ELSEVIER

Biochimica et Biophysica Acta 1279 (1996) 84–92

BBA Biochimica et Biophysica Acta

Expression of insulin-like growth factor (IGF)-I receptors, IGF-II/cation-independent mannose 6-phosphate receptors (CI-MPRs), and cation-dependent MPRs in polarized human intestinal Caco-2 cells

Nancy M. Dahms [a,*], Bellur Seetharam [a,b], Debra A. Wick [a]

[a] *Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA*
[b] *Department of Medicine, Division of Gastroenterology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*

Received 8 June 1995; accepted 12 September 1995

Abstract

We have analyzed the surface distribution and functional expression of the insulin-like growth factor I (IGF-I) receptor and the IGF-II/cation-independent mannose 6-phosphate receptor (IGF-II/CI-MPR) in the polarized human colon adenocarcinoma cell line, Caco-2. Domain-selective biotinylation of the apical and basolateral surfaces of Caco-2 cells grown on filter supports revealed a 3–4-fold enrichment of these receptors on basolateral membranes. In addition, the biotinylation studies revealed the presence of the cation-dependent MPR on both membrane surfaces, with a 3.4-fold enrichment on basolateral membranes. Binding of ^{125}I-IGF-I at 4°C confirmed similar higher levels of expression of the IGF-I receptor at the basolateral surface than at the apical surface. Cell surface-specific binding of the iodinated lysosomal enzyme β-glucuronidase was detected at 4°C on both plasma membrane domains. However, significant uptake of β-glucuronidase at 37°C was observed only from the basolateral surface. These results indicate that the MPRs and the IGF-I receptor are expressed in a polarized fashion in Caco-2 cells and that the IGF-II/CI-MPR present on apical membranes, unlike the IGF-II/CI-MPR expressed on the basolateral surface, is not functional in endocytosing lysosomal enzymes.

Keywords: Mannose 6-phosphate receptor; Insulin-like growth factor; Insulin-like growth factor receptor; Caco-2 cell

1. Introduction

Insulin-like growth factors (IGFs) I and II are polypeptide hormones that exert insulin-like metabolic and growth-promoting effects on a variety of tissues through their interaction with specific receptors (for reviews, see [1,2]). The IGF-I receptor, like the structurally homologous insulin receptor, is a tetrameric glycoprotein composed of two disulfide-linked $\alpha\beta$ dimers [3]. The α subunit (molecular mass ~ 135 kDa) is entirely extracellular and contains the ligand binding site whereas the β subunit (molecular mass ~ 90 kDa) is a transmembrane protein that contains a cytoplasmic tyrosine kinase domain. Binding of ligand to the IGF-I receptor activates the tyrosine kinase domain of the β subunit [4]. In contrast, the ~ 300 kDa membrane-bound IGF-II/cation-independent mannose 6-phosphate receptor (IGF-II/CI-MPR) is composed of a single polypeptide chain, the vast majority of which is exposed extracellularly, and lacks tyrosine kinase activity. In mammalian cells, the IGF-II/CI-MPR binds two distinct ligands with high affinity, IGF-II and mannose 6-phosphate (Man-6-*P*) residues on Asn-linked oligosaccharides of lysosomal enzymes (for recent review, see [5]). The ability to bind lysosomal enzymes via their Man-6-*P* recognition marker is shared by a second MPR, the ~ 46 kDa cation-dependent MPR (CD-MPR), that does not bind IGF-II [6,7].

In the presence of ligand, cell surface IGF-I receptors are internalized to an intracellular pool, an effect which is reversed upon removal of the ligand [8,9], and IGF-I is subsequently targeted for degradation [10]. It is well established that the targeting of lysosomal enzymes to lyso-

Abbreviations: IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; Man-6-*P*, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; IGF-II/CI-MPR, IGF-II/cation-independent MPR; CD-MPR, cation-dependent MPR; DSS, disuccinimidyl suberate; S-NHS-biotin, sulfosuccinimidobiotin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

* Corresponding author. Fax: +1 (414) 2668497.

SSDI 0005-2736(95)00234-0

somes can occur by either of two pathways (for reviews, see [5,11,12]). The major route is the biosynthetic pathway in which IGF-II/CI-MPRs transport newly synthesized lysosomal enzymes from the *trans* Golgi network to acidified late endosomes where the lysosomal enzymes are released for packaging into lysosomes and the receptors are then recycled back to the Golgi. In the endocytic pathway, the IGF-II/CI-MPR binds and internalizes extracellular lysosomal enzymes or IGF-II from the cell surface, resulting in the delivery of these ligands to the lysosome [13]. At steady state, the majority of the IGF-II/CI-MPR is present in late endosomes while a small fraction ($< 10\%$) is found on the plasma membrane [14,15]. The CD-MPR appears to have a similar intracellular distribution and trafficking pattern as the IGF-II/CI-MPR [15,16]. However, unlike the IGF-II/CI-MPR, the CD-MPR is extremely inefficient in binding and internalizing exogenous lysosomal enzymes [17,18].

Previous studies have described the existence of IGF-I, IGF-II, and the IGF receptors in gastrointestinal epithelium [19,20]. However, little information is available on the subcellular localization and functional expression of IGF receptors or MPRs in polarized epithelial cells. In order to address this issue, we have investigated the expression of IGF receptors and MPRs in the well-characterized human colon adenocarcinoma cell line, Caco-2. After confluence, Caco-2 cells exhibit morphological and functional characteristics of intestinal absorbing cells [21]. Their ability to grow into a polarized monolayer, composed of distinct apical and basolateral surfaces separated by tight junctions, on permeable filter supports has allowed Caco-2 cells to be used in numerous intestinal transport studies and in studies characterizing the intracellular sorting of proteins destined for the apical or basolateral membrane ([22] and references therein). In this report, we have utilized both domain-selective biotinylation and ligand binding studies to determine the surface distribution and functional expression of the IGF-II/CI-MPR and the IGF-I receptor in Caco-2 cells. Our results show that the majority of cell surface IGF-II/CI-MPRs and IGF-I receptors are localized at the basolateral membrane of Caco-2 cells. Although there is a significant amount of the IGF-II/CI-MPR on the apical membrane, the receptor can internalize lysosomal enzymes only from the basolateral surface. In addition, our studies also demonstrate the synthesis and basolateral enrichment of CD-MPRs in Caco-2 cells. A preliminary report of these findings has been published [23].

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially as indicated: EN^{3}HANCE and EXPRE^{35}S^{35}S ^{35}S-protein labeling mix which contains $\sim 70\%$ [^{35}S]methionine and $\sim 20\%$ [^{35}S]cysteine (1200 Ci/mmol, DuPont-New England Nuclear), Na^{125}I, carrier-free (Amersham), protein A-Sepharose and lactoperoxidase (Sigma), Immobilon-P transfer membrane (Millipore), human recombinant IGF-I (Upstate Biotechnology or Bachem), Bio-Rad protein assay (Bio-Rad), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and streptavidin (Gibco-BRL Life Technologies), disuccinimidyl suberate (DSS), and sulfosuccinimidobiotin (S-NHS-biotin) (Pierce), fetal bovine serum (HyClone Laboratories). Caco-2 cells [21] were kindly provided by Dr. Ward Olsen of Veterans Administration Hospital (Madison, WI, USA). L-MPR 13-2-1, MTX 3.2 cells over-expressing human β-glucuronidase were generously provided by Dr. William S. Sly of St. Louis University Medical School (St. Louis, MO, USA). Phosphomannan from *Hansenula holstii* was a gift of Dr. M.E. Slodki of the Northern Regional Research Center (Peoria, IL, USA).

2.2. Cell culture

Caco-2 cells (passages 76–90) were grown in DMEM (25 mM glucose) supplemented with 20% heat-inactivated fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Confluent monolayers were subcultured every 7 days by treatment with 0.05% trypsin and 0.53 mM EDTA in phosphate-buffered saline (PBS). For polarity experiments, cells were grown as epithelial layers by high density seeding ($1.5 \cdot 10^6$ cells/filter) onto nitrocellulose membrane filter inserts (Millicell-HA, 30 mm diameter, 0.45 μm pore size, Millipore). The formation and integrity of monolayers were assessed by the development of a significant transepithelial electrical resistance, which increased from $\sim 360\ \Omega\ cm^2$ to a maximum of $\sim 700\ \Omega\ cm^2$ 3 days and 14 days, respectively, after plating (values were measured with a Millicell-ERS voltohmmeter (Millipore) and were corrected for the resistance of filter inserts alone). All polarity studies were performed 14 days after plating. For uptake studies using radiolabeled ligands, only those monolayers showing permeability of $< 3\%$ for the radiolabeled ligand were utilized. Labeling of Caco-2 cells with EXPRE^{35}S^{35}S ^{35}S-protein labeling mix (50–100 μCi/ml) was performed in methionine-free DMEM containing 10% heat-inactivated fetal bovine serum for 16–20 h. In some experiments, cells were solubilized in buffer containing Triton X-100 (1%, v/v) and sodium deoxycholate (0.1%, w/v). To purify the MPRs, the resulting cell lysate was subjected to pentamannosyl phosphate-agarose affinity chromatography as described previously [24].

2.3. Immunoprecipitations

Cell lysate or affinity column fractions were incubated at 4°C for 16–24 h with protein A-Sepharose plus one of

the following antibodies: mouse anti-human IGF-I receptor α-subunit monoclonal antibody (αIR-3), 0.33–1.0 μg/ml (Oncogene Science) plus rabbit anti-mouse IgG polyclonal antibody, 0.33–1.0 μg/ml (ICN Biomedicals), rabbit anti-human insulin receptor α-subunit polyclonal antibody, 0.5 μg/ml (Upstate Biotechnology) or anti-MPR polyclonal antibodies (1:1000 dilution) [24]. Following recovery by centrifugation, protein A-Sepharose beads were washed four times with buffer containing 0.1 M Tris (pH 8.0), 0.1 M NaCl, 10 mM EDTA, and 1% Triton X-100 and once in buffer containing 20 mM Tris (pH 8.0) and 20 mM NaCl. Bound proteins were eluted by the addition of Laemmli sample buffer.

2.4. Iodination of ligands

Human recombinant IGF-I was iodinated as described previously [25] to specific activities of 50–100 μCi/μg. Human β-glucuronidase was purified from collections of serum-free conditioned medium from the cell line L-MPR 13-2-1 MTX 3.2, which is known to over-express and secrete human β-glucuronidase, by chromatography on an IGF-II/CI-MPR affinity column [26]. The specific activity of the purified β-glucuronidase was 544 U/μg as determined using *p*-nitrophenyl β-D-glucuronide as the substrate [27]. β-Glucuronidase (20 μg) was iodinated with 1 mCi of $Na^{125}I$ using soluble lactoperoxidase as described by Jadot et al. [26] except that following gel filtration on Sephadex G-150, ^{125}I-β-glucuronidase was further purified on an IGF-II/CI-MPR affinity column. The purified β-glucuronidase was then dialyzed exhaustively against PBS to remove Man-6-*P* and its specific radioactivity (1.5–7.0 μCi/μg) was determined using *p*-nitrophenyl-β-D-glucuronide as the substrate [27]. Streptavidin (50 μg) was iodinated using Iodogen as recommended by the manufacturer (Pierce), and separated from $Na^{125}I$ on a Sephadex G-25 column equilibrated in 10 mM $NaPO_4$ (pH 7.2), and 150 mM NaCl. Assuming 80% recovery, the specific radioactivity of the streptavidin was 3.8–6.2 μCi/μg. Protein concentrations were determined using the Bradford assay with bovine serum albumin (BSA) as the standard [28].

2.5. Cell surface biotinylation

Biotinylation of cell surface proteins was carried out by adding S-NHS-biotin (0.5 mg/ml) to the apical or basolateral compartments of filter-grown Caco-2 monolayers (14 day growth) and was performed a total of three times for 20 min each at 4°C in PBS containing 0.1 mM $CaCl_2$ and 1.0 mM MgCl [29]. The cells were then washed and the reaction quenched 6 times in PBS containing 0.1 mM $CaCl_2$, 1.0 mM MgCl, and 50 mM glycine (5 min each) at 4°C [30]. Following removal from the filter membrane, the cells were solubilized in detergent and subjected to either pentamannosyl phosphate-agarose chromatography or immunoprecipitation.

2.6. Streptavidin blotting

Samples biotinylated with S-NHS-biotin, after either immunoprecipitation or purification by pentamannosyl phosphate-agarose affinity chromatography, were subjected to SDS-PAGE under reducing conditions using the buffer conditions of Laemmli [31] and transferred to Immobilon-P using the conditions described by Burnette [32]. Immobilon-P membranes were blocked in PBS containing 0.5% Tween-20, 10% glycerol (v/v), 1.0 M glucose, 2% nonfat dry milk (Carnation), and 3% BSA for 2 h. After incubation with ^{125}I-streptavidin ($6.0 \cdot 10^7$ cpm/blot) in PBS containing 0.5% Tween-20, 10% glycerol (v/v), 1.0 M glucose, and 0.3% BSA for 2 h, the blots were washed (six times for 5 min each) in PBS plus 0.5% Tween-20 and dried [29]. Biotinylated proteins were visualized by autoradiography at −70°C using Kodak XAR-5 film and Cronex Lightning Plus intensifying screens (DuPont). To quantify the amount of biotinylated proteins, radiolabeled receptor bands were excised from membranes and the radioactivity of the bound ^{125}I-streptavidin was measured using a γ-counter. The results were then normalized to total protein.

2.7. Surface binding of ^{125}I-IGF-I and ^{125}I-β-glucuronidase

Caco-2 cells grown on filters were prepared for ligand binding studies with ^{125}I-β-glucuronidase by a 5 min incubation with 10 mM Man-6-*P* in PBS at 4°C to dissociate any endogenous lysosomal enzymes. Caco-2 cells were incubated for 10 min with an acidic pH buffer (12 mM citrate phosphate, pH 5.0, and 0.15 M NaCl) at 4°C to dissociate any endogenous ligands [6,33] prior to ligand binding studies with ^{125}I-IGF-I. For all surface binding studies, cells were washed thoroughly with PBS. Following addition of iodinated ligand (0.6 nM IGF-I or 0.2 nM β-glucuronidase) in serum-free DMEM containing 1% BSA and 20 mM Hepes (pH 7.2) to either the apical or basolateral chamber, the cells were incubated at 4°C for 24 h. To determine specific binding to the IGF-I receptor, ^{125}I-IGF-I ligand binding studies were performed in the absence and presence of αIR-3, a monoclonal antibody which specifically blocks the binding of IGF-I to the IGF-I receptor [34]. Preliminary experiments were performed at a range of αIR-3 concentrations to demonstrate that maximum inhibition of ^{125}I-IGF-I binding to Caco-2 cells was reached at 1 μg/ml (data not shown), a level consistent with that seen previously for maximal inhibition of IGF-I binding to the IGF-I receptor [34]. Identical concentrations of mouse IgG were used in the controls to account for any nonspecific effects that the presence of IgG may have on ligand binding. Nonspecific binding was determined in the pres-

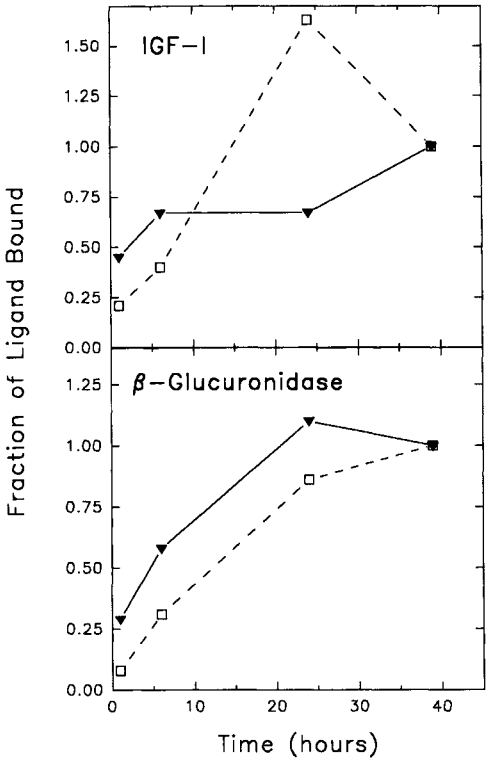

Fig. 1. Surface binding of iodinated ligands. Surface binding of iodinated IGF-I and β-glucuronidase to Caco-2 cells grown on filters was performed as described in Section 2. Specific binding to the apical (▼) and basolateral (□) surface is shown as a function of time and is represented as a fraction of the specific binding observed at the 40 h time point.

ence of 10 mM Man-6-*P* for ^{125}I-β-glucuronidase binding studies. Incubation of Caco-2 cells with each of the ligands at 4°C for various times indicated that equilibrium had been reached by 24 h (Fig. 1). Following incubation with ligands, apical and basolateral media were collected, precipitated with 10% trichloroacetic acid, and the radioactivity in the pellet and supernatant were measured separately in a γ-counter to assay for ligand degradation. The cells were washed with PBS, solubilized in PBS containing 1.0% Triton X-100 and 0.1% sodium deoxycholate, assayed for total protein, and the radioactivity measured in a γ-counter. Specific binding was calculated by subtracting nonspecific binding from total binding, and two-tailed unpaired Student's *t*-tests were performed to determine whether the differences between their means were significant.

2.8. *Internalization of ^{125}I-IGF-I and ^{125}I-β-glucuronidase*

Caco-2 cells grown on filters were prepared for ligand internalization studies in the same manner as the surface binding studies except that all washing and incubations were performed at 37°C. The iodinated ligands in serum-free DMEM containing 1% BSA were added to the apical or basolateral chamber and incubated for 2 or 6 h at 37°C. Nonspecific binding was determined as described in the above surface binding studies. Following incubation with ligand, the plate containing the cells on filter supports was placed in an ice water bath for 1 h. Apical and basolateral media were collected, precipitated with 10% trichloroacetic acid, and the radioactivity in the pellet and supernatant was measured separately in a γ-counter to assay for ligand degradation. To remove surface bound IGF-I, the cells were washed three times for 15 min each with PBS containing 1% BSA followed by three washes for 15 min each with an acidic pH buffer (12 mM citrate phosphate, pH 5.0, and 0.15 M NaCl). To remove surface bound β-glucuronidase, the cells were washed three times for 15 min each with PBS containing 1% BSA plus 10 mM glucose 6-phosphate followed by three washes for 15 min each with PBS containing 1% BSA plus 10 mM Man-6-*P*. The cells were washed three times with PBS and then scraped from the filter. The cells were solubilized in PBS containing 1.0% Triton X-100 and 0.1% sodium deoxycholate, assayed for total protein, and the radioactivity representing internalized ligand was measured in a γ-counter. Specific binding was calculated by subtracting nonspecific binding from total binding, and two-tailed unpaired Student's *t*-tests were performed to determine whether the differences between their means were significant.

3. Results

3.1. *Caco-2 cells synthesize IGF-II / CI-MPRs, CD-MPRs, and IGF-I receptors*

To demonstrate the presence of the MPRs as well as the IGF-I receptor, Caco-2 cells were labeled with [^{35}S]methionine/cysteine. Following labeling, the cells were extracted with detergent and then subjected to pentamannosyl phosphate-agarose affinity chromatography. Fig. 2A shows that Caco-2 cells synthesize proteins of ~230 kDa and ~42 kDa that bind to the pentamannosyl phosphate-agarose affinity column, corresponding to the apparent M_r of the IGF-II/CI-MPR and CD-MPR, respectively [5,11,12]. The absence of the proteins in the glucose 6-phosphate eluate as well as their presence in the Man-6-*P* eluate demonstrates ligand specificity, thereby confirming their identity as MPRs. In addition, identical results were seen when immunoprecipitations of cell lysates were carried out with antibodies specific for the MPRs (data not shown). In a separate experiment, detergent-solubilized [^{35}S]methionine/cysteine-labeled Caco-2 cells were immunoprecipitated with a monoclonal antibody specific for the IGF-I receptor and analyzed by SDS-PAGE. Both the α- and β-subunits of the IGF-I receptor are observed at ~135 kDa and ~90 kDa, respectively (Fig. 2B), which coincide with the apparent M_r previously reported for this receptor [3]. When immunoprecipitations were performed with insulin receptor-specific antisera, the insulin receptor was observed at extremely low levels (data not shown).

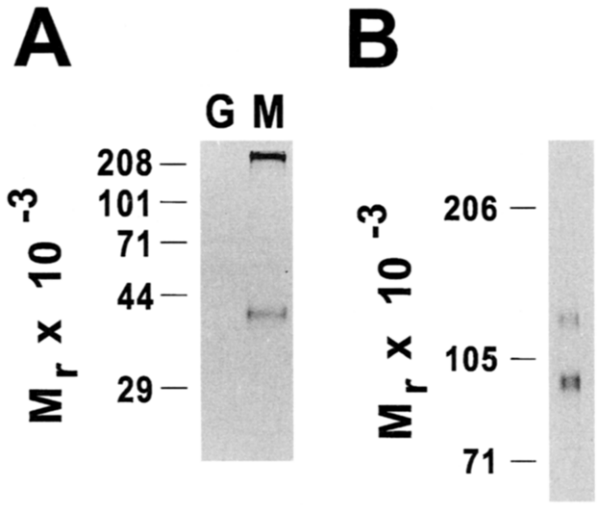

Fig. 2. Biosynthesis of MPRs and IGF-I receptor. (a) Detergent-solubilized cell lysates from [^{35}S]methionine/cysteine-labeled Caco-2 cells were passed over a pentamannosyl phosphate-agarose column. Following washing, the column was eluted first with 5 mM glucose 6-phosphate (nonspecific ligand) and then with 5 mM Man-6-*P* (specific ligand). The glucose 6-phosphate (G) and Man-6-*P* (M) eluates were precipitated with 10% trichloroacetic acid and analyzed on a 10% SDS-polyacrylamide gel under reducing conditions. (b) Detergent-solubilized cell lysates from [^{35}S]methionine/cysteine-labeled Caco-2 cells were immunoprecipitated with IGF-I receptor-specific antisera (αIR-3) and analyzed on a 7.5% SDS-polyacrylamide gel under reducing conditions. Radiolabeled bands were visualized by fluorography of gels impregnated with EN^{3}HANCE. The migration of molecular weight standards is indicated.

3.2. Polarized expression of MPRs and the IGF-I receptor

Selective labeling of apical or basolateral cell surfaces was performed using S-NHS-biotin, a water soluble biotin analog that has been shown to be impermeable to cell membranes and tight junctions [29]. Following biotinylation, the MPRs and IGF-I receptor were purified, subjected to SDS-PAGE, and transferred to Immobilon-P membranes. The membranes were then probed with ^{125}I-streptavidin to visualize the biotinylated proteins. The basolateral-to-apical surface ratio for each receptor was determined and the results are summarized in Table 1. The MPRs and IGF-I receptor were detected on both the apical and basolateral surfaces, with a basolateral enrichment of 3 for the IGF-II/CI-MPR (Fig. 3A), 3.4 for the CD-MPR (Fig. 3B), and 3.6 for IGF-I receptor (Fig. 3C). When biotinylated samples were immunoprecipitated with insulin receptor-specific antisera, insulin receptors were detected in levels too low to be accurately quantified (data not shown).

3.3. Binding and internalization of IGF-I

To assay for functional IGF-I receptors on the cell surface of Caco-2 cells, either apical or basolateral surfaces of cell monolayers were exposed to iodinated ligand. Precipitation of conditioned medium with 10% trichloroacetic acid demonstrated that there was no change (< 1%) in the percentage of precipitable counts from that seen for the iodinated ligand before incubation with the cells, indicating that no significant degradation of IGF-I had occurred during the incubation with Caco-2 cells (data not shown). Cells were incubated in the continual presence of ^{125}I-IGF-I for either 24 h at 4°C to measure binding to cell surface IGF-I receptors, or for 2 h at 37°C to measure internalization of the ligand. Specific binding of IGF-I by the IGF-I receptor was determined by subtracting the amount bound in the presence of αIR-3, a monoclonal antibody which specifically blocks the binding of IGF-I to the IGF-I receptor [34], from the amount of ligand bound in the presence of normal mouse IgG. Specific surface binding of ^{125}I-IGF-I by the IGF-I receptor measured at 4°C was 3.2-fold greater at the basolateral surface (Table 2), a result which is consistent with the observed basolateral enrichment of the IGF-I receptor as determined by cell surface biotinylation studies (Table 1). Specific internalization of ^{125}I-IGF-I at 37°C was also observed at both surfaces, with a 1.2-fold greater internalization occurring from the basolateral surface (Table 2). Similar results were observed when ^{125}I-IGF-I binding studies were performed on Caco-2 cells that were not pre-washed with an acidic pH buffer (data not shown). To confirm that the IGF-I receptor was in fact mediating the binding and internalization of IGF-I on the cell surface, intact Caco-2 cells were incubated in the presence of ^{125}I-IGF-I at concentrations similar to that used in the previous binding and internalization studies (Table 2), and DSS, followed by preparation of total membranes. Analysis by SDS-PAGE indicated that the α-subunit of the IGF-I receptor at ~ 135 kDa bound iodinated IGF-I, and this binding was effectively inhibited in the presence of excess unlabeled IGF-I (data not shown).

3.4. Internalization of β-glucuronidase occurs at the basolateral but not the apical surface

To assay for functional IGF-II/CI-MPRs on the cell surface we used a ligand specific for the IGF-II/CI-MPR. Iodinated β-glucuronidase, a lysosomal enzyme, was used as the specific ligand following purification on an IGF-

Table 1
Surface distribution of receptors

Receptor	Basolateral/apical [a]	*n* [b]
IGF-II/CI-MPR	3.0 ± 0.3	4
CD-MPR	3.4 ± 1.0	2
IGF-I receptor	3.6 ± 0.5 [c]	4

[a] Values represent the mean ± S.E. of the ^{125}I-streptavidin measured in receptor bands excised from Immobilon P membranes. The radioactivity determined was normalized to total protein and the basolateral-to-apical ratio of the receptors is shown.

[b] Number of independent measurements performed.

[c] Values were determined by measuring the radioactivity of the ~ 135 kDa α-subunit of the IGF-I receptor.

Table 2
Ligand binding and internalization

		cpm/mg total protein [a]						
		cell surface [b]				internalized [c]		
		− [d]	+ [e]	specific [f]		− [d]	+ [e]	specific [f]
^{125}I-GF-I [g]	apical (Ap)	5600 ± 123	2084 ± 90	3516	2 h:	20328 ± 778	8061 ± 302	12267
	basolateral (Bl)	15222 ± 1310	4080 ± 153	11142	2 h:	20480 ± 1774	5843 ± 414	14637
	Bl/Ap			3.2	2 h:			1.2
^{125}I-Glucuronidase [h]	apical (Ap)	2548 ± 186	1220 ± 212	1328	2 h:	946 ± 112	281 ± 33	665
					6 h:	2553 ± 282	708 ± 69	1846
	basolateral (Bl)	4280 ± 988	1010 ± 112	3270	2 h:	37214 ± 5541	217 ± 19	36998
					6 h:	114302 ± 3792	504 ± 21	113797
	Bl/Ap			2.5	2 h:			55.6
					6 h:			61.6

[a] Values represent mean ± S.E. Each filter of confluent, polarized Caco-2 cells contains ~0.3–0.4 mg total protein.
[b] Experiment performed at 4°C for 24 h.
[c] Experiment performed at 37°C for either 2 h or 6 h, followed by stripping of surface binding at 4°C.
[d] Values represent binding in the absence of competition (for β-glucuronidase), or in the presence of IgG (for IGF-I).
[e] Values represent binding in the presence of αIR-3 (IGF-I) or 10 mM Man-6-*P* (β-glucuronidase).
[f] Specific binding or internalization was calculated by subtracting competed values from noncompeted values ($P < 0.05$ in all cases).
[g] Values represent three independent measurements for cell surface and internalization studies.
[h] Values represent two independent measurements for cell surface and three for internalization studies.

II/CI-MPR affinity column to ensure that all of the ligand would contain Man-6-*P* residues. Because the CD-MPR is inefficient in binding and internalizing exogenous lysosomal enzymes [17,18], uptake of β-glucuronidase would be due only to the IGF-II/CI-MPR. Surface-specific binding of β-glucuronidase at 4°C was observed to occur at both surfaces with 2.5-fold greater binding at the basolateral surface (Table 2), which is consistent with the basolateral enrichment of the IGF-II/CI-MPR as determined by cell surface biotinylation studies (Table 1). In addition, a 55.6-fold greater uptake occurred from the basolateral surface

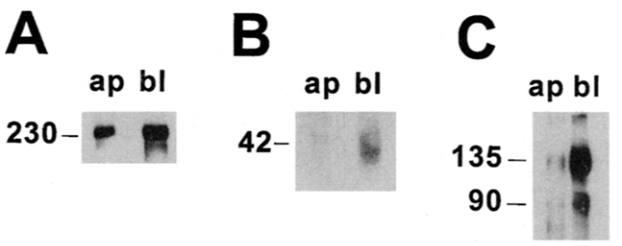

Fig. 3. Domain-selective labeling of Caco-2 monolayers with S-NHS-biotin. Caco-2 cells grown on filters were incubated with S-NHS-biotin present in either the apical (ap) or basolateral (bl) medium, solubilized and processed as described below. Samples were then analyzed on 9% SDS-polyacrylamide gels, transferred to Immobilon-P, and biotinylated proteins were visualized by probing with ^{125}I-streptavidin. (a) Man-6-*P* eluate from a pentamannosyl phosphate-agarose column containing IGF-II/CI-MPRs was precipitated with 10% trichloroacetic acid. (b) Following immunoprecipitation with IGF-II/CI-MPR-specific antisera, the cell lysate was subjected to pentamannosyl phosphate-agarose affinity chromatography and the Man-6-*P* eluate containing CD-MPRs was precipitated with 10% trichloroacetic acid. (c) The run-through fraction from pentamannosyl phosphate-agarose affinity chromatography was immunoprecipitated with IGF-I receptor-specific antisera (αIR-3). The migration of molecular weight standards is indicated.

when the cells were exposed to β-glucuronidase for 2 h at 37°C (Table 2). When the cells were exposed to β-glucuronidase for 6 h at 37°C, three times more ligand was internalized than during the 2 h incubation and a similar (61.6-fold) greater uptake was observed from the basolateral surface. In contrast, little internalization of β-glucuronidase was observed when the cells were exposed apically to ^{125}I-β-glucuronidase for either 2 h or 6 h at 37°C (Table 2). These results indicate that although the IGF-II/CI-MPR is present on both surfaces (Fig. 3A, Table 2), the receptor can internalize lysosomal enzymes only from the basolateral surface. To determine whether the lack of internalization at the apical surface was due to degradation or removal of the Man-6-*P* recognition marker from ^{125}I-β-glucuronidase, several experiments were performed. First, conditioned medium was precipitated with 10% trichloroacetic acid and the percentage of precipitable counts was found to be the same (< 3% difference) as that found with ^{125}I-β-glucuronidase prior to incubation with the cells (data not shown). Second, conditioned medium was subjected to IGF-II/CI-MPR affinity chromatography. SDS-PAGE analysis of the column run-through and the Man-6-*P* eluates indicated that the same percentage of binding to the receptor column occurred as that observed for ^{125}I-β-glucuronidase prior to incubation with the cells (data not shown). Third, after 2 h of 37°C exposure of ^{125}I-β-glucuronidase to the apical or basolateral surface of Caco-2 cells, the conditioned medium was removed, added to the basolateral chamber of Caco-2 cells grown on filters that had not been previously exposed to ligand, and incubated for another 2 h. The results indicated that the amount of internalized ^{125}I-β-glucuronidase from the basolateral surface was the same whether the radiolabeled ligand medium

was unconditioned, conditioned apically, or conditioned basolaterally (data not shown). Taken together, these results demonstrate that the lack of internalization of ^{125}I-β-glucuronidase at the apical surface is not due to degradation or loss of Man-6-*P* from ^{125}I-β-glucuronidase.

4. Discussion

Epithelial cells are characterized by the polarized distribution of plasma membrane proteins and lipids into two distinct surface domains: an apical domain which faces the exterior of the organism and a basolateral domain which faces the internal environment. The tight junctions that separate these two domains form a barrier between the two environments, allowing epithelial cells to carry out a variety of vectorial functions such as the transport of nutrients, ions, and other macromolecules [35]. Since IGFs are found in human milk [36], saliva [37], and serum [1], intestinal epithelial cells are exposed to IGFs on both their apical and basolateral surfaces. In addition, the preferential expression of the IGF receptors in proliferative crypt cells of the intestinal epithelium [19,38] suggests an important function for these receptors in regulating cell proliferation. To begin to address the roles of the IGF-I receptor and the IGF-II/CI-MPR in intestinal epithelial cells, we have utilized domain-selective biotinylation and ligand binding studies to determine the steady state surface distribution of these receptors in the enterocyte-like Caco-2 cell line.

Analysis of surface biotinylated proteins revealed a 3.6-fold enrichment of the IGF-I receptor on the basolateral surface (Fig. 3 and Table 1). Consistent with this finding were ligand binding studies which demonstrated a 3.2-fold higher level of binding at the basolateral surface (Table 2). IGF-I binding and its subsequent uptake observed at 37°C was shown to be due solely to the IGF-I receptor since the specificity of binding was demonstrated by inhibition with a well-characterized monoclonal antibody (αIR-3) that specifically inhibits IGF-I binding to the IGF-I receptor [34]. In addition, only low levels of the insulin receptor were detected in Caco-2 cells. Thus, these results indicate that functional IGF-I receptors exist on both the apical and basolateral surface domains of Caco-2 cells. It is unclear why the observed basolateral to apical ratio of IGF-I internalization (1.2, see Table 2) is not representative of the steady state surface levels of the IGF-I receptor. One possibility is that the rates of internalization of the apical and basolateral IGF-I receptors differ. Thus, additional experiments will be required to test this hypothesis.

Surface biotinylation (Fig. 3 and Table 1) and ^{125}I-β-glucuronidase binding studies (Table 2) also showed a 3-fold and 2.5-fold, respectively, enrichment of the IGF-II/CI-MPR on the basolateral surface of Caco-2 cells. These results are consistent with the immunohistochemical localization of IGF-II/CI-MPRs in both the apical and basolateral membranes of the intact rat and human colon [39]. In contrast, electron microscopic immunocytochemistry studies of Caco-2 cells showed that plasma membrane IGF-II/CI-MPRs are localized primarily to the apical domain, with little or no label present on the basolateral surface. However, IGF-II/CI-MPRs were observed in the vicinity of the basolateral membrane in smooth and coated dense vesicles as well as in electron-lucent vesicles [40]. It is not clear why the earlier immunocytochemistry studies [40] failed to detect significant levels of the receptor on the basolateral surface of Caco-2 cells. One possibility is that the significantly greater passage number (passage 130–170) of the Caco-2 cell line used in the earlier study [40] caused subtle changes in the cells that influenced the surface distribution of the IGF-II/CI-MPR. It has also been observed that the surface distribution of the IGF-II/CI-MPR varies among different types of polarized cells. For example, the IGF-II/CI-MPR was shown by domain-selective labeling methods and confocal microscopy to be present exclusively on the basolateral surface of MDCK cells [41] while immunocytochemistry of rat kidney indicated an apical localization of the receptor in proximal tubule cells [42]. The functional significance of distinct patterns of surface expression of the IGF-II/CI-MPR in these different polarized epithelial cells remains to be determined. In addition, our results also demonstrate that Caco-2 cells synthesize both MPRs and that the CD-MPR is enriched 3.4-fold on basolateral membranes (Table 1). In contrast, the CD-MPR could not be detected on the surface of polarized MDCK cells [41], indicating tissue and/or cell type differences in the expression of the CD-MPR on the cell surface. Caco-2 cells have been shown to exhibit marked polarity with respect to plasma membrane enzymes [21] and transport processes [43]. However, the MPRs and IGF-I receptor exhibit only a partial polarization, as has been reported for the epidermal growth factor receptor which exhibits a 2.5-fold enrichment on basolateral membranes of Caco-2 cells [44]. This partial polarization may be indicative of the need for these receptors to interact with their respective growth factors which are present in both the serum and the intestinal lumen [1,36,37,44].

Unexpectedly, our results showed that the IGF-II/CI-MPR expressed on the apical surface is functionally distinct from that of the receptor expressed on the basolateral surface. A similar observation has been made for the EGF receptor expressed in Caco-2 cells. Although the EGF receptor is found on both apical and basolateral membranes, only the basolateral EGF receptor increased its tyrosine kinase activity upon exposure to EGF [45]. Our results showed that Man-6-*P* inhibitable internalization of the lysosomal enzyme β-glucuronidase occurred almost exclusively from the basolateral side (Table 2). Analysis of the β-glucuronidase in conditioned medium demonstrated that the lysosomal enzyme and its Man-6-*P* recognition marker were intact, ruling out preferential degradation of the ligand in the apical medium. Although β-glucuroni-

dase is not internalized by the IGF-II/CI-MPR present on the apical surface, the apical receptor is capable of binding Man-6-*P*. This was demonstrated by specific binding of β-glucuronidase at 4°C on the apical surface (Table 2) and by the observation that the receptor that had been biotinylated on the apical surface bound to a pentamannosyl phosphate-agarose column (Fig. 3A). One explanation for these observations is that the IGF-II/CI-MPR cannot undergo endocytosis from the apical surface. This is not a general feature of Caco-2 cells since IGF-I (see Table 2), as well as epidermal growth factor [44], can be internalized from the apical surface. In addition, cobalamin bound to intrinsic factor has been shown to undergo transcytosis exclusively from the apical to basolateral surface [43]. Impaired internalization of lysosomal enzymes has been observed in another intestinal epithelial cell line. A recent report by Braulke et al. [46] showed that the human colon adenocarcinoma cell line SW1116 was defective in the endocytosis of lysosomal enzymes, and this effect was attributed to a defect in the endocytosis of the IGF-II/CI-MPR rather than to a general defect in membrane internalization. Thus, additional studies will be required to determine whether the IGF-II/CI-MPR expressed in apical membranes is structurally different from the receptor found in basolateral membranes.

In preliminary experiments, we have detected soluble, truncated forms of the IGF-II/CI-MPR in both the apical and basolateral medium of Caco-2 cells, with higher amounts seen in the apical medium (unpublished observation, Wick and Dahms). Thus, it is possible that the majority of the receptor that reaches the apical surface is processed for release into the apical medium. Truncated, soluble forms of the IGF-II/CI-MPR have been detected in serum [5] and have been shown to be synthesized by several different rat tissue explants in culture [47]. In addition, pulse-chase labeling experiments of several cell lines in culture suggest that extracellular release into the medium serves as a major degradative pathway for the IGF-II/CI-MPR [48]. Therefore, additional experiments will be required to determine whether the IGF-II/CI-MPR is targeted to the apical surface of Caco-2 cells for subsequent proteolysis and release into the medium. In addition, since it has been demonstrated that in Caco-2 cells protein sorting to the apical plasma membrane can occur either in the *trans* Golgi network prior to vesicular transport to the cell surface or by transcytosis after delivery to the basolateral cell surface [49], our future studies will be directed towards determining the route by which MPRs and IGF-I receptors are delivered to apical membranes.

Acknowledgements

We thank Mary Brzycki-Wessell for critical comments on the manuscript. This work was supported by Grant DK44200 from the National Institutes of Health.

References

[1] Humbel, R.E. (1990) Eur. J. Biochem. 190, 445–462.
[2] Czech, M.P. (1989) Cell 59, 235–238.
[3] Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. and Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2503–2512.
[4] Sasaki, N., Rees-Jones, R.W., Zick, Y., Nissley, S.P. and Rechler, M.M. (1985) J. Biol. Chem. 260, 9793–9804.
[5] Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330.
[6] Tong, P.Y., Tollefsen, S.E. and Kornfeld, S. (1988) J. Biol. Chem. 263, 2585–2588.
[7] Kiess, W., Blickenstaff, G.D., Sklar, M.M., Thomas, C.L., Nissley, S.P. and Sahagian, G.G. (1988) J. Biol. Chem. 263, 9339–9344.
[8] Lammers, R., Gray, A., Schlessinger, J. and Ullrich, A. (1989) EMBO J. 8, 1369–1375.
[9] Yamamoto, H., Prager, D., Yamasaki, H. and Melmed, S. (1993) Endocrinology 133, 1420–1425.
[10] Furlanetto, R.W. (1988) Endocrinology 122, 2044–2053.
[11] von Figura, K. and Hasilik, A. (1986) Annu. Rev. Biochem. 55, 167–193.
[12] Nolan, C.M. and Sly, W. S. (1987) Adv. Exp. Med. Biol. 225, 199–212.
[13] Oka, Y., Rozek, L.M. and Czech, M.P. (1985) J. Biol. Chem. 260, 9435–9442.
[14] Griffiths, G., Matteoni, R., Back, R. and Hoflack, B. (1990) J. Cell Sci, 95, 441–461.
[15] Klumperman, J., Hille, A., Veenendaal, T., Oorschot, V., Stoorvogel, W., Von Figura, K. and Geuze, H.J. (1993) J. Cell Biol. 121, 997–1010.
[16] Duncan, J.R. and Kornfeld, S. (1988) J. Cell Biol. 106, 617–628.
[17] Stein, M., Zijderhand-Bleekemolen, J.E., Geuze, H., Hasilik, A. and Von Figura, K. (1987) EMBO J. 6, 2677–2681.
[18] Ma, Z.M., Grubb, J.H. and Sly, W.S. (1991) J. Biol. Chem. 266, 10589–10595.
[19] Laburthe, M., Rouyer-Fessard, C. and Gammeltoft, S. (1988) Am. J. Physiol. 254, G457–462.
[20] Lund, P.K., Moats-Staats, B.M., Hynes, M.A., Simmons, J.G., Jansen, M., D'Ercole, A.J. and Van Wyk, J.J. (1986) J. Biol. Chem. 261, 14539–14544.
[21] Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Biol. Cell 47, 323–330.
[22] Ogier-Denis, E., Blais, A., Houri, J.-J., Voisin, T., Trugnan, G. and Codogno, P. (1994) J. Biol. Chem. 269, 4285–4290.
[23] Wick, D.A., Seetharam, B. and Dahms, N.M. (1994) FASEB J. 8, A1405 (abstract #853).
[24] Dahms, N.M., Rose, P.A., Molkentin, J.D., Zhang, Y. and Brzycki, M.A. (1993) J. Biol. Chem. 268, 5457–5463.
[25] Dahms, N.M., Wick, D.A. and Brzycki-Wessell, M.A. (1994) J. Biol. Chem. 269, 3802–3809.
[26] Jadot, M., Canfield, W.M., Gregory, W. and Kornfeld, S. (1992) J. Biol. Chem. 267, 11069–11077.
[27] Glaser, J.H. and Sly, W.S. (1973) J. Lab. Clin. Med. 82, 969–977.
[28] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[29] Sargiacomo, M., Lisanti, M., Graeve, L., Le Bivic, A. and Rodriguez-Boulan, E. (1989) J. Membr. Biol. 107, 277–286.
[30] Harter, C. and Mellman, I. (1992) J. Cell Biol. 117, 311–325.
[31] Laemmli, U.K. (1970) Nature 227, 680–685.
[32] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[33] Tong, P.Y., Gregory, W. and Kornfeld, S. (1989) J. Biol. Chem. 264, 7962–7969.
[34] Casella, S.J., Han, V.K., D'Ercole, A.J., Svoboda, M.E. and Van Wyk, J.J. (1986) J. Biol. Chem. 261, 9268–9273.
[35] Simons, K. and Fuller, S.D. (1985) Annu. Rev. Cell Biol. 1, 243–288.

[36] Corps, A.N., Brown, K.D., Rees, L.H., Carr, J. and Prosser, C.G. (1988) J. Clin. Endocrinol. Metab. 67, 25–29.
[37] Costigan, D.C., Guyda H.J. and Posner, B.I. (1988) J. Clin. Endocrinol. Metab. 66, 1014–1018.
[38] Heinz-Erian, P., Kessler, U., Funk, B., Gais, P. and Kiess, W. (1991) Endocrinology 129, 1769–1778.
[39] Pillion, D.J., Grizzle, W.E., Yang, M., Meezan, E., Stockard, C.R., Ganapathy, V., Leibach, F.H., Myers, R.B. and Haskell, J.F. (1993) Am. J. Physiol. 264, R1101–1110.
[40] Klumperman, J., Fransen, J.A.M., Tager, J.M. and Ginsel, L.A. (1992) Eur. J. Cell Biol. 57, 147–154.
[41] Prydz, K., Brändli, A.W., Bomsel, M. and Simons, K. (1990) J. Biol. Chem. 265, 12629–12635.
[42] Cui, S., Flyvbjerg, A., Nielsen, S., Kiess, W. and Christensen, E.I. (1993) Kidney Int. 43, 796–807.
[43] Ramanujam, K.S., Seetharam, S., Ramasamy, M. and Seetharam, B. (1991) Am. J. Physiol. 260, G416–422.
[44] Hidalgo, I.J., Kato, A. and Borchardt, R.T. (1989) Biochem. Biophys. Res. Commun. 160, 317–324.
[45] Bishop, W.P. and Wen, J.T. (1994) Am. J. Physiol. (Gastrointest. Liver Physiol. 30), G892–G900.
[46] Braulke, T., Mach, L., Hoflack, B. and Glössl, J. (1992) Arch. Biochem. Biophys. 298, 176–181.
[47] Bobek, G., Scott, C.D. and Baxter, R.C. (1991) Endocrinology 128, 2204–2206.
[48] Clairmont, K.B. and Czech, M.P. (1991) J. Biol. Chem. 266, 12131–12134.
[49] Matter, K., Brauchbar, M., Bucher, K. and Hauri, H.-P. (1990) Cell 60, 429–437.