Glycomics and Lipidopics

Extended Abstract

Ribosomal protein S3 secreted from cancer cell lines is N-glycosylated

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Abstract:

Ribosomal protein S3(rpS3) is a component of the 40S ribosomal small subunit but has multiple other extra-ribosomal functions like apoptosis, cell cycle control, DNA repair etc. It has a DNA repair endonuclease activity which is related with various cancers. Recently, we have discovered that this protein is secreted only from various cancer cell lines as a homodimer but not in normal cells. We also confirmed that rpS3 is secreted more into media from the more invasive cancer cell lines. Presently we confirmed that the secreted protein is glycosylated at the Asn 165 residue and point mutation on this site is defective for the secretion. The secretion pathway turned out to be a ER-Golgi dependent pathway. We propose that glysosylated rpS3 could be used as a useful cancer marker.

To determine whether glycosylation is necessary for the secretion of rpS3, HT1080 cells were treated with Tunicamycin to inhibit the attachment of the precursor N-linked glycan chain to the nascent peptide, and an immunoblot assay and ELISA assay were performed. The level of rpS3 secretion decreased in the presence of Tunicamycin indicating that N-glycosylation is required for the secretion of rpS3. Tubulin and C23 were probed with the relevant antibodies to exclude the possibility that the rpS3 detected in the culture media could be derived from necrosis or rupture. C23 (NCL/ Nucleolin), a nuclear marker, was not detected in the cell culture media. Since MIF is secreted through an unconventional secretory pathway and is not glycosylated, it was employed as a negative control. MIF secretion was not inhibited by Brefeldin A or Tunicamycin, and cellular apoptosis was not induced in the presence of Brefeldin A or Tunicamycin . These results suggest that the secreted rpS3 is N-glycosylated through the typical ER-Golgi route. Secreted rpS3 is N-glycosylated To confirm the N-linked glycosylation of the secreted rpS3, the concentrated cell culture media were immunoprecipitated using anti-rpS3 antibody and treated with or without PNGase F, which can remove N-linked glycans. The band equivalent to the secreted rpS3 displayed a slight downward shift when digested with PNGase F, indicating that the secreted rpS3 was N-glycosylated. Lectin binding analysis was further performed to confirm N-linked glycosylation of the secreted rpS3. Con A is a lectin that recognizes alpha-linked mannose and terminal glucose residues, conferring the ability to isolate N-glycosylated proteins in cell lysates and concentrated culture media. As shown in, Con A bound to rpS3 in both cell lysates and media, indicating the presence of N-linked glycans. Another ribosomal protein, receptor of activated protein kinase C1 (RACK1) was not detected by Con A lectin, suggesting that ribosomes do not interact with Con A and that RACK1 is not N-glycosylated. MIF was used as a marker of unglycosylated protein, while tumor necrosis factor receptor superfamily member 6 (FAS) was employed as a marker of N-glycosylated protein. The results confirmed that secreted rpS3 is N-glycosylated. Identification of N-linked glycosylation sites in rpS3 by mass spectrometry analysis To identify the sites of N-linked glycosylation in rpS3, a large amount of secreted rpS3 protein was prepared. The enriched HT1080 cell culture media was subjected to immunoprecipitation using the rpS3 antibody. The isolated rpS3 was separated by large SDS-PAGE and then stained with Coomassie brilliant blue. MS was carried out to identify which Asn residues were the sites of glycosylation. The purified rpS3 band was subjected to PNGase F digestion. Since PNGase F is an amidase, the Asn residues from which glycans were removed and became deaminated to Asp residues, resulting in an increase in the peptide mass of one1 unit. Following trypsin digestion, the deglycosylated tryptic peptides were selectively identified by LC-MS/MS. The 1 Da increase in peptide mass for each Asn-to-Asp conversion was used as a diagnostic signature to identify the glycosylated peptides. The mass spectra were searched for deamination of the Asn residues using Mascot software. The ion score was –10* Log (P), where P is the probability that the observed match is a random event. Individual ion scores exceeding 4 indicate identity or extensive homology ($P < 0.05$) the sequence coverage map of the identified protein. The observed peptide ions accounted for 46% sequence coverage. Two (Asn22 and Asn165) of the three Asn residues in rpS3 were detected, while Asn57 peptide was not detected by MS. Therefore, we constructed N57G and NNGG as a double mutation of both Asn57 and Asn165. The values of the molecular weight of the peptide, which can be ionized in various ways, are indicated in Supplementary Table S2. The molecular weight observed by LC-MS/MS is represented in red. Native Asn22 was detected, showing the values for the Phe11- Arg40

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peptide molecular weight. The molecular weight after removal of the oligosaccharides with PNGase F is shown in Supplementary Table S2B. While the molecular mass of Asn22 was detected as 779.4046 in the glycosylated samples, it had the expected value of 780.3886 in the deglycosylated samples, which did not match. Supplementary Tables 2C and D show the molecular weight of the Phe152-Arg173 peptide with and without PNGase F treatment. The molecular mass of Asn165 was observed to be 1100.5357 in the presence of glycans, while the value of the peptide ion was replaced to 1101.5211 in the deglycosylated samples. This means the increase of 1 Da was due to the Asn-to-Asp conversion. Taken together, the LC-MS/MS data suggest that secreted rpS3 is N-glycosylated at Asn165, not Asn22. However, Asn57 remains uncertain because its fragment was not detected. Also, the result of glycosylation on Asn165 site of rpS3 protein was exactly confirmed through immunoblot assay after glycoprotein isolation with stably FLAG-rpS3 or FLAG-N165G expressed cells Asn165 is the site of N-glycosylation for rpS3 secretion To further examine the effect of the N-glycosylation sites on the secretion of rpS3, the N-glycosylation sites of rpS3 were modified by site-directed mutagenesis. RpS3 wild-type and the mutants (N57G mutated on Asn57, N165G mutated on Asn165 and NNGG as double mutation on Asn57 and Asn165) were then stably transfected into HT1080 cells. Cell lysates and concentrated cell culture media were analyzed by immunoblotting with the antibodies indicated in The expression rate of rpS3 mutants to wild type rpS3 were determined by densitometry scans. The experiments were repeated four times . In the cell lysates, except in the NNGG mutant, the expression of N57G and N165G mutants were comparable to that of wild type . Also, in our previous study [31], we confirmed that heat-shock protein 90 (Hsp90) regulates rpS3 stability by binding on the N- and C-termini of the rpS3 protein. But, the binding site was not determined. So, the reason for the decrease of NNGG is the possibility that the binding of rpS3 and Hsp90 protein is related with these two domains (Asn57 and Asn165) of rpS3 protein. Interestingly, as shown in , the N165G mutant displayed reduced secretion (approximately 44%) compared to the wild-type. However, the N57G mutant showed an even greater secretion rate (144%) than the wild-type. The NNGG mutant had significantly reduced expression and secretion, suggesting that the reduced secretion was due to degradation within the cell. Analysis of the secretion by mutants and wild type was also carried out using ELISA. The results suggest that N-glycosylation of Asn165, but not Asn57, is required for the secretion of rpS3. Glycosylation of rpS3 is mediated with cancer migration and the invasive phenotype We previously confirmed that the level of secreted rpS3 protein is increased in malignant cells [7]. Because secreted rpS3 requires N-glycosylation, we sought to confirm whether glycosylation of rpS3 is related to cancer malignancy. Migration ability and cell shape for invasion were identified using wound healing and three-dimensional (3D) culture assays with wild-type rpS3 (FLAG-rpS3) or rpS3 mutant (FLAG-N165G). After incubation of 3 days in Matrigel, the N165G mutant rpS3 showed noticeable decreases in glycosylation . N165G cells had a round shape, while FLAG-rpS3 expressing cells exhibited directional alignment at their leading edges. The wound healing assay showed the N165G mutation of rpS3 had reduced cancer cell migration. Incubation of 7 days, as in the 3D culture assays, was performed on two malignant cancer cell lines: HT1080 fibrosarcoma cell line, and WM-115 human melanoma cell line after knock-downof endogenous rpS3 by si-RNAs (human si-rpS3/777 and si-rpS3/796). Decreased rpS3 protein levels in cells induced changes in the morphology of invasive malignant cancer cells to that of normal cells . These results suggest that the glycosylation of rpS3 regulates the migration and invasive phenotype of cancer cells. Secretion and glycosylation of rpS3 is related with malignant phenotype of cancer cells To confirm that glycosylated and secreted rpS3 regulates the invasiveness of cancer cells, we identified the glycosylation status by glycoprotein isolation, cell morphology by 3D culture assay after confirmation of secretion status of rpS3 protein in a normal cell line (human dermal fibroblasts, HDFs), NIH3T3 mouse immortalized embryonic fibroblasts and a leukemia cancer cell line (RBL-2H3 rat peripheral blood fibroblasts). RpS3 secretion was increased in RBL-2H3 cells and glycosylation was increased in RBL-2H3 and HT1080 cells, but not in the immortalized cell line. Also, invasiveness of RBL-2H3, HT1080 and WM-115 cell lines, which are aggressive cancer cell lines, was dramatically increased comparing to HDF, a normal cell line and NIH3T3, a less-aggressive cell line . Especially, thenormal cell line HDF, which displayed a non-invasive cell phenotyp did not secrete rpS3 or glycosylated rpS3. These results suggest that glycosylated and secreted rpS3 proteins are related to invasive cell phenotypes such as malignant cancer cells.

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