Identification of Uteroglobin-Related Protein 1 and Macrophage Scavenger Receptor with Collagenous Structure as a Lung-Specific Ligand-Receptor Pair

Liang-Hua Bin, Larry D. Nielson, Xinqi Liu, Robert J. Mason and Hong-Bing Shu

*J Immunol* 2003; 171:924-930; doi: 10.4049/jimmunol.171.2.924
http://www.jimmunol.org/content/171/2/924

**References**

This article cites 29 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/171/2/924.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Identification of Uteroglobin-Related Protein 1 and Macrophage Scavenger Receptor with Collagenous Structure as a Lung-Specific Ligand-Receptor Pair

Liang-Hua Bin,* Larry D. Nielson,† Xinqi Liu,‡ Robert J. Mason,† and Hong-Bing Shu2*†§

High in normal (HIN)-1 is a secreted protein highly expressed in normal human breast epithelium and down-regulated in breast carcinomas. By searching GenBank expressed sequence tag databases, we identified HIN-2, a protein homologous to HIN-1. HIN-2 is identical with a recently identified protein called uteroglobin-related protein 1 (UGRP1). Northern blot analysis demonstrated that UGRP1 is specifically expressed by lung, not by the other tissues examined. By in situ hybridization experiments, UGRP1 was shown to be expressed by lung Clara-like cells in the bronchial epithelium and to be up-regulated in cystic fibrosis. In a mammalian expression system, secreted recombinant UGRP1 was copurified with apolipoprotein A-I. Using a retroviral vector-mediated expression cloning approach, we identified macrophage scavenger receptor with collagenous structure (MARCO) as a receptor for UGRP1. Northern blot and in situ hybridization experiments indicated that MARCO is expressed by alveolar macrophages in the lung. UGRP1 also bound to bacteria and yeast. LPS, a previously identified MARCO ligand, competed with UGRP1 for binding to MARCO and bacteria. Our findings suggest that UGRP1-MARCO is a ligand-receptor pair that is probably involved in inflammation and pathogen clearance in the lung.

UGRP1 was identified in a suppressive-subtractive hybridization experiment using lung mRNAs from normal and TTF-1 (also called NKX2.1 and T/EBP) knockout mice (2). TTF-1 is a homeodomain-containing transcription factor that is critically involved in thyroid- and lung-specific expression of genes, such as the thyroid-specific thyroglobulin, thyroid peroxidase, and thyroid-stimulating hormone receptor, and the lung-specific surfactant proteins-A, -B, and -C (4–10). Targeted disruption of the TTF-1 gene results in a deficiency of the thyroid and pituitary glands and the immediate postnatal death of the knockout mice due to respiratory failure caused by severe hypoplastic lungs (11). These studies indicate critical roles for TTF-1 and its downstream genes in physiological and pathological processes of the lung, thyroid, and pituitary gland.

In this paper, we describe the further characterization of HIN-2/UGRP1. Using an expression cloning approach, we also identified macrophage scavenger receptor with collagenous structure (MARCO) (12–17) as a receptor for HIN-2/UGRP1. Our findings suggest that HIN-2/UGRP1 and MARCO are a novel ligand-receptor pair involved in lung inflammation and pathogen clearance in the lung.

Materials and Methods

Cells and reagents

293 and U937 (American Type Culture Collection, Manassas, VA), 292–10A1 (Imgenex, San Diego, CA), and lung cancer A549, H28, H157, H290, H322, H441, H460, and H661 cells (Colorado University Cancer Center, Denver, CO) were obtained. U937 cells were cultured in RPMI 1640 medium containing 10% FBS. All other cell lines were cultured in DMEM medium containing 10% FBS. The bacteria strains Listeria monocytogenes NP1.0 (Dr. T. Potter (National Jewish Medical and Research Center, Denver, CO)) were provided. The anti-FLAG mAb was purchased from Sigma-Aldrich (St. Louis, MO). N-terminal FLAG-tagged soluble TALL-1 (sTALL-1-FLAG) was produced in 293 cells and purified by anti-FLAG Ab affinity columns as described previously (18).

Plasmids

To construct the mammalian expression plasmid for FLAG-UGRP1, a cDNA fragment encoding full-length human UGRP1 was amplified from a...
UGRP1 EST clone by PCR with the following two primers: 5′-cggaatct-cccggaattc-3′ and 5′-acatctagacaccaagtgtgatagc-3′. To construct the mammalian expression plasmid for FLAG-HIN-1, a cDNA fragment encoding full-length human HIN-1 was amplified from a HIN-1 EST clone by PCR from full-length cDNAs that were previously cloned into plasmid pGEM-T easy (Promega, Madison, WI).Sections were hybridized and processed as described previously (19).

Transfection

Transfection of 293 and 293–10A1 cells was performed with a standard calcium phosphate precipitation method (20).

Western blotting

To detect UGRPI-FLAG expression, 293 cells (~3 × 10^6) were transfected with 10 μg of pRK-C-FLAG-UGRP1 or an empty control plasmid. Forty-eight hours after transfection, 5 ml of cell culture medium was collected and lyophilized. The lyophilized powder was dissolved in 500 μl of 1× SDS-PAGE loading buffer. Ten microliters of the sample was fractionated on SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and Western blot analysis was performed with a monoclonal anti-FLAG Ab.

Protein purification

To purify C-terminal FLAG-tagged UGRPI (UGRPI-FLAG) or HIN-1 (HIN-1-FLAG), ~2 liters of conditioned medium of 293 cells transfected with pRK-C-FLAG-UGRP1 or pRK-C-FLAG-HIN-1 were collected and concentrated using ultracentrifugation (Sigma-Aldrich). The conditioned medium was loaded into a 2-ml anti-FLAG Ab affinity chromatography column. The column was washed with 12 ml of TBS three times. The bound proteins were eluted with 100 μg/ml FLAG peptide (Sigma-Aldrich) and quantitated by SDS-PAGE and Coomassie blue staining.

Protein sequencing

Two micrograms of the purified proteins were fractionated on SDS-PAGE, transferred onto polyvinylidene difluoride membrane in 1× CAPS buffer (10 mM CAPS [pH 11] and 10% methanol), and the blot was stained with 0.1% Coomassie blue R-250 in 40% methanol/1% acetic acid for 1 min and then washed with water. The bands were subjected to N-terminal protein sequencing, which was performed by National Jewish Medical and Research Center protein sequencing core facility.

Flow cytometry analysis

Cells (~1 × 10^6) were incubated with 100 ng of the purified UGRPI-FLAG or HIN-1-FLAG in 200 μl of staining buffer (PBS containing 2% FBS, 0.1% NaN3, 0.15% sodium azide, and 0.5% bovine serum albumin) at 4°C for 20 min, and then sequentially incubated with anti-FLAG mAb (1 μg/ml) and RPE-conjugated goat anti-mouse IgG (1/200 dilution) in staining buffer, each for 30 min. Cells were washed twice with staining buffer after each incubation. Cellular fluorescence was measured with a BD Biosciences FACScan flow cytometer.

Expression cloning

Human lung cDNA retroviral expression library was purchased from Stratagene (La Jolla, CA). This library contains 1.8 × 10^8 independent clones. Plasmid DNA (150 μg) prepared from the library was transfected into 293–10A1 packaging cells (~5 × 10^5) by electroporation. Two micrograms of the purified proteins were fractionated on SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and Western blot analysis was performed with a monoclonal anti-FLAG Ab.

In situ hybridization

In situ hybridization was performed as previously described (19). Briefly, the lungs were fixed in freshly prepared 4% paraformaldehyde and embedded in paraffin. Radiolabeled sense and antisense riboprobes were transcribed with [32P]UTP from full-length cDNAs that were previously cloned into plasmid pGEM-T easy (Promega, Madison, WI). Sections were hybridized and processed as described previously (19).

Results

Identification of HIN-2/UGRP1 as an HIN-1 homologous protein

To identify a potential protein homologous to HIN-1 protein, we searched GenBank EST databases. We identified several human EST clones that encode a novel HIN-1-homologous protein, which we designated HIN-2. Because HIN-2 is identical to the recently identified UGRPI (2), we refer to HIN-2 as UGRPI below to avoid confusion. Human UGRPI protein contains 93 aa and shares 43% sequence identity with HIN-1 (Fig. 1A). Structural analysis using pSignal software program suggests that UGRPI contains a putative signal peptide at its N terminus.

Expression of human UGRPI

Recently, it has been shown that mouse UGRPI mRNA is predominantly expressed in the lung and is expressed at a low level in the thyroid (2, 3). We examined the tissue distribution of human UGRPI mRNA expression. Human UGRPI mRNA was specifically expressed in the lung as a single ~0.8-kb band and not detected in other tissues examined, which included the heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, small intestine, ovary, colon, and peripheral blood leukocytes (Fig. 1B). To determine the cell types in the lung that express UGRPI, we performed in situ hybridization experiments. The result suggests that human UGRPI is specifically expressed by the epithelial cells (Fig. 1Ca). Based on the spatial distribution pattern, the UGRPI-expressing epithelial cells are probably Clara cells of bronchioles. Furthermore, we found that human UGRPI was up-regulated in the lung epithelial cells of patients with cystic fibrosis compared with normal lung (Fig. 1Cb).

To determine whether human UGRPI is a secreted protein, we made a mammalian expression plasmid for C-terminal FLAG-tagged UGRPI. This plasmid transiently transfected into 293 cells, and secretion of UGRPI-FLAG in the cell culture medium was confirmed by Western blot analysis with anti-FLAG Ab (data...
To facilitate functional analysis of UGRP1, we purified UGRP1-FLAG from the conditioned medium with an anti-FLAG Ab affinity column. Coomassie blue staining of the purified proteins and Western blot analysis with anti-FLAG Ab suggest that UGRP1 exists as two forms: a ~9-kDa unmodified form and a ~13-kDa modified form (Fig. 2). To determine whether the putative N-terminal signal peptide is cleaved and the exact natural cleavage site, we performed N-terminal amino acid sequencing of the two forms of purified UGRP1-FLAG proteins. These experiments suggest that the first amino acid of the secreted UGRP1-FLAG proteins is phenylalanine at amino acid position 22. Our results suggest that the N-terminal 21 aa of UGRP1 is an authentic signal peptide and the mature UGRP1 protein contains aa 22–93 (Fig. 1A).

In the purification experiments, a ~26-kDa protein consistently copurified with UGRP1-FLAG (Fig. 2). N-terminal amino acid sequencing analysis indicates that this protein is apolipoprotein A-I (ApoA-I), a major protein component of high density lipoprotein (HDL). Similarly, we expressed and purified HIN-1-FLAG and found that ApoA-I was also copurified with HIN-1-FLAG (data not shown). However, ApoA-I did not copurify with the soluble TNF family member sTALL-1-FLAG under similar conditions, suggesting that ApoA-I is specifically associated with UGRP1 and HIN-1.

### Identification of MARCO as a receptor for UGRP1 by expression cloning

Because UGRP1 is a secreted protein, we determined whether it binds to cell surface receptor(s). To do this, we incubated UGRP1-FLAG with several cancer cell lines and performed flow cytometry analysis with anti-FLAG Ab. UGRP1-FLAG bound to the plasma membrane of several lung-derived cancer cell lines, including A549, H290, H322, and H292. In the same experiments, UGRP1-FLAG did not bind to monocytic U937 cells (data not shown). These data suggest...
that membrane receptor(s) exists for UGRP1. To identify the receptor(s) for human UGRP1, we performed expression cloning. Briefly, we transfected −5 × 10⁵ of 293–10A1 packaging cells with −150 μg of human lung cDNA retroviral expression library plasmid by calcium phosphate precipitation. Forty-eight hours after transfection, the recombinant virus-containing medium was collected to infect −5 × 10⁷ of U937 cells, which do not express UGRP1 receptor(s). The infected cells were incubated sequentially with UGRP1-FLAG and a monoclonal anti-FLAG Ab. The UGRP1-FLAG-bound cells were isolated by panning with goat anti-mouse IgG microbeads in a MiniMACS separation column. The isolated cells were amplified and subjected to another round of panning. After two rounds of panning, the majority of cells stained positive with UGRP1-FLAG (Fig. 3A), suggesting that these cells expressed UGRP1 receptor(s). We then performed RT-PCR to recovery the cDNAs in the retroviral vectors integrated into the UGRP1-FLAG-staining positive U937 cells. Two specific bands, 2.1 and 1.2 kb, respectively, were obtained from these experiments (Fig. 3B). DNA-sequencing analysis suggested that these two bands represent cDNAs encoding full-length human MARCO and lung surfactant protein C (SP-C), respectively (Fig. 3B).

To determine whether MARCO and SP-C are true UGRP1 receptors, we subcloned their full-length cDNAs into the MSCV-IRES-GFP retroviral vector, which contains an IRES element followed by the GFP cDNA. We transduced these vectors into U937 cells by retroviral-mediated gene transfer and sorted the GFP-positive cells. Flow cytometry analysis of the GFP-positive cells with
anti-FLAG Ab indicated that UGRP1-FLAG bound to MARCO-but not SP-C-transduced cells (Fig. 4). In these experiments, sTALL-1-FLAG did not bind to either MARCO- or SP-C-transduced cells (data not shown). These data indicate that MARCO, but not SP-C, is a specific receptor for UGRP1.

**MARCO is also a receptor for HIN-1**

Because HIN-1 is highly homologous to UGRP1, we determined whether HIN-1 can also bind to MARCO. We transduced MARCO into U937 cells and found that HIN-1-FLAG could bind to MARCO as suggested by flow cytometric analysis with anti-FLAG Ab (Fig. 4). These data suggest that UGRP1 and HIN-1 can bind to the same receptor.

**MARCO is expressed by alveolar macrophages in the lung**

It has been reported that mouse MARCO is expressed mostly in subsets of macrophages located in the peritoneum, marginal zone of the spleen, and the medullary cord of lymph nodes (12–14, 16). Consistent with a previous report (15), we found that human MARCO is expressed at the highest level in lung and liver, and barely detectable in other examined tissues, including the heart, brain, placenta, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, small intestine, ovary, and colon (Fig. 5A). MARCO is also expressed by primary human monocytes but not by T lymphocytes (Fig. 5A).

To determine the cell types in the lung that express MARCO, we performed in situ hybridization experiments. MARCO was detected in alveolar macrophages, but not other cell types (Fig. 5B).

**UGRP1 binds to bacteria and yeast**

Previously, it has been shown that LPS is a ligand for MARCO, and MARCO is involved in clearance of bacteria by binding to bacteria (12–14, 16). In flow cytometric experiments, we found that LPS could compete with UGRP1 for binding to MARCO (Fig. 6A).

We then examined whether UGRP1 can bind to bacteria. We used two lung-tropic bacteria strains, the Gram-positive *L. monocytogenes* NP 1.0 strain and the Gram-negative *P. aeruginosa* 01 strain. We incubated UGRP1-FLAG protein with live and heat-killed bacteria and then determined whether UGRP1-FLAG binds to bacteria by Western blot analysis with anti-FLAG Ab. The results indicate that UGRP1 binds to both live and heat-killed stains (Fig. 6B). Interestingly, UGRP1 also binds to live or heat-killed yeast strain Y190 (Fig. 6B). In the same experiments, sTALL-1-FLAG did not bind to bacteria or yeast (Fig. 6C), suggesting that the binding of UGRP1 to bacteria and yeast is specific.

Because UGRP1 binds to Gram-negative bacteria, we determined whether UGRP1 directly binds to LPS. To test this, we determined whether addition of LPS can block binding of UGRP1 to *P. aeruginosa*. As shown in Fig. 6C, LPS inhibited UGRP1 binding to *P. aeruginosa* in a dose-dependent manner, suggesting UGRP1 binds to LPS.

UGRP1 can bind to Gram-positive bacteria and yeast, which do not contain LPS, suggesting UGRP1 can also bind to other microbiological substances in addition to LPS. Interestingly, it has been demonstrated previously that MARCO can also bind to Gram-positive bacteria and unopsonized environmental particles. It is possible that UGRP1 and MARCO are involved in host defense against a wide range of microorganisms and other pathogens.

**Discussion**

In the present study, we identified human UGRP1 and demonstrated that human UGRP1 is specifically expressed by epithelial cells of the bronchioles. Human UGRP1 is a secreted protein, and the mature protein contains aa 22–93. Using expression cloning approach, we identified MARCO as a UGRP1 receptor. In Northern blot and in situ hybridization experiments, we found that...
MARCO is a member of the class A scavenger receptor family (12–17). The protein contains a short intracellular N-terminal domain, a transmembrane domain, an extracellular region with a short spacer, a collagenous domain, and a C-terminal cysteine-rich domain. Previously, MARCO has been shown to bind to Gram-positive and -negative bacteria and unopsonized environmental dusts and is critically involved in the clearance of these pathogens in vivo (12–17). Our experiments suggest that UGRP1 can bind to MARCO, and this binding is inhibited by LPS. We also found that UGR P1 can bind to Gram-positive and -negative bacteria and yeast. In addition, our in situ hybridization experiments indicated that UGRP1 is up-regulated in the lung epithelial cells of a patient with cystic fibrosis compared with normal lung. Taken together, these data point to the possibility that UGRP1 acts as an opsonin, and the UGRP1–MARCO ligand-receptor pair is involved in clearance of pathogens in the lung.

In our biochemical purification experiments, we found that ApoA-I was copurified with UGRP1. This association is specific, because ApoA-I did not bind to the control sTALL-1-FLAG. ApoA-I is the major component of HDL (21). Interestingly, it has been shown previously that ApoA-I binds to scavenger receptor class B type I, a receptor that belongs to the scavenger receptor family as MARCO does (21). In addition, HDL has been found to neutralize LPS activity in vitro and in animals, including LPS-induced release of proinflammatory cytokine (22, 23). It is possible that UGRP1 is one mediator of HDL-induced regulation of lung inflammation. Our studies also suggest that UGRP1 is up-regulated in the lung of patients with cystic fibrosis, further supporting a role of UGRP1 in the modulation of lung inflammation.

The lung epithelium is the first barrier for defense against inhaled pathogens in the lung. The lung epithelial cells secrete abundant antimicrobial peptides such as β-defensins and LL37 to kill bacteria (24–26). Other proteins, such as the uteroglobin/Clara cell secretory protein (CCSP) family members, are also secreted by the airway epithelial cells and abundantly expressed in the lung (27–29). CCSP is a 16-kDa protein that is secreted by Clara cells of the lung. Gene knockout experiments have shown that CCSP is an important modulator of lung inflammation following infection or injury (27, 29, 30). Interestingly, UGRP1 has ~25% amino acid sequence identity with CCSP, and both are secreted by Clara-like cells in the lung. It is possible that UGRP1 is functionally related to CCSP. The exact roles of UGRP1 in lung physiological and pathological processes need to be further investigated. Our current study provides an important basis for this future effort.

Acknowledgments
We thank Roberta Pelanda, David Riches, Ed Chan, Philippa Marrack, Terry Potter, and Mike Vasil for advice and discussion, and Shirley Sobus and Bill Townend for help with cell-sorting experiments.

References


