ANALYSIS OF LYSOSOMAL Neu4 SIALIDASE ASSOCIATED PROTEINS BY USING MASS SPECTROMETRY (MS/MS)

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To my Grandmother Dursen EFECAN

ABSTRACT

ANALYSIS OF LYSOSOMAL Neu4 SIALIDASE ASSOCIATED PROTEINS BY USING MASS SPECTROMETRY (MS/MS)

Sialidases are glycohydrolytic enzymes which remove sialic acid residues from glycoproteins, oligosaccharides and glycolipids. There are 4 different sialidases known in mammalians. These are Neu1 (lysosomal), Neu2 (cytoplasmic), Neu3 (cell membrane) and Neu4 (lysosomal/mitochondrial) sialidase. Sialidases are involved in many metabolic and cellular processes interactioning with another proteins or work together in multiprotein complexes. For example, Neu1 is only active with beta-galactosidase and cathepsin A enzyme in lysosome. Interactions of sialidases Neu2, Neu3 and Neu4 enzyme with other proteins remain unknown

In our study, we aimed to identify proteins which have interaction with sialidase Neu4 as well as Neu1 by using mass spectrometry analysis to find new possible roles of sialidases. Our bait protein's cDNA was tagged with calmodulin binding protein as well as streptavidin binding protein. After transfection and expression of vectors to mammalian cells, proteins were purified using tandem affinity purification (TAP). We identified some associated proteins with sialidase Neu1 and Neu4 by using MS/MS analysis and bioinformatics.

ÖZET

LİZOZOMAL Neu4 SİALİDAZIN ETKİLEŞİME GİRDİĞİ PROTEİNLERİN KÜTLE SPEKTROMETRİSİ (MS/MS) İLE ANALİZİ

Sialidazlar glikoproteinlerden, oligosakkaritlerden, ve glikolipitlerden sialik asit gruplarını uzaklaştıran glikohidrolazlardır. Memelilerde 4 adet sialidaz karakterize edilmiştir. Bunlar Neu1 (lizozomal), Neu2 (sitoplazmik), Neu3 (hücre membranı), Neu4 (mitokondriyel/lizozomal) sialidazdır. Sialidazlar birçok metabolik yolakta ve hücresel işleyişlerde rol almaktadır. Sialidazlar başka proteinlerle ilişki kurabilir ya da multi-protein kompleksleri oluşturarak beraber çalışabilir. Örnek olarak, Neu1 hücrede beta-galaktosidaz ve katepsin A enzimi ile beraber lizozomda aktiftir. Diğer Neu2, Neu3 ve Neu4 sialidaz enzimlerinin proteinlerle ilişkileri tam olarak tanımlanamamıştır.

Çalışmamızda lizozomal Neu4 enziminin ve yanında Neu1 enziminin ilişkili olduğu proteinleri kütle spektrometrisi ile belirleme ve sialidazların yeni rollerini bulmak amaçlandı. Proteinimizin cDNA'sı kalmodulin bağlanma bölgesi ve streptavidin bağlanma bölgesi ile işaretlendi. Anlatım vektörü memeli hücrelerine transfeksiyon yapıldıktan sonra sıralı afinite saflaştırması ile proteinler saflaştırıldı. Sonuç olarak çalışmamızda sialidaz Neu4 ve Neu1 ile ilişkili olan proteinleri, MS/MS ve biyoinformatik analizleri kullanarak tanımladık.

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CHAPTER 1

INTRODUCTION

1.1. Sialidases

Sialidases (EC 3.2.1.18), also known as neuraminidases, are glycohydrolatic enzymes which remove sialic acid residues from glycoproteins (mucin), oligosaccharides (siallylactose) and glycolipids (GD1a) (Saito and Yu et al. 1995) (Figure1.1). Sialidases are found in all organisms such as virus, bacteria, protozoa and vertebrate (Saito and Yu et al. 1995). Sialidases have roles in metabolic pathways like cell proliferation, adhesion, differentiation, membrane fusion and fluidity, catabolism of gangliosides and glycoproteins (Saito and Yu et al. 1995). Substrates which carry sialic acid residues can affect and induce metabolic pathways when these residues were degraded by sialidases. (Miyagi et al. 2008).

Figure 1.1. GD1a molecule carries sialic acid (SA) residues.

In mammals, 4 different sialidases were identified according to subcellular distribution, substrate specificity and stability. These sialidases are lysosomal Neu1 (Bonten et al. 1996), cytosolic Neu2 (Monti et al. 1999), plasma membrane Neu3 (Monti et al. 2000) and lysosomal or mitochondrial membrane Neu4 (Monti et al. 2004). Human sialidases contain conserved F/YRIV/P (-Phe (Tyr)-Arg-Ileu-Val (Pro)-) motif and Asp boxes (–Ser–X– Asp–X–Gly–X–Thr–Trp–) in the N-terminal part of the enzyme (Roggentin et al. 1993).

Sialidase expression in cells is lower than other genes and detection of sialidases in tissues is difficult when compared to other proteins (Minami et al. 2011). Neu1 has the highest expression level in cells about 10- 20 times higher than Neu3 and Neu4. Neu2 has extremely low expression (Hata et al. 2008). Neu1 shows low homology to other sialidases (nearly 19-24 % homology) but Neu2, Neu3 and Neu4 have 34-40 % homology when compared to each other.

1.1.1. Neu1

Neu1 is the lysosomal sialidase which is expressed from *NEU1* on human chromosome 6p21.3, locus of the major histocompatibility complex (Bonten et al. 1996). Neu1 is 43-46 kDa protein and locates on lysosomal membrane, lysosomal lumen, cell membrane as an integral membrane protein (Lukong and Seyrantepe et al. 2001). It is also found in vesicular traffic system (Vinogradova et al. 1998). It is ubiquitously expressed in vertebrate cells with the highest expression levels in the kidney, pancreas, skeletal muscle, liver, lungs, placenta and brain (Bonten et al. 1996). One of the well-known lysosomal multienzyme complex composing of Neu1 is β-galactosidase (β-GAL) and Cathepsin A protein complex (Bonten et al. 2009).

Sialidase Neu1 removes sialic acid residues from glycoprotein and glycolipids (Pshezhetsky et al. 2001). Deficiency in NEU1 causes metobolic disorder called sialidosis and accumulation of sialylated oligosaccharides are observed in lysosomes of sialidosis patients (Thomas et al. 2001). Neu1 also has role in immune response which causes monocyte differentiation by transporting to the cell membrane from lysosome (Liang and Seyrantepe et al. 2006). Furthermore Neu1 initiates signal pathways by removing sialic acid residues from receptors like insulin, TOLL-like receptor (Figure 1.2) and regulates metabolic processes (Arabkhari et al. 2010; Stamatos et al. 2010; Amith et al. 2009).

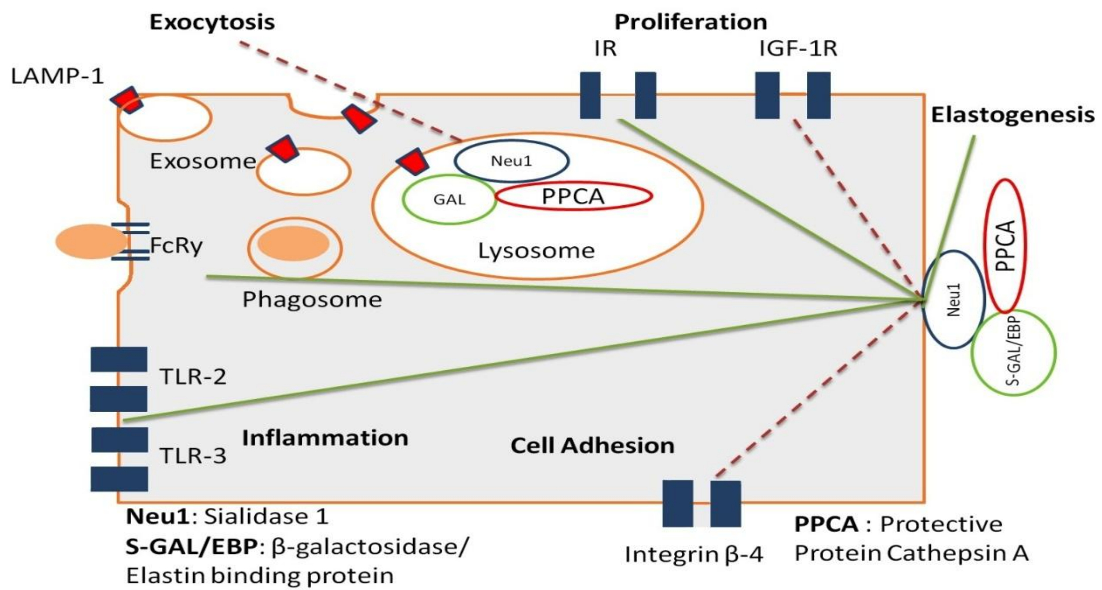

Figure 1.2. Interactions of Neu1 in cellular pathways. TLR: TOLL like receptor, LAMP: Lysosomal-associated membrane protein, IR: Insulin receptor, IGF-1R: Insulin growth factor 1 receptor, FcRy: fc receptor-related protein Y (Adapted from Alexey V. Pshezhetsky et al. 2010).

1.1.2. Neu2

Neu2 is cytosolic sialidase which is expressed on human chromosome 2q37. It was isolated from rat skelatal muscle by cDNA analysis as a mammalian sialidase (Miyagi et al. 1993). Neu2 locates on cytoplasma and has an activity on glycopeptides and gangliosides and mostly expressed in muscle cells in human. (Miyagi and Tsuiki et al. 1985; Monti et al.1999; Tringali et al. 2004). The human NEU2 gene up-regulation in leukemic K562 cells (human myelogenous leukemia) induced a marked decrease in anti-apoptotic factors Bcl-XL and Bcl-2 (Tringali et al. 2007).

1.1.3. Neu3

Neu3 is known as a membrane sialidase and is expressed on human chromosome 11q13.5. It has a role in caveolar domain of membrane (Wada et al. 1999, Wang et al. 2002). Neu3 affects ganglioside's oligosaccharide composition on the cell membrane. Neu3 also initiates induction of cell transformation, differentiation and formation of cell interactions (Kopitz et al. 1996; Kopitz et al. 1998).

1.1.4. Neu4

Neu4 is lysosomal/mitochondrial sialidase and is expressed from telomeric region of the long arm of human chromosome 2q37. Sialidase Neu4 enzyme has two isoforms which are generated by alternative splicing (Figure 1.3). Short form contains 484 amino acids (Monti et al. 2004) and long form containing 496 amino acids (Bigi et al. 2010). Mouse Neu4 has two isoforms Neu4a and Neu4b (Shiozaki, Koseki et al. 2009). It has a F/YRVP sequence motif in N terminal site and Asp boxes downstream. It is shown that Neu4 localizes on lysosomes (Seyrantepe et al. 2004), mitochondria (Yamaguchi et al. 2005) and endoplasmic reticulum, (Bigi et al. 2010). The long form of Neu4 carries additional 12 amino-acid sequence on N-terminal and it is predicted to be a mitochondrial sorting signal (Yamaguchi et al. 2005). Mannose 6-phospate tagging directs Neu4 to lysosome (Monti et al. 2004). Neu4 is active in acidic pH and degrades gangliosides suggesting that it is a lysosomal enzyme and it locates lysosomes although Neu4 sialidase enzymes carry any signal on neither C terminal nor N terminal of amino acid sequence. However it was observed that long form of Neu4 locates on mitochondria , short form locates on endoplasmic reticulum (Bigi et al. 2010).

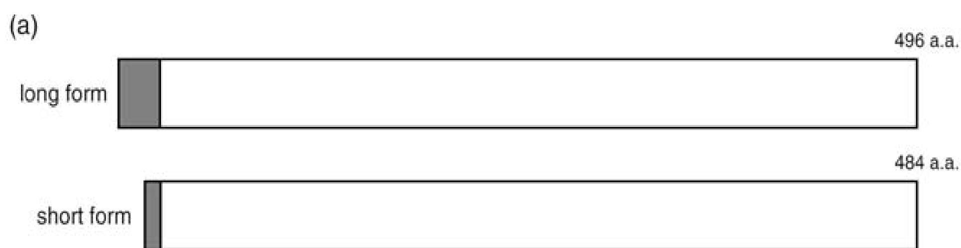

Figure 1.3. Human Neu4 sialidases isoforms
(Source: Yamaguchi et al. 2005)

Neu4 is ubiquitously expressed in human brain, colon, small intestine, kidney, heart, skeletal muscle. Human Neu4 sialidase enzyme has the highest expression level in liver. Mouse brain has the highest expression level of Neu4 sialidase compared to other tissues and organs (Comelli et al. 2003). Synthetic substrates 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (4-MU-NANA or 4MU-NeuAc) can be degraded by Neu4 sialidase (Monti et al. 2004). Murine Neu4 sialidase

hydrolyses polysialic acid residues efficiently degrading oligoSia and polySia chains as substrates *in vitro* (Takahashi et al. 2012).

Neu4 sialidase shows optimum activity in acidic pH and degrades GM1, GM2 and GD1a gangliosides *in vitro* (Figure 1.4). Overexpression of sialidase Neu4 converts lysosomal storage in human skin fibroblasts which have deficiency of lysosomal Neu1 sialidase. (Seyrantepe et al. 2004). It means Neu4 sialidase is biologically active enzyme and can be useful for novel therapeutic purposes such as in sialidosis and galactosialidosis.

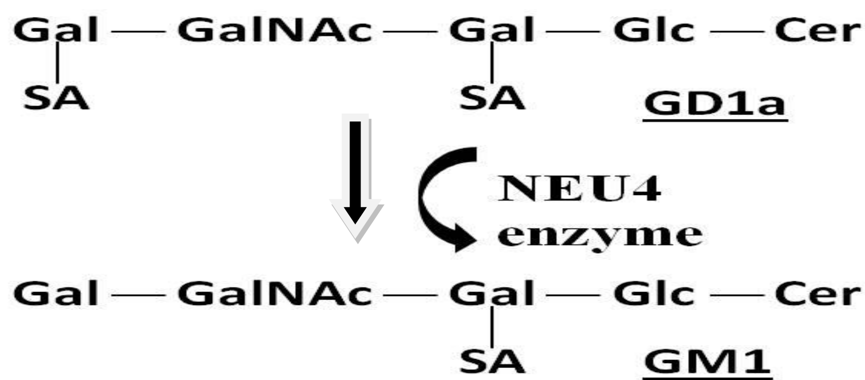

Figure 1.4. Degredation GD1a to GM1 by sialidase activity

It was observed that Neu4 knocked-out mouse has decreased GM1 and increased GD1a levels in brain tissue (Seyrantepe et al. 2008). Neu4 sialidase and HexA (hexaminidase A) knock-out mouse showed abnormal behaviors like epileptic seizures, rapid neuronal loss and accumulation of ganglioside GM2 (Seyrantepe et al. 2010). Neu4 has a role in degradation of gangliosides in mouse.

Expression of Neu4 decreases in macrophage differentiation (Stamatos et al. 2005). In human colon cancer cells Neu4 has low expression levels and overexpression of Neu4 induced apoptosis causing reduced invasiveness and motility (Miyagi et al. 2008). Neu4 acts on Sialy Lewis antigens, known as a colon cancer marker, and decreases level of Sialy Lewis antigens (Kazuhiro et al. 2011). Expression of long form Neu4 is dramatically down-regulated in the early course of apoptosis. On the other hand, overexpression of long Neu4 activates neuroblastoma proliferation via Wnt/β catenin signal pathway (Tringali et al. 2012). Thymoquinone induced Neu4 activate NFκB activation via G-protein coupled receptor (GPCR) and matrix metalloproteinase (MMP) protein complex (Finlay et al. 2010)

1.2.Tandem Affinity Purifications (TAP)

Detection of complex protein-protein interactions are important to understand biological processes in cells. Co-immunoprecipitation method can be with two-hybrid studies to detect proteins which interact each other. However it does not give information about proteins complexes with more than two proteins. To detect protein complexes in yeast, another technique was developed (Puig et al. 2001; Rigaut et al. 1999). Tandem affinity purification (TAP) provides us to detect associated protein complexes by using special tag which is added to protein amino acid sequence and we can detect proteins using mass spectrometry analysis. Two or more tags are added to DNA sequence that express bait protein. Furthermore protein and its complex are purificated by affinity steps gently with non-disrupted protein complex . Two tags are sufficent to elute protein and its complexes furthermore the method allow clean purification.

1.2.1. Interplay Mammalian Tandem Affinity Purification System

TAP system for mammalian cell (Braman et al. 2007) was developed to purify protein complex using affinity of Streptavidin Binding Peptide and Calmodulin Binding Peptide (Figure 1.5).

Streptavidin binding peptide (SBP, 4,91 kDa) which shows high affinity to streptavidin resin and can be eluted with biotin (Wilson et al. 2001; Keefe et al. 2001). Calmodulin binding peptide (CBP, 2,96 kDa) which shows high affinity to calmodulin resin in calcium solution and removing of calcium from environment releases protein complex from calmodulin resin via chelating agent. SBP and CBP are useful for tandem affinity purification and can be attached to proteins and these proteins can be eluted easily using only affinity without protease digestion (Figure 1.6).

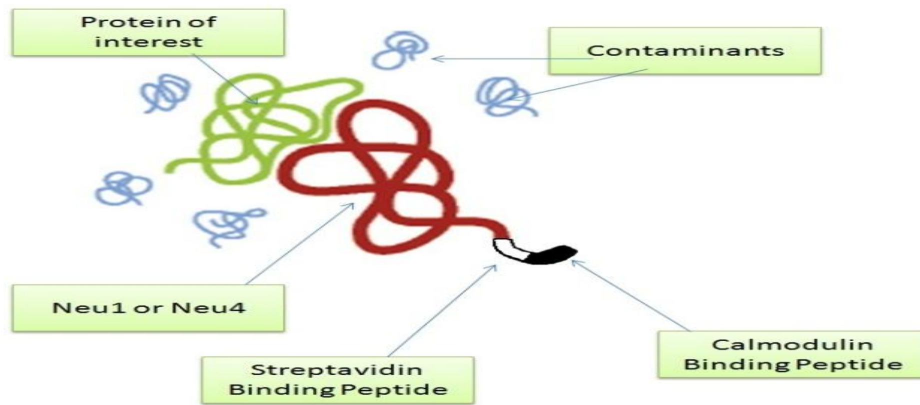

Figure 1.5. Bait protein with calmodulin binding peptide, streptavidin binding peptide and associated protein in cell (Adapted from Agilent website)

First purification step is very important because mammalian cells include a lot of contaminant which has interactions with CBP and these proteins can be purified if we choose Calmodulin resin purification.

SBP tag	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG
CBP tag	KRRWKKNFIAVSAANRFKKISSSGAL

Figure 1.6. Streptavidin Binding Peptide (SBP; 4,91 kDa) and Calmodulin Binding Peptide (CBP; 2,96 kDa) (Adapted from Agilent Kit Manual)

1.3. Mass Spectrometry (MS) Analysis

Molecules have different molecular weights and ionic charges. MS is based on weight/charge ratio of molecules because MS measures this ratio. MS is powerful technique to determine molecules and can determine sample composition, compounds and its shape, and also post-translational modifications such as phosphorylation, methylation, acetylation, and ubiquitination. Complex protein mixtures and purifed proteins can be detected by using different MS analysis depending on the studies and proteins can be identified after database searching of MS results (Figure 1.7).

Molecules must be in ionic form to be analyzed in MS. MS instrument includes ionizer, mass analyzer and mass detector. When MS/MS is applied, results give us our

proteins' fragments which is amino acid sequence depending on charge and molecular weight.

Ionized molecules are turned in gas form in ionizer module and sent to mass analyzer which have magnetic field. ESI (Electro Spray Ionization) and MALDI (Matrix Assisted Laser Desorption-Ionization) techniques are used (Domon and Aebersold et al. 2005) to produce ions.

Mass analyzers separate peptides depending on their molecular weight/charge ratio. If more than one mass analyzer is used, result is named as MS/MS and gives detailed amino acid sequence of interested protein (Domon and Aebersold et al. 2005). Quadrupole, ion traps, orbitraps, fourier transform ion cyclotron resonance (FTICR) and time of flight (TOF) are the mass analyzers.

FTICR and orbitraps do not have ion detector. Ions hit detector and ion detector give some peaks as a signal depending on molecular weight/charge ratio. Secondary electron multipliers (SEMs) and 18 microchannel plate (MCP) detectors are the most common ion detectors (Dubois et al. 1999).

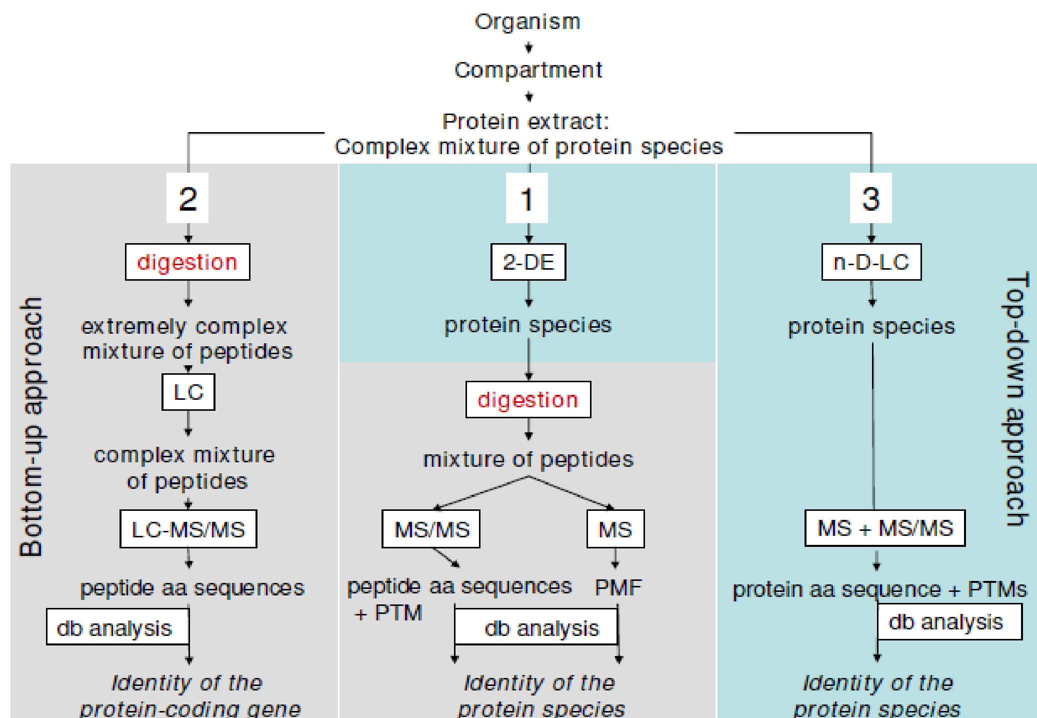

Figure 1.7. Processes to prepare a sample for mass spectrometry analysis and identification; 2-DE: two-dimensional electrophoresis; LC: liquid chromatography; n-D: ndimensional; db: database; MS: mass spectrometry; PMF: peptide mass fingerprint; PTM: post-translational modification (Source: Schlüter et al. 2009).

1.4. Aim of the Project

Our aim is to identify lysosomal Neu4 as well as Neu1 sialidase associated proteins by MS/MS analysis and find possible new roles of Neu4 sialidase in cellular events.

CHAPTER 2

MATERIALS AND METHODS

2.1. RNA Isolation and cDNA Synthesis

Total RNA was extracted from human fibroblast cell line using Trizol reagent (Invitrogen). Isolated RNA was used in RT-PCR for cDNA synthesis (Fermentas). Lysosomol Neu4 sialidase was amplified with forward primer containing EcoRI restriction site (5'-GAATTCCATGATGAGCTCTGCAGCCTTC-3') and reverse primer containing XhoI restriction site (5'-CTCGAGGGAGGGCCAGCAGCACCCCCGA-3') by DNA polymerase (NEB) removing stop codon on Neu4 sialidase cDNA.

2.2. TA Cloning

After PCR amplification, samples were run in 0,8% agarose gel and purified using Genejet Gel Extraction Kit (Fermentas, K0513). Purified 1,6 kb lysosomal Neu4 cDNA fragment was cloned into TA cloning vector (Invitrogen) (Figure 2.1) using Dh5α *E.coli* strain. Transformant cells were selected using blue-white screening. White colonies were cultured in LB-Broth medium containing 100 mg/ml kanamycin at 37 °C overnight in shaker incubator. Plasmids were isolated using alkaline lysis protocol and digested by EcoRI and XhoI enzyme at 37 °C for confirmation.

Cloned TOPO plasmids were sequenced at Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center as a service provider by different fluorescent dye labelled dideoxynucleotide chain terminating method. Using M13 primer and T7 primer. The sequences obtained from the sequencer were analysed using BLAST (Basic Local Alignment Search Tool).

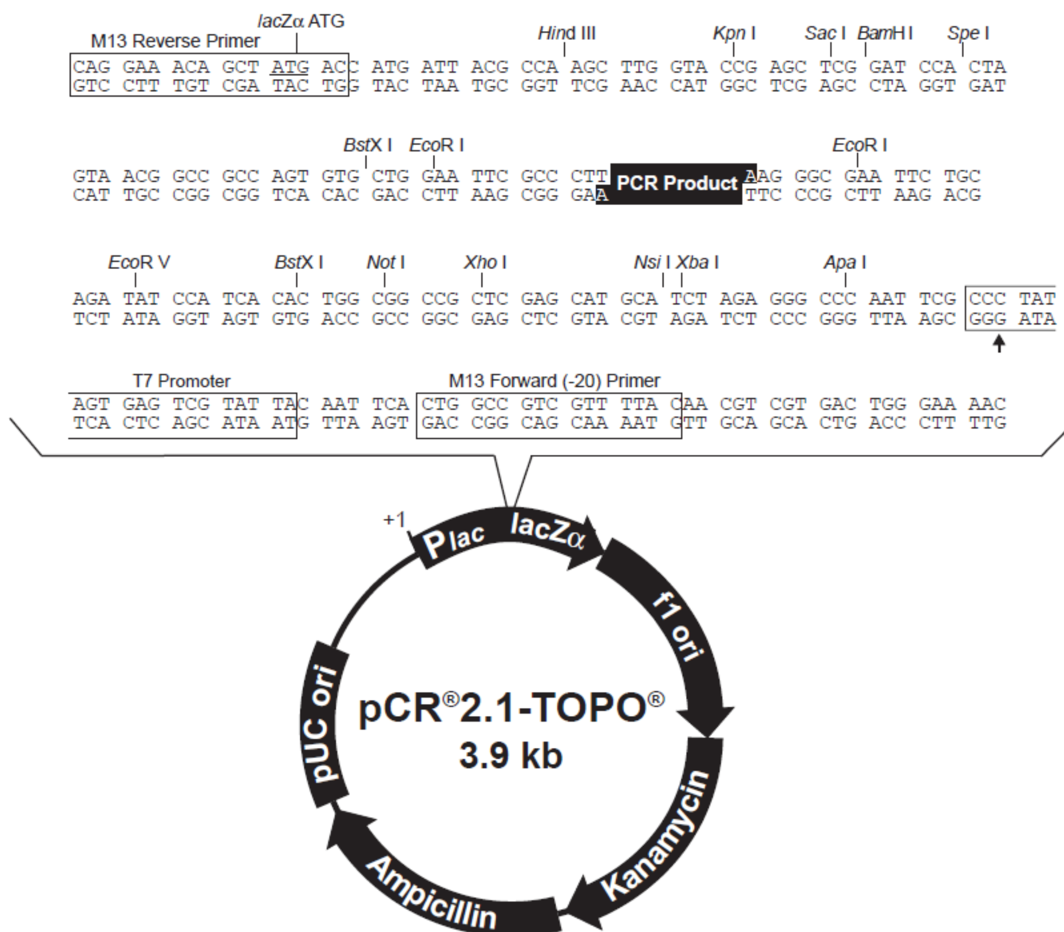

Figure 2.1. pCR 2.1 TOPO vector

2.3. Propagation of pCTAP-A and pCTAP-B Vector

Interplay Mammalian TAP Kit (Agilent) provided us 3 kinds of expression vectors named as pCTAP-A, pCTAP-B and pCTAP-C. pCTAP-B vector (Agilent) was suitable for protection of Neu4 cDNA frame and pCTAP-A for NEU1 cDNA frame preventing frame-shift mutations so they were propagated with PureLink™ Plasmid DNA Purification Kit (Midiprep, Invitrogen, K2100-04) and confirmed using Hind3 enzyme (Fermentas). Vector was confirmed using HindIII enzyme at 37 °C overnight and loaded on 1% agarose gel.

2.4. Cloning of NEU4 cDNA into pCTAP-B Vector and NEU1 into pCTAP-A

To enhance cloning efficiency, we prepared double digestion for sticky ends at the ends of the vectors and inserts. TOPO-Neu4 and PCTAP-B vectors (Figure 2.2) were double digested using EcoRI and XhoI enzyme. Enzyme units and buffer selection were determined as a recommendation of Fermentas Double Digestion Tool (http://www. fermentas.com/en/tools/doubledigest). After 2 hours digestion at 37 °C, digestion mixture loaded on 0,8% agarose gel and run. Neu4 cDNA and pCTAP-B vector with sticky ends, were purified by Genejet Gel Extraction Kit (Fermentas).

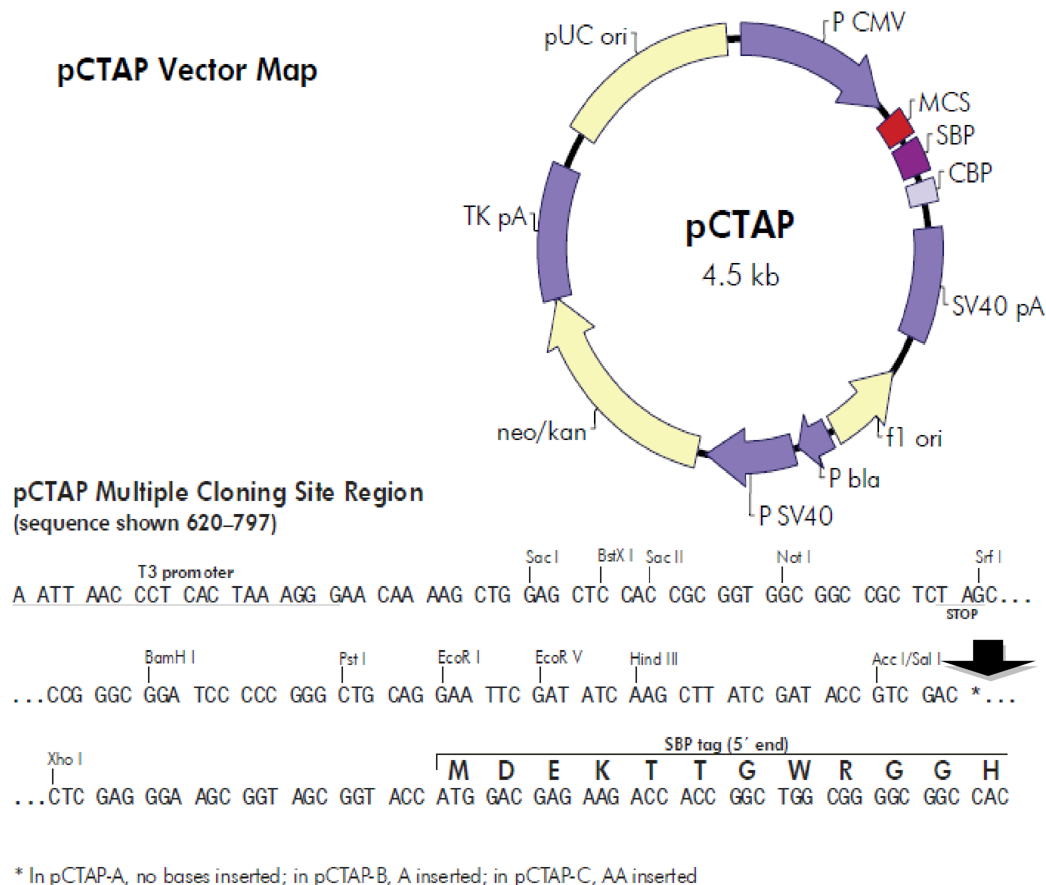

Figure 2.2. pCTAP Vector : In pCTAP-A, no bases inserted; in pCTAP-B, A inserted; in pCTAP-C, AA inserted on arrowed site.

Concentrations of samples were measured with nanodrop (NanoDrop Technologies, Inc. ND-1000 spectrophotometer). Purified and double digested Neu4 cDNA and pCTAP-B were ligated with T4 DNA Ligase (Invitrogen, 15224-017) according to manufacturer's instructions.

After ligation 1 µl of ligation mixture were transformed into chemically competent Dh5α *E.coli* cells using heat shock method. Cells were plated on 50 mg/ml kanamycin LB-Agar plates and incubated at 37 °C for 16 hours. Following day, transformant cells were selected and inoculated in 50 mg/ml kanamycin LB-Broth. After overnight incubation, plasmid purification was performed using alkaline lysis method. Plasmid was confirmed by double digestion method with EcoRI and XhoI restriction enzymes at 37 °C for 2 hours and digestion mixture was run on 1% agarose gel. Later, we prepared large scale inoculation in 500 ml LB-Brotth containing 50 mg/ml kanamycin at 37 °C for 16 hours. After incubation, plasmids were purified by plasmid purification kit (PureLink™ HiPure Plasmid Filter MaxiPrep Kit K210017 (Invitrogen)).

2.5. Transfection Reagent Polyethylimine (PEI)

PEI is a cationic lipid and it can be used as a lipid based transfection reagent. It has affordable price and has a great transfection efficiency. It is also less cell toxic when compared to other reagents. PEI is prepared as 1 mg/ml stock solution with sterile distilled water and stored at -20 °C. It remains fresh and does not lose transfection efficiency more than 1 year at -20 °C pH must be between 6,4-7,0.

2.6. Optimization of Transfection with pEGFP-N2 (Green Fluorescent Protein)

COS7 (African green monkey kidney cell line) cells were cultured in T25 flasks in MEM which includes 10% FBS, 1% 1X non-essential amino acid, 1% streptomycin/penicillin (100 unit) and inoculated to 6 well plates. Cells were grown up until they reach about 80% confluency. Transfection were performed with "blank

MEM" that does not contain FBS, amino acids and antibiotics. Medium was changed at transfection time with blank MEM. 1 ml blank MEM was used for 6 well plate.

Polyethylimine (PEI 1 mg/ml) was used as a transfection reagent and pEGFP-N2 vector was transfected to COS7 as well as HeLa cell lines. Different ratios of DNA:PEI were tested on COS7 cells (1:1, 1:2, 1:3, 1:4, 1:5 and mock control). Vector DNA and PEI were added into 100 µl of 150 mM NaCl solution seperately and both mixtures were incubated for 10 minutes at room temperature. NaCl solution was used to enhance transfection efficiency. PEI is cationic lipid and NaCl solution serves best condition to make complex with DNA for PEI. PEI in NaCl solution was combined with vector DNA in NaCl solution preventing DNA loss and incubated for 15 minutes at room temperature. Mixture was drop wised to blank medium of cells and gently shaken.

Cells were incubated for 3 hours at 37 °C, 5% CO_2. After 3 hours MEM including only 10% FBS was added into cells in the same quantity with blank MEM. Cell were incubated for 3 hours additional and medium was changed with MEM including 10% FBS, 1X amino acids, 1% penicillin-streptomycin. GFP flourescence was visualized under flurorescent microscope after 24 hours. The best transfection efficiency was achieved with 1:3 ratio of DNA:PEI, respectively (3µg DNA with 9 µl PEI (1 µg/µl)). Optimization of large scale transient transfection which was performed with a 150 mm cell culture dish and 72 µg of DNA in the same ratio was used.

2.7. Transfection of COS7 Cells and Protein Purification for Western Blot Optimization

After optimization of transfection, we transfected our construct, pCMV-NEU1 which was used in previous study, to COS7 cells to confirm expression of vectors in 6 well plate using western blotting. After 48 hours of transfection, cells were collected and washed with cold PBS 3 times. Cells were gently mixed with lysis buffer including protease inhibitor coctail (Sigma) and Phenylmethanesulfonyl fluoride (PMSF, Sigma) and lysed using freeze and thaw technique 3 times. Cells were frozen at -80 °C and thawed on ice. Lysate was centrifuged on 14.000 rpm for 10 minutes at 4 °C. Supernatant was collected, aliquated and stored at -80 °C.

2.8. Quantification of Protein Amount

We used Bradford Assay to determine protein concentration. Firstly we prepared different BSA concentrations ranging between 10 and 200 µg/ml to draw standard graphic for absorbance-protein concentration diluted isolated proteins with Bradford solution in ratio of 1:10. Absorbance was measured at 520 nm wavelenght and protein concentrations were determined using standart graphic.

2.9. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

First we optimized western blot protocol in our laboratory then we detected our expressed Neu1 proteins using western blot technique. We prepared SDS-PAGE gel to run and separate our proteins. We prepared 6% upper gel and 10% resolving gel (dH_20, upper buffer or resolving buffer, %30 Acrylamide (Sigma), 10% SDS (Applichem), TEMED (Sigma) , 10% APS (Applichem)). 20 µg protein mixture was mixed with protein loading dye and heated at 90 °C at 5 minutes. Protein mixture was loaded on gel and run 6 hours at 60 V as vertical electrophoresis in running buffer (0.25M Tris, 1,92 M Glycine, 1% SDS (w/v)).

Another gel was prepared to stain proteins with 1 mg/ml Coomasie Blue (Sigma), 10% acetic acid, 30% methanol or ethanol. Gel was incubated in Coomasie Blue for 1 hour and incubated in the same solution without Coomasie Blue to visualize protein bands in order to confirm whether lysis method was worked or not.

After electrophoresis, gel was placed between whatmann sheet and nitrocellulose membrane. Nitrocellulose membrane was activated in transfer buffer (48mM Tris, 39mM Glycine, 20% ethanol (v/v), 1L dH_2O).

Proteins were transfered to nitrocellulose membrane (Roche) at 60 V for 3 hours. Membrane was tested using Ponceau S (Sigma) dye and washed ultrapure water and washing buffer (1X PBS, 0.05% Tween20).

Membrane was blocked with blocking buffer (1X PBS, 0.05% Tween20, 0,5% non-fat dry milk) for 1 hour at room temperature. After blocking, membrane was incubated with washing buffer including 10% blocking buffer with anti-NEU1 (Santa Cruz Antibodies) and beta-actin primer antibodies (1:5000) in different containers for 1

hour and shaking gently. Membranes were washed 6 times with washing buffer for 5 minutes each and membrane was incubated in washing buffer including 10% blocking buffer with anti-rabbit HRP (Horseradish peroxide) seconder antibody (Santa Cruz 1:2000) for 1 hour and shaking gently. Membranes were washed 6 times for 5 minutes each. After last washing, 500 µl enhanced chemiluminescent substrate for HRP (Thermo) and 500 ul enhancer solution (Thermo) were spread on membrane and incubated for 2 minutes. Membranes releasing chemiluminescence were visualized using VersaDoc Imager in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

2.10. Detecting Recombinant Proteins using Western Blotting

We transfected our constructs pCTAP-A-NEU1 and pCTAP-B-NEU4 to COS7 cells in 6 well plates to detect their expressions. Cells were lysed and total protein extract was collected. 20 µg of protein was loaded on SDS-PAGE and transfered nitrocellulose membrane using electroblotting. Although Neu4 antibody commercially is available, it is not trustworthy. Therefore we used anti-NEU1 antibody to detect recombinant Neu1 and anti-calmodulin antibody (Upstate, Milipore 1:5000) to detect recombinant Neu4. Anti rabbit-HRP (Horseradish peroxide) antibody was used as a seconder antibody. Membranes were visualized using VersaDoc Imager in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

2.11. Transfection for Tandem Affinity Purification

Since $1x10^8$ cells were required we cultured COS7 and HeLa cells in 150 mm culture dishes in 10 replicas for each TAP transfection. Cells were passaged one day before the experiment to maintain 80-90% confluency. Cells were transfected with expression vectors pCTAP-A-NEU1 and pCTAP-B-NEU4 using optimized method with 72 µg DNA for 1 culture dish and mock-transfected cells were used as control.

2.12. Purification of Bait and Associated Proteins

Cells were washed with cold PBS 3 times and scaled-up, pooled, and processed concurrently on ice after 48h post transfection. Cells were lysed with lysis buffer (provided by Agilent) using freeze and thaw technique. Proteins (Figure 2.3) were purified using Interplay Mammalian Tap System according to manufacturer's instructions using calmodulin and streptavidin affinity of tagged protein (Figure 2.4) and stored at -20 °C for further mass spectrometry analysis.

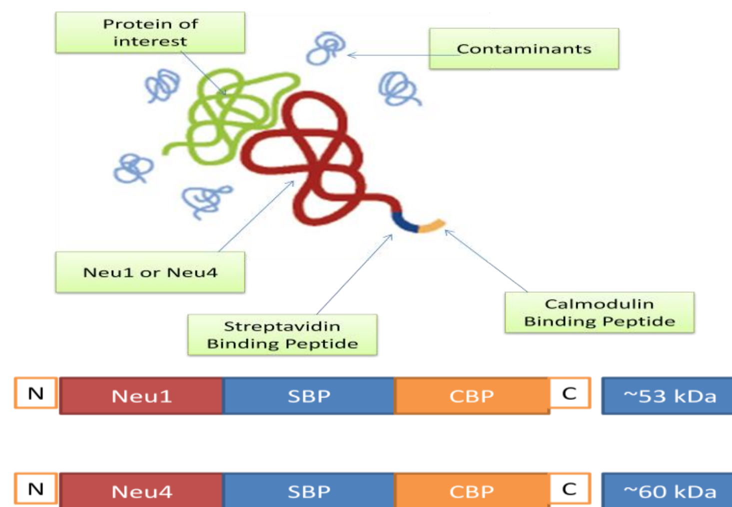

Figure 2.3. Schematic presentation of tagged Neu1 (53 kDa) and Neu4 (60kDa) protein

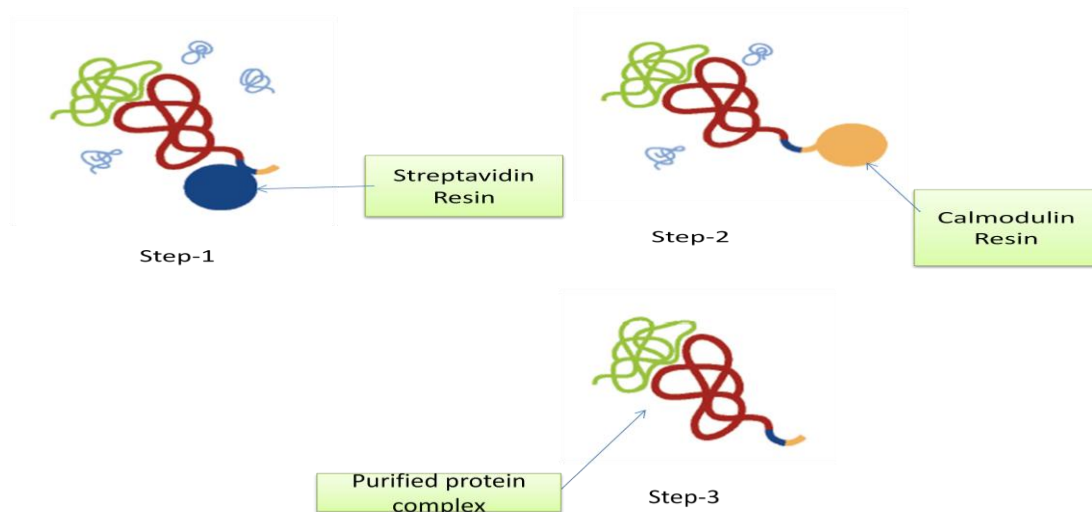

Figure 2.4. Schematic presentation of purification steps of tagged Neu1 and Neu4 protein.

2.13. MS/MS Analysis

We made collaboration with Dr. Ahmet Tarık Baykal from Gene Engineering and Biotechnology Institute, TUBITAK-MAM and Prof. Dr. Talat Yalçın from IYTE Mass Spectrometry Laboratory.

Purified proteins from transfected COS7 cells were analysed in TUBITAK-MAM. First, proteins were concentrated using dialysis method overnight (3-7 IDa cut off). Uncolored mixture was centrifuged at 15000 rcf for 10 minutes. Concentration of proteins were measured using bradford reagent. 50 µg protein was added into test tube and added dH_2O to make volume 50 µl. Mixture was incubated at 80 °C for 15 minutes. 100 mM DTT (Dithiothreitol) dissolved in 50 mM ammonium bicarbonate, was added and incubated at 60 °C for 15 minutes. 200 mM IAA (Iodoacetamide) dissolved in 50 mM ammonium bicarbonate, was added and incubated at room temperature in dark. 1 µg of trypsin, dissolved in 50 mM ammonium bicarbonate (1:50) was added and incubated at 37 °C overnight.

500 ng of trypsinized peptides were used for analysis in the presence of 2 ul TFA (Trifluoroacetic acid) and 2 µl ACN (Acetonitril). HDMS (High Definition Mass Spectrometry) and nano Acquity UPLC (Ultra-High Performance Liquid Chromatography) system were used to prepare samples for MS/MS. Control protein was added to mixture as a positive control like enolase yeast protein. These peptides were separated using acetonitril gradient (5-40%) for 90 minutes and using 1D-reverse phase separation. Finally, samples were analysed in ESI-MS/MS to detect protein composition (Figure 2.5).

Purified proteins from transfected HeLa were analyzed in IYTE Mass Spectrometry Laboratory. Proteins were run into non-linear (pH gradient : 3-10) strip. Strip was loaded to SDS-PAGE gel for 2-dimensional gel electrophoresis. Proteins were run into gel for 5 hours. Polyacrylamide gel was incubated in Coomassie solution and incubated for 16 hours at 4 °C and washed with water. Gel was incubated in destaining solution for 2 hours. Protein dots were cut from gel and washed. Proteins were trypsinized overnight. Peptides were zip tipped and loaded on matrix before MALDI-TOF analysis.

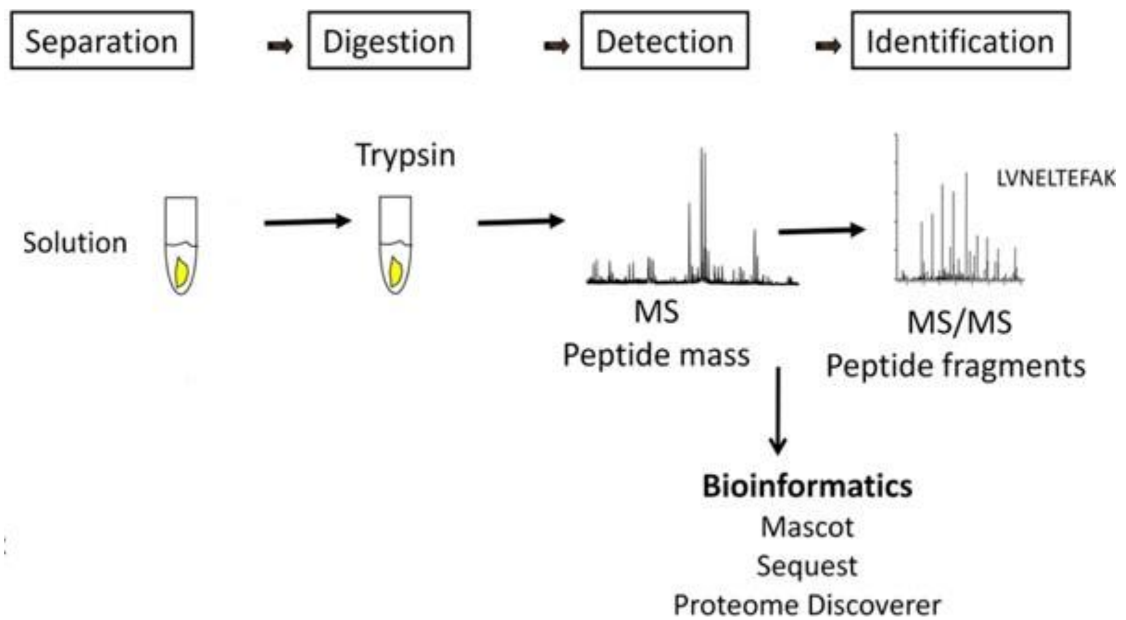

Figure 2.5. In-solution trypsinization of proteins and downstream process of MS/MS (Adapted from Gucek 2012)

2.14. Bioinformatic Analysis

Dr.Tarık Baykal processed MS/MS data using bioinformatic in PGLS (ProteinLynx Global Server) . Also "PKL" data were analyzed by using Mascott programme and searched in protein database (Swissprot) to find releated protein candidates according to peptide composition which was purified by TAP system. HeLa proteins were analyzed by using Mascott programme in IYTE Mass Spectromety Laboratory.

CHAPTER3

RESULTS

3.1. Cloning, Confirmation by Restriction Enzymes and Sequence Analysis

Human Neu4 cDNA was cloned into pCTAP-B vector and purified by using miniprep plasmid purificaion kit (Fermentas). pCTAP-B-NEU4 and previous prepared pCTAP-A-NEU1 were confirmed by double digestion with EcoRI and XhoI restriction enzymes, also with NotI and BamHI retriction enzymes. Confirmation was also performed with KpnI (Figure 3.1, Figure 3.2).

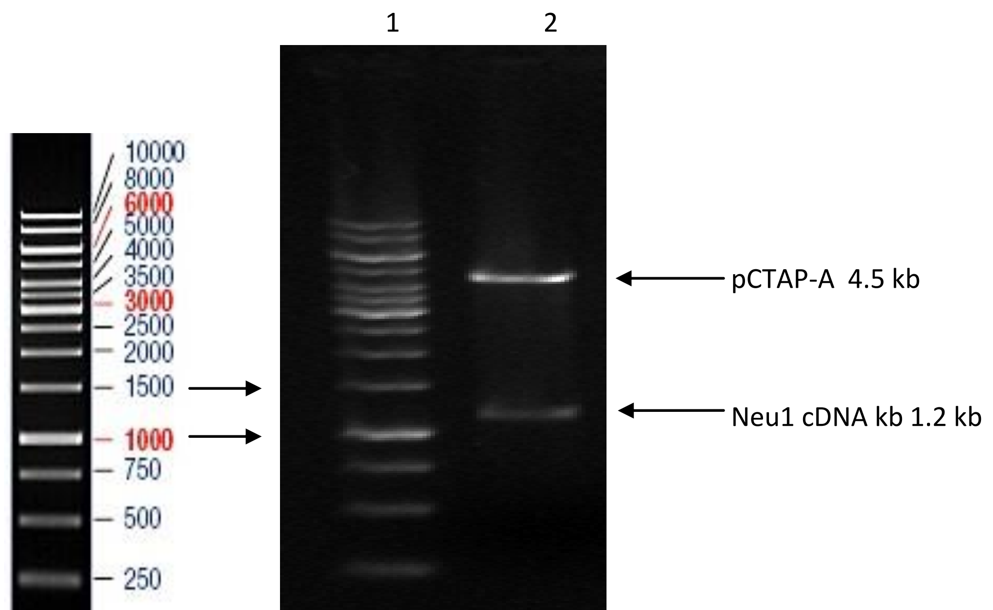

Figure 3.1. Agarose gel electrophoresis of double digestion of pCTAP-A-NEU1 with NotI-BamHI. 1) 1 kb DNA marker. 2) Gel image indicates 4.5 kb fragment of pCTAP-A vector and 1.2 kb fragment of Neu1 cDNA.

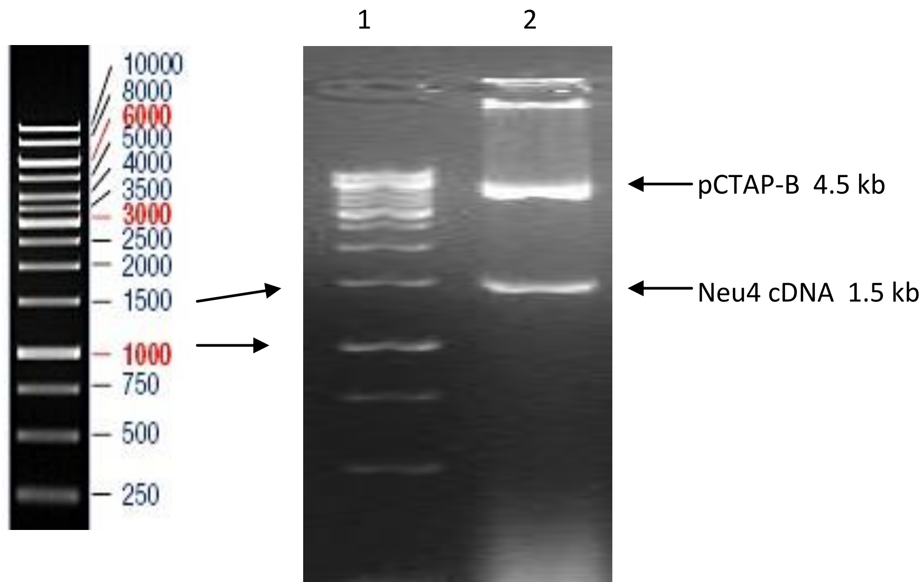

Figure 3.2. Agarose gel electrophoresis of double digestion of pCTAP- B-NEU4 with EcoRI-XhoI 1) 1 kb DNA ladder , 2) Gel image indicates 4,5 kb fragment of pCTAP-B and 1,5 kb Neu4 cDNA.

Neu1 and Neu4 DNA were sequenced in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center and neither DNA polymerase error nor stop codons were detected (Figure 3.3). We used KpnI enzyme digestion to make additional confirmation (Figure 3.4).

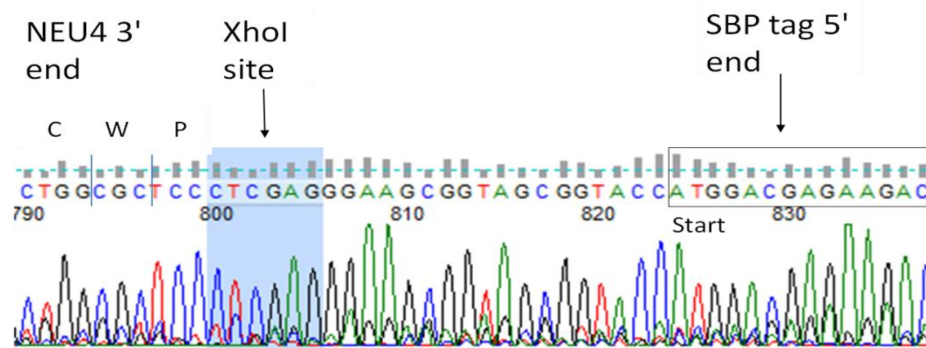

Figure 3.3. Sequence Analysis of pCTAP-B-Neu4 showing no-stop codon.

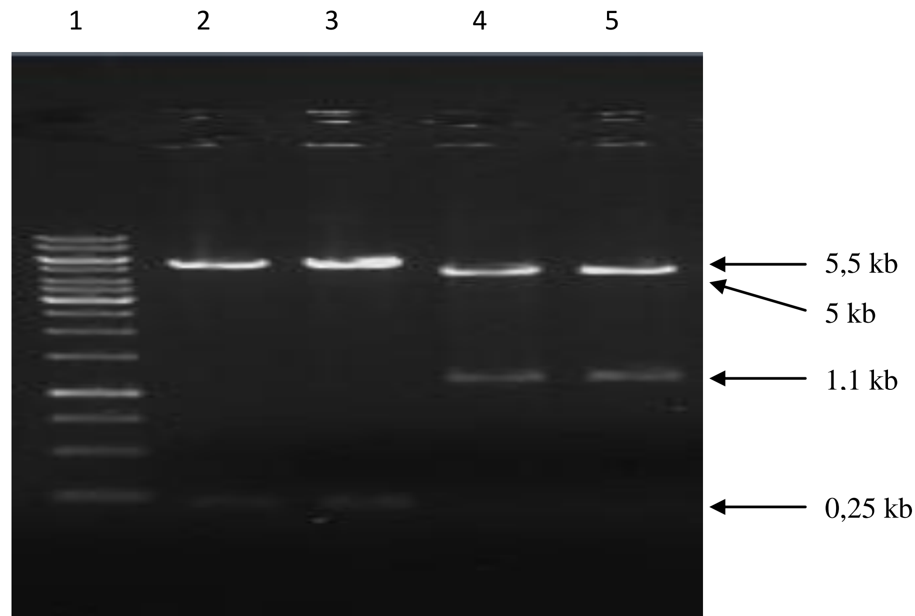

Figure 3.4. Agarose gel electrophoresis of KpnI digestion of pCTAP-A-NEU1 and pCTAP-B-NEU4. 1) 1kb DNA ladder 2-3) KpnI digestion of pCTAP-A-NEU1 cuts insert and vector and gel image indicates ~5,5 kb and 0,25 kb DNA bands 4-5) Confirmation of pCTAP-B-NEU4 with KpnI enzyme, KpnI enzyme cuts insert and vector and gel image indicates ~5kb and 1,1 kb DNA bands.

3.2. Optimization of Transfection

pEGFP-N2 vector was used to optimize transfection. The best transfection efficiency was observed with 1:3 ratio of DNA:PEI. GFP was visualized by fluorescent microscope and cells that expressed GFP were observed (Figure 3.5).

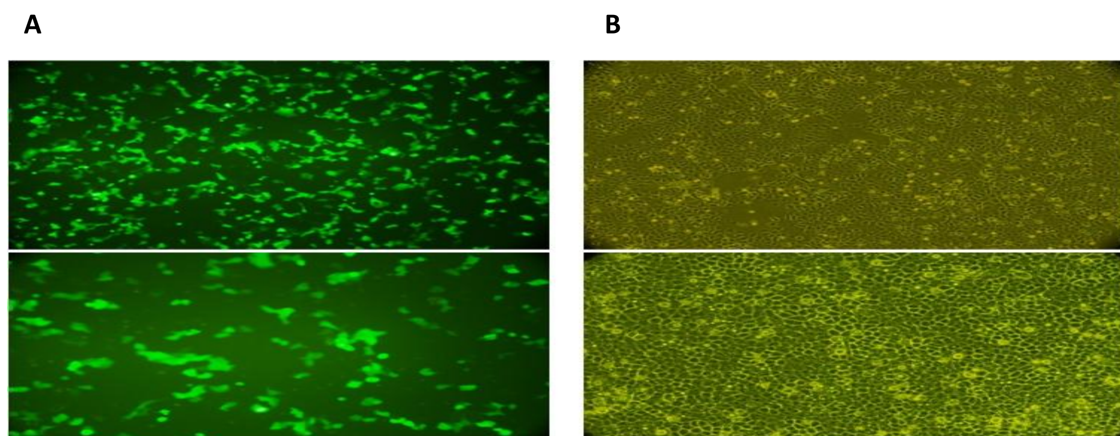

Figure 3.5. Transfection Optimization. A : Transfected COS7 cells (up) and HeLa cells (down) under fluorescent light. B : Same area of COS7 and HeLa cells under white light.

3.3. Optimization of Western Blotting

To optimize Western Blotting we used proteins from non-transfected COS7 cells (Figure 3.6) as well as pCMV-NEU1 and pCTAP-A-NEU1 transfected COS7 cells (Figure 3.7). We used anti-Neu1 (Santa Cruz), anti-beta actin (Santa Cruz) antibodies.

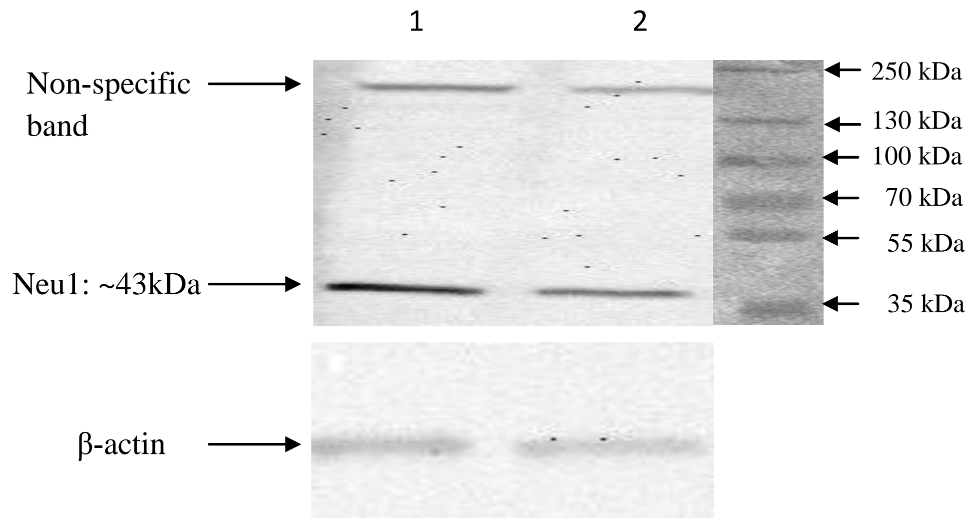

Figure 3.6. Detection of endogenous Neu1 protein in COS7 (1) and HeLa (2) cells by Western Blotting

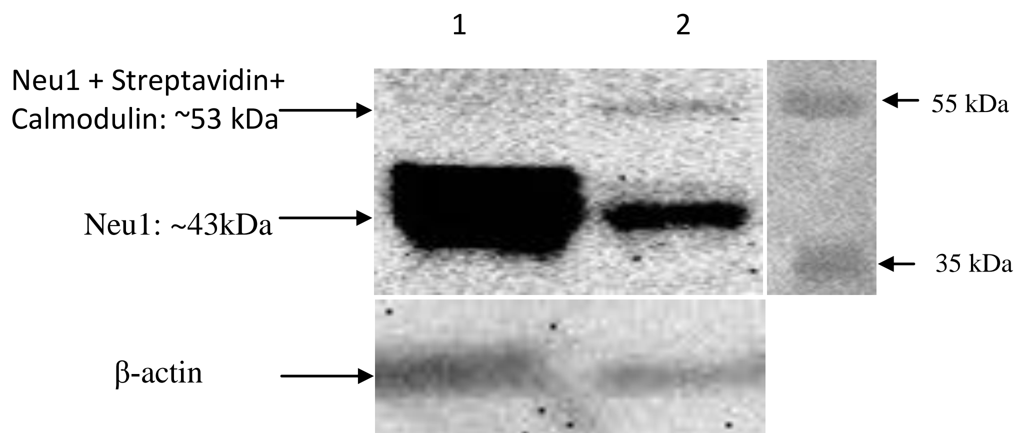

Figure 3.7. Detection of tagged Neu1 protein in COS7 cells by Western Blotting. Neu1 expression of pCMV-NEU1 (1) and pCTAP-A-NEU1 (2) in COS7 cells.

3.4. Western Blotting Analysis of Purified Tagged Proteins

Transfected COS7 cells were lysed and total protein purification was performed. Expression of pCTAP-A-NEU1 was detected with anti-calmodulin epitope tag antibody (Upstate, Millipore) and anti-Neu1 antibody (Santa Cruz). Expression of pCTAP-B-NEU4 and pCTAP-A-NEU1 were detected using anti-calmodulin epitope tag antibody (Figure 3.8, Figure 3.9).

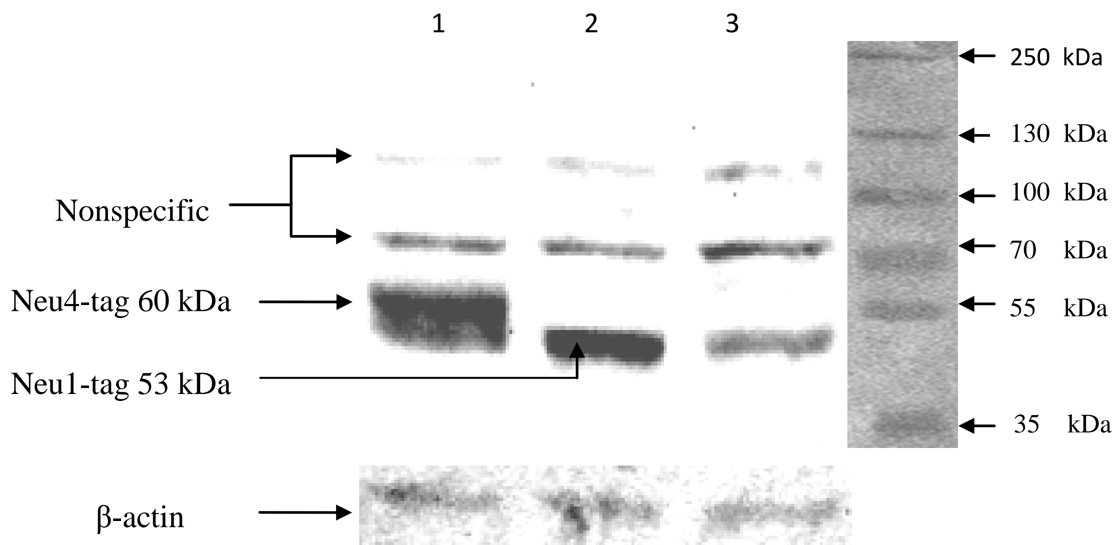

Figure 3.8. Detection of tagged proteins using anti-calmodulin epitope tag antibody 1) Expression of Neu4+SBP+CBP, 60 kDa 2) Expression of Neu1+SBP+CBP, 53 kDa 3) Mock transfected control

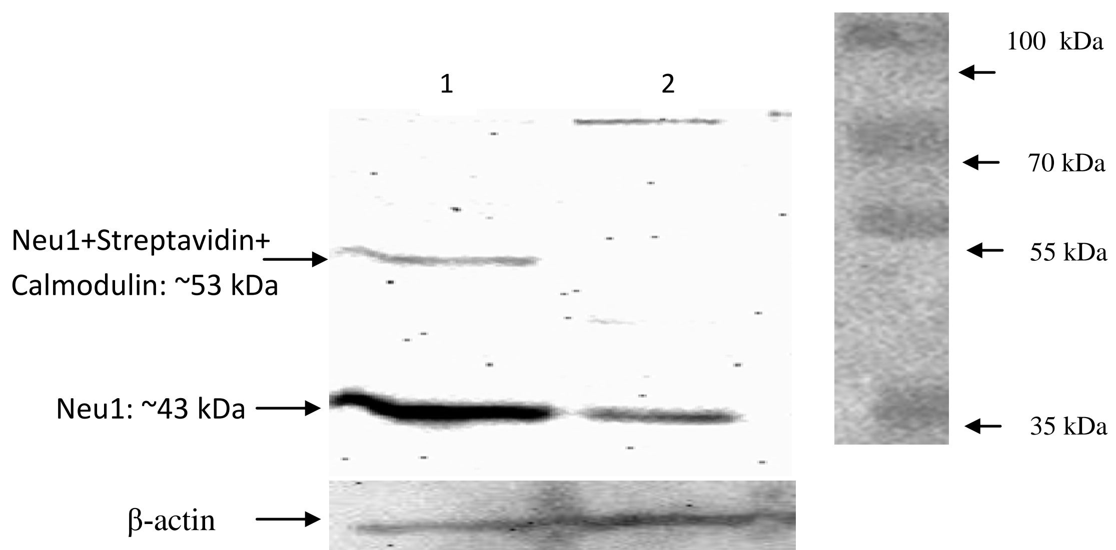

Figure 3.9. 1) Detection of expression of pCTAP-A-NEU1 with anti-Neu1 antibody. 2) Mock transfected control.

3.5. Coomasie Staining of Tandem Affinity Purification

Proteins were stained with coomasie brilliant blue (Sigma) to detect cell lysis and protein purification from total lysate. COS7 proteins were run into SDS-PAGE gel and dyed (Figure 3.10). HeLa proteins were run into 2D gel electrophoresis (Figure 3.11).

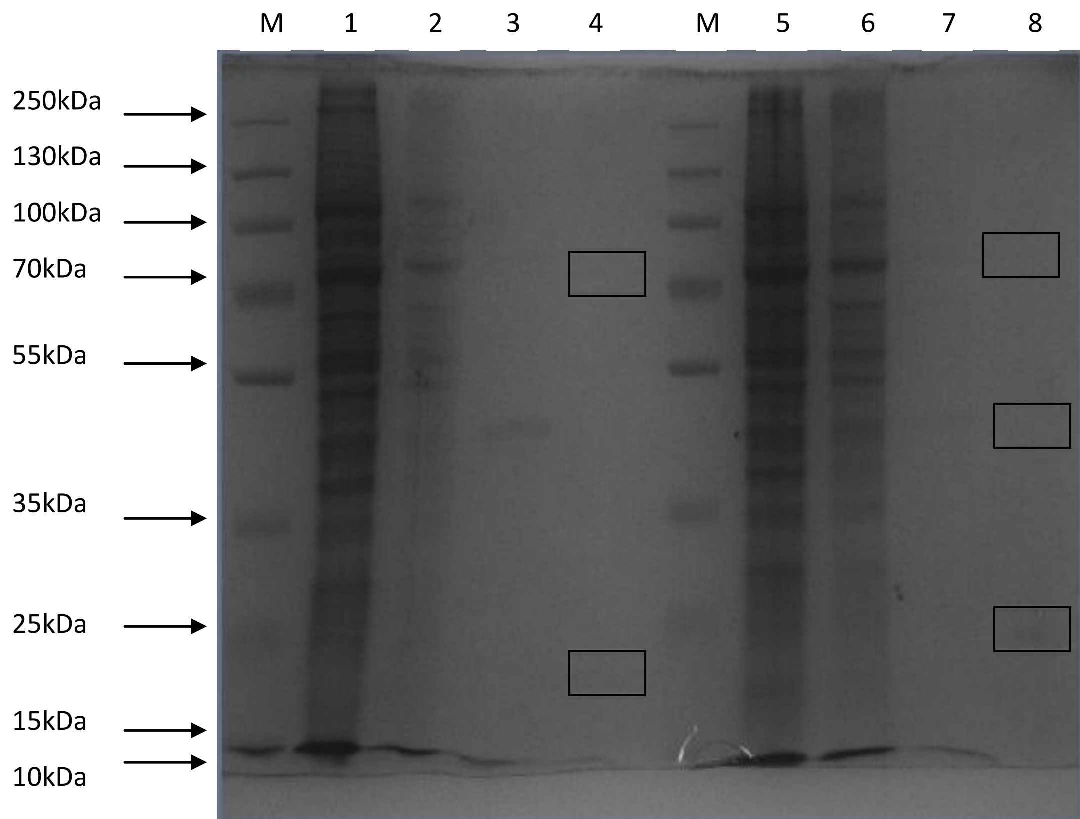

Figure 3.10. Coomasie staining of purified proteins from COS7 cells. M: marker 1) Mock Transfection 2) 5 µl of total lysate pCTAP-A-NEU1 transfection 3) 10 µl SEB (Streptavidin Elution Buffer) with eluted proteins with tagged Neu1 4) 10 µl CEB (Calmodulin Elution Buffer) with eluted proteins with tagged Neu1. 5) Mock transfection 6) 5 µl total lysate pCTAP-B-NEU4 transfection 7) 10 µl SEB with eluted proteins 8) 10 µl CEB with eluted proteins. Protein amount loaded on SDS-PAGE was determined according to Agilent; TAP kit manufacture's recommendations.

pH 3 pH 10

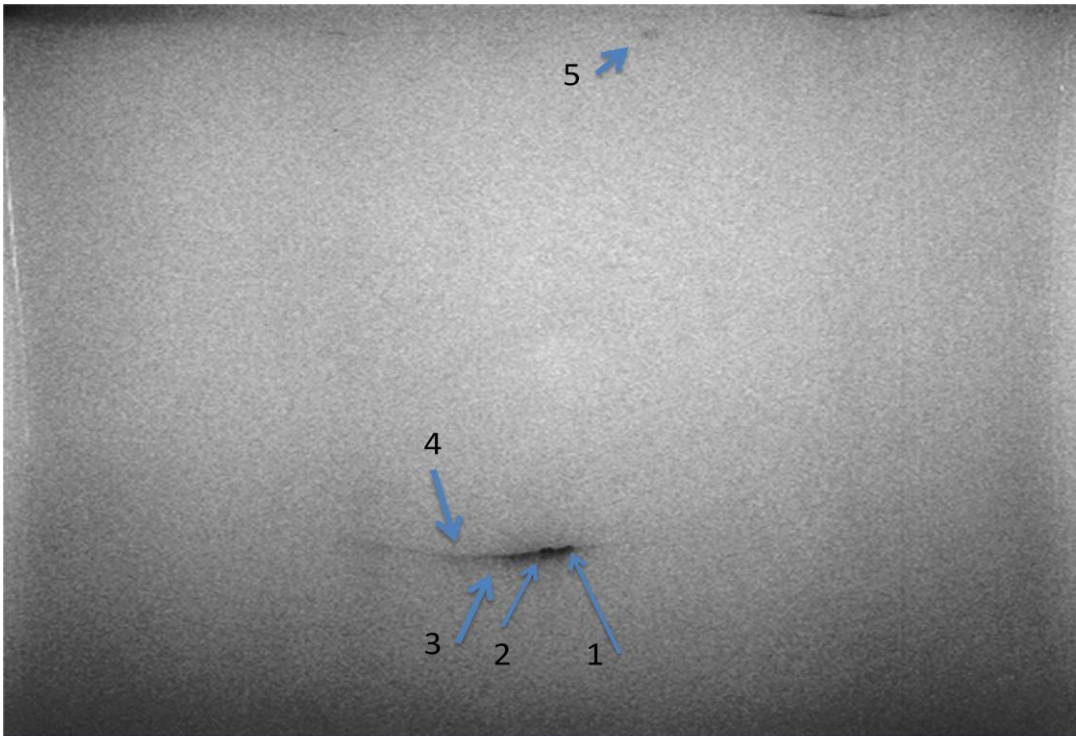

Figure 3.11 Purified HeLa proteins 2D-PAGE

3.6. BSA Standard Curve

We prepared standard curve which shows proportion between absorbance (595 nm) and protein density using control BSA samples (Figure 3.12). All protein density calculations were done using standart curve after measurement absorbance at 595 nm.

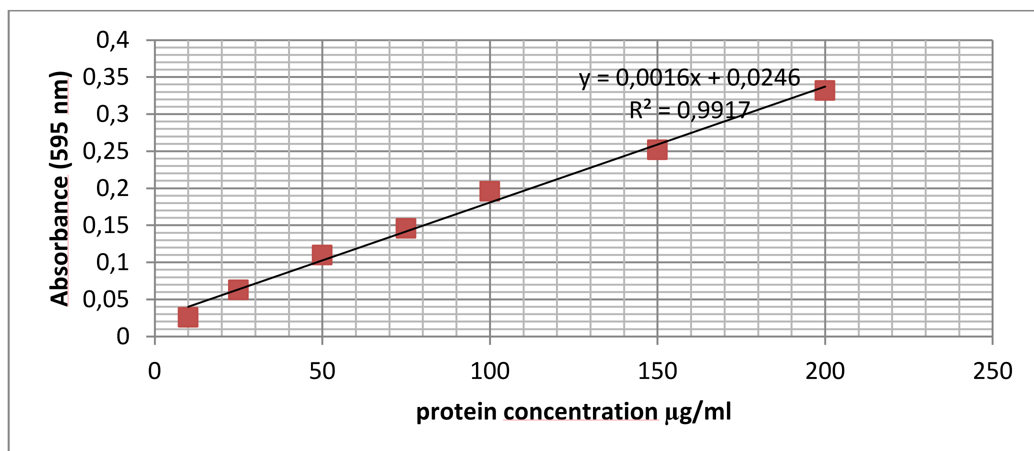

Figure 3.12. BSA standart curve

3.7. MS/MS results

Dr.Tarık Baykal (TÜBİTAK) performed MS/MS analysis of purifed proteins from pCTAP-A-NEU1 and pCTAP-B-NEU4 transfected COS7 cells. ESI-MS/MS (ESI-QTOF) method was used to detect proteins. Data-independent acquisition (DIA) was used as a file format. Some candidate proteins were found (Table 3.1, Table 3.2). Trypsin, Calmodulin and Keratin proteins are contaminant in protein samples.

Table 3.1. MS/MS analysis of purifed proteins in Calmodulin Elution Buffer from pCTAP-A-NEU1 transfected COS7 cells

Accession	Description	mW (Da)	pI (pH)	PLGS Score	Peptides	Theoretical Peptides	Coverage %
P62158	CALM HUMAN Calmodulin	16826	3,9	12981	35	23	96
P00924	ENO1 YEAST Enolase 1	46787	6,2	2041	14	28	26
P0CG48	UBC HUMAN Polyubiquitin C	76991	7,8	1601	9	62	21
P16402	H13 HUMAN Histone H1 3	22336	11,5	494	5	21	14
P04264	K2C1 HUMAN Keratin type II cytoskeletal 1	65998	8,3	369	7	45	9
Q8NHM4	TRY6 HUMAN Putative trypsin 6	26522	5,8	365	3	15	8

Table 3.2. MS/MS analysis of purified proteins in Calmodulin Elution Buffer from pCTAP-B-NEU4 transfected COS7 cells.

Accession	Description	mW (Da)	pI (pH)	PLGS Score	Peptides	Theoretical Peptides	Coverage (%)
P00924	ENO1 YEAST Enolase 1	46787	6,2	3110	18	28	30
P62158	CALM HUMAN Calmodulin	16826	3,9	3006	10	23	72
P10412	H14 HUMAN Histone H1 4	21852	11,5	987	7	19	22
P07477	TRY1 HUMAN Trypsin 1	26541	6,1	477	5	14	8
Q8WWR8-2	**NEUR4 HUMAN Isoform 2 of Sialidase 4**	52904	7,8	259	4	31	14

PKL files were sent to our laboratory and we also used Mascot MS/MS Ion Search program on internet. Proteins were searched in Swissprot protein database as a human (Table 3.3 and Table 3.4) and primate (Table 3.5 and Table 3.6) protein. Some candidate proteins were found. Calmodulin and keratin are contaminant proteins.

Table 3.3. MS/MS analysis, by Mascot program on internet, of purified proteins in Calmodulin Elution Buffer from pCTAP-A-NEU1 transfected COS7 cells as human protein. Scores > 32 indicate identity or extensive homology.

Description	Protein Name	Score
CALM_HUMAN	Calmodulin	358
K2C1_HUMAN	Keratin, Type II cytoskeletal	142
NEUR1_HUMAN	**Sialidase-1**	54
K1C9_HUMAN	Keratin, Type I cytoskeletal	53
ACTBL_HUMAN	Beta-actin-like protein 2	46
K1C10_HUMAN	Keratin, Type I cytoskeletal	43
H12_HUMAN	Histone H1.2	34
FRM4B_HUMAN	FERM domain-containing protein 4B	23
PLAP_HUMAN	Phospholipase A-2 activating protein	21

Table 3.4. MS/MS analysis, by Mascot program, of purified proteins in Calmodulin Elution Buffer from pCTAP-B-NEU4 transfected COS7 cells as human protein. Scores > 33 indicate identity or extensive homology.

Description	Protein Name	Score
CALM_HUMAN	Calmodulin	100
H12_HUMAN	Histone H1.2	87
K2C1_HUMAN	Keratin, type II cytoskeletal	71
IL4_HUMAN	Interleukin 4	23
WNT3_HUMAN	Proto-oncogene Wnt-3	22
K1C10_HUMAN	Keratin, type I cytoskeletal	18
AT11C_HUMAN	Probable phospholipid-transporting ATPase	13

Table 3.5. MS/MS analysis, by Mascot program, of purified proteins in Calmodulin Elution Buffer from pCTAP-A-NEU1 transfected COS7 cells as primate protein. Scores > 33 indicate identity or extensive homology.

Description	Protein Name	Score
CALM_HUMAN	Calmodulin	332
K2C1_HUMAN	Keratin, Type II cytoskeletal	143
NEUR1_HUMAN	Sialidase-1	54
K1C9_HUMAN	Keratin, Type I cytoskeletal	47
ACTBL_HUMAN	Beta-actin-like protein 2	46
K1C10_HUMAN	Keratin, Type I cytoskeletal	35
ZMY19_HUMAN	Zinc finger MYND domain-containing protein	33
FRM4B_HUMAN	FERM domain-containing protein 4B	23

Table 3.6. MS/MS analysis, by Mascot program, of purified proteins in Calmodulin Elution Buffer from pCTAP-B-NEU4 transfected COS7 cells as primate protein. Scores > 33 indicate identity or extensive homology.

Description	Protein Name	Score
CALM_HUMAN	Calmodulin	96
H12_HUMAN	Histone H1.2	86
K2C1_HUMAN	Keratin, type II cytoskeletal	70
ZMY19_HUMAN	Zinc Finger MYND domain-containing protein	34
IL4_HUMAN	Interleukin-4	23
K1C10_HUMAN	Keratin, type I cytoskeletal	18
AT11C_HUMAN	Probable phospholipid-transporting ATPase	13

Prof. Dr. Talat Yalçın performed (IYTE Mass Spectrometry Laboratory) MS/MS analysis of purified proteins from pCTAP-B-NEU4 transfected HeLa cells. MALDI-TOF/TOF system was used to detect proteins. 4 significant MS peaks were chosen and MS/MS analyses were performed. MS/MS results were searched in Swissport protein database by using Mascot Search Program. We found some high score protein in human database. Also we saw same proteins when we searched MS/MS results in all organisms. High score proteins were highlighted in tables (Table 3.7, Table 3.8, Table 3.9 and Table 3.10) and scores of proteins were given under tables (Figure 3.13, Figure 3.14, Figure 3.15, and Figure 3.16).

Table 3.7. MALDI-TOF/TOF (MS/MS) results of purified HeLa proteins from pCTAP B-NEU4 transfection.

CND3_HUMAN	**Condensin complex subunit 3**
PRP31_HUMAN	U4/U6 small nuclear ribonucleoprotein
TGFR2_HUMAN	TGF-beta receptor
TITIN_HUMAN	Titin
RB6I2_HUMAN	ELKS/Rab6-interacting/CAST family member 1
TMM70_HUMAN	Transmembrane protein 70, mitochondrial
GCST_HUMAN	Aminomethyltransferase, mitochondrial
CP2J2_HUMAN	Cytochrome P450 2J2
PRO3_HUMAN	Profilin-3
SYNE2_HUMAN	Nesprin-2

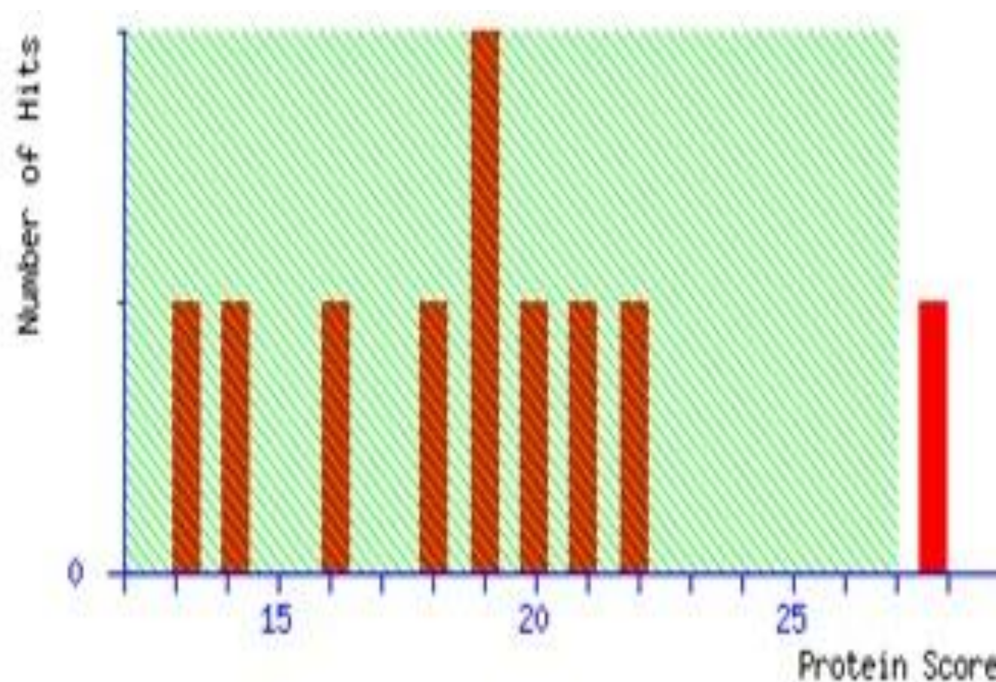

Figure 3.13. Protein scores of the MS/MS results of Table 3.5

Table 3.8. MALDI-TOF/TOF (MS/MS) results of purified HeLa proteins from pCTAP B-NEU4 transfection.

Description	Protein Name
CND3_HUMAN	**Condensin complex subunit 3**
CG0B3_HUMAN	Uncharacterized protein c7orf63
GNN_HUMAN	Tetratricopeptide repeat protein GNN
TMM70_HUMAN	Transmembrane protein 70, mitochondrial
MACF1_HUMAN	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
CP2J2_HUMAN	Cytochrome P450 2J2
SESN2_HUMAN	Sestrin-2
FOXN4_HUMAN	Forkhead box protein N4
TRIO_HUMAN	Triple function domain protein
TGFR2_HUMAN	TGF-beta receptor type-2

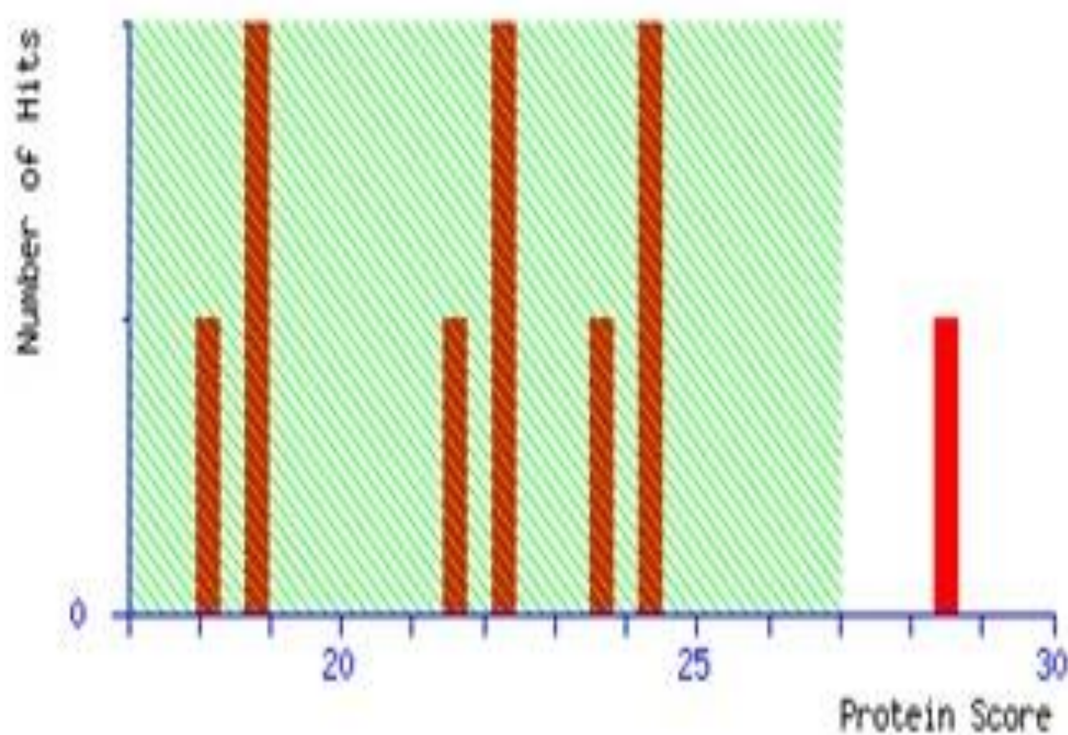

Figure 3.14. Protein scores of the MS/MS results of Table 3.6

Table 3.9. MALDI-TOF/TOF (MS/MS) results of purified HeLa proteins from pCTAP B-NEU4 transfection.

Description	Protein Name
NMD3A_HUMAN	**Glutamate (NMDA) receptor subunit 3A**
TRM44_HUMAN	Probable tRNA (uracil-O(2)-) methyltransferase
CCD87_HUMAN	Coiled-coil domain containing protein 87
T3HPD_HUMAN	Trans-L-3-hydroxyproline dehydratase
NFX1_HUMAN	Transcriptional repressor NF-X1
KDM8_HUMAN	Lysine-specific demethylase 8
SHSA8_HUMAN	Putative protein shisa-8
LM07_HUMAN	LIM domain only protein 7
CHST7_HUMAN	Carbohydrate sulfotransferase 7
CP21A_HUMAN	Steroid 21-hydroxylase

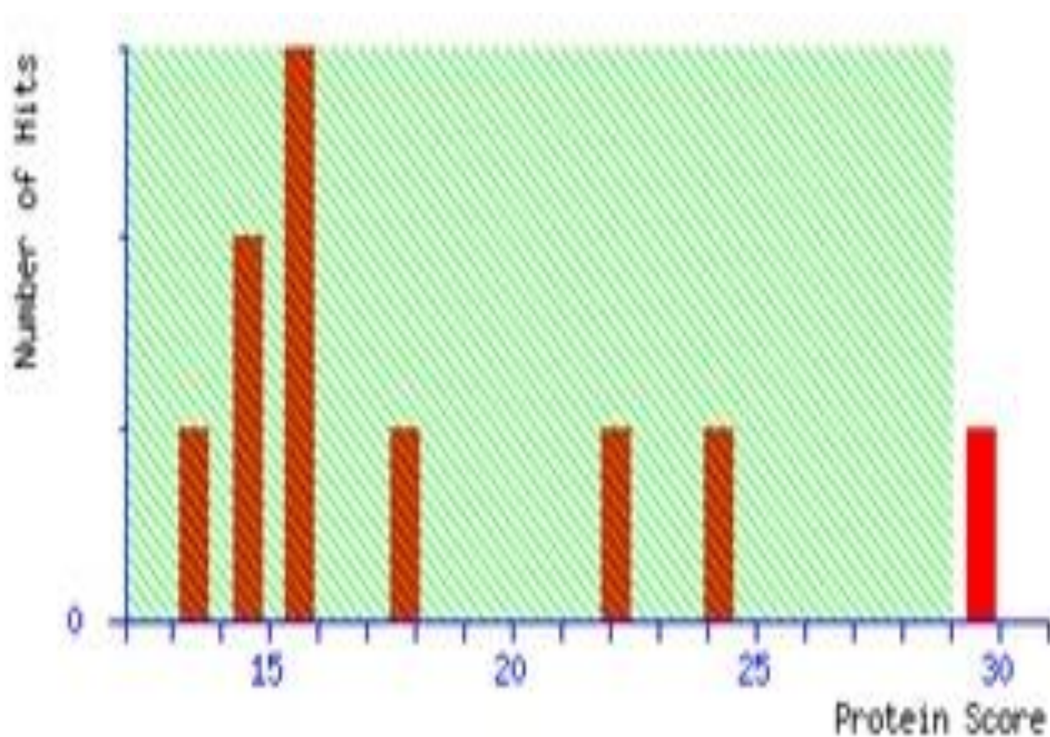

Figure 3.15. Protein scores of the MS/MS results of Table 3.7

Table 3.10. MALDI-TOF/TOF (MS/MS) results of purified HeLa proteins from pCTAP-B-NEU4 transfection.

Description	Protein Name
GIMA2_HUMAN	**GTPase IMAP family member 2**
RP25L_HUMAN	Ribonuclease P protein subunit p25-like protein

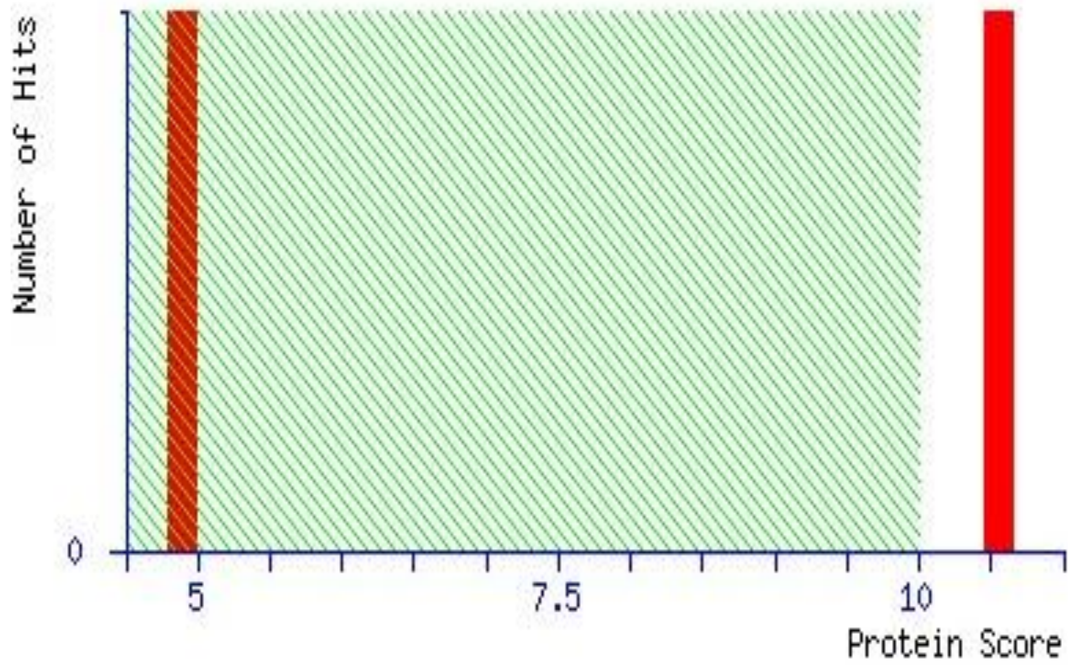

Figure 3.16. Protein scores of the MS/MS results of Table 3.8

CHAPTER 4

DISCUSSION

Sialidases remove sialic acid residues from substrates such as glycoproteins, gangliosides and glycolipids. They are involved in several metabolic pathways and may initiate signal transduction removing sialic acid residues from receptors.

Sialidases work in different conditions and localize different regions of cells. For example Neu1 makes complex with beta-galactosidase and cathepsin A enzyme in lysosomes. If dissociation occurs in protein complex, sialidase Neu1 loses its enzymatic activity causing lysosomal storage disease "sialidosis". However, proteins associated with other sialidases remain unknown. Therefore, in this study we aimed to tag Neu4 and Neu1 sialidase using Calmodulin Binding Peptide and Streptavidin Binding Peptide and elute proteins interacted with sialidase Neu4 and Neu1 and identify associated proteins by using mass spectrometry.

MS/MS data shows that there are candidate proteins which may interact sialidase Neu4 and Neu1. We also found that nuclear proteins are in protein composition which eluted in Calmodulin Elution Buffer, but most of these proteins' scores including nucleus proteins are too low to find significant associated protein. Surprisingly we did not see cathepsin A and beta-galactosidase protein in tagged Neu1 protein composition. The reason of this situation that probably protein interaction was not strong with tagged protein.

CHAPTER 5

CONCLUSION

In this study, sialidase Neu4 and Neu1 associated proteins were analysed by using tandem affinity purification and MS/MS methods. In TUBITAK-MAM, PGLS database was searched after ESI MS/MS analysis of purified proteins from pCTAP-A-NEU1 and PCTAP-B-NEU4 transfected COS7 cells. We found calmodulin, keratin, polyubiqutinin C, histone 1.3 and trypsin in calmodulin elution buffer of pCTAP-A-NEU1 transfected COS7 cells. We found isoform 2 of Neu4, trypsin, histone 1.4, calmodulin in calmodulin elution buffer of pCTAP-B-NEU4 transfected COS7 cells. Yeast enolase was control protein. Keratin and calmodulin were detected as major contaminants.

Primate and Human database of Swissprot were searched for COS7 proteins purified from PCTAP-A-NEU1 and pCTAP-B-NEU4 transfection by using "PKL" files on Mascot. We have found sialidase-1, calmodulin, keratin, beta-actin like protein 2, histon 1.2, FERM domain containing protein, phospholipase -2 activating protein in calmodulin elution buffer of pCTAP-A-NEU1 transfected COS7 cells with higher score. We also detected calmodulin, histon 1.2, keratin, interleukin 4, proto-oncogene WNT3, probable phospholipid transporting ATPase IG proteins in calmodulin elution buffer of pCTAP-B-NEU4 transfected COS7 cells. Keratin and calmodulin were detected as major contaminants. Primate database results were the same as Human database except zinc finger MYND domain containing protein 19. Also this protein was found in both of calmodulin elution buffer of pCTAP-A-NEU1 and pCTAP-B-NEU4 transfected COS7 cells sample.

IYTE Mass Spetrometry Laboratory performed MALDI-TOF-TOF analysis for purified HeLa proteins expressed from pCTAP-B-NEU4. We detected condensin complex subunit 3, GTPase ITAP family member 2 and glutamate (NMDA) receptor subunit 3A proteins as high score proteins.

As a further study, we plan to make purification of tagged proteins from their specific environment like membrane, lysosome, mitochondria (organel specific purification). In our study we chose to tag C-terminal of sialidase Neu4 and Neu1. C-terminal tagging might have affected protein interactions and this problem could be

solved by tagging N-terminal side of proteins. Other mammalian cell lines could be transfected with our expression vectors to compare proteins. As a future aspect, we are planning to analyze proteins from HeLa cell purification in TUBITAK-MAM and proteins from COS7 cell purification in Mass Spectrometry Laboratory in IYTE to compare same results in parallel.

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