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Antisense Down-regulation of Lipocalin-interacting Membrane Receptor Expression Inhibits Cellular Internalization of Lipocalin-1 in Human NT2 Cells*

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Petra Wojnar, Markus Lechner, and Bernhard Redl‡

From the Department of Molecular Biology, University of Innsbruck, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria

There is increasing experimental evidence demonstrating that many lipocalins bind to specific cell surface receptors. However, whereas the binding of lipocalins to their lipophilic ligands has now been characterized in much detail, there is a lack of knowledge about the nature of lipocalin receptors, the physiological role of receptor binding, and the molecular mechanism of ligand delivery. We previously identified a novel human membrane protein (lipocalin-1-interacting membrane receptor (LIMR)), which interacts with lipocalin-1 (Wojnar, P., Lechner, M., Merschak, P., and Redl, B. (2001) *J. Biol. Chem.* 276, 20206–20212). In the present study, we investigated the physiological role of LIMR and found this protein to be essential for mediating internalization of lipocalin-1 (Lcn-1) in NT2 cells, leading to its degradation. Whereas control NT2 cells rapidly internalized ^{125}I-Lcn-1 or fluorescein isothiocyanate-labeled Lcn-1, NT2 cells that were made LIMR deficient by cDNA antisense expression greatly accumulated Lcn-1 in the culture medium but did not internalize it. Because sequence and structure analysis indicated that proteins similar to LIMR are present in several organisms and at least two closely related orthologues are found in human and mouse, we suggest LIMR to be the prototype of a new family of endocytic receptors, which are topographically characterized by nine putative transmembrane domains and a characteristic large central cytoplasmic loop.

Lipocalins were found to be important extracellular carriers of lipophilic compounds in vertebrates, invertebrates, plants, and bacteria (1–4). There is increasing evidence that this group of proteins is involved in a variety of physiological processes including retinoid, fatty acid, and pheromone signaling; immunomodulation; inflammation; detoxification; modulation of growth and metabolism; tissue development; apoptosis; and even behavior processes (1, 5–7). Whereas the structural basis of lipocalin-ligand binding is now well understood (8), there is a major lack of knowledge regarding the mechanisms by which lipocalins exert their biological effects. It is well accepted that many, if not all, of these proteins are able to bind to specific cell receptors (9), although only two of these receptors have been identified thus far (10, 11). Due to limited data concerning the structure of the lipocalin receptors themselves, the molecular mechanisms beyond this receptor binding are very unclear at the moment. One hypothesis is that the holo-lipocalin releases its ligand upon receptor binding, and this ligand diffuses through the cell membrane to interact with an intracellular fatty acid-binding protein or an intracellular receptor. There is also some evidence that lipocalins undergo internalization by receptor-mediated endocytosis. Another plausible mechanism might be that the lipocalin-receptor interaction creates a direct signal inducing various physiological processes (9).

We have recently identified and characterized LIMR,[1] a novel human 57-kDa cell membrane protein (11), which interacts with Lcn-1, a lipocalin member produced by a number of secretoric glands and tissues and known to bind a variety of lipophilic compounds (12–14). Because we found expression of LIMR in the human NT2 cell line (11), we used an antisense gene knockout technology to investigate the role of this receptor in the mechanism of ligand delivery in more detail. The results of our study clearly demonstrated that LIMR is essential for cellular internalization of Lcn-1. Thus, it has to be classified as a novel endocytic receptor. Because sequence and structure analysis indicated that proteins similar to LIMR are present in several organisms and at least two closely related orthologues are found in human and mouse, we suggest LIMR to be the prototype of a new family of endocytic receptors.

EXPERIMENTAL PROCEDURES

Materials—Human NT2 precursor cells were obtained from Stratagene (La Jolla, CA), and human T-47D breast cells (ATCC HTB-133) were obtained from American Type Culture Collection (Manassas, VA). Cell culture media, fetal bovine serum, and other cell culture materials were purchased from PAA Laboratories Inc. (Parker Ford, PA).

Cell Culture—NT2 cells were propagated in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12K supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in 75-cm^2 culture dishes under 5% CO_2 at 37 °C. T-47D cells were grown at 37 °C in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.2 unit/ml bovine insulin and 10% fetal bovine serum.

Vector Construction—To construct a vector expressing LIMR antisense RNA, a cDNA fragment corresponding to nucleotides 332–2349 (1917 nucleotides) of LIMR (11) was amplified by PCR using primers to which *Xba*I/*Xho*I restriction enzyme cutting sites were attached (5′-CTAGCGTCTAGAATGGAAGCACCTGACTAC-3′ and 5′- TTTATCTCGAGTCAGGTGGTCCAAAGCCC-3′). The PCR product was gel-purified, digested with *Xba*I/*Xho*I restriction enzymes, and ligated with *Xba*I/*Xho*I-digested vector pOPRSVI/MCS (Stratagene) in an antisense orientation. The resulting plasmid (pOPRSVI-AS-LIMR) was verified by DNA sequencing. A religated plasmid backbone pOPRSVI/MCS was used as a transfection control.

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‡ To whom correspondence should be addressed. Tel.: 43-512/507-3603; Fax: 43-512/507-2866; E-mail: bernhard.redl@uibk.ac.at.

[1] The abbreviations used are: LIMR, lipocalin-1-interacting membrane receptor; AS, antisense; FITC, fluorescein isothiocyanate; Lcn-1, lipocalin-1 (identical to tear lipocalin or human von Ebner's gland protein); RBP, retinal-binding protein; MEM, maintenance medium.

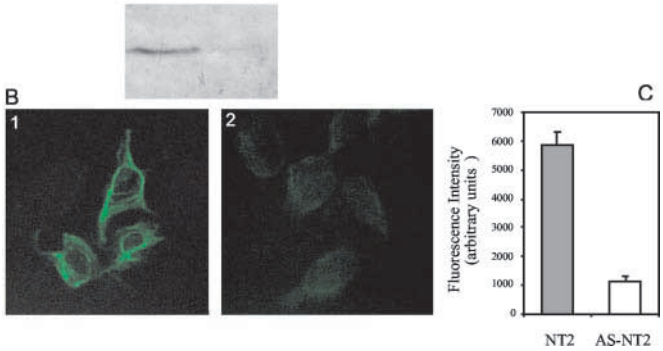

FIG. 1. **LIMR antisense expression reduces LIMR protein expression in NT2 cells.** *A*, equal amounts of proteins from membrane preparations were separated by SDS-PAGE (10%) and analyzed by immunoblotting using a LIMR-specific antiserum. *Lane 1*, NT2-mock cell membranes; *lane 2*, AS-NT2 cell membranes. *B*, LIMR immunostaining of NT2 cells transfected with basic vector (NT2-mock) or with AS-LIMR. *C*, bar graphs showing the reduction of LIMR expression in AS-NT2-transfected cells, indicated as the mean ± S.E. of fluorescence intensities. The plotted data are mean values of pixel intensity derived from analysis with ImageQuant software (Amersham Biosciences). The LIMR-specific fluorescence intensity in AS-NT2 cells (□) is 80.56% lower than that in control cells (▨).

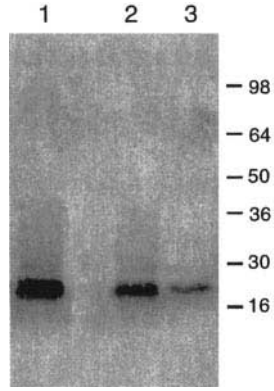

FIG. 2. **Accumulation of Lcn-1 in medium from AS-NT2 cells as detected by Western blot analysis using Lcn-1-specific antibodies.** Cells were grown to 5×10^4 cells/ml, and 25 μl of each medium was subjected to SDS-PAGE (14%) and immunoblotting. *Lane 1*, recombinant Lcn-1 protein (2 μg); *lane 2*, medium from AS-NT2 cells; *lane 3*, medium from NT2 cells.

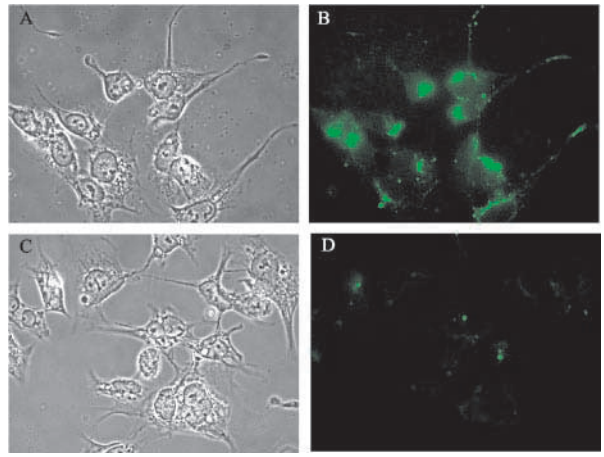

FIG. 3. **Internalization of FITC-Lcn-1 by NT2 cells.** *A* and *C*, phase-contrast micrographs of NT2 cells. *B*, micrograph of control NT2 cells incubated with FITC-Lcn-1. *D*, micrograph of LIMR-deficient AS-NT2 cells. FITC-labeled Lcn-1 was added to a final concentration of 50 μg/ml. Cell surface-bound ligand that had not been internalized was removed with 50 mM glycine, 150 mM NaCl, pH 3.0 buffer. Control cells incubated with unconjugated FITC showed no fluorescence (data not shown). Immunofluorescence slides were viewed with a ×63 objective on a Carl Zeiss Axioplan2 microscope.

Establishment of an Antisense-transfected NT2 Cell Line—Stable transfection of NT2 cells was carried out using MBS Mammalian Transfection Kit (Stratagene) according to the manufacturer's recommendation. Forty-eight h after transfection, cells were selected in culture medium containing 400 μg/ml G418. Twenty-five days after selection, individual G418-resistant colonies were subcloned. Five subclones of LIMR antisense- and mock-transfected cells were analyzed by immunoblotting analysis and immunofluorescence for LIMR expression.

Protein Labeling—Purified Lcn-1 was labeled with $Na^{125}I$ (Amersham Biosciences) using IODO-BEAD Iodination Reagent as described by the supplier (Pierce). To remove excess $Na^{125}I$ or unincorporated $^{125}I_2$ from the iodinated protein, dialysis against phosphate-buffered saline was performed using dialysis cassettes (Pierce). The proteins had specific activities ranging from 0.33 to 0.99 GBq/mg.

FITC-labeled Lcn-1 (FITC-Lcn-1) was prepared by incubating purified Lcn-1 (5 mg/ml) with 5 mg/ml FITC (Sigma-Aldrich) in 200 mM sodium bicarbonate buffer, pH 9.0, for 20 h at 4 °C. Unconjugated FITC was removed by gel filtration on PD-10 columns (Amersham Biosciences).

FITC-Lcn-1 Uptake of Control NT2, AS-NT2, and T-47D Cells Analyzed by Fluorescence Microscopy—Cells were grown on coverslips for 18 h at 37 °C in complete medium (minimal essential medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 for AS-NT2 cells; RPMI 1640 medium for T-47D cells). The cells were washed once with serum-free medium and incubated for 3 h in serum-free medium at 37 °C. FITC-labeled Lcn-1 was added (final concentration, 50 μg/ml each) and incubated for 1 h at 37 °C. To remove cell surface-bound ligand that had not been internalized, cells were washed twice with phosphate-buffered saline and treated with 50 mM glycine, 150 mM NaCl, pH 3.0, for 2 min. The cells were washed with Dulbecco's phosphate-buffered saline, fixed in 100% ice-cold acetone for 5 min, and analyzed with a ×63 objective on a Carl Zeiss Axioplan2 microscope. The images were resized and saved as 8-bit.tif files by using the commercially available software MetaMorph® Imaging System (Universal Imaging Corp., Visitron GmbH, Germany).

Ligand Internalization and Degradation Experiments Using Radiolabeled Lcn-1—Cells were seeded into 2.0-cm^2 wells and grown to 90% confluence with ~3×10^5 cells attached/cm^2. The cells were then washed with maintenance medium (MEM) and incubated in the same medium containing ^{125}I-labeled Lcn-1 (5 nM) at 4 °C for 2 h. Afterward, cells were washed three times with cold MEM (0.5 ml), warmed up to 37 °C by the addition of prewarmed MEM, and incubated at 37 °C for selected intervals. At each time point, the cells were washed once with ice-cold MEM and treated with a solution containing trypsin (0.5 mg/ml), proteinase K (0.5 mg/ml), and 5 mM EDTA in MEM for 15 min at 4 °C to strip cell surface proteins (15, 16). The cell suspension was then centrifuged, and the radioactivity associated with cell pellets (defining internalized ^{125}I-labeled Lcn-1) was measured. For determination of degradation rate, cells were incubated with 5 nM ^{125}I-Lcn-1/well, and the culture medium was collected at various times. The radioactivity appearing in the cell culture medium that was soluble in 10% trichloroacetic acid was corrected for non-cellular-mediated degradation by subtracting the amount of degradation in control wells lacking cells and was taken to represent degraded ligands.

Immunocytochemistry—Conventional immunohistochemistry was performed as described previously (11) using LIMR-specific primary antibodies at a dilution of 1:100 and FITC-labeled secondary antibodies (DAKO, Copenhagen, Denmark).

Membrane Preparation—Cell membranes were prepared using the Mem-Per Mammalian Membrane Protein Extraction Kit (Pierce) according to the manufacturer's recommendations.

RESULTS

Lack of LIMR Production in Antisense-transfected Cells—After transfection with pOPRSVI-AS-LIMR or a control vector, NT2 precursor cells were selected using G418. The presence of the antisense plasmid in genomic DNA was verified by PCR using plasmid-specific primers (data not shown). In a first step, the expression of LIMR in plasmid-transfected and control cells was investigated by Western blot analysis. As indicated in Fig. 1*A*, no significant expression of LIMR could be detected in AS-NT2 cells. To confirm this result, we performed immunofluorescence analysis using LIMR-specific antibodies. Whereas

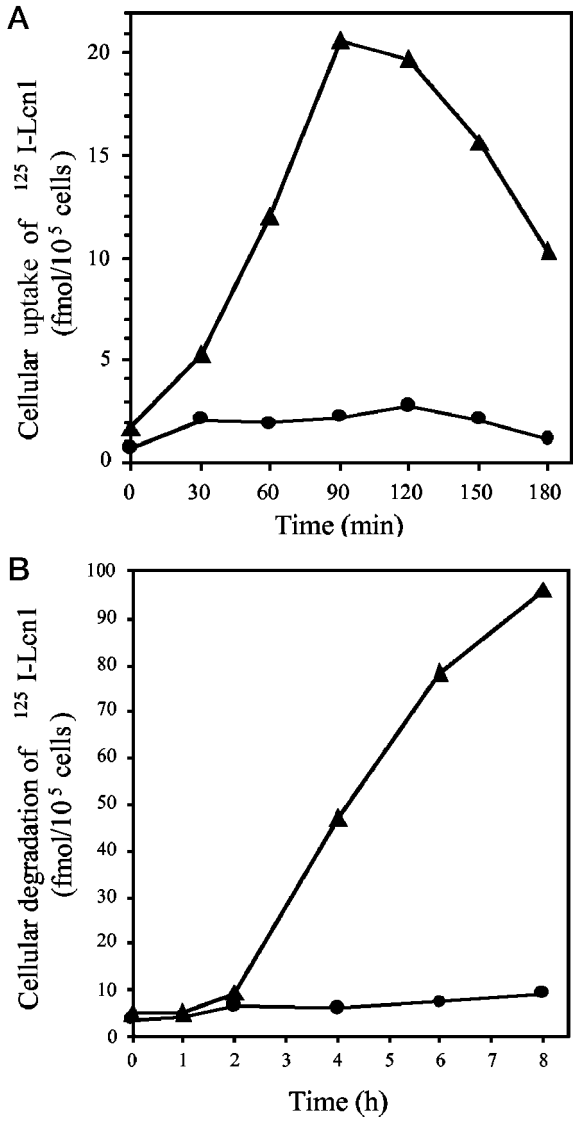

FIG. 4. **LIMR antisense-transfected NT2 cells do not internalize and degrade ^{125}I-Lcn-1.** *A*, internalization of ^{125}I-Lcn-1 by control NT2 cells (▲) and antisense-transfected NT2 cells (●). Control NT2 cells and AS-NT2 cells were preincubated in the presence of ^{125}I-labeled Lcn-1 (5 nM) for 2 h. After washing, cells were warmed to 37 °C and treated at the time points indicated with trypsin/proteinase K to remove cell surface-bound radioactivity. The remainder was collected, and radioactivity was counted. *B*, degradation of ^{125}I-Lcn-1 by control NT2 cells (▲) and antisense-transfected NT2 cells (●). Control NT2 cells and AS-NT2 cells were incubated with ^{125}I-Lcn-1 (5 nM) for selected time intervals at 37 °C. At the indicated times, the amount of degraded radioligand was determined by counting the radioactivity appearing in the cell culture medium that was soluble in 10% trichloroacetic acid.

the control cells showed a clear and specific staining of the cell membrane, a highly reduced staining was observed with the antisense-transfected cells (Fig. 1*B*). We used ImageQuant software (Amersham Biosciences) to compare the immunofluorescence intensity of pOPRSVI-AS-LIMR-transfected cells and control NT2 cells. We found that the fluorescence intensity of the signal corresponding to the expression of LIMR was reduced by more than 80% in antisense-transfected cells relative to the intensity of the LIMR signal in mock-transfected cells (Fig. 1*C*).

Lcn-1 Accumulation in the Medium of LIMR-deficient Cells—As described previously, NT2 precursor cells produce a small amount of Lcn-1 present in the medium under the conditions used (17). To test whether there is an effect on the presence of Lcn-1 in the medium of LIMR-deficient cells, we

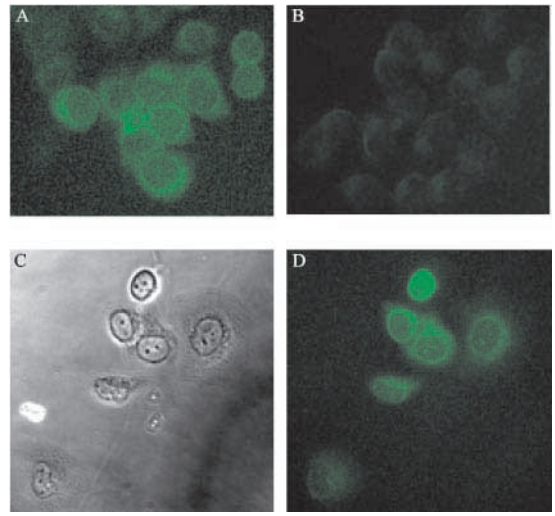

FIG. 5. **LIMR expression and internalization of FITC-Lcn-1 by human T-47D breast cells.** Immunofluorescence showing T-47D cells incubated with LIMR antiserum (*A*) and T-47D cells incubated with only FITC-conjugated secondary antibodies as a control (*B*). *C*, phase-contrast micrograph of T-47D cells. *D*, micrograph of T-47D cells incubated with FITC-Lcn-1. FITC-Lcn-1 incubated cells were treated as described in the Fig. 3 legend. Control cells incubated with unconjugated FITC showed no fluorescence (data not shown). Immunofluorescence slides were viewed with a ×63 objective on a Carl Zeiss Axioplan2 microscope.

compared this medium with medium from an equal amount of wild type or mock-transfected cells by Western blotting. As shown in Fig. 2, a significant increase in the presence of Lcn-1 was found in the medium of LIMR-deficient cells compared with the controls. This result gave us a first indication that there was an accumulation of Lcn-1 that was probably associated with a block in endocytosis, similar to that described for major urinary protein in megalin-deficient mice (10), and that LIMR might be an essential factor for Lcn-1 endocytosis.

Inhibition of Lcn-1 Internalization in LIMR-deficient Cells—To determine whether LIMR functions to mediate endocytosis of Lcn-1, we compared the ability of NT2 control cells and LIMR-deficient cells to internalize Lcn-1. To visualize the uptake, FITC-Lcn-1 was added to the cultured cells. The cells were fixed and examined by fluorescence microscopy. As demonstrated in Fig. 3, fluorescence was seen in discrete granules of the NT2 cells but not in LIMR-deficient cells.

To confirm these results and to study the kinetics of endocytosis, cells were incubated in the presence of ^{125}I-Lcn-1 for 2 h at 4 °C to allow binding of radiolabeled ligand. After washing and incubation for various times, the radioactivity associated with the cell pellet was counted. As indicated in Fig. 4*A*, the bound radioactivity was internalized within 2 h in the NT2 control cells. In contrast to NT2 control cells, no significant uptake of ^{125}I-Lcn-1 could be found in AS-NT2 cells (Fig. 4*A*), thus demonstrating that LIMR is essential for endocytosis of Lcn-1 and supporting the result obtained with FITC-Lcn-1.

Uptake of ^{125}I-Lcn-1 in NT2 control cells was followed by successive degradation, as indicated by the increasing amount of radioactivity that was secreted into the cell culture medium and soluble in 10% trichloroacetic acid (Fig. 4*B*).

Lcn-1 Internalization Is Not Unique to NT2 Cells—To investigate whether Lcn-1 internalization is a specific feature of NT2 cells or a more general mechanism, we tested another cell line. Because it is known that both LIMR and Lcn-1 are expressed in mammary gland (11, 18), we searched for human breast cells expressing LIMR. It is evident from immunostaining using LIMR antibodies that human T-47D cells (19) express LIMR (Fig. 5*A*). Therefore, this cell line was tested for uptake of

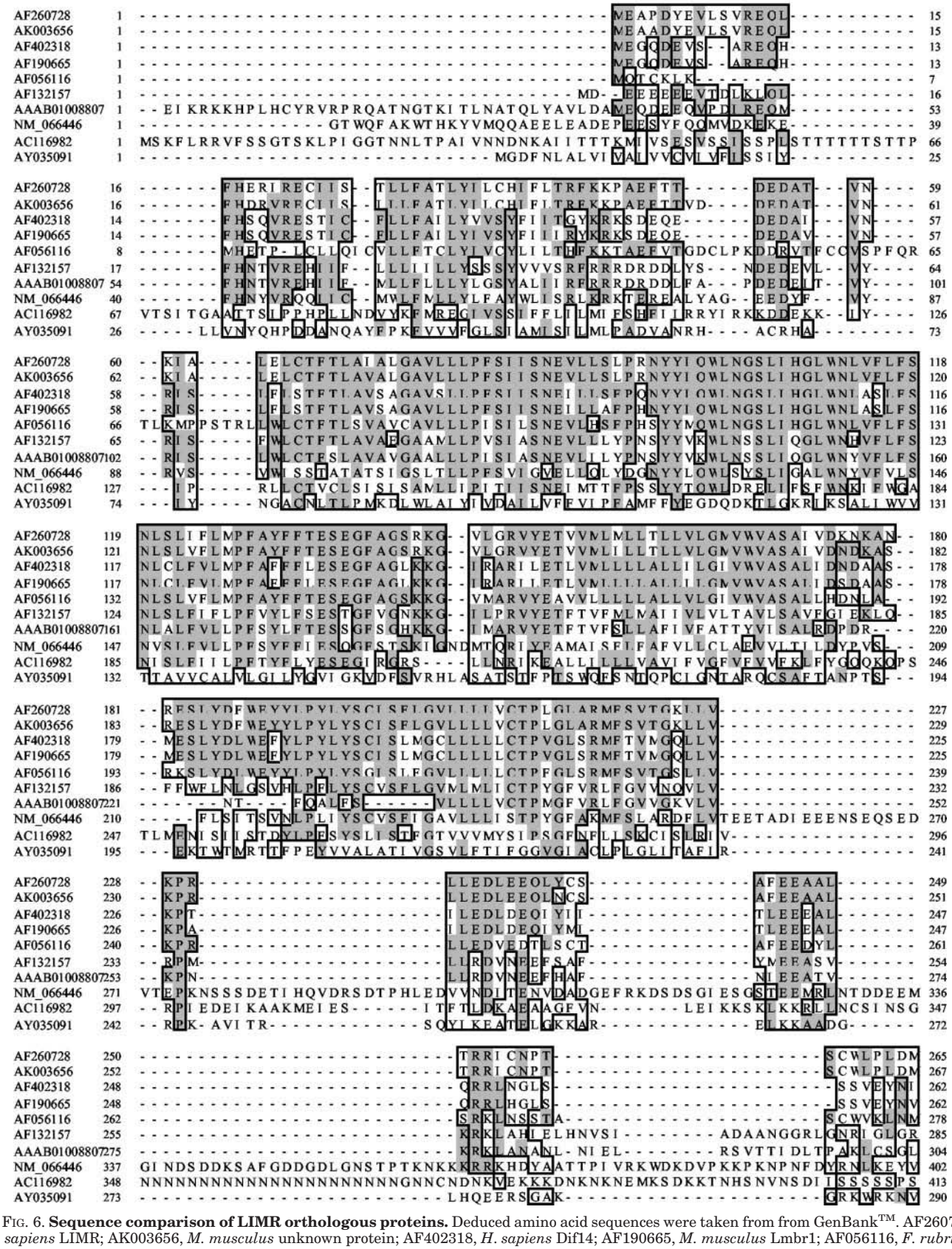

FIG. 6. **Sequence comparison of LIMR orthologous proteins.** Deduced amino acid sequences were taken from from GenBank™. AF260728, *H. sapiens* LIMR; AK003656, *M. musculus* unknown protein; AF402318, *H. sapiens* Dif14; AF190665, *M. musculus* Lmbr1; AF056116, *F. rubripes* unknown protein; AF132157, *D. melanogaster* unknown protein; AAAB01008807, *A. gambiae* strain PEST unknown protein; NM_066446, *C. elegans* unknown protein; AC116982, *D. discoideum* unknown protein; AY035091, *A. thaliana* unknown protein. Identical residues are *gray-shaded*, homologous residues are *boxed*.

FITC-Lcn-1. Fig. 5*D* demonstrates that incubation with FITC-Lcn-1 resulted in an intracellular immunostaining similar to that obtained with NT2 cells. Thus, it is evident that Lcn-1 is also internalized by T-47D cells.

LIMR Is the Prototype of a New Family of Membrane Proteins—LIMR was described to be a novel protein with no sig-

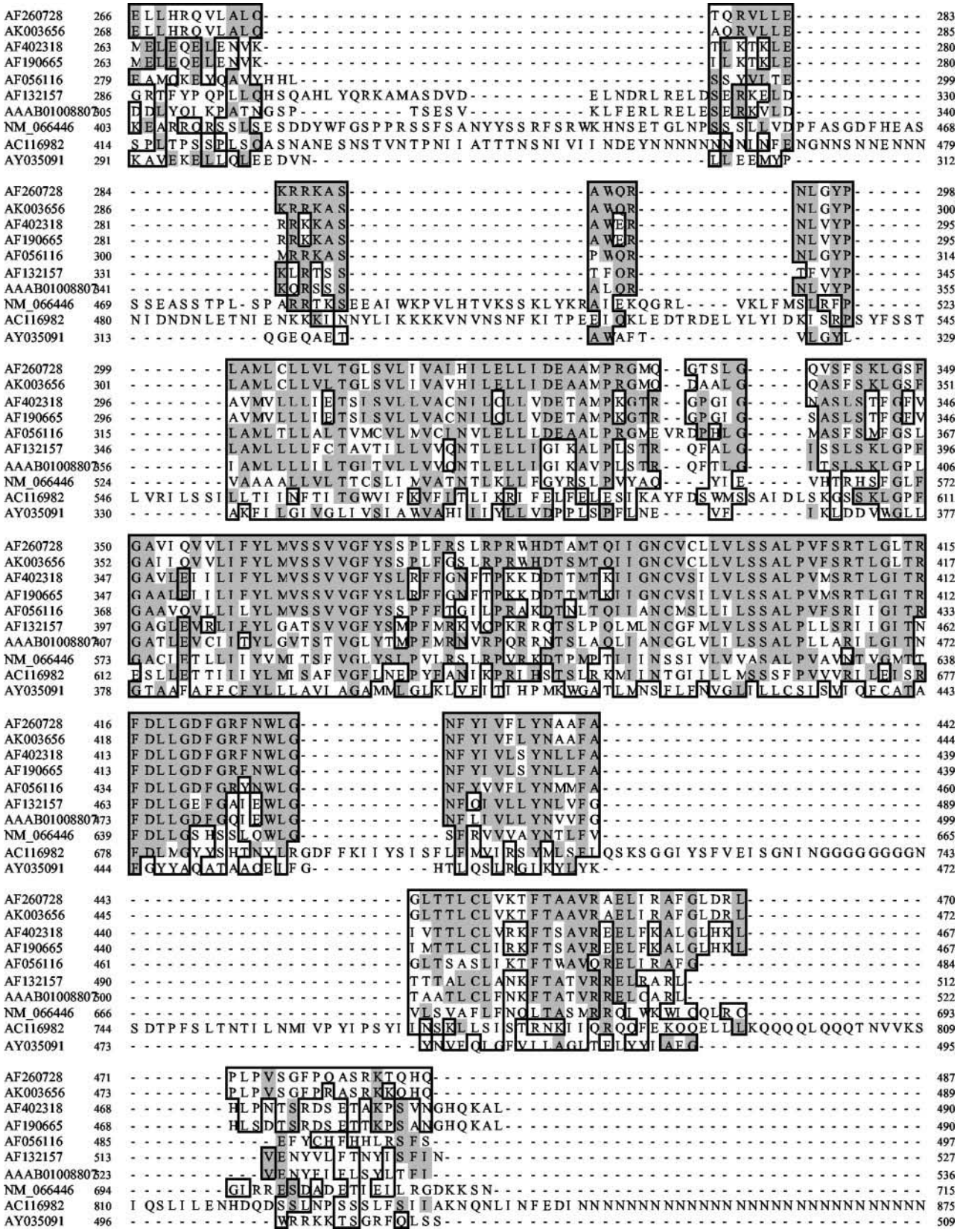

FIG. 6—*continued*

nificant similarity to known proteins (11). However, we found a number of thus far uncharacterized genes with statistically significant deduced amino acid similarities (Fig. 6). These include two proteins from *Mus musculus*, an uncharacterized protein (GenBank™ accession number AK003656) with an amino acid sequence identity of 94.46% and the Lmbr1 protein with an identity of 57.91% (GenBank™ accession number AF190665). Dif14 from *Homo sapiens* (GenBank™ accession number AF402318), the human counterpart to *M. musculus* Lmbr1, shows 58.32% identity to LIMR. In addition, putative

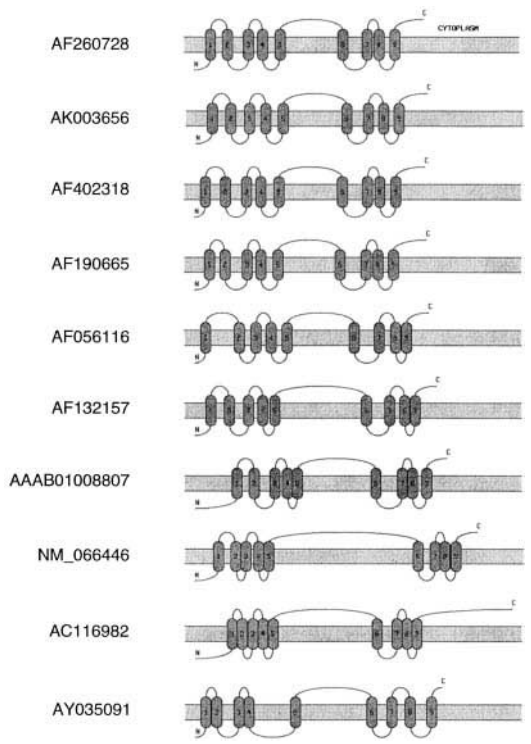

FIG. 7. **Putative topography of LIMR orthologous proteins.** The analysis was performed using TopPred 2 (22), the topology prediction program of membrane proteins at Stockholm University. Proteins are numbered as described in the Fig. 6 legend.

proteins from *Fugu rubripes* (GenBank™ accession number AF056116), *Caenorhabditis elegans* (GenBank™ accession number NM066446), *Drosophila melanogaster* (GenBank™ accession number AF132157), *Anopheles gambiae* strain PEST (GenBank™ accession number AAAB01008807), *Dictyostelium discoideum* (GenBank™ accession number AC116982), and *Arabidopsis thaliana* (GenBank™ accession number AY035091) were found to show amino acid sequence identities of 56.26%, 28.96%, 42.51%, 42.09%, 20.33% and 9.89%, respectively. Interestingly, a detailed comparison of the N terminus of LIMR, which was found by phage-display to be the Lcn-1-interacting domain (11), with the orthologous human Dif14 and mouse Lmbr1 indicated this region to be less conserved than the rest of the proteins. Within this region, there is an amino acid identity of only 12.5% between human LIMR and human Dif14, whereas the overall identity is 58.32%. A similar result is also found comparing the mouse LIMR and the orthologous Lmbr1, with an identity of 37.5% within the N terminus and 57.91% with the entire protein.

Because LIMR was clearly characterized as a membrane protein (11), we performed a secondary structure prediction and prediction of putative transmembrane regions of all the orthologues found using three different prediction programs (20–24). The results presented in Fig. 7 indicate a striking similarity in the topography of these proteins. All of them consist of nine transmembrane regions with an outside N terminus and a C terminus orientated toward the cytoplasm. Most noteworthy, all of these proteins contain a large central intracellular loop consisting of 90–150 amino acids. Due to the highly conserved topography and the fact that, at least in human and mouse, two distinct orthologues are present, we propose this group of proteins to be a novel family of membrane proteins. Because LIMR, which is suggested to be the prototype of this protein family, is essential for endocytosis of Lcn-1, it might be speculated that all of these proteins function as endocytic receptors.

DISCUSSION

A prerequisite for understanding the physiological function of lipocalins is some knowledge about the mode of delivering the bound ligands. There is increasing evidence that many lipocalins bind to specific cell receptors. Receptor binding has been demonstrated for α-1-microglobulin (25, 26), glycodelin (27), insecticyanin (28), α-1-acid glycoprotein (29, 30), β-lactoglobulin (31, 32), and odorant-binding protein (33). Although this is still a controversial area, there is considerable evidence that retinol-binding protein (RBP) binds to its target cells via specific surface receptors (34, 35). In addition, major urinary protein has been found to bind to megalin (10), a member of the low density lipoprotein receptor-related protein family (36). However, with the exception of megalin, none of these cell receptors has been isolated or characterized on a molecular level. We have recently isolated a novel human cell membrane protein, LIMR, which interacts with Lcn-1 (11). Lcn-1 is a lipocalin member produced by a number of human secretory glands and tissues that is known to bind a variety of ligands, including fatty acids, fatty alcohols, cholesterol, retinol, retinoic acid, phosphatidylcholine, and arachidonic acid and its peroxidation products (12, 13). Several biological functions have been proposed for this lipocalin, but its main function seems to be clearance or detoxification of lipophilic, potentially harmful compounds (14, 17). We have therefore suggested LIMR to be directly involved in this process. However, the exact role of LIMR had to be established. The data presented in this paper clearly demonstrate that LIMR mediates cellular uptake of Lcn-1, thus it has to be characterized as a novel endocytic receptor.

In general, several models have been discussed for the ligand transfer from lipocalins to the cell interior, including releases of the ligand upon receptor binding and transport or diffusion through the cell membrane or internalization of the lipocalin-ligand complex by receptor-mediated endocytosis. Another model suggests that the lipocalin-receptor interaction creates a direct signal inducing various physiological processes (37). Most experimental data concerning the uptake mechanism of lipocalin ligands came from studies on RBP and its physiological ligand, retinol. Although there are some conflicting reports, it seems that there is a cell type-specific difference in the release of retinol from RBP to the cell interior. In retinal pigment epithelial cells and placental brush-border membranes, it was suggested that RBP remains external to the cell, with only retinol internalized (38, 39). Therefore the RBP receptor is supposed to act as a channel for retinol. By contrast, in hepatocytes and epithelial kidney cells, RBP was clearly demonstrated to undergo internalization via receptor-mediated endocytosis and subsequent degradation, thereby releasing the retinol (40, 41). Megalin seems to be involved in the epithelial endocytic uptake of RBP (42). Endocytic uptake mediated by megalin was also demonstrated for major urinary protein (10), a lipocalin member that binds small natural odorants and is highly secreted by the liver and filtered by the kidney into the urine of adult male mice and rats (43).

Although both LIMR and megalin are involved in endocytic uptake of lipocalins, there is no structural similarity between these proteins. Whereas megalin has the typical structure of the low density lipoprotein receptor family consisting of a large extracellular domain containing different ligand-binding clusters, a single transmembrane domain, and a short cytoplasmic tail (44), LIMR consists of a short extracellular domain, nine transmembrane domains interrupted by a large intracellular loop, and an intermediate-length cytoplasmic tail (11).

Considering that Lcn-1 binds physiological ligands of a number of different chemical classes, cellular internalization of the

whole protein-ligand complex would be superior to a mechanism where the ligand is delivered to a receptor. However, as indicated by the results obtained with RBP, it seems that internalization of the protein-ligand complex is also preferred by a lipocalin that is very specific for binding of retinol. In addition, α-1-acid glycoprotein is another lipocalin that was found to be endocytosed by a specific receptor (26). Therefore, internalization of the whole lipocalin-ligand complex seems to be the preferential mechanism.

Whether LIMR is a specific lipocalin receptor or rather a multifunctional receptor, similar to megalin, remains to be proven by additional studies. However, in contrast to megalin, which consists of a large ligand binding cluster (45), the N-terminal ligand binding domain of Lcn-1 is small, indicating some specificity. In this context, the low amino acid similarity within the N-terminal region of LIMR, which contains the Lcn-1 binding domain, and that of human Dif14 supports this suggestion.

It is novel, but not unexpected, that there are a number of genes encoding LIMR orthologous proteins in other organisms, including *M. musculus*, *F. rubripes*, *D. melanogaster*, *A. gambiae*, *C. elegans*, and *D. disccoideum*. Partial cDNA sequences with significant similarities were also found in expressed sequence tag databases from *Macaca mulatta* (GenBank™ accession number BQ807894), *Bos taurus* (GenBank™ accession number BE685341), *Sus scrofa* (GenBank™ accession number BE014550), *Rattus norvegicus* (GenBank™ accession number BF565677), and *Xenopus laevis* (GenBank™ accession number BQ732574). Most interestingly, in the human, there is another LIMR orthologous protein called Dif14, encoded on chromosome 7q36 (46), whereas LIMR is encoded on chromosome 12p11 (11). As in human, in mouse there are also two closely related proteins; one is highly similar to human LIMR, whereas the other, Lmbr1, is more similar to human Dif14. Human and mouse Dif14/Lmbr1 is of considerable interest in genetics because it was suggested to be involved in preaxial polydactyly, one of the most frequently observed congenital limb malformations, whereby a disruption of the Dif14/Lmbr1 gene was speculated to be the basis of this malformation (46, 47). However, more consistent with the function of an endocytosis receptor, as proposed here, recent work demonstrated that misexpression of a cis-acting regulator located within the same respective intron of the Dif14/Lmbr1 gene is the basis for preaxial polydactyly and that the Lmbr1 gene is incidental to the phenotype (48).

The fact that there are orthologous proteins in one and the same organism that are encoded by different chromosomes prompted us to define these proteins as a novel family of putative endocytic receptors. Thus far, this family may be grouped into two branches. According to the amino acid similarities and the deduced dendrogram of a CLUSTAL-W-PHYLIP analysis, the first group consists of *H. sapiens* LIMR, *M. musculus* AK003656, and the putative proteins from *F. rubripes* (AF056116), *D. melanogaster* (AF132157), and *C. elegans* (NM066446), whereas the second group consists of *H. sapiens* Dif14, *M. musculus* Lmbr1, and probably the proteins from *A. gambiae* strain PEST (AAAB01008807), *D. discoideum* (AC116982), and *A. thaliana* (AY035091).

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