The N1 Domain of Human Lactoferrin Is Required for Internalization by Caco-2 Cells and Targeting to the Nucleus †

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The N1 Domain of Human Lactoferrin is Required For Internalization By Caco-2 Cells and Targeting to the Nucleus

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Abstract

Human lactoferrin (hLf) has been shown to interact with cells from the Caco-2 human small intestinal cell line. There currently is little information on the molecular details of its interaction. As a first step toward detailed characterization of this interaction, we used a series of Lf chimeras to analyze which part of Lf is responsible for the interaction with Caco-2 cells. Recombinant chimeric proteins consisting of segments of human Lf (hLf) and bovine transferrin (bTf) were produced in a baculovirus-insect cell system, and purified by a combination of cation exchange chromatography and immobilized bTf antibody affinity chromatography. Each chimera was labeled with a green fluorescent dye to monitor its interaction with Caco-2 cells. Similarly the intestinal Lf receptor (LfR), also known as intelectin, was probed with an anti-LfR antibody that was detected with a secondary antibody conjugated with red-color fluorescent dye. The results demonstrated that chimeric proteins containing the N-lobe or the N 1.1 subdomain of Lf bound equally as well as intact Lf to Caco-2 cells. Confocal microscopy analysis revealed that these proteins, along with the LfR, were internalized and targeted to the nucleus. These results indicate that the N1.1 subdomain of human Lf is sufficient for binding, internalization and targeting to the nucleus of Caco-2 cells.

Lactoferrin (Lf) is a single-chain, iron-binding glycoprotein that is abundant in milk of some species such as humans, rhesus monkeys, pigs, and mice. It has been suggested to facilitate iron absorption in infants [1]. Lf is also found in high concentration in most exocrine secretions and in the secondary granules of neutrophils, from which it is released following activation of these cells [2,3]. A growing body of evidence supports the role of Lf in a variety of biologically important activities such as defending against a variety of pathogens, modulating the immune system, and stimulating cell proliferation [1]. However, there is little information available on the molecular mechanisms by which Lf mediates these physiological effects. Trans-activation of various genes, such as activation of AP-1 through the JNK and p38 MAPK pathways [4], may be induced by binding of Lf to DNA [5,6], but the signal for nuclear localization of Lf and whether endogenous or external Lf trans-activates gene expression are still not clear. Recently, delta-Lf, a cytoplasmic Lf isoform that is the product of alternative splicing of the Lf gene, was found to provoke anti-proliferative effects and cell cycle arrest in the S phase [7]. Delta-Lf enters the nucleus, and binds to the Skp1 (S-phase kinase-associated protein 1) promoter, suggesting that delta-Lf may regulate cell cycle progression by increasing Skp1 gene expression [8]. The nuclear localization signal sequence of delta-Lf was identified in the C-lobe. For exogenous Lf exerting any effects on the cell, interaction with a specific receptor is likely to be involved.

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Kinetic studies have demonstrated the presence of receptors for Lf (LfR) on the mucosa of the small intestine from various species such as rabbits, piglets, rhesus monkeys, and mice [9-12]. The presence of intact Lf in the infant small intestine [13], a reflection of its relative resistance to proteolysis [14], indicates that ligand for the receptor would be available at least under certain circumstances. The gene encoding the human LfR from fetal small intestinal brush border membrane, also known as intelectin, has been cloned and expressed in Caco-2 cells, conferring increased uptake of iron from Lf [15]. RT-PCR studies revealed that the gene is expressed in a wide variety of tissues, raising the question of whether this receptor may be responsible for mediating other physiological activities of Lf, possibly by eliciting cell signaling pathways. Other types of LfRs have also been characterized in different cell types, including LDL receptor related protein (LRP), asialoglycoprotein receptor (ASGPR), CD14 [16], and nucleolin [17]. LRP is abundant in hepatocytes, neurons, smooth muscle cells, and fibroblasts. CD14 has been postulated as the monocyte LfR, and ASGPR as the liver LfR. Nucleolin was recently characterized as the lymphocyte LfR. Thus, different types of cells appear to express different molecules as receptors for Lf, and therefore, to have their own mechanisms triggered by exogenous Lf.

This study was initiated to probe the interaction between Lf and the intestinal LfR, intelectin. The overall approach was to test series of hybrid proteins containing portions of human Lf and bovine Tf for their ability to interact with Caco-2 cells expressing the LfR. These proteins were designed to maintain overall structural integrity so that the regions of human Lf would be likely to retain their native structure. The interaction was assessed by monitoring the presence and location of the hybrid proteins, the intestinal LfR and other cellular constituents by fluorescence microscopy.

Experimental Procedures

Antibodies

An antibody (P2AB) was purified from the antiserum P247AS that had previously been raised in rabbits against synthetic peptides corresponding to parts of amino acid sequences of the intestinal LfR [15] by using an immobilized antigen peptide (CTVGDRWSSQQGSKAD: peptide 2). Briefly, Peptide 2 (~0.5 mg) was immobilized to 1 mL of Affigel 15 (Bio-Rad, Richmond, CA). P247AS (5 mL) was applied to the column and the column was washed with PBS. P2AB was then eluted by 0.2 M glycine buffer, pH 2.7. The eluate was immediately adjusted to pH 7.4 with 1M NaOH.

Fifty μg of affinity purified sheep anti-bTf antibody (Bethyl Laboratories, Montogomery, TX) were coupled to 1 mL of Affigel-Hz resin (Bio-Rad) according to the manufacturer's instruction for purification of chimeras.

HRP-conjugated rabbit anti-hLf antibody (Biodesign, Kennebunk, MA), rabbit anti-bTf antibody (ICN Pharmaceuticals, Aurora, OH), and HRP-conjugated anti-rabbit IgG antibody (Amersham Biosciences, Piscataway, NJ) were used for detection of chimeras.

Design and preparation of hybrid hLf/bTf cDNAs

The genes encoding the various chimeric proteins were prepared as described previously with unique restriction sites engineered in the sequences encoding junctions between the domains and subdomains [18]. The set of constructed hybrid hLf/bTf is illustrated in Figure 1. The Bac-to-Bac Expression System (Life Technologies Inc., Rockville, MD) was used for expression of chimeras. Each construct was subcloned from the pGEMT vector into pFASTBAC1. The recombinant baculoviruses were generated according to the manufacturer's instructions.

Tricoplusia ni (*T. ni*) cells were routinely maintained at 28°C in 250-ml Erlenmeyer flasks containing 100 ml of a serum free medium (ESF921; Expression System, Woodland, CA) and sub-cultured every 3-4 days. For expression of recombinant proteins, *T. ni* cells were seeded at 6×10^5 cells/ml and infected by the recombinant baculovirus at a multiplicity of infection of 0.1. At 3 days post-infection, the infected cell culture was harvested and centrifuged at 1,000 × g for 10 min at 4°C to separate supernatant and cells.

Isolation of chimeric proteins

The supernatant of infected *T. ni* cells was further centrifuged at 10,000 × g for 15 min and filtered through a Nylon filter cartridge with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). The filtered supernatant of infected *T. ni* cells was loaded onto a Hi-Trap SP (Amersham Biosciences)column equilibrated with 50 mM Tris buffer, pH 8.0. The bound proteins were eluted by a gradient of 1 M NaCl. The eluted fractions were subjected to SDS-PAGE and Western blot to check purity and immunogenic specificity. Chimeras lacking the N 1.1 region were not sufficiently purified by the ion exchange chromatography, and were applied to an anti-bTf antibody immobilized affinity column equilibrated with phosphate buffer (pH 7.0), containing 0.3 M NaCl. Recombinant chimeras were eluted by an elution buffer (0.2M glycine buffer, pH 2.7), and the eluate was neutralized immediately by addition of 1M Tris, pH 8.8. Typical yields of the soluble chimeric proteins when expressed in *T. ni* cells were 50 ~ 200 μg from 100 mL culture supernatant.

Gel electrophoresis and Western blot

Purified proteins (1 μg) were subjected to SDS-PAGE under reducing conditions. Gels were stained with Coomassie Brilliant Blue R solution. For Western blotting, electrophoresed proteins were transferred to PVDF (Bio-Rad) membrane. Non-specific binding sites on the membrane were blocked by incubation in a blocking buffer (PBS containing 0.05% Tween-20 and 5% skim milk). The membrane was washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween) for 7 min each. For hLf detection, blots were incubated with HRP-conjugated anti-hLf antibody (1:20,000 dilution) for 1 h. For bTf detection, blots were incubated with rabbit anti-bTf antibody (1:500 dilution) for 1 h, and then incubated with HRP-conjugated anti-rabbit IgG antibody (1:20,000 dilution). Bound antibody was detected by SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL).

Labeling of chimeras

Twenty μl of 1M $NaHCO_3$ and 5 μl of 10 mg/mL Alexa Fluor 488 (Molecular Probes, city, Eugene, OR) dissolved in DMSO were added to 10 μg of each chimera that was prepared in 200 μl PBS. The mixture was incubated for 1 h at room temperature and dialyzed against PBS. Coupling efficiencies were examined according to the manufacturer's instructions to verify constant amount of fluorescent dye (3-4 molecule/protein) were attached to each chimera.

Fluorescent microscopy analysis

Caco-2 cells were grown on glass slides until 2 weeks post-confluence. Cells were incubated with 100 μl of 50 μg/ml chimera labeled with fluorescent dye (Alexa Fluor 488) for 30 min at 37°C in the presence of excess human Tf. Cells were fixed with 4% paraformaldehyde, blocked with 10% FBS and 1% BSA in PBS, and then incubated with P2Ab (1 μg/mL) for 1 h. Cells were incubated with 2.5 μg/mL of secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG) for 45 min, mounted, and then analyzed under fluorescence microscopy.

Confocal laser microscopy analysis

Caco-2 cells were cultured on 0.33-cm^2 cell culture inserts (Transwell-COL), with 0.4 μm pore size membranes. Cells were then incubated with 100 μl of 50 μg/ml Alexa Fluor 488-conjugated

hLf chimera from the apical side for 30 min at 37°C. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Nuclei of cells were stained with 1 μM TO-PRO-3/PBS(-) for 1 h at room temperature. Coverslips were mounted in ProLong Antifade (Molecular Probes). Confocal laser microscopy was performed using an Olympus BX50WI (Olympus America Inc, Melville, NY), and digital images were captured using a Bio-Rad Radiance 2100 confocal system.

Results

Production and purification of chimeric proteins

Bovine transferrin was used as the partner for development of hybrid proteins with human Lf since it possesses none of the receptor binding properties of hLf but is sufficiently similar in overall structure and sequence (61 % amino acid identity) to be suitable for generating functional hybrids. The splice sites between the gene fragments were situated between structural domains to avoid any interference with folding of the individual domains (Figure 1). Expression of the hybrid genes in insect cells resulted in proper processing, export and secretion of the chimeric proteins into the culture medium. Previous studies have shown that recombinant Lf produced by this system is fully functional in metal and receptor binding [19] and that hybrid proteins retain binding properties to their respective bacterial receptors [18].

Purification of hLf and chimeras containing the N1.1 domain of hLf was achieved in one step with cation exchange chromatography. An additional step involving anti-bTf antibody affinity chromatography was required for chimeras not containing the N 1.1 domain. SDS-PAGE analyses confirmed that the recombinant protein preparations were relatively pure (Figure 2, Panel A). As expected, the chimeric proteins reacted with both anti-Lf antibody (Panel B) and anti-bTf antibody (Panel C) in contrast to recombinant hLf and bTf, which only reacted with their respective antisera. Recombinant bTf and the chimeric proteins containing the intact N-lobe from bTf (C, C1, C2) consistently migrated faster than recombinant hLf and the chimeric proteins lacking the intact N-lobe from bTf (N, N 1.1, N1.2, N2). The reason for the difference in migration rates has not been determined but could be related to the fact that there are glycosylation sites in both lobes of hLf but only a single glycosylation site in the bTf C-lobe.

Fluorescent microscopy analysis

We examined the association of the isolated chimeric proteins with Caco-2 cells. Results of fluorescent microscopy analysis are shown in Figure 3. Column I shows images filtered to detect only green fluorescence, indicating localization of exogenously applied chimeric proteins. Fluorescence was clearly detected for cells exposed to recombinant hLf and the N and N1.1 chimeric proteins (Figure 3, Rows A, B and C), suggesting that the N1.1 region is required for association with Caco-2 cells.

Column II shows images filtered only for red fluorescence, indicating localization of endogenous intestinal LfR. Red fluorescence was associated with a limited number of the available cells in all of the samples (Figure 3, column II, rows A–I), suggesting that intestinal LfR is only expressed in a subpopulation of Caco-2 cells. Column III shows merged images of columns I and II. Green fluorescence was always associated with cells that were also labeled with the red fluorescent dye indicating that recombinant hLf and the chimeric proteins only interacted with the cells expressing intestinal LfR. The co-localization under fluorescence microscopy suggests that the N1.1 region mediates interaction with the intestinal LfR in Caco-2 cells.

Confocal laser microscopy analysis

Confocal laser microscopy was performed to determine whether the interaction of hLf or the chimeras resulted in internalization into the Caco-2 cells. The experiments with intact recombinant hLf are illustrated in Figure 4, Panels A-D. The blue color (Figure 4A) represents the nucleus, indicating that the image at this depth (Z-axis) is inside the cell. The paired nuclei are from immediately adjacent cells. Figures 4B, 4C, and 4D are images at the same position of the Z-axis. The red color in Figure 4C represents the intestinal LfR and suggests that it is localized in the nucleus. The green color in Figure 4B represents recombinant hLf, which appears to be localized in the nucleus of the bottom but not the top cell. The merged image (Figure 4D) was obtained by overlaying three images from Figure 4A to Figure 4C. The results demonstrate that the intestinal LfR is localized inside of the nucleus of both the upper (pink color) and lower cell (white color). Internalization of hLf in the nucleus of the lower cell is confirmed by the white color of the merged image. Thus, these results indicate that hLf and the intestinal LfR are co-localized in the nucleus of the Caco-2 cells.

The results with the N and N1.1 chimeric proteins were essentially the same as with intact hLf. Figure 4 E is a merged image of an experiment with the N1.1 protein, and clearly illustrates that the N1.1 protein and the intestinal LfR are co-localized in the nucleus. The results with recombinant bTf and all of the other chimeric proteins were different from hLf, N and N1.1 in that there was no localization of the proteins or the intestinal LfR in the nucleus of the cells. The results for the N1.2 protein, illustrated in Figure 4F, are representative of the results with all of the other proteins. In this image, which is a merged image comprised of all images of the entire Z-axis from the top to the bottom, there is no association of the intestinal LfR and the nucleus, as well as no evident green fluorescence.

Discussion

Chimeric proteins comprised of segments of hLf and bTf were used in this study to identify the region of hLf involved in interacting with Caco-2 cells. Previous studies have shown that recombinant Lfs and Tfs produced from a baculovirus-insect cell expression system are fully functional with respect to metal binding and interacting with receptors on eukaryotic and bacterial cells [18,19] indicating that this would be a suitable production system for this study. Similarly, chimeric proteins of human/bovine Tfs have previously been used successfully for identifying the domains of Tfs involved in binding to specific bacterial Tf receptors involved in iron acquisition from host proteins [20,21]. Thus we were confident that our ability to identify the binding domains in this study would not be compromised by misfolding of the chimeric proteins. In this respect it is noteworthy to mention that several of the chimeric proteins that were defective in binding to Caco-2 cells in this study (C, C1, C2) were the ones capable of interacting with bacterial lactoferrin receptors in a prior study [18], which strongly argues that the lack of interaction with Caco-2 cells would not be due to defects in proper folding. It is not at all surprising that the regions of hLf recognized by the bacterial and host receptors are different (C-lobe and N1.1 subdomain, respectively) since there is no significant homology between bacterial LfRs and human intestinal LfR [15].

Since the recombinant chimeric proteins were not evaluated for the presence and nature of the oligosaccharide side chains, we cannot totally exclude their contribution to differences in the observed binding properties. However, it has previously been demonstrated that a deglycosylated form of native hLf was unaltered in binding to the intestinal LfR [22], indicating that the oligosaccharide side chains do not participate in binding to the intestinal LfR. Similarly, although the oligosaccharide chains from insect-cell-derived hLf [19] as well as those from rice-produced hLf [23,24] have been shown to be different from those of native hLf, the binding properties were retained. Thus we are confident that the differences in binding properties observed in this study can primarily be attributed to differences in the protein backbone.

These studies do not directly demonstrate that there is an interaction between the intestinal lactoferrin receptor and hLf or the N1.1 chimera but it is strongly inferred from their co-localization. Previous studies have shown that uptake of iron from hLf was significantly enhanced in Caco-2 cells overexpressing the intestinal LfR [15] suggesting a functional interaction between hLf and the intestinal LfR. Together with the current findings, these results indicate that N1.1 is likely to retain capacity to interact with the intestinal LfR and the Caco-2 cells.

It should be noted that the green fluorescence was observed in a limited number of the available cells in both fluorescent microscopy analysis and confocal laser microscopy analysis, indicating that Lf is selectively associated with specific cells. Our previous studies in Caco-2 cells revealed that recombinant hLf was localized in only a subset of cells when it was added to the apical side, but that no localization was observed when it was added to the basolateral side [25]. In contrast, human Tf (hTf) was localized in a majority of Caco-2 cells when it was added to the basolateral side, although no localization was observed when it was added to the apical side, indicating that hLf localization in Caco-2 is not Tf receptor dependent but depends on a specific LfR on the apical membrane [25]. The previous confocal microscopy analysis revealed that hTf, as a control, was internalized and localized to an endosomal compartment only from the basolateral side [25], supporting that our analytical methods work appropriately.

The region of hLf involved in recognizing the lymphocyte LfR has previously been investigated using proteolytic fragments of hLf [26,27] and derivatives that were blocked on specific Lys residues of hLf [26,28]. Using inhibition of binding of hLf to activated lymphocytes as an assay, the binding region was initially localized to an N-terminal tryptic fragment (residues 4-281) [27] and subsequently further localized to a proteolytic subfragment (residues 4 – 52) obtained from digestion with *Staphylococcus aureus* V8 protease [26]. In the same study, it was observed that modification of hLf with the reagent SASD, which primarily reacted with Lys 74, eliminated its ability to block binding. The authors attempted to reconcile the two results by suggesting that the SASD moiety attached to Lys 74 was potentially capable of masking residues 4-6, 28-34 and 38-45 based on molecular modeling analyses. The fact that the first three residues were removed by tryptic cleavage suggests that the N-terminal four Arg cluster of hLf at positions 2-5 was not essential for receptor binding, which was confirmed by studies with site-directed mutant proteins [29] and is comparable to results obtained for the receptor in liver cells where binding was retained after removal of 14 N-terminal amino acids of hLf [30].

In this study, the binding determinant for the intestinal LfR was localized to the N 1.1 subdomain (residues 1-90), suggesting that the lymphocyte and intestinal LfRs may recognize the same determinants on hLf. The lymphocyte LfR has recently been identified as nucleolin, which does not have any significant homology with the intestinal LfR. Thus, it would seem logical to perform further characterization of the binding properties of lymphocytes, intestinal epithelial cells (Caco-2 cells) and possibly other cell lines in parallel using more refined chimeric proteins or site-directed mutant proteins to confirm whether they truly recognize the same determinants. We demonstrated that the N 1.1 subdomain is not only sufficient for binding to Caco-2 cells (Figure 3), but also is sufficient for mediating its internalization and localization to the nucleus (Figure 4). The internalization of the LfR to the nucleus was only observed in cells exposed to hLf, N, and N1.1, indicating that binding to the receptor is likely triggering the internalization process. The targeting of a receptor and its cognate ligand to the nucleus appears to be quite novel and thus the mechanism for this process is uncertain. An obvious question is whether this phenomenon occurs in other cell types and may serve as the general mechanism by which hLf is targeted to the nucleus in order to perform its proposed role in transcriptional regulation [5,6].

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Abbreviations

Lf
: lactoferrin

hLf
: human lactoferrin

Tf
: transferrin

bTf
: bovine transferrin

LfR
: lactoferrin receptor

LRP
: LDL receptor related protein

ASGPR

asialoglycoprotein receptor

HRP

horse radish peroxidase

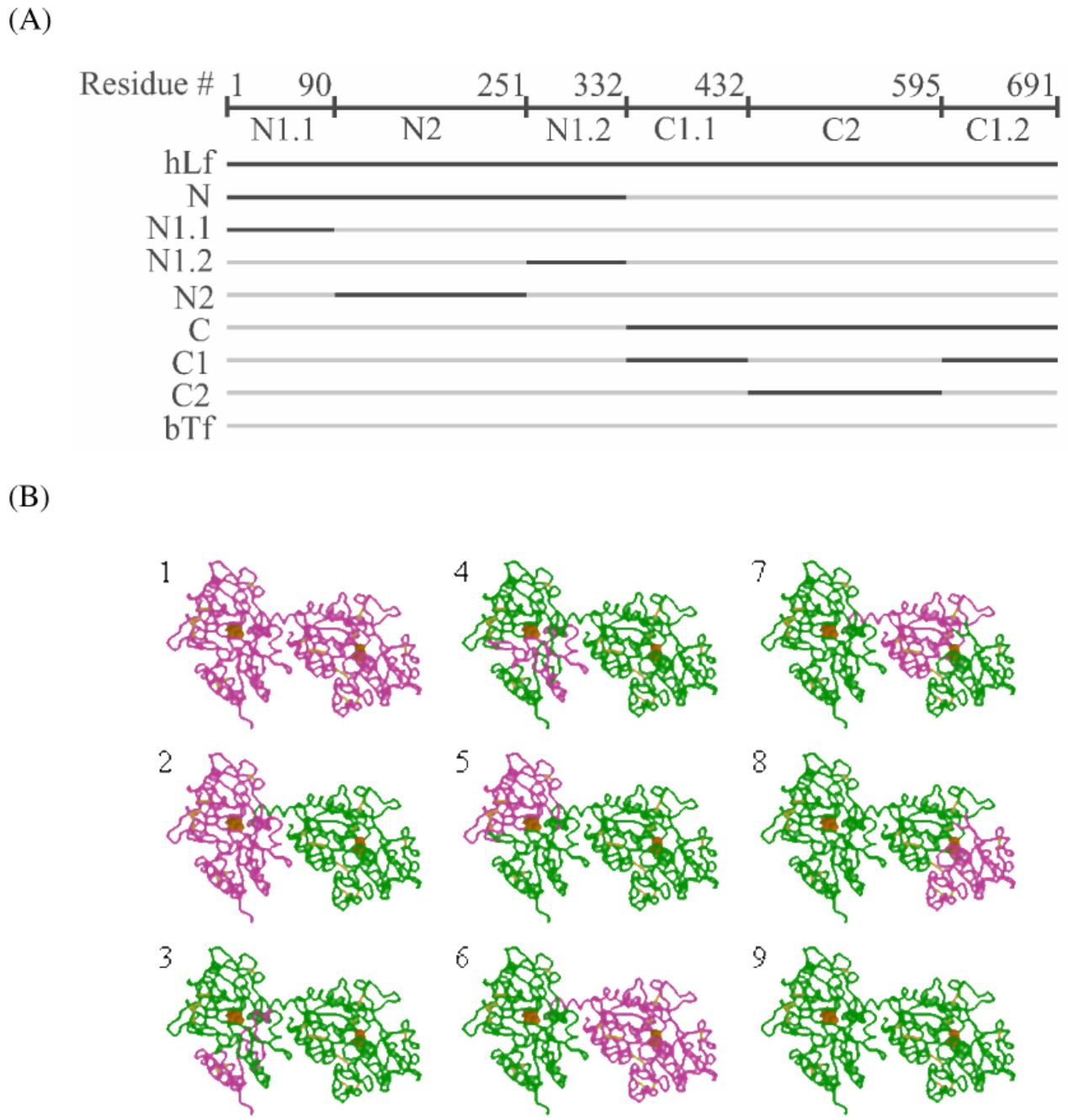

Figure 1.
Schematic representation of constructed chimeras. The composition of the chimeric constructs is shown with the hLf sequence in black and the bTf sequence in gray (Panel A). The molecular images of the chimeric proteins are shown with the hLf region in pink and the bTf region in green (Panel B). Image 1: hLf, image 2: N, image 3: N1.1, image 4: N1.2, image 5: N2, image 6: C, image 7: C1, image 8: C2, image 9: bTf

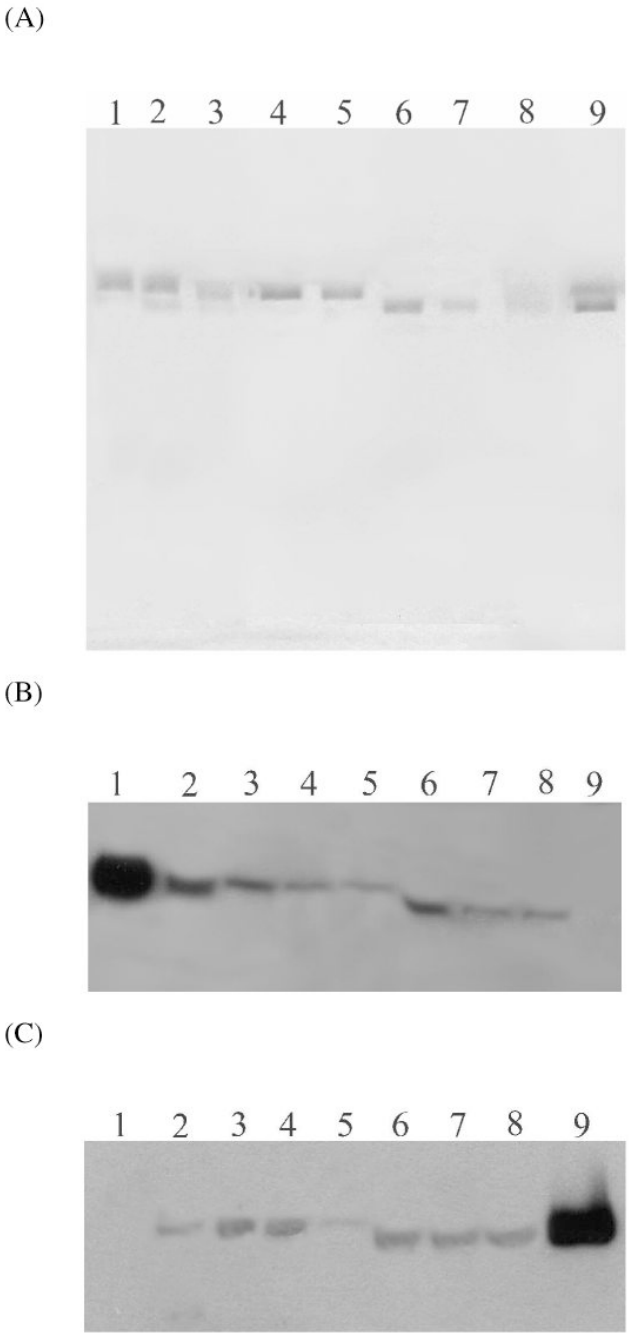

Figure 2.
SDS-PAGE analysis of the purified recombinant proteins. 1 μg samples of the indicated proteins were resolved on a standard SDS-PAGE gel and either stained for protein (Panel A) or electroblotted and detected by anti-hLf antibody (Panel B) or anti-bTf antibody (Panel C). Lane 1: hLf, lane 2: N, lane 3: N1.1, lane 4: N1.2, lane 5: N2, lane 6: C, lane 7: C1, lane 8: C2, lane 9: bTf

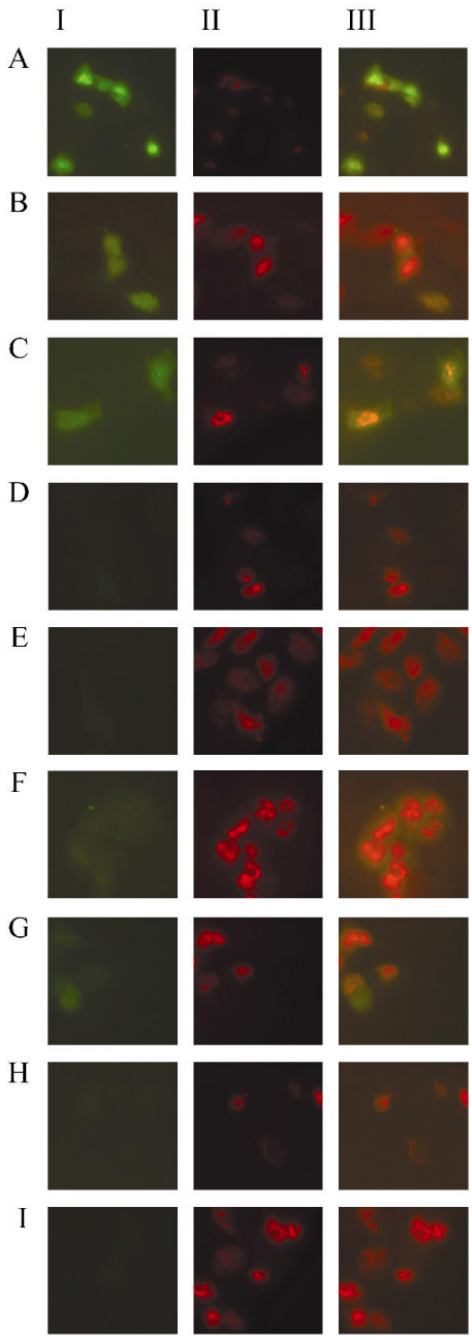

Figure 3.
Fluorescent microscopy of Caco-2 cells. Chimera is represented by green color in column I, LfR is represented by the red color in column II, and the merged image is in column III. Recombinant hLf (A), N (B), N1.1 (C), N1.2 (D), N2 (E), C (F), C1 (G), C2 (H), recombinant bTf (I) were used as ligands.

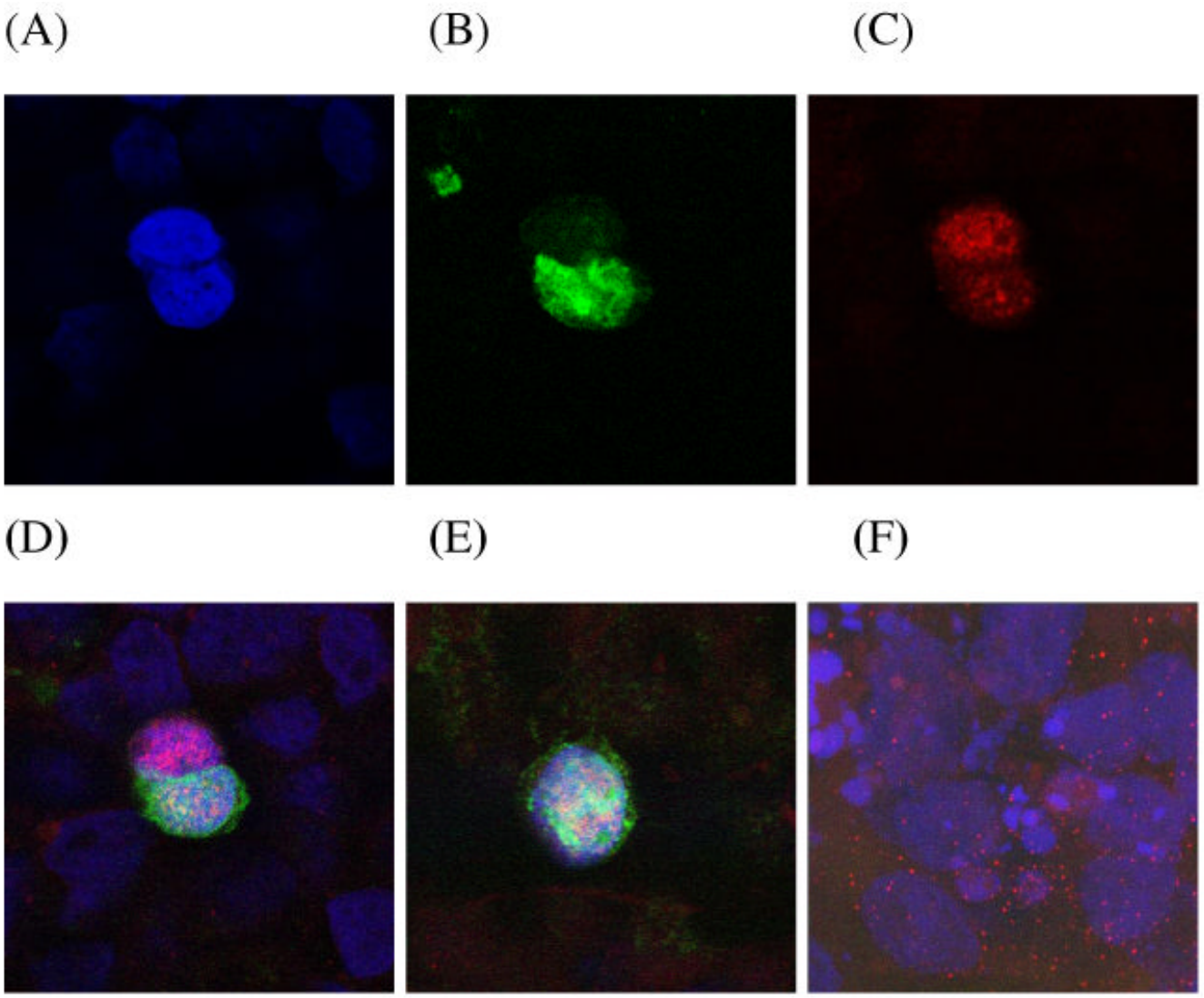

Figure 4.

Confocal laser microscopy analysis of Caco-2 cells. Results from recombinant hLf as a ligand (A)-(D). Image through blue filter, indicating nucleus (A), image through green color represent hLf (B), and image through red color represent LfR (C). Merged image of (A), (B), and (C), representing co-localization of hLf and LfR in nucleus (D). Result from N1.1 chimeric protein as a ligand (E). Merged image of blue, green and red color (E). Results from N1.2 chimeric protein as a ligand. Merged and pressed image (pressed all images of entire Z-axis from the top to the bottom together into one plain image) of blue, green, and red color (F).