 nm in the circular dichroism spectrum (Fig. 1*E*). Therefore, helices A and B known from the crystal structure of intact IL-10 are also preserved in the IL-10AB peptide. In contrast to IL-10AB, neither IL-26AB, nor IL-26B exhibited a pronounced tendency to adopt a helical secondary structure (Fig. 1, *F* and *G*). The ellipticity at 208 and 222 nm is significantly lower than in IL-10AB and there is only one distinct minimum present at ~200 nm, indicating the predominance of coil elements and an increased flexibility in IL-26AB and IL-26B. These structural differences between IL-26AB and IL-10AB are consistent with their differences in amino acid composition, suggesting that this region in IL-26 may exhibit unique binding properties.

The addition of heparin up to a 3-fold molar excess to IL-10AB did not result in any significant changes in the circular dichroism spectrum, suggesting that IL-10AB and heparin do not interact (Fig. 1*E*). In contrast, strong changes were observed in the circular dichroism spectrum for IL-26AB upon addition of heparin, showing that this peptide is capable of binding heparin. The most prominent changes were observed in the first titration step corresponding to equivalent amounts of IL-26AB and heparin, suggesting that this peptide has a distinct heparin binding capability and that saturation or nonspecific interactions occur at higher heparin concentrations (Fig. 1*F*). Unexpectedly, the shorter IL-26B peptide containing a total of eight positively charged amino acids did not show relevant structural changes in the titration experiment, suggesting that this stretch of IL-26 on its own is not sufficient for the interaction with heparin and that additional structural

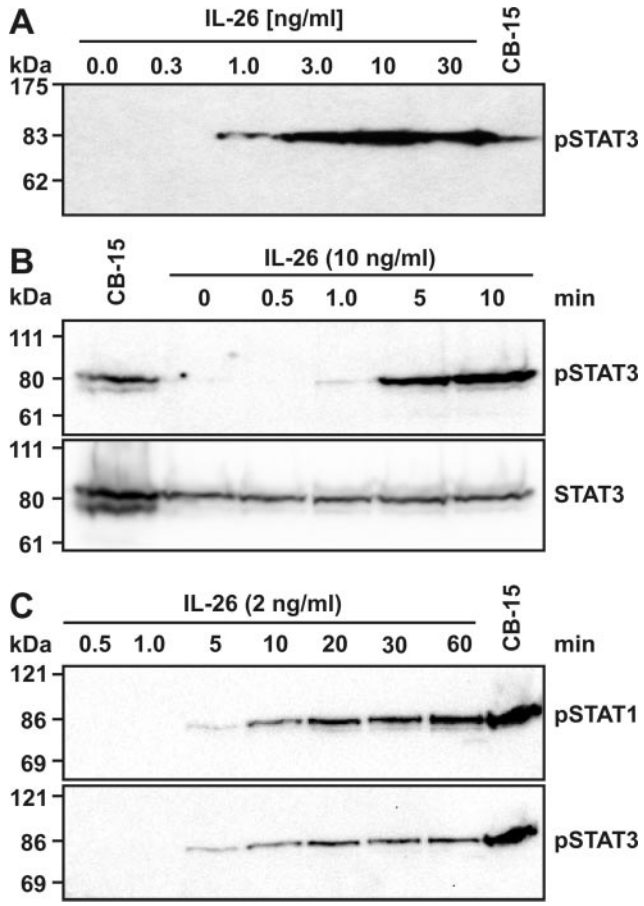

FIG. 2. **IL-26-mediated induction of STAT1 and STAT3 phosphorylation.** *A*, IL-26 titration for induction of STAT3 phosphorylation. Colo-205 carcinoma cells were treated with purified recombinant His-IL-26 (up to 30 ng/ml) for 20 min in 24-well plates. Whereas no signal was detectable at 0.3 ng/ml, ~1 ng/ml was sufficient to induce STAT3 phosphorylation as determined by Western blot specific for tyrosine-phosphorylated STAT3 (*pSTAT3*). As a control, a lysate of the HVS-transformed T-cell line CB-15 was used that constitutively expresses phosphorylated STAT3. *B*, kinetics of IL-26-induced STAT3 phosphorylation. Colo-205 carcinoma cells were treated with 10 ng/ml purified recombinant His-IL-26 for 0.5, 1.0, 5, and 10 min. STAT3 phosphorylation was determined by pSTAT3-specific Western blotting. A faint signal was already detectable after 1 min, whereas 5 min were sufficient for strong STAT3 phosphorylation signals. The *lower panel* shows total STAT3 protein amounts irrespective of phosphorylation (restained membrane). *C*, IL-26-induced phosphorylation of STAT1 and STAT3. Colo-205 carcinoma cells were treated with a low concentration of recombinant His-IL-26 (2 ng/ml) for 0.5, 1.0, 5, 10, 20, 30, and 60 min. Phosphorylation of STAT1 and STAT3 were determined by Western blotting and staining with antibodies against the phosphorylated forms. After pSTAT1 staining, the same membrane was reprobed for pSTAT3. The phosphorylation of both factors occurred synchronously.

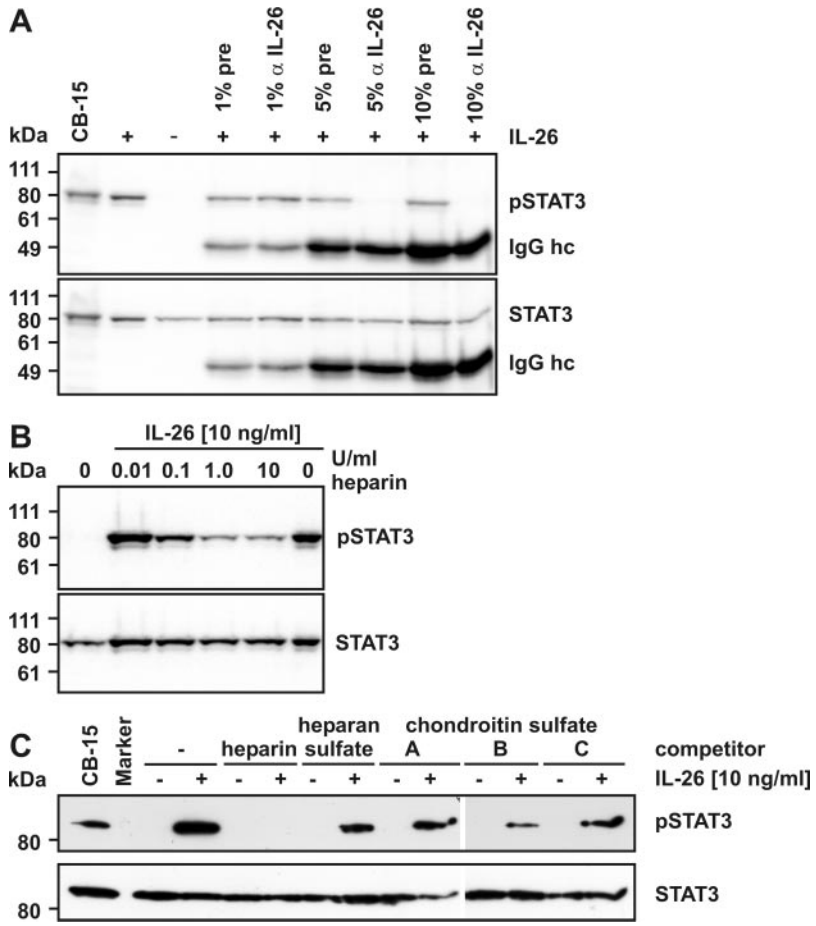

FIG. 3. **Inhibition of IL-26-mediated STAT3 phosphorylation by antiserum and heparin.** *A*, antibody blockade of IL-26-mediated STAT3 phosphorylation. In order to demonstrate the specificity of the IL-26-mediated STAT3 phosphorylation, a rabbit anti-IL-26 antiserum and the preimmune serum as a negative control were preincubated with 10 ng/ml purified recombinant His-IL-26 and then applied to Colo-205 cells. Just 5% (v/v) of rabbit anti-IL-26 antiserum was sufficient to inhibit STAT3 phosphorylation, whereas the preimmune serum did not show an effect. In the lanes that resulted from antisera treatment, an additional band corresponding to immunoglobulin heavy chains (*IgG hc*) is visible. The *bottom panel* shows a restain of the same membrane as a control for the presence of equal total STAT3 amounts. *B*, inhibition of IL-26-mediated STAT3 phosphorylation by heparin. Purified recombinant IL-26 (10 ng/ml) was preincubated for 15 min with heparin, and Colo-205 cells were then incubated for 20 min before lysis. A Western blot for phosphorylated STAT3 indicated that already 0.1 unit/ml heparin considerably reduced STAT3 phosphorylation in Colo-205. At the *bottom*, total STAT3 Western blots of the same membranes are shown as control for equal protein loading. *C*, inhibitory activity of various glycosaminoglycans on IL-26. After preincubation with the glycosaminoglycans heparin, heparan sulfate, and chondroitin sulfates A, B, and C (100 μg/ml each, corresponding to ~20 units of heparin/ml) for 30 min, the effect of purified recombinant His-IL-26 (10 ng/ml) on Colo-205 cells was analyzed by Western blotting for phosphorylated STAT3. As a control, a total STAT3 Western blot from the same lysates is shown. Whereas heparin completely blocked STAT3 phosphorylation, a weaker inhibitory effect was observed for chondroitin B sulfate (dermatan sulfate). In contrast, heparan sulfate and chondroitin A and C sulfate had little influence on the STAT3 phosphorylation levels.

elements present in the N-terminally adjacent sequence are required (Fig. 1*G*). Such additional structural features may include the presence of particular non-conserved amino acids in IL-26AB or the observation that the N-terminal helices of IL-26 have a decreased tendency to adopt a helical structure, which might be a prerequisite for heparin binding.

Interleukin-26 Activates STAT Signaling in Epithelial Cells—A variety of cell lines were tested for responsiveness to IL-26 stimulation by analysis of STAT phosphorylation. In the epithelial line Colo-205, derived from a human adenocarcinoma of the colon, 1 ng/ml purified, recombinant IL-26 was sufficient to induce STAT3 phosphorylation, and maximal plateau levels of phosphorylated STAT3 were reached at 3–5 ng/ml (Fig. 2*A* and data not shown). STAT3 phosphorylation could be detected as early as 1 min after addition of the cytokine at 10 ng/ml (Fig. 2*B*). Phosphorylation of both STAT3 and STAT1 followed similar kinetics (Fig. 2*C*). STAT phosphorylation was specific for IL-26, because preincubation of IL-26 with 5% (v/v) polyclonal anti-IL-26 antiserum completely blocked the effect, whereas equal amounts of preimmune sera did not (Fig. 3*A*). Similarly, an affinity-purified polyclonal goat antibody against human IL-26 (5 μg/ml; AF1375, R&D Systems) efficiently blocked IL-26-mediated STAT3 phosphorylation, whereas antibodies to IL-10 or IL-20 had no influence (data not shown). In line with the efficient binding of IL-26 to heparin, the IL-26-induced STAT phosphorylation was shown to be blocked in the presence of low heparin concentrations (Fig. 3*B*). The heparin-dependent blockade of IL-26 effects was most pronounced when low concentrations of IL-26 such as 2 ng/ml were used (data not shown). Upon comparison of various glycosaminoglycans at a 100 μg/ml concentration corresponding to ~20 units/ml heparin (Fig. 3*C*), heparin had the most pronounced blocking effects, whereas the structurally most similar heparan sulfate showed weak effects. Among the three classes of chondroitin sulfates, only chondroitin sulfate B (dermatan sulfate) showed an obvious inhibition of IL-26-induced STAT3 phosphorylation (Fig. 3*C*).

In addition to Colo-205, the colon carcinoma lines Lovo and SW-403 responded to IL-26, albeit at a weaker intensity (Fig. 4*A*). The human epithelial keratinocyte line HaCaT (38) reacted to IL-26 treatment, however mainly during early passages of this cell line (Fig. 4*B*). In all these cell lines,

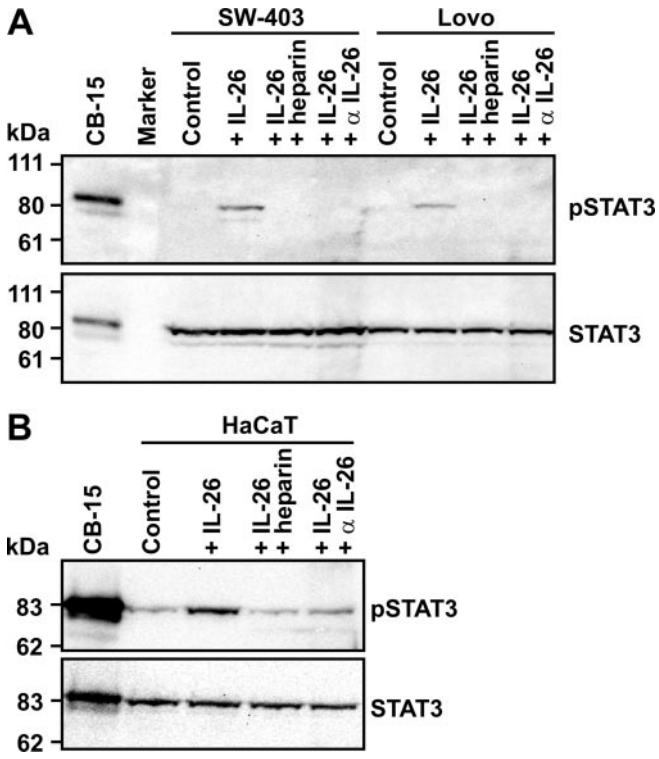

FIG. 4. **IL-26-responsive colon carcinoma cells and keratinocytes.** The human colon carcinoma cell lines SW-403 and Lovo (*A*) and the human keratinocyte cell line HaCaT (*B*), which is known to react to IL-20, responded to treatment with purified, recombinant His-IL-26 (10 ng/ml; 20 min) with STAT3 phosphorylation as detected by phospho-STAT3 specific staining in Western blots. The STAT3 phosphorylation could be blocked by preincubation of the cytokine with heparin (10 units/ml) and by a rabbit antiserum (10% v/v) against IL-26. Lysates from HVS-transformed CB-15 cells served as a positive control. In contrast to the differential pSTAT3 signals, the total STAT3 control Western blots demonstrated equal protein loadings.

IL-26-mediated STAT3 phosphorylation could be blocked by IL-26-specific antibodies or by heparin. In contrast to these cell types, a variety of other cell types did not respond to IL-26 treatment, among them the endocrine colon carcinoma line Colo-320, the pancreatic carcinoma line Panc-I, the cervical cancer line HeLa, the skin carcinoma lines RTS3b and SCC12F, the hepatoma line HepG2, the embryonic kidney line 293T, primary human umbilical vein endothelial cells, as well as the hematopoetic lines Molt-4, KMH-2, and K562 (data not shown).

In the colon carcinoma line Colo-205, IL-10 secretion was observed to be stimulated in a dose-dependent way by IL-26, however rather weakly (Fig. 5*A*). IL-8 levels were moderately inducible in Colo-205 and found more strongly enhanced in Lovo cells, depending on the variable levels of endogenous IL-8 production in Lovo cells. In early passage HaCaT keratinocytes, a strong induction of IL-8 secretion to high levels was observed (Fig. 5*B*). This effect disappeared after prolonged cultivation of the cells. In addition to the IL-26 effects on cytokine secretion, we observed reproducibly that the level of CD54 (ICAM-1) expression on the Colo-205 cell surface was enhanced after treatment with IL-26 for 24 h (Fig. 5*C*). In contrast, neither CD102 (ICAM-2), nor CD50 (ICAM-3) expression were influenced (data not shown).

Components of the IL-26 Receptor—The family of heterodimeric type II cytokine receptors comprises a series of candidate molecules for the IL-26R. In an expression to phenotype correlation study, we analyzed the expression patterns of the candidate receptor molecules IL-10R1, IL-10R2, IL-20R1, IL-20R2, IL-22R, and IL-28R (LICR-2) by RT-PCR and correlated the expression patterns with the IL-26 sensitivity phenotype. Whereas IL-10R1 transcripts showed the expected specificity for hematopoetic cell types, the expression of IL-20R1 strictly correlated with the IL-26 sensitivity of SW-403, Lovo, Colo-205, and HaCaT cells, therefore making IL-20R1 the best candidate for the long receptor chain. The expression patterns of the alternative long chains IL-22R and IL-28R were largely ubiquitous. Whereas IL-20R2 expression was, as expected, specific for keratinocytes (HaCaT), IL-10R2 was ubiquitously expressed and remained the only candidate for the short receptor chain being involved (Fig. 6*A*). This suggested that IL-20R1 and IL-10R2 were the best candidates for forming the IL-26R.

The involvement of these molecules in IL-26 signaling was further investigated using blocking antibodies. Whereas IL-10R1 antibodies did not alter IL-26-mediated induction of STAT3 phosphorylation in Colo-205, this was strongly diminished by the addition of blocking monoclonal antibodies against IL-10R2 prior to IL-26 treatment (Fig. 6*B*). A murine antiserum raised against IL-20R1 had a strong blocking effect after a short preincubation time of 15 min (Fig. 6*C*), whereas it did not affect IL-22-mediated signaling in Colo-205 (data not shown). In addition, solid phase interaction assays indicated a direct binding between the recombinant extracellular domain of IL-20R1 and IL-26 (Fig. 6*D*). Thus, both IL-20R1 and IL-10R2 appear to participate in forming the IL-26R. This hypothesis was finally functionally verified by the transient cotransfection of IL-26 non-responsive Chinese hamster ovary cells with expression constructs for the human IL-20R1 and IL-10R2 chains (Fig. 6*E*). Only if both receptor chains were cotransfected, a strong increase of STAT3 phosphorylation was observed after treatment with IL-26. Therefore, we conclude that IL-26 uses IL-20R1 and IL-10R2 for signal transduction.

DISCUSSION

Whereas IL-26 and the other members of the IL-10 family show sequence conservation with ~25% amino acid identity and 50% similarity, the receptor usage and functions of the various cellular IL-10 paralogs appear diverse (8–12). Among the IL-10 paralogs, the functional context of IL-26 (AK155) remained to be defined (4).

In contrast to the other IL-10 family members, IL-26 is characterized by an unusual number of positively charged amino acids, mainly within helix B. Whereas IL-10 carries similar numbers of positively and negatively charged residues, this balance is shifted in IL-26, which contains 30 Lys or Arg and only 12 Asp or Glu residues, resulting in a predicted isoelectric point of 10.7. The recombinant His-IL-26 protein used in this study was purified from *Escherichia coli* after denaturation via nickel chelate chromatography. The protein did not contain detectable levels of lipopolysaccharides and formed homodimers as shown by non-denaturing gel electrophoresis (4). Moreover, circular dichroism spectra of full-length His-IL-26 showed a maximum at 190 nm and a minimum at 220 nm, characteristic of an intact folded protein (data not shown).

Various attempts to produce recombinant IL-26 using 293T cells, yeast, or insect cells have remained unsuccessful. Endogenous and recombinantly expressed IL-26 protein remained mostly cell-associated and was detectable only in small amounts in the supernatant (4). This is compatible with our observation of an efficient interaction of IL-26 with heparin, presumably because of the strong positive charge of IL-26. In culture supernatants of endogenously expressing or transiently transfected cells, much more IL-26 was detectable in the presence of heparin. Moreover, IL-26 could be enriched on heparin columns and eluted under high salt conditions, and a direct interaction between IL-26 and heparin was suggested by solid

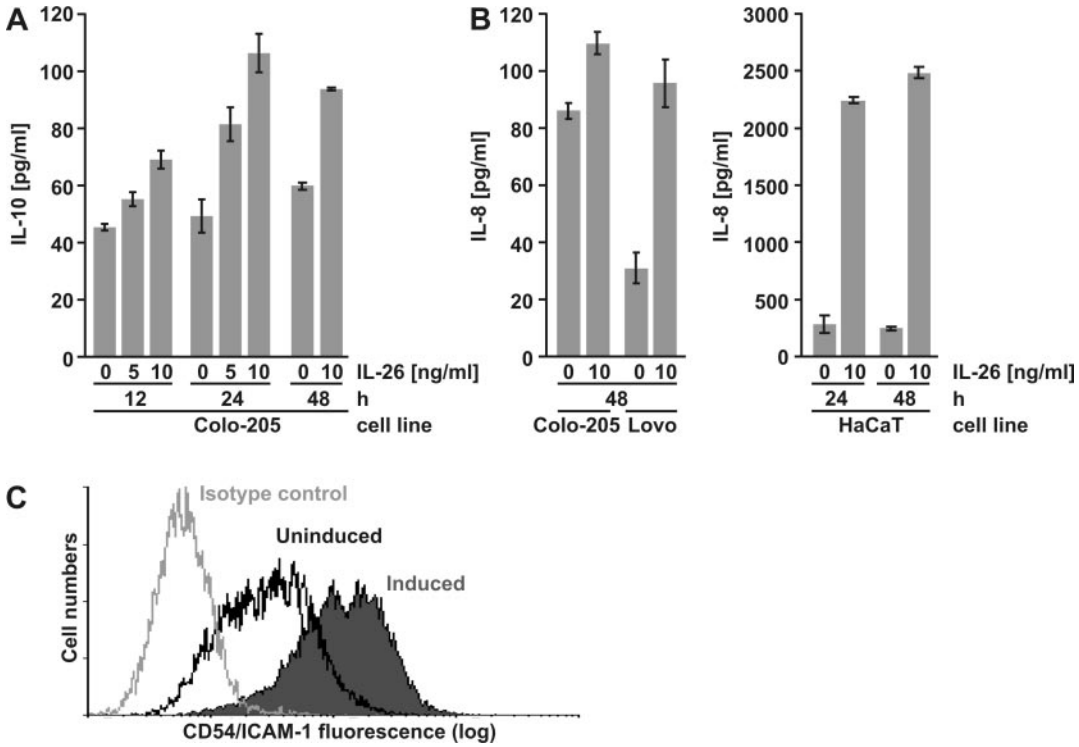

FIG. 5. **Induction of cytokine secretion and CD54 expression by IL-26 in Colo-205 cells.** *A*, induction of IL-10 secretion by IL-26. Colo-205 cells were treated with purified recombinant His-IL-26 (up to 10 ng/ml) for up to 48 h. As measured by ELISA from culture supernatants, IL-26 induced IL-10 secretion in a dose-dependent manner. IL-10 ELISA results are shown at 12, 24, and 48 h incubation time after addition of IL-26. Mean results of triplicate experiments and the standard deviation are shown. Maximal effects were observed at 24 h after stimulation. *B*, induction of IL-8 secretion by IL-26. IL-8 levels were determined by ELISA from culture supernatants from Colo-205, Lovo, and HaCaT cells after treatment with IL-26 (10 ng/ml). Mean results of triplicate experiments and the standard deviation are shown. In Colo-205 cells, IL-26 weakly induced IL-8 secretion. In Lovo colon carcinoma cells, a stronger induction rate was observed, however also only to rather low maximal concentrations. In contrast, the IL-8 concentrations in the supernatants of early passage HaCaT cells were strongly enhanced by IL-26 stimulation. ELISA results are shown for 48 h incubation time in the case of Colo-205 and Lovo, and in HaCaT for 24 and 48 h incubation times. *C*, induction of CD54 surface expression by IL-26. Colo-205 colon carcinoma cells were treated with purified, recombinant His-IL-26 (10 ng/ml). As measured by flow cytometry, the surface expression of CD54 (ICAM-1) was induced after IL-26 treatment with maximal values after 24 h. The *empty gray curve* shows a negative isotype control, the *black curve* represents uninduced Colo-205 cells, whereas the *filled curve* shows CD54 surface expression after IL-26 treatment.

phase binding assays (Fig. 1). Similar effects have also been described for IL-10 and IFN-γ (42, 43); however, apparently at lower affinity, probably because of lower net positive charges of these proteins. Heparin is a glycosaminoglycan, which is rich in negatively charged sulfate groups. Glycosaminoglycans form the polysaccharide moieties of proteoglycans on cell surfaces. The affinity of IL-26 to heparin could result in an immediate binding of secreted IL-26 to surface glycosaminoglycans either of the secreting or of adjacent cells. Thus, cell surface-bound IL-26 could cause local or contact-dependent effects, which may show cell type specificity because of the cell type-specific pattern of proteoglycans.

The cytokine-heparin interaction was further investigated by circular dichroism spectroscopy. Heparin-dependent structural changes were predominantly observed for a peptide representing the helices A and B of IL-26 (Fig. 1*F*), which show considerable flexibility. In contrast, helix B of IL-26 alone was not sufficient for binding heparin (Fig. 1*G*), suggesting that the strongly basic region needs N-terminally adjacent residues for an efficient interaction. Thus, the helix AB region can be suggested as the specific heparin binding domain of IL-26.

On the basis of the almost complete sequence information of the human genome, a series of novel receptors were discovered which belong to the family of heterodimeric type II cytokine receptors (29). With the exception of IL-26, all members of the IL-10 cytokine family had been attributed to one or more receptor molecule combinations: The long IL-20R1 chain also recognizes IL-19 and IL-24, whereas IL-10R2 is utilized by IL-10 and IL-22 (17, 34–36). It is noteworthy that the combination of IL-20R1 and IL-10R2, which is proposed as IL-26R in this study, had not yet been described as a functional receptor combination for any other cytokine. The colon carcinoma line Colo-205 is a useful tool to study different IL-10 family members, because most type II receptor chains are expressed. Colo-205 cells reacted rapidly on treatment with recombinant IL-26 by phosphorylation of STAT3 and also STAT1 at comparable intensities and kinetics (Fig. 2). In addition to Colo-205, the colon carcinoma lines Lovo and SW-403, as well as the keratinocyte line HaCaT reacted to IL-26 stimulation with STAT phosphorylation (Fig. 4). STAT activation could be blocked with IL-26-specific polyclonal antibodies from rabbit and goat, and by low concentrations of heparin, which presumably competes with surface proteoglycans for IL-26 binding at low concentrations (1 unit/ml or 5 μg/ml heparin). Similar concentrations of heparin (4 μg/ml) were described to inhibit IFN-γ binding to Colo-205 cells by 50%, whereas IL-10 effects were reduced by 50% with 20–100-fold higher heparin concentrations (42, 44). Thus, IL-26 seems to utilize a mechanism that has been postulated before for IL-10 and IFN-γ (Ref. 42, and citations therein), however, presumably at comparably higher affinity. By blocking experiments, a preference of IL-26 to heparin and dermatan sulfate (chondroitin B sulfate) was noted in contrast to weak effects of heparan sulfate and chondroitin sulfates A and C. Thus, the IL-26 heparin interaction does not simply reflect the net charge, but also the glycosaminoglycan structure (Figs. 3 and 4). Glycosaminoglycan moieties on proteoglycans could serve as coreceptors, recruiting to and enriching the

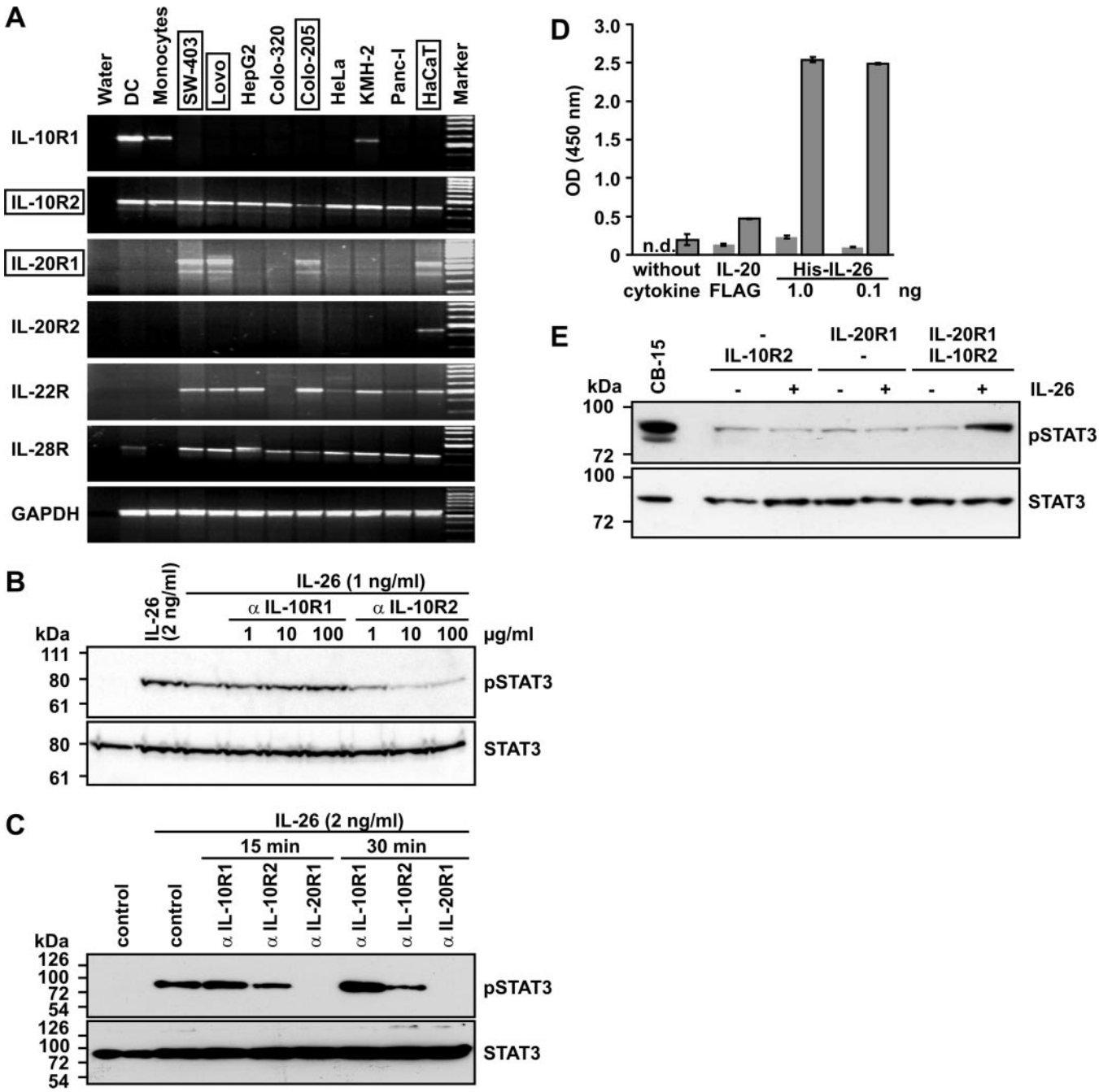

FIG. 6. **Cytokine receptor molecules involved in IL-26 responsiveness.** *A*, expression patterns of cytokine receptor family type II genes. A series of cell types were tested by RT-PCR for the transcription of various members of the cytokine receptor type II family (IL-10R1, IL-10R2, IL-20R1, IL-20R2, IL-22R, IL-28R, and glyceraldehyde-3-phosphate dehydrogenase as positive control): Dendritic cells (*DC*, differentiated *in vitro* from CD14+ monocytes), lipopolysaccharide-activated monocytes, SW-403 (colon carcinoma), Lovo (colon carcinoma), HepG2 (hepatoma), Colo-320 (colon carcinoma), Colo-205 (colon carcinoma), HeLa (cervical carcinoma), KMH-2 (Hodgkin's disease), Panc-I (pancreatic carcinoma), and HaCaT (keratinocytes). The IL-26 responsiveness (cell line designations in *boxes*) strictly correlated with the expression of IL-20R1. The ubiquitous expression pattern of IL-10R2 is compatible with its possible role in forming the IL-26R. *B*, involvement of IL-10R2 in the IL-26 response. Colo-205 cells were treated for 5 min with purified, recombinant His-IL-26 (1–2 ng/ml) without or with preincubation for 6 h with blocking antibodies to IL-10R1 and IL-10R2, respectively. Whereas a blocking monoclonal antibody against the IL-10R1 chain did not influence STAT3 phosphorylation levels of IL-26-treated Colo-205 cells, a dose-dependent (1–100 μg/ml) inhibition was achieved using a blocking monoclonal antibody against IL-10R2. Equal total STAT3 protein amounts were demonstrated by restaining the same membrane (*lower panel*). *C*, participation of IL-20R1 in the IL-26 response. Whereas IL-10R1 antibodies (10 μg/ml) did not influence and IL-10R2 (10 μg/ml) antibodies reduced STAT3 phosphorylation levels of His-IL-26-stimulated (1 ng/ml) Colo-205 cells, a polyclonal murine antiserum raised against IL-20R1 (diluted 1:500) completely blocked STAT3 activation. Equal total STAT3 protein amounts were demonstrated by restaining the same membrane (*lower panel*). *D*, solid-phase interaction test between IL-20R1 and IL-26. Polysterene microtiter plate wells were coated with supernatants from 293T cells (200 μl), which had been transfected with either a control vector (*gray bars*) or an expression construct for the extracellular region of IL-20R1 (*gray boxed bars*). Purified recombinant His-tagged IL-26 was detected via His tag-specific antibodies. 293T-cell supernatants expressing low amounts of FLAG-tagged IL-20, which was detected using a FLAG tag specific antibody, served as a control. Results of a triplicate experiment are shown. *n.d.*, not detectable. *E*, reconstitution of functional IL-26 receptors by transient cotransfection of a non-responsive cell line. Chinese hamster ovary cells were transfected with expression constructs for either IL-10R2 or IL-20R1 or both chains in combination. An IL-26-dependent activation of STAT3 phosphorylation was observed only if both chains were transiently expressed. In contrast to the regulated pSTAT3 signals, the total STAT3 control Western blot shows constant signal intensities (*lower panel*).

cytokine at the cell surface, before specific cytokine receptors are addressed in close local vicinity.

The high affinity of IL-26 to cell surfaces made it difficult to screen candidate IL-26R molecules by conventional affinity studies. We therefore compared the receptor expression patterns of various cell lines, among them the IL-10 reactive Molt-4 or the IL-22-sensitive HepG2, which are not stimulated by IL-26. IL-26 responsiveness correlated with the simultaneous transcription of IL-20R1 and IL-10R2, whereas IL-10R1 and IL-20R2 could be excluded to participate in the IL-26R because of their expression pattern (Fig. 6*A*). The contribution of the ubiquitously expressed IL-28R was excluded by functional experiments (37). Experiments with neutralizing antibodies confirmed the participation of IL-20R1 and IL-10R2 in forming the IL-26R. Whereas a blocking monoclonal antibody to IL-10R1 showed no effect, the preincubation of Colo-205 with anti-IL-10R2 antibodies resulted in a clearly reduced STAT3 phosphorylation after IL-26 treatment (Fig. 6, *B* and *C*) and an anti-IL-20R1 antiserum completely blocked STAT3 phosphorylation. Correspondingly, solid phase binding tests indicated a direct interaction between IL-26 and the extracellular domain of IL-20R1 (Fig. 6*D*), which is in line with recent data (45). After this article had been submitted, Sheikh *et al.* (45) published that the stable transfection of IL-20R1 expression constructs into the human mammary carcinoma cell line MCF-7 reconstituted functional IL-26R in drug-resistant cell clones. Unfortunately, we have been unable to confirm this observation upon transient transfections of MCF-7 or related tumor cell lines. However, by transient transfection of Chinese hamster ovary cells, we have reproducibly shown that IL-26-dependent STAT3 phosphorylation requires the transfection of simultaneously both receptor chains, IL-20R1 and IL-10R2 (Fig. 6*E*).

We thus propose IL-26 as a T-cell cytokine which targets proteoglycans as coreceptors and specific receptors consisting of IL-20R1 and IL-10R2. Receptor engagement induces phosphorylation of STAT1 and STAT3 and leads to altered target gene expression, among them IL-8, IL-10, and CD54 (ICAM-1). Further target genes of IL-26 remain to be determined. The present knowledge would argue in favor of a proinflammatory signal, which might play a role in the leukemogenesis by HVS in susceptible primate species. Because T-cells do not express IL-20R1 as the IL-26R α-chain, a direct autocrine growth-promoting effect of IL-26 upon T-cell transformation by HVS is unlikely. A close interaction of lymphocytes and epithelial cells is typical for γ-herpesvirus pathogenesis and epithelium-associated virus-induced lymphomas. IL-26 is a good candidate to play a role in this interaction as well as in cutaneous or mucosal immunity.

Acknowledgments—We thank Norbert Fusenig (Heidelberg) for providing early passage HaCaT, Irene Leigh (London) for RTS3b, Lawrence Young (Birmingham) for SCC12F cells, Armin Heiss (Heidelberg), and Marehalli Nagalakshmi (Palo Alto) for experimental advice, and Erika Ayllón Ortiz and Lorena Urda (Heidelberg) for technical assistance. We are grateful to Kerry Mills (Heidelberg) and Babak Javid (Cambridge) for critically reading the manuscript.

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