Original Article

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Susceptibility to proteases of anti-Tn-antigen MLS128 binding glycoproteins expressed in human colon cancer cells

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Summary **Anti-Tn antigen MLS128 monoclonal antibody was produced two decades ago by immunizing mice with "cancerous antigens" derived from LS180 colon cancer cells. Previous studies demonstrated that MLS128 bound to 110 kDa glycoprotein (GP) in colon cancer cells, thereby inhibiting cell growth. Extensive attempts have been made towards understanding the inhibitory action of MLS128 on colon cancer cell growth and solving the primary structure of 110 kDa GP. Since limited proteolysis of 110 kDa GP was observed in microdomain fractions that had been kept frozen for several years, susceptibility of 110 kDa GP to trypsin and other proteases as well as N-glycosidase F has been investigated. Furthermore, 110 kDa GP expression was examined in colon cancer cells independently cultured in Akiyama laboratory. In summary, 110 kDa GP contains N-glycans. It does not contain inter-disulfide bonds but appears to have intra-disulfides. It must contain multiple cleavage sites for trypsin and thermolysin since these proteases digested 110 kDa GP to MLS128-undetectable small fragments. It seems to contain cleavage sites for cathepsin D which could cause limited digestion. LS180 cells derived from Akiyama laboratory produced a limited proteolysis product-like 75 kDa GP. This study provides a structural basis for developing cancer diagnostics and therapeutics.**

Keywords: Mucin-type O-glycans, N-glycans, limited proteolysis, colon cancer cell lines

1. Introduction

Glycosylation is the major post-translational modification of proteins, which is usually required for their functions. Glycoproteins in humans contain two main types of glycans, N-linked and O-linked glycans. Mucin type O-linked glycosylation is the most common form of O-glycosylation which is covalently bound *via* O-linked N-acetylgalactosamine (GalNAc) to serine or threonine residues of glycoproteins. GalNAcα-Ser/Thr, known as Tn antigen, is thus the precursor for all mucin-type O-glycans. Extended glycosylation shields Tn-antigen in healthy and benign tissues. Tn antigens are, however, uncovered in approximately 90 % of carcinomas (*1*). Tn antigen is thus considered as a cancer-specific biomarker and a potential target for cancer therapeutics.

Anti-Tn antigen MLS128 monoclonal antibody (mAb) was produced two decades ago by immunizing mice with "cancerous antigens" derived from LS180 colon cancer cells (*2,3*). Previous studies demonstrated that MLS128 bound to 110 kDa glycoprotein (GP) in colon cancer cells, thereby inhibiting cell growth (*4,5*). To further understand the inhibitory action of MLS128 on colon cancer cell growth, the primary structure of 110 kDa GP must be identified. Various attempts have been made to isolate and identify the 110 kDa GP. MLS128-stainable spots separated by two-dimensional gel electrophoresis (2D EP) were subjected to in-gel digestion with trypsin. Tryptic peptides excised

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from the gel 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 have been successfully identified. This failure may be due to the 110 kDa GP's extremely low susceptibility to trypsin as the result of interference by abundant O-glycosylation, the lack of a database for glycosylated peptides, or the limited availability of samples (*6*).

An important finding from the previous study though was the limited proteolysis of 110 kDa GP observed in microdomain fractions that had been kept frozen for several years (*6*). 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, suggesting that unidentified proteases that are resistant to the added inhibitors are responsible for such a limited proteolysis of 110 kDa GP.

Based on the above-mentioned observations, susceptibility of 110 kDa GP to trypsin and other proteases as well as N-glycosidase F has been investigated. Furthermore, to examine whether or not colon cancer cells generally express the 110 kDa GP, cell lysates from 6 colon cancer and other cell lines derived from Akiyama laboratory, were subjected to Western blotting which revealed that 110 kDa GP is commonly expressed in colon cancer cells, and that the limited proteolysis product similar to that observed (*6*) seems to be produced in LS180 cells. This manuscript describes the results of further characterization of the 110 kDa GP which may likely lead to identification of the receptor for MLS128 in colon cancer cells.

2. Materials and Methods

2.1. *Materials*

Production and characterization of MLS128 were previously described (*2,3*). Goat anti-mouse IgG labeled with horseradish peroxidase (HRP) was from Jackson ImmunoResearch Lab. (West Grove, PA, USA). Cell culture media (DMEM and McCoy's 5A) were purchased from Gibco (Grand Island, NY, USA). Protease Inhibitor Cocktail (P2714), trypsin, and thermolysin were obtained from Sigma-Aldrich (St Louis, MO, USA). N-Glycosidase F and cathepsin D were purchased from Roche Applied Science (Indianapolis, IN, USA) and Merck Millipore (Temecula, CA, USA), respectively.

2.2. *Cell culture*

In Yamaguchi laboratory, LS180 colon cancer cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 4.5 mg/mL D-glucose and 110 μg/mL sodium pyruvate. HT29 cells were cultured in McCoy's 5A containing 10% FBS. In Akiyama laboratory, LS180 and WiDr colon cancer cells and 293FT cells were cultured in DMEM supplemented with 10% FBS. HT29 and HCT116 colon cancer cells were cultured in McCoy's 5A supplemented with 10% FBS. RKO colon cancer cells were cultured in MEM supplemented with 10% FBS, MEM NEAA (Gibco) and sodium pyruvate. DLD1 colon cancer cells and H1299 human non-small cell lung carcinoma cells were cultured in RPMI1640 supplemented with 10%FBS. All culture media included 1% Penicillin-Streptomycin solution (Sigma-Aldrich).

2.3. *Preparation of cell lysates*

LS180 and HT-29 cells as well as other cells were cultured in their respective media. Cells were collected by scraping, followed by centrifugation at 200 × g for 5 min, and then 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 PMSF and protease inhibitors (P2714, Sigma-Aldrich) (lysis buffer A) on ice for 15 min. Supernatants were obtained from solubilized cells by centrifugation at 17,000 × g for 10 min. To examine effects of protease inhibitors, LS180 and HT29 cell lysates were also prepared in the absence of 10 mM sodium orthovanadate, 1 mM PMSF and protease inhibitors (P2714, Sigma-Aldrich). Protein concentrations were measured by the Bradford method.

2.4. *Western blotting analyses*

Solubilized proteins (10 μg) from each cell line were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 3% BSA in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Tween 20 (TBST) for 1 h at room temperature. Western blotting was carried out with MLS128 as a primary antibody, and then bound primary antibody was detected with HRP-conjugated anti-mouse IgG and color development using Ez West blue (ATTO Co., Tokyo, Japan).

2.5. *Treatment of LS180 and HT29 cell lysates with enzymes and Western blotting*

N-Glycosidase F treatment was carried out using 200 μg of cell lysates in 40 μL of PBS containing 0.5% SDS and 1% β-mercaptoethanol. Cell lysates were incubated at 90°C for 3 min, then cooled down to room temperature to which 0, 1, 3 U of N-Glyosidase F and 10 μL of 5% NP-40 was added. After overnight incubation at 37°C, the reaction was stopped by addition of 5× SDS-PAGE

sample buffer. Ten µg of cell lysates from each reaction mixture were applied to SDS-PAGE and immunoblotted with MLS128 as described above. For digestions of 110 kDa GP by trypsin, thermolysin, and cathepsin D, cell lysates were denatured as described above. Cell lysates (200 µg) were digested with trypsin in 50 µL of 0.2 M Tris-HCl buffer, pH 8.0, at 37°C overnight whereas 100 µg of cell lysates were digested with cathepsin D in 25 µL of 0.1 M sodium acetate buffer, pH 3.5, at 37°C overnight. Furthermore, cell lysates (100 µg) were digested with thermolysin in 25 µL of 50 mM Tris-HCl buffer, pH 8.0, at 30°C overnight.

2.6. *Sucrose gradient fractionation of HT29 and LS180 cell lysates*

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 1,300 × 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 immunoblot analysis. Fractions were subjected to SDS-PAGE and immunoblotting with MLS128 as described bove.

3. Results

3.1. *Structural characterization*

3.1.1. *The 110 kDa GP contains N-glycans in addition to abundant O-glycans*

MLS128 binds to a 110 kDa glycoprotein expressed in LS180, HT29, and LS174T colon cancer cells as previously reported (*5*). Since it is known that MLS128 binds Tn-antigen motifs such as two or three consecutive Tn-antigens (Tn2 or Tn3) (*3,7*), 110 kDa GP is expected to contain abundant O-glycans, many of which must be clustered. In order to determine whether or not N-glycans exist in 110 kDa GP, cell lysates prepared from LS180 and HT29 cells were treated with N-glycosidase F, and subjected to Western blotting with anti-Tn antigen mAb (MLS128). The results clearly demonstrated production of one smaller 100 kDa GP from 110 kDa GP at 1 and 10 U doses in both LS180 and HT29 cells (Figure 1), which indicated that 110 kDa GP contains N-glycans.

3.1.2. *The 110 kDa GP does not contain inter-disulfide bonds but appears to have intra-disulfides*

SDS-PAGE analyses of 110 kDa GP derived from LS180 and HT29 cells under reducing and non-reducing conditions revealed a molecular size of nearly 110 kDa, which indicated that 110 kDa GP does not seem to contain inter-disulfide bonds (Figure 2). Under non-reducing conditions, however, MLS128-stained bands were broader and smaller, approximately 100 kDa, than those seen under reducing conditions, which indicated that 110kDa GP contains intra-disulfide bonds.

3.2. *Sensitivity to proteases*

3.2.1. *Effects of endogenous proteases on 110 kDa GP during preparation of cell lysates*

In the experiments shown in Figure 2, cell lysates prepared in the presence or absence of protease inhibitors were used to compare the effect of reduction on the structure of 110 kDa GP. In both LS180 and HT29 cells, proteolytic degradation of 110 kDa GP did not seem to occur during preparation of cell lysates since an intact 110 kDa GP was observed in both cell

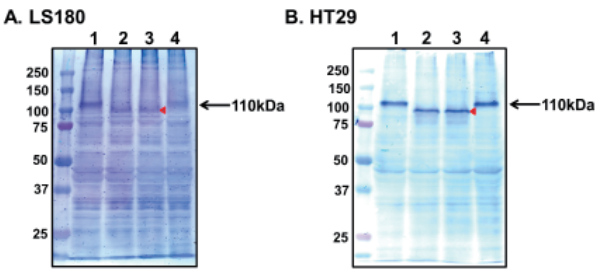

Figure 1. N-Glycosidase F digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 U (lane 1 & 4), 1 U (lane 2) and 3 U (lane 3) of N-glycosidase F at 37°C overnight, and then 10 µg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow heads indicate a 100 kDa fragment.

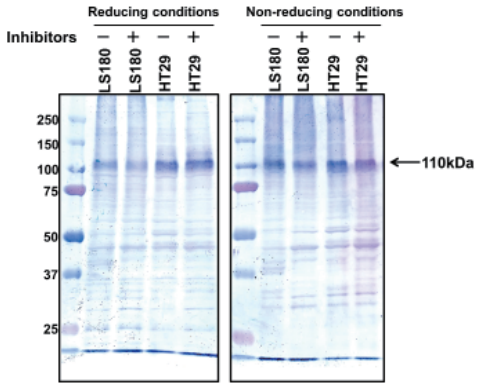

Figure 2. Comparison of non-reduced and reduced 110 kDa GP. Cell lysates were prepared from LS180 and HT29 cells in the presence (+) or absence (-) of inhibitors. Cell lysates, 10 µg each, were analyzed under reducing or non-reducing conditions by SDS-PAGE and immunoblotting as described in the Methods.

lysates prepared in the presence of protease inhibitors (Figure 2, lanes indicated with "Inhibitors +") and those prepared in the absence of them (Figure 2, lanes indicated with "Inhibitors −"). Such results suggested that 110 kDa GP is not particularly sensitive to endogenous proteases at least during the preparation of cell lysates.

3.2.2. *Digestions of 110 kDa GP by exogenous proteases*

We earlier suspected that the recovery of MLS128-detectable proteins from 2D EP gels may have been insufficient due to insensitivity of 110 kDa GP to trypsin digestion (*6*). Results that 110 kDa GP from both LS180 and HT29 cells were completely digested by trypsin at a concentration of 1 μg/μL (Figure 3B, lane 6 for HT29 cell lysates; data not shown for LS180 cell lysates) demonstrated that the low recovery of 110 kDa GP peptides from 2D EP gels was not likely due to its insensitivity to trypsin digestion. The results shown in Figures 3A and 3B were carried out to determine whether trypsin at lower concentrations is able to produce limited proteolysis fragments of 110 kDa GP as previously observed in microdomain fractions (*6*). Since LS180 lysates-derived 110 kDa GP was more sensitive to trypsin digestion than HT29 cell lysates-derived 110 kDa GP, different ranges of trypsin concentrations were used to establish partial digestions. Interestingly, trypsin at a concentration of 33 ng/μL produced a faint ~90 kDa fragment from LS180 cell lysates-derived 110 kDa GP (Figure 3A, lane 6).

Although effects of endogenous proteases on 110 kDa GP appear to be negligible while cell lysates were prepared, contamination of endogenous proteases must have caused limited proteolysis during the long-time storage of cell lysates in their partially-purified fractions. Since protease inhibitors used during sucrose gradient fractionation of LS180 and HT29 cell lysates did not include inhibitors for aspartyl proteases, there is a possibility that aspartic proteases such as cathepsin D may be responsible for the limited proteolysis observed in the microdomain fractions during 3-4 years of storage at –80°C. Results shown in Figure 4 indicated that cathepsin D treatment in fact produced a smaller size with approximately 90 kDa GP from both LS180 and HT29 cell lysates-derived 110 kDa GP. Although the 90 kDa is not exactly the same size as previously observed in the microdomain fractions, this result suggests a possible involvement of cathepsin D-like protease in the limited proteolysis of 110 kDa GP. Furthermore, although thermolysin at a concentration of 400 ng/μL completely digested LS180 and HT29 cell lysates-derived 110 kDa GP (Figures 5A and 5B, lane 3), it partially digested LS180 cell lysates-derived 110 kDa GP to about 90 kDa at a concentration of 40 ng/μL (Figure 5A, lane 2).

In summary, these results suggested that although 110 kDa GP is heavily O-glycosylated, there must be amino acid sequences accessible to those proteases, which may contribute to production of limited proteolysis as observed (*6*). In addition to cathepsin D which produced

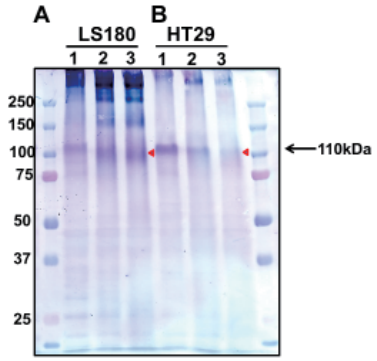

Figure 4. Cathpsin D digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 U (lane 1), 1 U (lane 2) and 10 U (lane 3) of cathepsin D at 37°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow heads indicate a 90 kDa fragment.

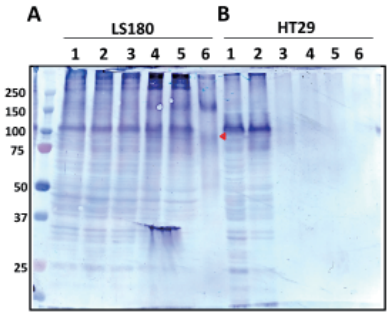

Figure 3. Trypsin digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by trypsin ; 0 ng/μL (**A** & **B**, lane 1), 2 ng/μL (**A**, lane 2), 5 ng/μL (**A**, lane 3), 11 ng/μL (**A**, lane 4), 25 ng/μL (**A**, lane 5), 33 ng/μL (**A**, lane 6 & **B**, lane 2), 100 ng/μL (**B**, lane 3), 200 ng/μL (**B**, lane 4), 500 ng/μL (**B**, lane 5), and 1000 ng/μL (**B**, lane 6) at 37°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow head indicates a 90 kDa fragment.

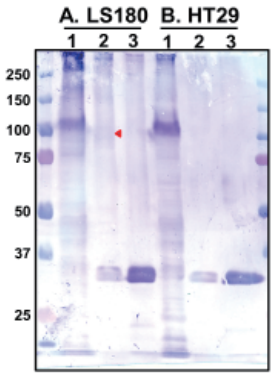

Figure 5. Thermolysin digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 μg/μL (lane 1), 40 ng/μL (lane 2) and 400 ng/μL (lane 3) of thermolysin at 30°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow head indicates a 90 kDa fragment.

the 90 kDa fragment in 110 kDa GP derived from both LS180 and HT29 cells, susceptibility of LS180 cell lysates to trypsin and thermolysin which appeared to produce a transient 90 kDa fragment was noticeable.

3.3. *Expression of 110 kDa GP in various colon cancer and control cancer cell lines*

3.3.1. *110 kDa GP is expressed in two additional colon cancer cell lines*

Six colon cancer and other types of cell lines cultured in Akiyama laboratory were analyzed together with LS180 and HT29 cells cultured in Yamaguchi laboratory to examine whether or not those cells cultured in Akiyama laboratory express 110 kDa GP (Figure 6A). In addition to LS180 and HT29 cells, DLD1 and WiDr colon cancer cells expressed 110 kDa GP at levels lower than those of LS180 and HT29 cells. In contrast, 110 kDa GP was not significantly expressed in two other colon cancer cell lines, RKO and HCT116, as well as non-colon cancer cell lines such as H1299 human non-small cell lung carcinoma cells and 293FT human embryonal kidney cells. This study thus revealed that two additional colon cancer cell lines expressed 110 kDa GP in addition to three colon cancer cell lines, LS180, LS174T, and HT29, previously reported (*5*). Five out of seven colon cancer cell lines examined have been found positive for the expression of 110 kDa GP. These results indicated a rather high incidence of 110 kDa GP expression in colon cancer cell lines.

3.3.2. *Limited proteolysis seems to occur in LS180 cells derived from Akiyama laboratory*

A notable difference was observed in the result shown in Figure 6A, that is, in contrast to 110 kDa GP that was clearly detected in LS180 cells cultured in Yamaguchi laboratory, an approximately 75 kDa GP was immunostained as a second major band in LS180 cells cultured in Akiyama laboratory. Western blotting was carried out again to closely compare the molecular sizes and expression levels of 110 kDa GP in LS180 and HT29 cells cultured in two independent laboratories. Results shown in Figure 6B confirmed the above-mentioned observation, that is, 110 kDa GP was found as the major binding protein for MLS128 in LS180 and HT29 cells cultured in either laboratory.

The fact that an additional 75 kDa fragment was found in LS180 cells cultured in Akiyama laboratory is intriguing. Proteolysis fragments with 70~75 kDa were previously identified by immunoblotting by MLS128 of sucrose density gradient fractions of both LS180 and HT29 cell lysates (*6*). The detection of such molecular masses was not definite in the previous experiments using biotin-labeled antibodies as secondary antibodies because endogenous biotin-containing enzymes with 125, 75, and 73 kDa in cell lysates were unavoidably stained (*8*). An alternative staining procedure was thus applied to distinguish MLS128 stainable fragment with 75 kDa, if it exists, from 75, and 73 kDa proteins immunostained in the previous study. Fractions 1-12 from sucrose density gradient fractionation of HT29 cell lysates were analyzed by loading 5 times more sample to SDS-PAGE gel than the previous experiments. MLS128-bound GPs blotted to a PVDF membrane was visualized by EZ-West staining (Figure 7A). The results demonstrated that two

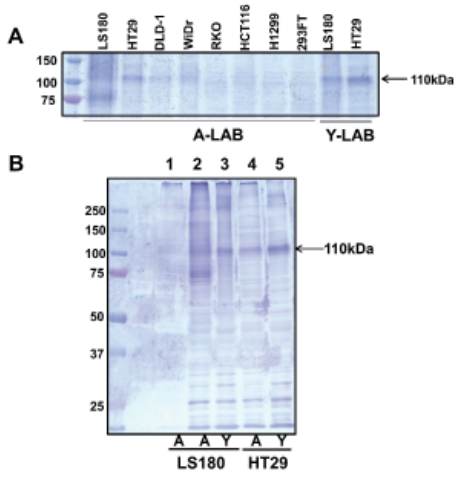

Figure 6. Binding of MLS128 to various colon cancer and other cell lines. In **A**, six colon cancer and other cell lines as indicated from Akiyama laboratory (A-LAB) were examined for expression of 110 kDa GP together with LS180 and HT29 cells cultured in Yamaguchi laboratory (Y-LAB). Other cell lines used were human non-small cell lung carcinoma cells (H1299) and human embryonal kidney cells (293FT). Ten μg of cell lysates per lane were immunoblotted as described in the Methods. In **B**, 2 and 10 μg of cell lysate prepared from LS180 cells from Akiyama (A) laboratory (lanes 1 and 2, respectively) were immunoblotted together with 10 μg of LS 180 cell lysate from Yamaguchi (Y) laboratory (lane 3) to closely compare expression levels of 110 kDa and 75 kDa immunoreactive bands. Likewise, 10 μg each of HT29 cell lysate from Akiyama and Yamaguchi laboratories were immunoblotted next each other (lanes 4 and 5, respectively).

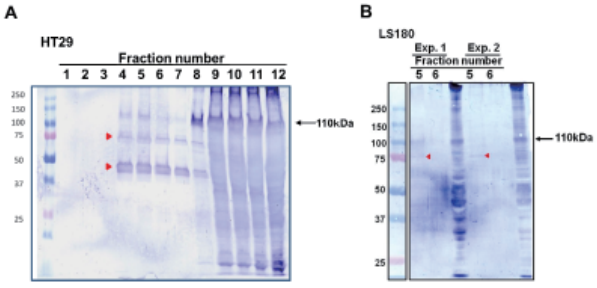

Figure 7. Immunoblotting of HT29 and LS180 cell lysates fractionated by sucrose gradient centrifugation. In **A**, fractions kept frozen for 4 years were analyzed by Western blotting and EZ-West staining using 5 times more sample than those used for the previous study (*6*). Red arrow heads indicate postions of 75 and 45 kDa fragments immunostained. In **B**, fraction numbers 5 and 6 in the microdomain from two independent sucrose gradient fractionation of LS180 cell which had been kept frozen for 4 years were analyzed by Western blotting and EZ-West staining using the same amount of sample as used for the previous study (*6*). The red arrow head indicates a 75 kDa fragment immunostained.

fragments with ~75 kDa and 45 kDa in addition to the intact 110 kDa GP were clearly seen in fractions 4-7. It is notable that fraction 8-12 in which most cellular proteins recovered contained an intact 110 kDa. This suggests so-called microdomain fractions 4-7 did not contain high levels of proteins including scaffolds which may protect 110 kDa from degradation. Furthermore, sucrose density gradient fractions 5 and 6 derived from LS180 cell lysates from two independent experiments were subjected to Western blotting and EZ-West staining. The results shown in Figure 7B demonstrated that MLS128 stainable 75 kDa fragments similar to that found in HT29 cell lysates were visible.

4. Discussion

Towards identification of 110 kDa GP, different strategies have been taken to date. After affinity purification of LS180 cell lysates by MLS128-immobilized agarose chromatography, the eluates were subjected to SDS-PAGE. The 110 kDa GP areas were analyzed by LS-MS/MS, which only revealed contaminated proteins (data not shown). Two-dimensional gel electrophoresis carried out using immunoprecipitation (IP) from HT29 cell lysates showed three distinctive MLS128-stainable spots. Tryptic peptides derived from these spots were analyzed using MALDI-TOF along with software to search data bases, which did not lead to identification of 110 kDa GP (*6*). In contrast, we found that those three spots were not detected in 2D EP of IP from LS180 cell lysates although they were visible by immunoblotting of 2D EP to which cell lysates were applied. Therefore, we speculated that possible differences exist in the distribution of Tn antigen clusters on 110 kDa GP derived from LS180 and HT29 cells. For example, if Tn antigens on 110 kDa GP derived from LS180 cells are less dense than those on 110 kDa GP derived from HT29 cells, IP with MLS128 would result in a low recovery of 110 kDa GP from LS180 cells. To further gain the structural information of 110 kDa GP, in this study, we took advantage of MLS128 by which 110 kDa GP and its fragments can be detected as long as consecutive Tn-antigen epitopes exist even after treatments with enzymes such as N-glycosidase F and proteases.

The present study provided the following structural information on 110 kDa GP. *i*) 110 kDa GP contains N-glycans. *ii*) It does not contain inter-disulfide bonds but appears to have intra-disulfides. *iii*) It must contain multiple cleavage sites for trypsin and thermolysin since these proteases digested 110 kDa GP to MLS128-undetectable small fragments. *iv*) It seems to contain cleavage sites for cathepsin D which could cause limited digestion. *v*) LS180 cells derived from Akiyama laboratory produced a limited proteolysis product-like 75 kDa GP.

First of all, this study revealed that 110 kDa GP derived from LS180 and HT29 cells contains some N-glycans in addition to many O-glycans, most of which are expected to form Tn-antigen clusters. The result suggests that 110 kDa GP belongs to well-known mucin-type glycoproteins such as glycophorin A and leukosialin (*9,10*). Glycophorin A consisting of 131 amino acid residues contains 15 O-glycans and one N-glycan at Asn^{45} whereas leukosialin consists of 381 amino acids in which 29 Ser/Thr are O-glycosylated and Asn^{239} is N-glycosylated. Apparent molecular masses of glycophoryn A and leukosialin were reported to be 36 and 50-150 kDa (*9-12*). Size wise, leukosialin is close to a 110 kDa GP. Different apparent molecular masses of 110-130 kDa have been reported for leukosialin expressed in various leukemic cell lines. The different molecular masses were shown to be caused by expression of distinct patterns of O-linked oligosaccharides that are specific to each cell type (*12*). Leukosialin, also known as CD43, was originally found in hematopoietic cells (*12*), but later identified in a variety of nonhematopoietic cancers including lung, breast, and colon (*11*). Aberrant glycosylation of leukosialin/CD43 in various cancers must have resulted in apparent molecular masses of 50-150 kDa (*11*). Monoclonal antibody called UN1 recognizes a heavily sialylated and O-glycosylated protein with apparent molecular weight of 100-120 kDa. The tumor antigen UN1 was later identified to be a different glycoform of leukosialin/CD43 (*13*). Based on these previous studies, it is probable that 110 kDa GP may share the same protein backbone as leukosialin/CD43 consisting of 381 amino acids. It should be noted, however, that 110 kDa GP is heavily O-glycosylated with GalNAc, but that extension of further glycosylation to GalNAc including sialylation does not often seem to occur. In fact, digestion of 110 kDa GP derived from LS180 and HT29 cells by sialidase did not change the molecular mass or the intensity of the 110 kDa band as judged by Western blotting (data not shown), suggesting a minimum involvement of sialic acid residues in 110 kDa GP. Earlier, immunoblotting with anti-CD43 antibody was carried out using LS180 and LS174T cell lysates, which resulted in several immunostained bands but did not recognize 110 kDa GP. To determine the amino acid sequence of 110 kDa GP, a new strategy has been initiated which takes advantage of a smaller fragment, 75 kDa GP, derived from LS180 cells cultured in Akiyama laboratory.

Since limited proteolysis was observed in the microdomain fractions after 3-4 years of storage at –80°C (*6*), susceptibility of 110 kDa GP to endogenous and exogenous proteases was analyzed. Although similar sizes of limited proteolysis fragmentation was not produced, an around 90 kDa GP was detected from LS180 and HT29 cell lysates digested by cathepsin D. Such a similar size fragment was observed in LS180 cell lysates, but not in HT29 cell lysates, digested by trypsin (Figure 3A, line 6) or thermolysin (Figure 5A, line 2). These results are consistent with our previous

results with IP using MLS128 which did not seem to precipitate LS180-derived 110 kDa GP as efficiently as HT29-derived 110 kDa GP, suggesting a possibility that differential O-glycosylations to 110 kDa GP may exist in two cell lines (*6*).

The most intriguing result in the present study was the finding of 75 kDa GP in LS180 cells cultured in Akiyama laboratory in addition to 110 kDa GP. LS180 cells originally obtained from ATCC were independently cultured in Yamaguchi and Akiyama laboratories. Over the years, differences in cell constituents must have developed in LS180 cells in Akiyama laboratory. It is possible that the region susceptible to contaminating endogenous proteases in 110 kDa GP is somehow cleaved in the cell or during lysate preparations from LS180 cells from Akiyama laboratory. The size of 75 kDa GP found in LS180 cells cultured in Akiyama laboratory (Figure 6) is very similar to the limited proteolysis fragments observed in newly immunoblotted microdomain fractions shown in Figure 7. This interesting result has given a new direction as to how to identify the primary structure of 110 kDa GP. Thus we are now analyzing the smaller size MLS128-stainable fragment using 2D EP, enzyme digestion, and sophisticated mass spectrometry available at City of Hope Mass Spectrometry & Proteomics Core equipped with an Orbitrap Fusion (Thermo) and a Triple Quadrupole (Agilent).

The present study also revealed that 110 kDa GP may be an oncoprotein expressed mainly in colon cancer cells. In addition to LS180, LS174T, and HT29 cell lines previously reported (*5*), the current study found that two out of four colon cancer cell lines newly examined expressed 110 kDa GP, which sums up to 71% of colon cancer cell lines being positive for the expression of 110 kDa GP. This is consistent with an earlier study on normal and malignant tissues by MLS128 histochemistry which revealed that positive immunostaining was detected with high frequency (75-100%) in carcinomas of the esophagus, stomach, colon, biliary tract and pancreas (*14*). Further studies on 110 kDa GP expressed in colon cancer cells would provide a fundamental basis for developing cancer diagnostics and therapeutics.

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