Original Article

Characterization of anti-Tn-antigen MLS128 binding proteins involved in inhibiting the growth of human colorectal cancer cells

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Summary   MLS128 monoclonal antibody, which binds an epitope consisting of two or three consecutive Tn-antigens, inhibits colon cancer cell growth by binding to a 110 kDa glycoprotein (GP). Previous studies suggested a possible association of insulin-like growth factor-I receptor (IGF-IR) signaling in the inhibition of colon cancer cell growth by MLS128 (Morita *et al.* *Biosci Trends. 3, 32-37, 2009*; Zamri *et al. ibid. 6, 303-312, 2012*). The current study thus investigated the nature of 110 kDa GP and its possible association with IGF-IR. MLS128 treatment for 3 days caused down-regulation of IGF-IR and disappearance of 110 kDa GP in HT29 colon cancer cells. Immunoprecipitation/immunoblotting experiments did not reveal a direct association between the two molecules in HT29 cells. In LS180 and HT29 cells, however, 110 kDa GP and IGF-IR were found in microdomains. Treatment of these cells with MLS128 for 3 days caused a reduction in the IGF-IR and 110 kDa GP associated with microdomains. Two-dimensional gel electrophoresis/MLS128 immunoblotting of HT29 and LS180 cell lysates and immunoprecipitates revealed three spots, from which tryptic peptides were recovered for protein sequencing. Identification of 110 kDa GP was unsuccessful due to its heterogeneity and resistance to tryptic digestion. During this study, however, limited proteolysis of 110 kDa GP was observed in the microdomain-associated 110 kDa GP from HT29 and LS180 cells, suggesting that protease-susceptible sites or domains exist in the middle of 110 kDa GP. This information on limited proteolysis may provide a clue to identifying 110 kDa GP.

Keywords: Mucin-type *O*-glycans, microdomains, limited proteolysis, immunoprecipitation, immunoblotting, two-dimensional gel electrophoresis

1. Introduction

A common *O*-linked glycosylation with *N*-acetyl-galactosamine (GalNAc) occurs at serine or threonine residues of glycoproteins (GPs), including mucins. GalNAcα-Ser/Thr, which is known as Tn-antigen, is the precursor for all mucin-type *O*-glycans. Tn-antigens

are shielded by extended glycosylation in healthy and benign tissues but are uncovered in approximately 90% of carcinomas (*1,2*). The anti-Tn monoclonal antibodies (mAbs) MLS128 and 83D4 were produced two decades ago by immunizing mice with "cancerous antigens" respectively derived from LS180 colon cancer cells and breast carcinoma tissue (*3-5*).

MLS128 is IgG$_3$ that recognizes the structure of two or three consecutively arranged GalNAc of Tn-antigens (*5*). Osinaga *et al.* used surface plasmon resonance (SPR) to measure the kinetic parameters for anti-Tn-antigen 83D4 and MLS128, and they showed that MLS128 binds to a synthetic glycopeptide, Tn3, with approximately 10 times higher affinity than for

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Tn2 whereas 83D4 binds to both glycopeptides with a similar level of affinity (*6*). Recent efforts by the current authors have focused on molecular cloning of the variable domains of MLS128 (*7*) and determining the thermodynamic properties of Tn3 affinity for MLS128 (*8*). The current authors previously reported that MLS128 significantly inhibits breast and colon cancer cell growth and suggested involvement of insulin-like growth factor-I receptor (IGF-IR) signaling in MLS128's inhibition of cancer cell growth (*9*). More recently, the current authors indicated that a 110 kDa GP is the receptor for MLS128 in colon cancer cells (*10*). That work yielded two significant findings.

First, treating three colon cancer cell lines, LS180, LS174T, and HT29, with MLS128 for 3 days resulted in inhibition of their cell growth and obvious disappearance of the 110 kDa band as indicated by immunoblotting with MLS128. The disappearance of the110 kDa band was clearly evident in HT29 cells on Day 1, 2, and 3 after the mAb treatment but the disappearance of the 110 kD band was seen only on Day 3 after the treatment of LS180 and LS174T cells. The disappearance of the 110 kDa band could be explained by either the loss of the Tn-antigen epitopes on the protein backbone or degradation of the protein backbone itself. Second, the growth of colon cancer cells depends in part on IGF-IR signaling, as suggested by anti-IGF-IR mAb treatment that inhibited cell growth *via* IGF-IR down-regulation. MLS128 treatment of LS180, LS174T, and HT29 cells did not significantly cause IGF-IR down-regulation as previously described except for HT29 cells on Day 3 (*10*). That study revealed that MLS128 bound specifically to 110 kDa GP in the three colon cancer cell lines examined and that IGF-IR signaling was not associated with MLS128's inhibition of the growth of LS180 and LS174T cells. Results, however, did not exclude a possible link between IGF-IR- and 110 kDa GP-mediated growth signaling pathways in HT29 cells.

Based on the above findings, the current study has further characterized the 110 kDa GP in order to understand MLS128's inhibitory action on cell growth in HT29 and LS180 colon cancer cells. Specifically, the original working hypothesis regarding the interaction between IGF-IR and the 110kDa GP has been intensively tested using HT29 colon cancer cells.

2. Materials and Methods

2.1. *Materials*

Production and characterization of MLS128 (anti-Tn antigen IgG$_3$) and 1H7 (anti-IGF-IR IgG$_1$) mAbs were previously described (*1-4* and *11-14*, respectively). LS180 and HT29 human colon adenocarcinoma cells were obtained from the American Tissue Type Culture Collection. Rabbit anti-IGF-IRβ (#3027) was purchased from Cell Signaling Technology (Beverly, MA, USA).

Mouse anti-actin clone C4 antibody was obtained from MP Biomedicals, LLC. (Santa Ana, CA, USA). Rabbit anti-Src family kinase (SFK) (SRC 2; sc-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit or -mouse secondary antibody labeled with biotin was from Kirkegaard & Perry Lab. (Gaithersburg, MD, USA). Cell culture media (DMEM and McCoy's 5A) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. *Cell culture*

LS180 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 4.5 mg/mL D-glucose and 110 μg/mL pyruvic acid. HT29 cells were cultured in McCoy's 5A containing 10% FBS. All culture media included a 1% penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA).

2.3. *Effects of 1H7 or MLS128 on the growth of colon cancer cells*

Cells (1×10^4) were plated in wells of a 96-well plate and cultured in 100 μL of respective media containing 10% FBS for 24 h. Attached cells were then washed twice with 10 mM phosphate buffer, pH 7.4, containing 0.14 M NaCl (PBS), and cultured in 100 μL of media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL) (*Note*: The mAb concentrations used were different due to the difference in their affinities for respective ligands or shed mucins which sequester MLS128 from binding to cell surface receptors). After culturing for 72 h, cell growth was determined using a CCK-8 cell counting kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Absorbance at 450 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA). Quadruple wells were prepared for each data point.

2.4. *Western blotting*

HT29 colon cancer cells were collected by scraping. They were then centrifuged at $200 \times g$ for 5 min and solubilized in 50 mM Tris-HCl buffer, pH 7.4, containing 1% NP40, 2 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (Sigma-Aldrich P2714) (Lysis buffer A) on ice for 15 min. Supernatants were obtained from solubilized cells by centrifugation at $17,000 \times g$ for 10 min. Protein concentrations were measured using the Bradford method. The solubilized proteins (2 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 3% bovine serum albumin (BSA) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Tween 20 for 1 h at

room temperature. Western blotting was carried out with anti-IGF-IR and MLS128 as primary antibodies. Bound primary antibodies were then detected with biotin-labeled secondary antibodies using the Vectastain ABC-Amp kit and an alkaline phosphatase kit (Vector Laboratories, Inc. Burlingame, CA, USA).

2.5. Effects of 1H7 or MLS128 treatment on IGF-IR and 110kDa GP levels in HT29 colon cancer cells

HT29 colon cancer cells (8×10^5) were cultured in the media containing 10% FBS for 24 h in wells of 6-well plates. Cells were then cultured in the media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL). After culturing for 24, 48, and 72 h, cells were collected and solubilized in 50 μL of the lysis buffer as described above. The solubilized proteins (2 μg per lane) were separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed thereafter as described above using primary antibodies against 110 kDa GP (1.6 μg/mL MLS128), IGF-IR β subunit (1000-fold dilution), and β-actin (20,000-fold dilution). Bound primary antibodies were visualized and then analyzed using an NIH Image 1.63 Analysis system (Research Services Branch, the US National Institute of Mental Health).

2.6. Immunoprecipitation (IP) and immunoblotting (IB) of IGF-IR or 110 kDa GP using 1H7, MLS128, and control antibodies from HT29 cell lysates

Whether IGF-IR and 110 kDa are associated or not was determined by IP with anti-IGF-IR, or MLS128, or control IgG$_3$ from cell lysates followed by IB. HT29 colon cancer cells cultured in the media containing 10% FBS in 150 mm dishes were collected by scraping and then centrifuged at $200 \times g$ for 5 min. After they were washed with PBS, the cells were suspended in 1 mL of the lysis buffer on ice for 15 min. Supernatants obtained by centrifugation at $17,000 \times g$ for 15 min were incubated with 20 μL of protein G Sepharose at 4°C for 1 h by rotating. After centrifugation at $17,000 \times g$ for 10 min, protein concentrations of the supernatants were determined. Lysis buffer A was added to yield a concentration of 500 μg protein/200 μL, to which 10 μg of anti-IGF-IR, MLS128, or IgG$_3$ was mixed by rotation at 4°C overnight. To bring down associated protein complexes, 25 μL of 50% protein G Sepharose was added to each tube and the tubes were rotated for 30 min. The gels were recovered by centrifugation at $17,000 \times g$ for 10 min and then suspended in 1 mL of 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20. After this washing process was repeated 5 times, the gels were suspended in 30 μL of 1× SDS-PAGE sample buffer and then subjected to heating at 100°C for 5 min. IP samples were analyzed by Western blotting using anti-IGF-IR, MLS128, or anti-IgG$_3$ as described above.

2.7. Sucrose gradient fractionation of HT29 and LS180 cell lysates

All steps were carried out at 4°C as previously described (*15,16*). HT29 and LS180 cells grown in 3 ~ 5 150 mm-dishes were washed with chilled PBS and lysed in 2 mL lysis buffer B (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, containing protease inhibitors and 1 mM PMSF, 10 mM sodium vanadate, and 0.1% NP40) on ice for 20 min. After centrifugation for 5 min at $1300 \times g$, supernatants (2 mL) were diluted with 2 mL of 85% (w/v) sucrose in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM EDTA (TNE buffer). The diluted lysates were overlaid with 4 mL of 30% (w/v) sucrose and then with 4 mL of 5% (w/v) sucrose in TNE buffer in an ultracentrifuge tube. The samples were centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA), and fractions were collected from the top for IB analysis. Fractions were immunoblotted with anti-Src family kinase (SFK) which served as an internal marker for microdomains (*17*).

2.8. Two-dimensional gel electrophoresis

HT29 and LS180 cells (2×10^7) were lysed with 200 μL of 7 M urea, 2 M thiourea, 2% 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2% sulfobetaine 10, 1% protease inhibitor cocktail (Sigma-Aldrich), and 65 mM dithiothreitol (DTT); 40 μL of the lysates were then subjected to two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to previous methods (*18*). Briefly, isoelectric focusing was carried out on Immobiline DryStrips (pH 4-7, 18 cm; GE Healthcare, Buckinghamshire, UK) using a CoolPhoreStar IPG-IEF type-P apparatus (Anatech, Tokyo, Japan) for 18 h. The strips were equilibrated in 50 mM Tris-HCl, pH 6.8, containing 6 M urea, 2% SDS, 30% glycerol, and 2% DTT and then electrophoresed on 10% polyacrylamide gels according to Laemmli's method. Proteins were visualized by Coomassie brilliant blue (CBB) and silver staining. For immunostaining, two-dimensional gels were transferred to an Immobilon-P Membrane (Millipore, Bedford, MA, USA) and the membrane was stained with 0.4 μg/mL of MLS128 antibody followed by alkaline phosphatase-conjugated anti-mouse IgG (Zymed Laboratories, San Francisco, CA, USA). Binding of MLS128 was visualized *via* a chromogenic method using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium (NBT) color development substrate (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Immunoprecipitates from HT29 and LS180 cell lysates by MLS128 were also separated on two-dimensional gels and immunoblotted under the same conditions described above.

2.9. *Statistical analyses*

Levels of cell growth and protein bands in Western blotting were expressed as means ± S.E. from 3 or more experiments. An unpaired Student's *t*-test was used to compare the growth or intensity of the bands in two groups of experiments performed in the absence and presence of MLS128.

3. Results

3.1. *HT29 cell growth is inhibited by 1H7 or MLS128 mAb treatment*

The current authors previously found that 1H7 or MLS128 treatment of three colon cancer cell lines,

LS180, LS174T, and HT29, for three days significantly inhibited cell growth (*10*). Although the original hypothesis regarding a possible interaction between IGF-IR and 110 kDa GP-mediated signaling in LS180 cells (*9*) was not confirmed, a possible link between IGF-IR and 110 kDa GP signaling in HT29 cells has not been excluded (*10*). As a first step to test this hypothesis, the time-course effects of 1H7 or MLS128 mAb treatment on cell growth were measured on Day 1, 2, and 3 (Figure 1). 1H7 significantly inhibited cell growth from Day1 to Day 3. While HT29 cell growth was inhibited by MLS128 on Day 1, 2, and 3, this inhibition was only significant on Day 3. These results confirm that both mAb treatments inhibit cell growth for 3 days, as was previously reported (*10*), and they also demonstrate that 1H7 treatment has a more
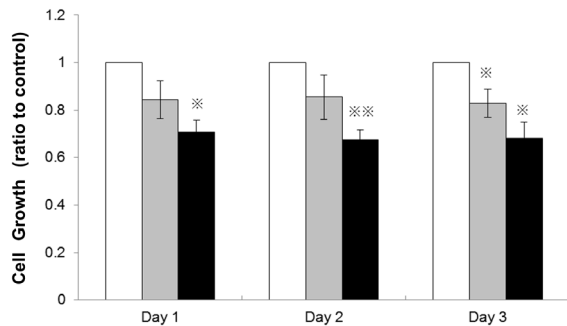
Figure 1. Effects of anti-IGF-IR 1H7 or anti-Tn-antigen MLS128 mAb on the growth of HT29 colon cancer cells. HT29 cells were cultured in the absence (control white bars) or presence of 1H7 (black bars) or MLS128 (grey bars) as described in the Methods. Cell proliferation was measured on Day 1, 2, and 3 using a CCK-8 cell counting kit (Dojindo, Kumamoto, Japan). Cell growth in the presence of either mAb on Day 1 to 3 was compared to the control (without Ab treatment): average ± S.D. (*n* = 5). * *p* < 0.05; ** *p* < 0.01.
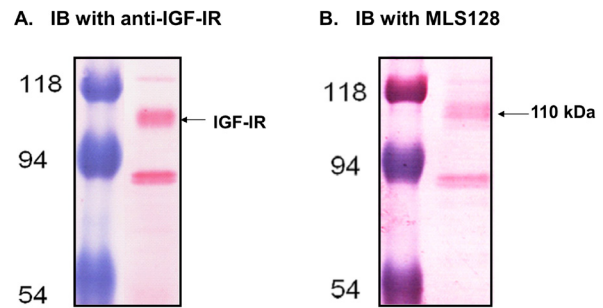
Figure 2. Expression of IGF-IR and 110 kDa GP (the receptor for MLS128) in HT29 cells. Solubilized proteins (2 μg) prepared from HT29 cells were separated by SDS-PAGE and immunoblotted with anti-IGF-IRβ **(A)** or MLS128 **(B)** as described in the Methods. The 75 and 73 kDa proteins stained are endogenous biotin-containing enzymes (*19*).
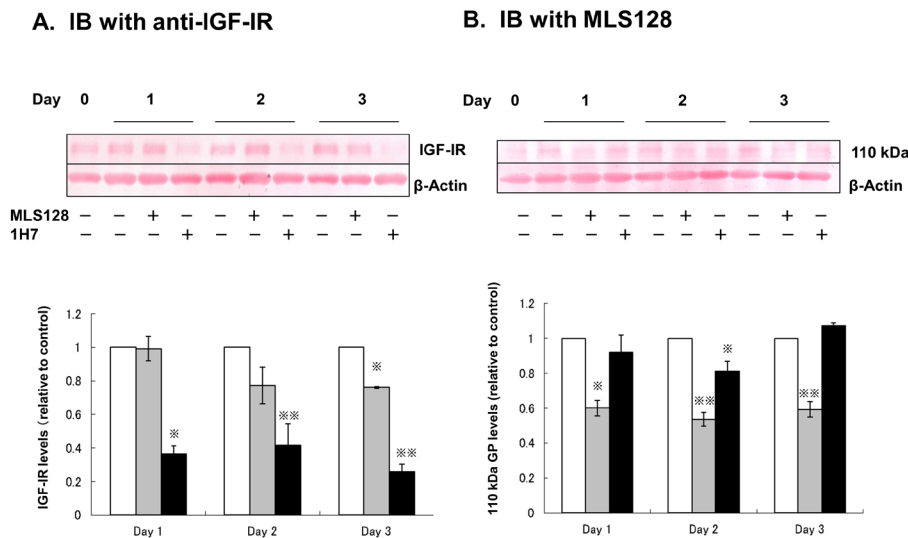
Figure 3. Effects of 1H7 or MLS128 treatment on cellular levels of IGF-IR and 110 kDa GP in HT29 colon cancer cells. HT29 cells were cultured in the absence of mAb (control; white bars) or presence of mAb (MLS128; grey bars, 1H7; black bars) for three days. Cells were harvested on Day 1, 2, and 3 and solubilized for Western blot analyses as described in the Methods. Shown are typical immunoblots from three experiments for IGF-IR **(A)** and 110 kDa GP **(B)**. Average levels of IGF-IR **(A)** and 110 kDa GP **(B)** on Day 1, 2, and 3 were calculated as ratios to the respective control. * *p* < 0.05; ** *p* < 0.01.

substantial effect than that of MLS128 treatment.

3.2. *Effects of mAb treatment on IGF-IR or 110 kDa GP in HT29 cells*

The receptors for 1H7 and MLS128 on HT29 cells were visualized by Western blotting with anti-IGF-IRβ (Figure 2A) and MLS128 (Figure 2B). The bands seen below the 94 kDa marker protein are 75 and 73 kDa endogenous biotin-containing enzymes (*19*), which served as an internal control. There were comparable levels of IGF-IR and 110 kDa GP expression in HT29 cells.

Treatment of HT29 cells with 1H7 resulted in the down-regulation of IGF-IR on Day 1-3 (Figure 3A, black bars). The levels of 110kDa GP decreased significantly in HT29 cells treated with MLS128 for 1-3 days (Figure 3B, grey bars). These results are consistent with those previously described for three colon cancer cell lines (*10*). MLS128 treatment caused down-regulation of IGF-IR on Day 2 and 3 (Figure 3A, grey bars). The effects were statistically significant on Day 3 but were not significant on Day 2. This result confirms the previous finding in LS180 cells (*9*) to some extent and provided the impetus to perform the following experiments.

3.3. *Association of IGF-IR and 110 kDa GP examined using IP/IB*

To test whether IGF-IR and 110 kDa GP are associated in HT29 cells, IP/IB experiments were carried out using combinations of anti-IGF-IR, MLS128, and control IgG₃. Since one of two independent experiments originally performed indicated a possible co-immunoprecipitation of the two molecules, IP/IB was performed using 4 times the quantity of HT29 cell lysates used in previous experiments. In Figure 4A, IGF-IR (lane 1) was visible as expected. A band

was seen at the IGF-IR position in MLS128-IP (lane 2), but a band was also seen in the control-IP (lane 3), suggesting that it must be non-specific. In anti-IGF-IR IP (Figure 4B, lane 1), no band corresponding to 110 kDa GP (lane 2) was detected. These results thus suggested no direct association between IGF-IR and 110 kDa GP (Figure 4).
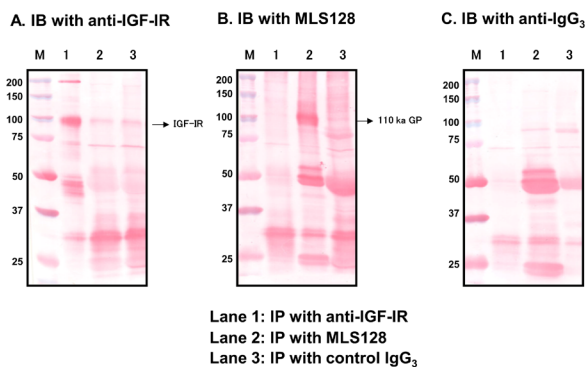
3.4. *Characterization of IGF-IR and 110 kDa GP in microdomains examined using sucrose gradient centrifugation*

Although there does not appear to be a direct association between IGF-IR and 110 kDa GP in HT29 cells, both molecules may exist in close proximity, such as in microdomains where receptors for various signaling have apparently been localized (*15-17*). Sucrose gradient fractionation of HT29 and LS180 cell lysates was performed to determine whether or not IGF-IR and 110 kDa GP exist in microdomains, and if so, to determine whether or not the treatment of cells with MLS128 for 3 days affects the distribution of 110 kDa GP and/or IGF-IR.

In HT29 cells, both IGF-IR and 110 kDa GP were localized in microdomains as shown in Figure 5A and B, respectively. The same results were obtained with LS180 cells (Figure 6B -MLS128). Anti-Src family kinase (SFK) blots indicated that the fractions 4-6 represent microdomains (Figure 5C, Figure 6 SFK).

Next, whether or not changes in the distribution of IGF-IR or 110 kDa GP occurred in HT29 and LS180 colon cancer cells after MLS128 treatment was examined. The results shown in Figure 6A (-MLS128) are, in fact, immunoblots derived from sucrose gradient centrifugation fractions of HT29 cells shown in Figures 5A, B, and C. The sucrose gradient fractionation experiments were actually performed in parallel, one for HT29 cells without MLS128 treatment (-MLS128) and the other for HT29 cells treated with MLS128 for 3 days (+MLS128), the results of which are summarized in Figure 6A. The same sets of data for LS180 cells are shown in Figure 6B. In both HT29 and LS180 cells, treatment with MLS128 for 3 days was followed by a reduction in band intensities of IGF-IR, 110 kDa GP, and control SFK in microdomains, suggested that the MLS128 treatment may have modulated microdomain organization.

During characterization of HT29 cells using sucrose gradient fractionation, an interesting phenomenon unexpectedly became apparent. Western blotting experiments were usually carried out immediately after sucrose gradient fractionation. The results shown in Figures 5A and D are, however, immunoblot experiments using the same fractions that had been kept frozen for 3 years. IB with anti-IGF-IR shown in Figure 5A was repeated 3 years after fractionation since the background of the original immunoblot was too high to

A. IB with anti-IGF-IR   **B. IB with MLS128**   **C. IB with anti-IgG₃**

Lane 1: IP with anti-IGF-IR
Lane 2: IP with MLS128
Lane 3: IP with control IgG₃

Figure 4. Immunoprecipitation (IP) and immunoblotting (IB) of IGF-IR or 110 kDa GP using 1H7, MLS128, and control antibodies from HT29 cell lysates. IP of HT29 cell lysates by anti-IGF-IR (**1**), MLS128 (**2**), or control IgG₃ (**3**) and IB with anti-IGF-IR (**A**), MLS128 (**B**), or anti-IgG₃ (**C**) were done as described in the Methods.
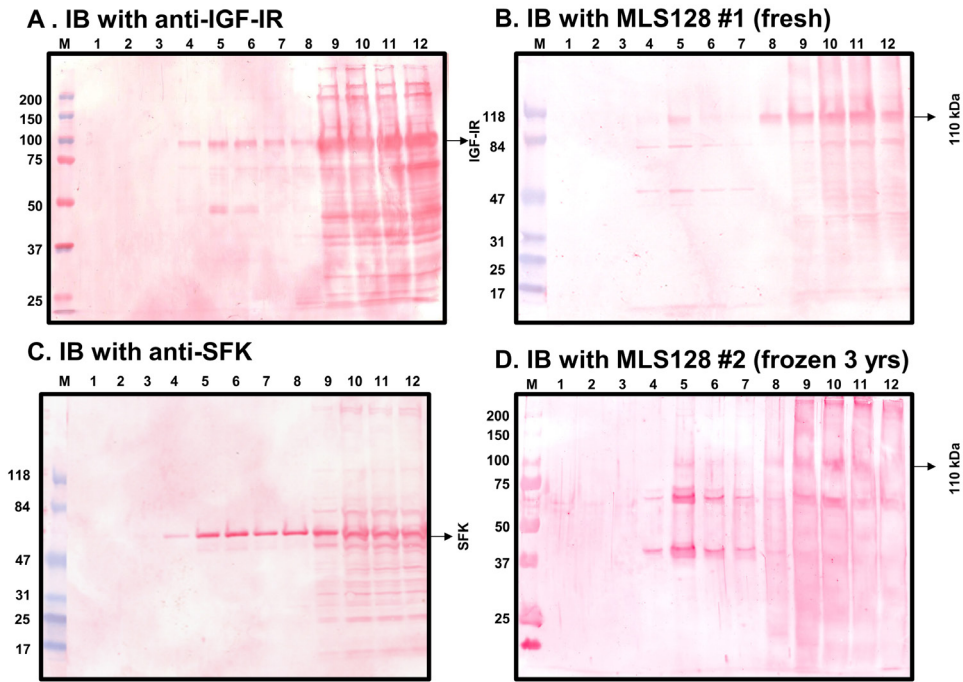
Figure 5. Characterization of HT29 cells by sucrose gradient fractionation. HT29 cell lysates were fractionated by sucrose gradient centrifugation, and then fractions were immunoblotted as described in the Methods. **(A)**, IB of fractions 1-12 stored at −80°C for 3 years with anti-IGF-IR. **(B)**, IB of fresh fractions 1-12 with MLS128. **(C)**, IB of fresh fractions 1-12 with anti-SFK. **(D)**, IB of fractions 1-12 stored at −80°C for 3 years with MLS128.
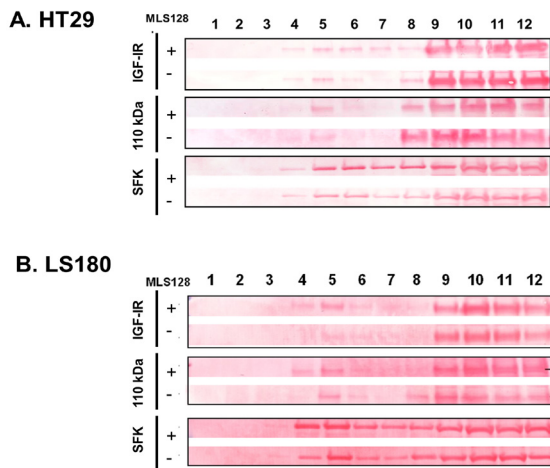

Figure 6. Effects of MLS128 treatment on IGF-IR and 110 kDa GP in the microdomains of HT29 and LS180 cells. Sucrose gradient fractionations of HT29 **(A)** and LS180 **(B)** cells that were treated with PBS (-MLS128) or MLS128 (+MLS128) were immunoblotted with anti-IGF-IR, MLS128, and anti-SFK antibodies as labeled.

clearly identify the IGF-IR band in microdomains. The IGF-IR blot redone with the frozen fractions revealed that IGF-IR is still intact after storage for 3 years (Figure 5A). In contrast, the MLS128 immunoblot of frozen samples shown in Figure 5D revealed degradation products with molecular masses of 66 and 41 kDa, which would add up to approximately 110 kDa, as seen in the immunoblot of fresh samples (Figure 5B). This observation suggested that a protease-susceptible region may exist in the middle of 110 kDa GP. Similar

degradation products of MLS128-stainable 46-48 kDa were observed in the microdomains of LS180 cell lysate fractions that had been frozen for 4 years (data not shown). The aforementioned phenomena suggested that the 110 kDa GP became susceptible to microdomain-associated proteases during storage, freezing, and thawing.

3.5. Identification of 110 kDa GP in HT29 and LS180 colon cancer cells

Extensive efforts have been made to identify the 110 kDa GP that was found to be the receptor for MLS128 in three colon cancer cell lines (*10*). It represents, after all, the first step in understanding the mechanisms by which MLS128 inhibits colon cancer cell growth. Figures 7A and B summarize two-dimensional gel electrophoresis of HT29 and LS180 cell lysates, respectively. Silver staining resulted in numerous proteins (Figure 7, A1 and B1) whereas CBB staining depicted around 100 abundant proteins (Figure 7, A2 and B2). Some of these were stained by MLS128 (Figure 7, A3 and B3), indicating that Tn-antigen epitopes are present in quite a few proteins. Spots depicting the candidate 110 kDa GP are indicated by arrows in Figure 7, A3 and B3. The position is identified by comparing the immunoblot of HT29 immunoprecipitates, which only revealed three MLS128-stainable spots in the area (Figure 7, C1). In two-dimensional electrophoresis, the same GPs with different numbers of sialic acid residues are most often separated on a scale with evenly
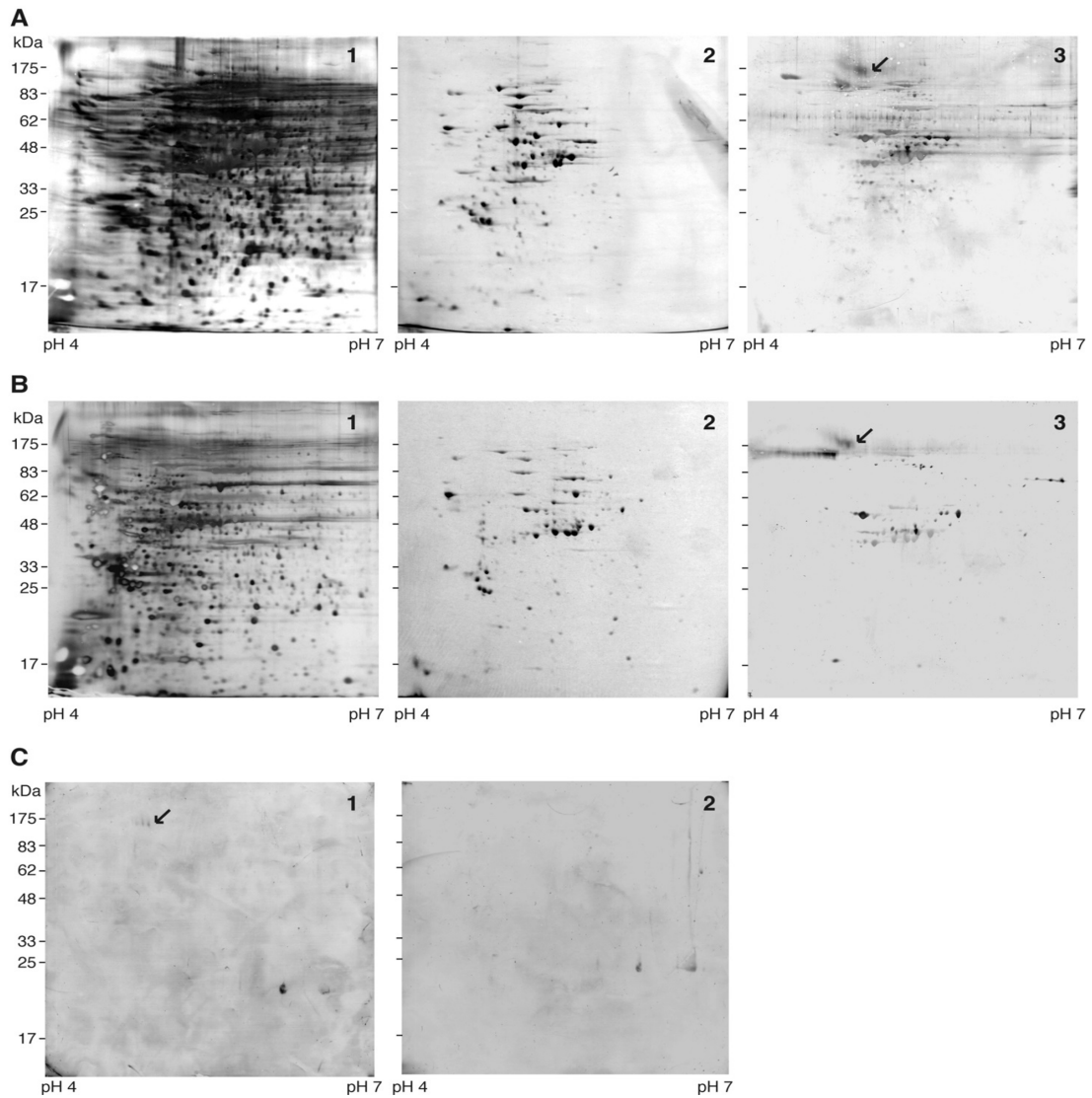
Figure 7. Two-dimensional gel electrophoresis of HT29 and LS180 cell lysates, and immunoprecipitates of those lysates.
Cell lysates prepared from HT29 **(A)** and LS180 **(B)** were subjected to two-dimensional gel electrophoresis as described in the Methods. Silver staining (**1**), CBB staining (**2**), and IB with MLS128 (**3**) are shown. **(C)** shows results of two-dimensional gel electrophoresis of immunoprecipitates from HT29 lysates (**1**) and LS180 cell lysates (**2**) immunostained with MLS128. An arrow points to three spots in C1, identifying the location of 110 kDa GP in the immunoblots of HT29 and LS180 cell lysates (**A3** and **B3**, respectively).

spaced intervals by isoelectric focusing. Since 110 kDa GP is expected to contain clustered *O*-glycans based on its reactivity with MLS128, these three spots may represent the same protein with microheterogeneity of their glycans. In contrast, these spots of 110 kDa GP were not observed in the immunoprecipitates from LS180 cell lysate (Figure 7, C2), although spots of 110 kDa were found in the lysate (indicated with an arrow in Figure 7, B3). The reason why the spots seen in the HT29 cell lysate IP were not detected in the two-dimensional electrophoresis of LS180 cell lysate IP may be because of the difference in the density of Tn-antigen clusters on 110 kDa GP. A triplet Tn glycoepitope on the 110 kDa GP from LS180 cells might be less abundant than that of 110 kDa GP from HT29 cells. In IB, target molecules are immobilized on a membrane, allowing multivalent binding with

antibodies, while they may have weaker interaction in solution in IP. This could cause a low recovery of 110 kDa GP in the immunoprecipitates from LS180 cell lysates.

To identify the 110 kDa proteins, these corresponding spots revealed by two-dimensional gel electrophoresis were subjected to in-gel digestion with trypsin. Tryptic peptides derived from these spots were then analyzed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry along with software to search databases. Despite repeated attempts, the 110 kDa protein could not be successfully identified. This failure may be due to the extremely low susceptibility to trypsin of 110 kDa GP as a result of interference by abundant *O*-glycosylation, the lack of a database for glycosylated peptides, and the limited availability of samples.

4. Discussion

Previous studies (*9,10*) and the current study found that: (*i*) MLS128 inhibits LS180, LS174T, and HT29 colon cancer cell growth, (*ii*) MLS128 binds to 110 kDa GP on the surface of colon cancer cells, (*iii*) although IGF-IR signaling was not associated with MLS128's inhibition of LS180 cell growth as was originally proposed, a possible link between IGF-IR- and 110 kDa GP-mediated signaling pathways was still suggested in HT29 cells, (*iv*) the 110 kDa band diminished significantly in colon cancer cells treated with MLS128 for 3 days, (*v*) 110 kDa GP along with tyrosine protein kinases such as IGF-IR and SFK was found in microdomains, and (*vi*) the levels of 110 kDa GP and the tyrosine protein kinases in microdomains were modulated by MLS128 treatment of HT29 and LS180 cells. The 110 kDa GP in microdomains was susceptible to limited proteolytic degradation, resulting in two domains.

The major question asked in this study was whether or not IGF-IR and 110 kDa GP are associated in HT29 cells. Given the results of IP/IB experiments, this possibility was excluded. Co-localization of IGF-IR and the MLS128 receptor in microdomains was, however, indicated by sucrose gradient centrifugation and IB using HT29 and LS180 cell lysates. A reduction in 110 kDa GP along with tyrosine protein kinases such as IGF-IR and SFK in microdomains was noted after HT29 and LS180 cells were treated with MLS128 for 3 days, which suggested that MLS128 binding to colon cancer cells resulted in modulation of the signaling molecules associated with the microdomains. Whether or not the modulation of microdomain-associated signaling molecules plays a role in the ability of MLS128 to inhibit cancer cell growth remains to be answered.

Despite extensive efforts to identify the 110 kDa GP in HT29 and LS180 cell lysates and immunoprecipitates, this identification was unsuccessful. Although Western blotting as shown in Figure 2B revealed a broad but distinct band, two-dimensional electrophoresis and MLS128 IB revealed the heterogeneous nature of 110 kDa GP (Figure 7, A3 and B3). The three spots stained are characteristic of having a differing number of sialic acid residues on the same protein backbone. Identification of the 110 kDa protein was unsuccessful because of its microheterogeneity and extremely low susceptibility to trypsin due to its heavy glycosylation, both of which resulted in a low recovery of 110 kDa GP tryptic peptides. Methods of removing and purifying tryptic peptides from protein bands or spots on gels are sophisticated and well established. For mucin-type GPs, however, trypsin digestion as is commonly used may not have worked efficiently due to abundant *O*-glycosylation, which would clearly render 110 kDa GP resistant to trypsin digestion. Procedures must be modified to optimize conditions for mucin-type GPs in order to identify 110 kDa GP.

A promising finding is that limited proteolysis of 110 kDa GP occurred in microdomains after freezing and storage. Two independent observations involving HT29 and LS180 cells suggested the existence of protease-susceptible sites in the 110 kDa GP. The first was the microdomain-associated 110 kDa GP degradation to two fragments with 66 and 41 kDa in HT29 cells. The second observation was that 46-48 kDa fragments were produced in the microdomains derived from LS180 cells stored at –80°C. Since those bands were generated from 110 kDa GP in cells that had not been treated with MLS128, conformational relaxation during storage, freezing, and thawing must have exposed the cleavage site(s) to contaminating proteases, resulting in limited proteolysis as was observed. Although protease inhibitor cocktails were added to cell lysates and during sucrose gradient fractionation, degradation of 110 kDa still occurred, which suggests that unidentified proteases that are resistant to the added inhibitors are responsible for the limited proteolysis of 110 kDa GP. Degradation products in HT29 and LS180 cell microdomains had differing sizes of 66/41 kDa and 46-48 kDa, respectively. This finding may support the notion that there are differences in the abundance and/or heterogeneity of *O*-glycosylation of the 110 kDa GP produced by HT29 or LS180 cells.

In summary, the current study provided new insights into the nature of 110 kDa GP. Although 110 kDa GP is not directly associated with IGF-IR, it is found in microdomains together with IGF-IR. MLS128 treatment may modulate receptors in microdomains. The 110 kDa GP was apparently heterogenous due to different degrees of its sialylation. Its heterogeneity and resistance to trypsin digestion were apparently major obstacles to its successful identification. During this study, however, 110 kDa GP was found to have sites or domains in the middle of the molecule that are susceptible to proteases. There was limited proteolysis of 110 kDa GP in the microdomains of cells that had been frozen for 3-4 years. Different sizes found in microdomain-associated 110 kDa GPs of HT29 and LS180 cells may provide clues to help identify 110 kDa GP.

Acknowledgements

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