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1．研究テーマ

ガングリオシドシアリダーゼのカベオラ膜ドメインにおける局在とカベオリンとの相互作用

2．本年度の研究業績

(1) 学会・研究会等における発表　有　・　無　（学会名・演題）

第74回　日本生化学会大会

シグナル伝達経路における形質膜シアリダーゼ

(2) 学会誌等に発表した論文　有　・　無　（雑誌名・論文名）

Eur.J.Biochem. 268, 2201-2208 (2001)

Site-directed mutagenesis of human membrane-associated ganglioside sialidase: Identification of amino-acid residues contributing to substrate specificity

3．今後の研究計画

Neu3 はカベオラ膜ドメインに局在し、そこでカベオリンと相互作用することがわかった。Neu3 遺伝子にあるカベオリン結合モチーフによって、本酵素は他のシグナル伝達分子のように、カベオリンと相互作用しながら活性制御を受けている可能性が推察された。これらの結果は Neu3 がガングリオンドの発現を調節して、シグナル伝達分子としての役割を果していることを示す。Neu 3 はカベオリンシグナル伝達経路にての役割及びそのメカニズムを今後の計画として研究を進めようと計画している。

4．指導責任者の意見

動物シアリダーゼについては近年、細胞質性、リソソーム性、形質膜性シアリダーゼの cDNA が相次いでクローン化され、分子レベルの研究が端緒についたところである。それらの機能の重要性については推察されているものの、明らかな実験的証拠はほとんど挙がっていない。本研究は、先に宮城県立がんセンター研究所でクローン化されたヒト Neu3 遺伝子および同研究所で作成された単クローン抗体を用いて蛋白分子レベルで、本酵素の機能の一端を明らかにしたものである。本酵素がカベオラ膜ドメインに存在し、シグナル伝達分子カベオリンと相互作用することを示した最初の報告である。本研究の成果を第 74 回日本生化学学会にて発表した。

指導責任者氏名　田村　真理　㊞

5．研 究 報 告 書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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－日中医学協会助成事業－

ガングリオシドシアリダーゼのカベオラ膜ドメインにおける局在とカベオリンとの相互作用

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(Summary)

The ganglioside-specific sialidase, Neu 3, has been suggested to play essential roles in regulation of cell surface functions, because of its major localization in the plasma membrane and strict substrate preference for gangliosides involved in signal transduction. Here we show that human Neu 3 sialidase is enriched in caveolae microdomains and interacts with caveolin, like other caveolin-binding signaling molecules. Using HeLa cells and Neu 3-transfected COS-1 cells, endogenous and exogenous Neu 3 was found to concentrate together with caveolin-1 in low density Triton X-100 insoluble membrane fractions on sucrose density gradients of the respective cell extracts, as assessed by enzyme activity assays and immunobloting with a monoclonal antibody to human Neu 3. The presence of a putative caveolin-binding motif within Neu 3 prompted us to determine whether Neu 3 interacts directly with caveolin-1. In transfectants expressing a polyhistidine-tagged form of Neu 3, caveolin-1 co-eluted with Neu 3 on affinity column chromatography. A mutation with a single amino acid change in the caveolin-binding motif led to inhibition of recruitment of the sialidase to the microdomain, accompanied by loss of the enzyme activity. Interaction of Neu 3 with caveolin-1was supported further by co-immunoprecipitation of Neu 3 by anti-caveolin-1 antibody. These results indicate that Neu 3 functions as a caveolin-interacting protein within caveolin-rich microdomains.

Key Words Neu 3, Sialidase, Ganglioside, Caveolin, Lipid raft

(Introduction)

Sialidase catalyzes the removal of sialic acids from glycoproteins and gangliosides, an initial step of the degradation of these molecules. Mammalian forms have been demonstrated to differ from microbial sialidases in various aspects, especially in the presence of multiple types even in a single cell and in strict substrate specificity, this suggesting participation not only in lysosomal degradation but also in many cellular processes (1, 2). Although the functional roles of mammalian sialidases are not fully understood, recent progress in sialidase gene cloning has allowed partial clarification. Among three mammalian sialidases so far cloned (abbreviated to Neu 1, 2 and 3), ganglioside-specific sialidase (Neu 3) is a key enzyme for ganglioside hydrolysis and is unique in specifically hydrolyzing gangliosides and in its localization in the plasma membrane. To obtain functional evidence regarding Neu 3, we previously cloned and characterized the sialidase cDNAs of mammalian origin (3-6), and have employed a human Neu 3 cDNA (4) in the present work.

Gangliosides, sialic acid-containing glycosphingolipids, are present in surface membranes of cells and are thought to play important functional roles in regulating a wide range of biological processes, including cell surface interactions, cell differentiation and transmembrane signaling(7-9). The ganglioside-specific sialidase Neu 3 may thus have many functions. Accumulating evidence suggesting functional importance for plasma membrane-attached vesicular organelles called detergent–insoluble glycoshingolipid-enriched complexes (DIGs) or lipid rafts (10-13). DIGs are closely related to caveolae, 50-100 nm invaginations of the plasma membrane, in their lipid composition and resistance to detergent solubilization. A major structural protein of caveolae is caveolin, known to act as a scaffold for various signaling molecules (14). Recent studies have indicated that caveolin recruits transducer molecules into caveolae and regulates their functions by direct interaction with these proteins, including G protein alpha subunits, H-ras, Src family tyrosine kinases, MAP kinase, and endothelial nitric oxide synthase, which recognize a short cytosolic domain derived from the N-terminal region of caveolin-1, termed the caveolin scaffolding domain, by caveolin-binding common motifs present within their molecules (14). In addition, interaction of the caveolin scaffolding domain with caveolin-binding motifs of EGF-R (15) and protein kinase C (16) is known to inhibit their kinase activity. We now present evidence that Neu 3 is closely associated with caveolin-1 within caveolae microdomains, probably acting as a transducer molecule. Neu 3 is the first example of a carbohydrate-metabolizing enzyme to be shown to interact with caveolin. In this context it is of interest that, in the course of the present study, a paper (17) appeared describing ganglioside sialidase activity cofractionating with rafts from a

neuroblastoma cell line.

EXPERIMENTAL PROCEDURES

Cells and DNA Transfection--- HeLa cells and COS-1 cells were obtained from The Cell Bank of Tohoku University and RIKEN Cell Bank, respectively, and maintained in Dulbecco's modification of Eagle's minimum medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2. Sialidase expression vectors were constructed by subcloning a cDNA fragment containing the open reading frame (ORF) of human membrane-associated ganglioside sialidase (human Neu 3) or the mutated DNA into the EcoRI site of pME18S, an eukaryote expression vector under control of the SRα promotor, a generous gift from Dr. T. Maruyama, Tokyo Medical and Dental University. Introduction of mutations on human Neu 3 cDNA was performed by recombination PCR (18) with the following mutation primers: for the Y181A mutation 5'-GCGTATACCGCGTACATCCCTTCCTGG-3' and 5'-GGGATGTACGCGGT ATACGCAGGGATG-3', and for the F187R mutation, 5'-CCTTCCTGGCGCTTTT GCTTCCAGCTAC-3' and 5'-AAGCAAAAGCG CCAGGAAGGGATGTAG-3'. PCR reactions were carried out using the primers, a plasmid containing the wild type human Neu 3 ORF (pME-HmSD) as a template, and LA-Taq polymerase (Takara, Japan) under the conditions of 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 60°C for 30 sec, 72 °C for 5 min. Mutated DNAs, with sequences confirmed by the dideoxy chain termination method using an AutoRead Sequencing kit, were finally subcloned into the EcoRI site of the pME18S vector. As a control, a cytosolic sialidase expression plasmid was constructed by subcloning a cDNA fragment covering the ORF into pME18S and used for transfection.

COS-1 cells were transfected by electroporation by the procedures previously described (5). After 48 h of transfection, cells were collected and stored -80°C until use. To correct for transfection efficiency, a luciferase expression plasmid was co-transfected with the sialidase plasmid and the sialidase activity was then normalized to the luciferase activity as detailed eariler (19).

Sucrose density gradient analysis ---Low density Triton X-100-insoluble membrane domains were isolated from cultured cells by slight modifications of the method described (20). Briefly, cultured cells (-3 x 10^7) were collected in 1 ml of ice-cold lysis buffer containing 25 mM MES, pH6.5, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. After homogenization, the cell lysate was adjusted to 40% sucrose by addition of 1 ml of the above lysis buffer without Triton X-100 containing 80% sucrose. A linear sucrose gradient (5-30%) in the buffer without Triton X-100 was layered over the lysate and centrifuged at 190, 000 x g (39, 000 rpm) for 16-20 h at 4 °C in an SW-41 rotor. Ten fractions were collected as 1 ml-aliquots.

Construction, Expression, and Purification of Polyhistidine Tagged Human Neu3---The polyhistidine tag was incorporated into the C terminus of the wild type and Y181A mutants of the human Neu 3 cDNA, respectively, using the PCR primers and pME-HmSD as a template. The final construction was subcloned into the EcoRI site of the pME18S vector and used for transient expression in COS-1 cells. The transfectants were homogenized in the lysis buffer described above, assayed the sialidase activity , and dialyzed against the start buffer (20 mM phosphate buffer, pH 7.5, 10 mM imidazole, 0.5 M NaCl, and 1% Triton X-100) for 3 h at 4 °C. The dialyzed fraction was applied to 1 ml of affinity column for histidine-tagged proteins (HisTrap column; Pharmacia Biotech) pre-equilibrated with the start buffer. After washing with 10 ml of the buffer, bound proteins were eluted with 5 ml of the elution buffer (20 mM phosphate buffer, pH 7.5, 500 mM imidazole, 0.5 M NaCl and 1% Triton X-100). Washes and eluates were then subjected to immunoblot analysis.

Sialidase Assays---The cell lysate and the fractions from the sucrose density gradient were used for sialidase assays routinely using gangliosides as the substrate. The reaction mixture contained 50 nmol of substrate as bound sialic acid, 0.2 mg of bovine serum albumin, 10 μmol of sodium acetate (pH 4.6) and 0.2 mg of Triton X-100. After incubation at 37°C for 10-60 min, released sialic acid was determined by the thiobarbituric acid method after passing through an AG1X-2 mini-column, as described elsewhere (6). Protein was determined by dye-binding assay (Bio-rad). One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/h.

Preparation of Monoclonal Antibody to Human Neu 3 --- The foot pads of 6 week-old female BALB/c mice were injected every week with 2 mg protein of the particulate fraction of human Neu 3-transfected COS cells, emulsified with incomplete adjuvant. After five successive injections followed by a booster injection, spleens and bilateral inguinal and parietal abdominal lymphnodes of immunized mice were harvested, and B cells were fused with the mouse myeloma X 63 Ag8.653 (The Cancer Cell Repository, Tohoku University, Sendai, Japan) in 50% polyethylene glycol 1000 (Wako). Screening of hybridomas was carried out by examining the specific immunoreactivity with the particulate fraction of the Neu 3 transfectants in ELISA system (ZYMED) and with the membrane-permeabilized Neu 3 transfectants for FACS analysis. The particulate fractions from vehicle-transfected COS cells were also used to exclude false positives due to reactions with parent COS cell proteins. The positive clones were screened by dilution to obtain single specific clones. The subclass of monoclonal antibody (mAbs) was determined to be IgG_1 with a Mouse isotyping kit.

Immunoblotting and immunoprecipitation --- Cell lysates, sucrose density gradient fractions or fractions from HisTrap affinity columns were separated on SDS-polyacrylamide electrophoresis gels (10%) under reducing conditions. Proteins were transferred to PVDF membranes and immunoblotted with antibodies specific to respective proteins. The individual bands were detected with peroxidase-conjugated goat anti-mouse IgG or -rabbit IgG (Promega) and development was

achieved with an enhanced chemiluminescence system (ECL Plus Western blotting reagent; Amersham Pharmacia Biotech.), according to the manufacturer's recommendations. In immunoprecipitation experiments, cell lysates were incubated overnight at 4℃with Protein A Sepharose (Amersham Pharmacia Biotech.) and polyclonal anti-caveolin-1 (Santa Cruz Biotec.) or monoclonal anti-human Neu 3 antibody and the precipitates after washing were applied to SDS-polyacrylamide electrophoresis, and then immunoblotted as described above. Antibodies and their sources were as follows: anti-caveolin-1 IgG (mAb 2297, Transduction Labs) and anti-caveolin-1 (Santa Cruz Biotec.) ; anti-cSrc (Santa Cruz Biotec.); anti-MAPK (New England Biolabs); anti-14-3-3 (Santa Cruz Biotec.).

RESULTS

Co-fractionation of human Neu 3 with caveolin-1---To examine whether endogenous ganglioside-specific sialidase is associated with caveolin-1, we used HeLa cells having relatively high sialidase activity. Cell lysates were fractionated on a sucrose density gradient and the fractions were analyzed for protein concentration, caveolin-1 immuno-reactivity, and sialidase activity with gangliosides as substrates. Fig.1a shows ganglioside sialidase activity to co-fractionate in low density fractions 4-6 with caveolin-1 and cSrc, a signal transducer, as assessed by immunoblot analysis, while greater than 90% of the total protein loaded on the gradient was recovered in high density fractions 9-10. To obtain evidence that Neu 3 protein is present in the caveolin-rich fraction, we prepared mAb specific to human Neu 3 and observed the protein distribution by immunoblotting. The antibody was evaluated to be only reactive with Neu 3 (48 kD protein), from HeLa cells (in the experiments with homogenates in Fig.1b) as well as from Neu 3-transfected COS-1 cells. On sucrose gradient Neu 3 protein from HeLa cells was detected mainly in fractions 5-6, in parallel with sialidase activity together with caveolin-1. We then examined COS-1 cells transiently transfected with human Neu 3 cDNA. More than 70% of the ganglioside activity was found in fractions 4-6, rich in caveolin-1, and approximately 30% of the activity remained in the fractions 9-10 (Fig.2a). The activity detected in the high density fractions was not due to overloading, because re-loading on the gradient resulted in recovery of the activity in the same fractions. Neu 3 proteins were detected in the fractions having high activity in a manner similar to the case of HeLa cells. On the other hand, when we tested cytosolic sialidase (Neu 2)-transfected COS-1 cells by measuring sialidase activity at pH 6.0 with 4MU-NeuAc, all of the sialidase was found in the high density fractions (Fig.2b). In addition, endogenous lysosomal sialidase (Neu 1) in COS-1 cells, assayed with 4MU-NeuAc as substrate at pH 4.6, showed a neary identical pattern to that of the exogenously expressed cytosolic sialidase (data not shown). These results indicate human Neu 3 to indeed be localized in the caveolin-rich microdomains, in contrast to Neu 2 and Neu 1 sialidases.

Interaction of Human Neu 3 with Caveolin-1.--- To examine the possibility that Neu 3 interacts with caveolin in the microdomain, we searched for a functional binding site for caveolin-1 in the Neu 3 molecule. Caveolin binding motifs were recently deduced ($\Phi X \Phi XXXX \Phi$ and $\Phi XXXX \Phi XX \Phi$, where Φ is an aromatic residues W, F, or Y) (14). These motifs are present in most caveolae-associated proteins, including PKCa, MAP kinase, and EGFR. In the human Neu 3, we identified a possible region (YTYYIPSW, 179-186 residues) within the hydrophobic stretch of the putative transmembrane domain sequence (4). To determine whether the binding site in Neu 3 is functional, the following strategies were employed: introduction of mutations into Neu 3, affinity purification of polyhistidine (His)-tagged Neu 3 forms, and immunoprecipitation with antibody to either caveolin-1 or Neu 3.

Two mutants were expressed and the cell lysates were analyzed by fractionation on a sucrose density gradient (Fig.3). Mutant F187R with one amino acid change next to the motif sequence demonstrated reduction of ganglioside activity in the cell lysate to only 45% of the wild type level, and mutant Y181A with a single amino acid change within the motif showed complete loss of ganglioside activity, although Neu 3 protein level was similar to the wild type (Fig.3a). On sucrose gradient fractionation, mutant F187R exhibited only a low amount of Neu 3 protein in fraction 5 with low sialidase activity, much more protein and activity being evident in fractions 9-10. In mutant Y181A, Neu 3 protein was hardly recovered in the fractions 4-6 where caveolin was still concentrated. These results suggest that the predicted caveolin-binding motif in Neu 3 may be involved in binding to caveolin-1, loss of the binding ability leading to failure of recruitment to caveolae and subsequently to reduction of the enzyme activity.

To obtain further evidence for the direct interaction between Neu 3 and caveolin-1, we next used His-tagged forms of Neu 3 constructed by introducing the His-tag epitope into the C-terminus (Fig.4a and b). On transient expression in COS-1 cells, the His-tagged form could be purified by Ni^{2+} ion-charged HiTrap Chelating column chromatography, the bound proteins being specifically eluted by affinity elution with imidazole (21). As a control for non-specific binding, human Neu 3 without the tag epitope was also expressed. Both Neu 3 forms showed sialidase activity at the same level. After affinity purification on HiTrap column, fractions were analyzed by immunoblotting with antibodies to human Neu 3 and caveolin-1 (Fig. 4a). His-tagged Neu 3 was specifically eluted by affinity elution with imidazole, while no binding to the column was observed with Neu 3 lacking the His-tag. Under these conditions endogenous caveolin-1 and MAP-kinase co-eluted with His-tagged Neu 3, but 14-3-3 protein in the washing effluents. When the cell lysates from mutant Y181A with His-tag epitope were applied on a HiTrap column, most of caveolin-1 protein was detected in the washings, even though all of the Neu 3 protein in the affinity eluates (Fig. 4b). We further examined direct association of Neu 3 with caveolin-1 by immunoprecipitation with antibody to either caveolin-1 or Neu 3 (Fig. 4c). Under the conditions where almost equal amount of endogenous caveolin-1 was applied for immunoprecipitation (in the lower panel), wild type Neu 3

was co-immunoprecipitated with caveolin-1 by anti-caveolin-1 antibody, whereas much less amount of mutant Neu 3 protein was recovered compared to wild type level. Consistent with the sucrose gradient data described above, the protein level in the immunoprecipitates was higher in mutant F187R retaining some activity than in mutant Y181A. Caveolin-1 was not detected in anti-Neu 3 immunoprecipitates. These data together indicate that the predicted caveolin-binding motif sequence in human Neu 3 can serve as a ligand for the caveolin scaffolding domain and that loss of the capacity for binding to caveolin and subsequent failure of recruitment to caveolae results in reduced enzyme activity.

DISCUSSION

Ganglioside-specific sialidase Neu 3 is a unique glycosidase in its localization within the plasma membrane and the strict substrate specificity. Unlike lysosomes, the membranes do not contain a set of glycosidases to degradate glycoproteins and glycolipids (22), suggesting that Neu 3 is involved in cell surface events other than catabolism of glycoconjugates. In fact, there are several observations suggesting important functional roles of sialidase in processes like cell differentiation (5, 23), apoptosis (24), and malignant transformation (25-28). However, no clear evidence for the physiological functions of Neu 3 has hitherto been presented, to our knowledge.

In this study, we demonstrated that this sialidase interacts directly with caveolin-1 and that its activity is likely to be regulated by caveolin-mediated recruitment to caveolae-microdomains. Many caveolin-associated proteins are now known to contain caveolin-binding motifs and this is considered to be a general mechanism for caveolin-mediated sequestration and subsequent inactivation within caveolae. In the case of Neu 3, mutation of the putative caveolin-binding site led to not only blocking of the recruitment to caveolae but also suppression of the sialidase activity. This indicates that Neu 3 is a unique member of caveolin associated proteins which is activated rather than inactivated by caveolin-mediated sequestration, similar to the case of the insulin receptor promoting IRS-1 phosphorylation (29). To activate Neu 3 in vitro, detergents such as Triton X-100 are required. There is no endogenous activator located in the plasma membrane known at present which can be replaced by the detergent. Since we have observed that phospholipids can stimulate the Neu 3 sialidase activity (Wada, T. and Miyagi, T., manuscript in preparation), it is most feasible that phospholipids constituting caveolae may activate Neu 3 after recruitment.

We previously demonstrated that Neu 3 contains a hydrophobic but non-α-helical stretch as a putative transmembrane domain which faces the extracytoplasmic side at the N-terminus (3). On the other hand, our recent work showed possible active site amino acid residues to be distributed all over the Neu 3 molecule (4). These two results make uncertain whether this sialidase is catalytic in the membrane-anchored state or rather on the cytosolic side of the membrane, as it may be neccesary for an appropriate tertiary structure for activity (30). In this regard, based on the observation that caveolin is a cytoplasmically oriented integral protein, the human Neu 3 molecule may function on the cytosolic site of the membrane by interacting with caveolin, and cross-talk with other signal transducers. The regulation mechanisms require further analysis.

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Fig.1. Localization of Neu 3 of HeLa cells in low density sucrose gradient fractions rich in caveolin-1. Triton X-100 lysates of HeLa cells were fractionated on a sucrose density gradient as described in the "EXPERIMENTAL PROCEDURES", and the fractions were tested for ganglioside sialidase activity (a) and for caveolin-1 (a), cSrc (a), and Neu 3 (b) proteins by immunoblot analysis. The specificity of the mAb anti-human Neu 3 was evaluated using homogenates (fraction H) from HeLa cells and only a 48 kD band corresponding to the Neu 3 was detected by immuno blot analysis (b).

Fig.2. Localization of human Neu 3 expressed in COS-1 cells in caveolin-rich fractions. The fractions on a sucrose density gradient were assayed for ganglioside sialidase activity and immunoblotted for Neu 3 and caveolin proteins with mAb anti-human Neu3 and anti-caveolin-1, respectively (a). As a control, cytosolic sialidase expressed in COS-1 cells was assayed the activity for 4MU-Neu Ac as a substrate(b).

Fig. 3. Failure of recruitment to caveolae and reduction of sialidase activity by mutation of the caveolin-binding motif within Neu 3. Mutants (F187R and Y181A) and wild type were expressed in COS-1 cells and each Neu 3 protein was detected by immuno blot analysis (a). Cell lysates of the transfectants were fractionated on a sucrose density gradient. Suppression of sialidase activity and Neu 3 protein level was observed in caveolin-rich fractions from mutant cell lysates, irrespective of enrichment of caveolin –1 in low density sucrose gradient fractions (b).

Fig. 4. Co-purification of polyhistidine-tagged human Neu 3 with caveolin-1 by Ni^{2+} chelating column chromatography and co-immunoprecipitation of Neu 3 and caveolin-1. Human Neu 3 with or without a His-tag was expressed in COS-1 cells and purified by affinity chromatography as described in the "EXPERIMENTAL PROCEDURES"(a). In the upper panel, washings and eluates were tested for Neu 3, caveolin-1, MAP-kinase and 14-3-3 proteins by immunoblot analysis with respective antibodies. In the lower panel, the protein concentrations of the fractions from the column are shown and arrows indicate start of affinity elution with 50 mM imidazole. A His-tag form of mutant Y181A was subjected to affinity chromatography, and Neu 3 and caveolin-1 proteins were analyzed by immunoblotting (b). Cell lysates were collected for immunoprecipitation with anti-caveolin antibody (polyclonal), and Neu 3 or caveolin-1 in the immunoprecipitates was detected with antibody to human Neu 3 or caveolin-1 (monoclonal) (c).

Fig.5. Reduced Neu 3 sialidase activity by dissociation of Neu 3 from caveolin-1 with _-cyclodextrin treatment. Cells were treated with 10 mM _-cyclodextrin for 50 min at 37°C, and the lysates of collected cells were assayed for sialidase activity (a), and fractionated on a sucrose density gradient for detection of Neu 3 activity and the protein (b).

注：本研究は、2001 年 10 月 25 日『第 74 回日本生化学会大会』にてポスター発表。

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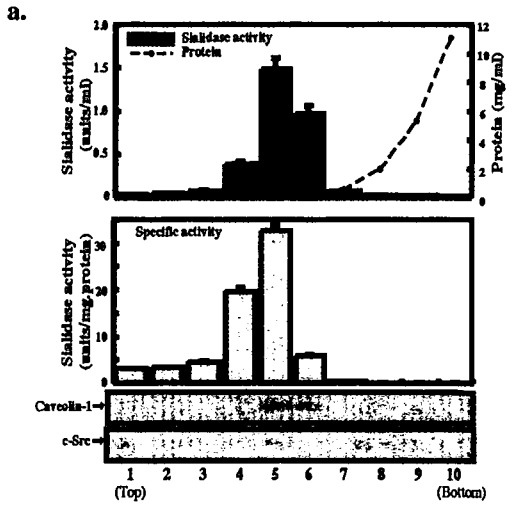

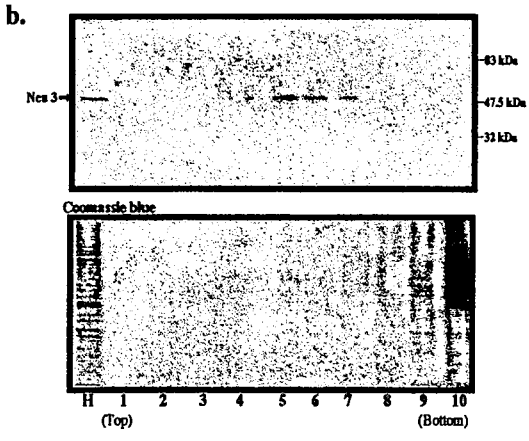

Fig.1

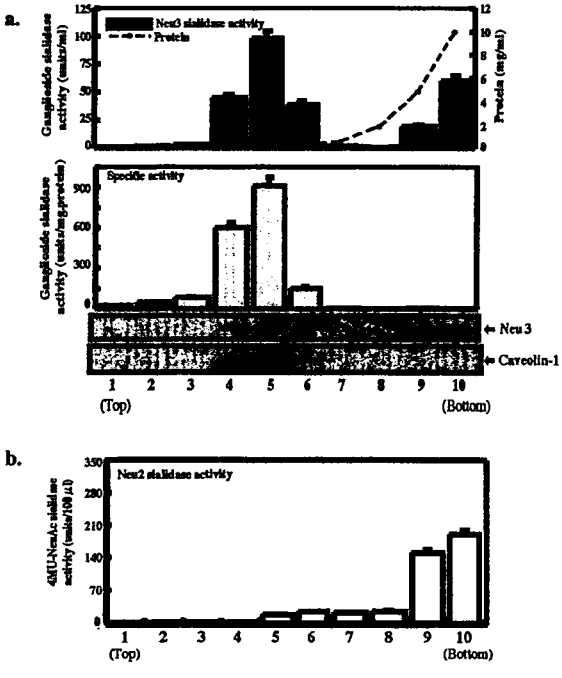

Fig.2

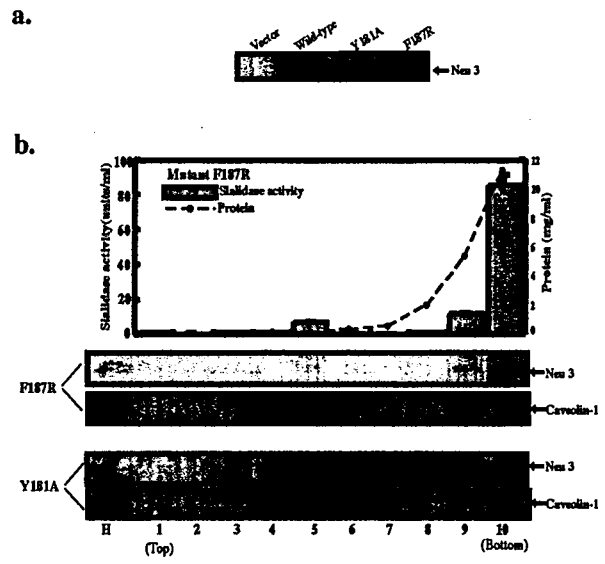

Fig.3

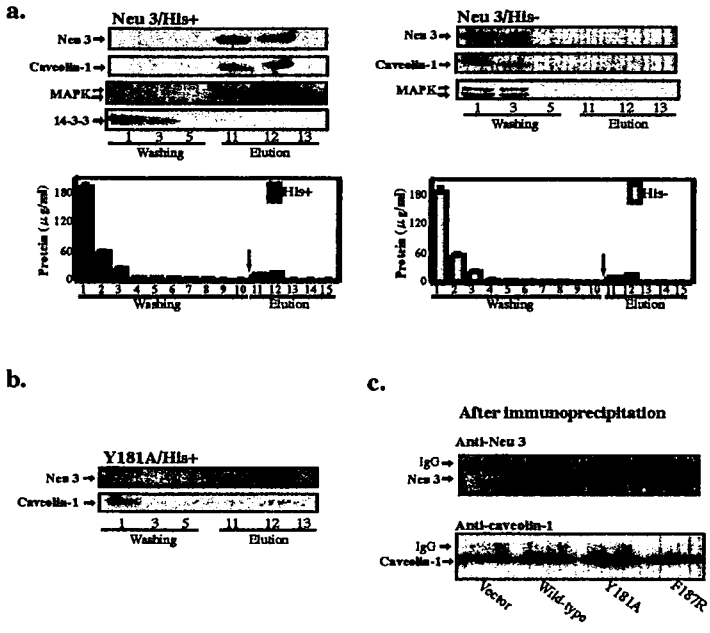

Fig.4

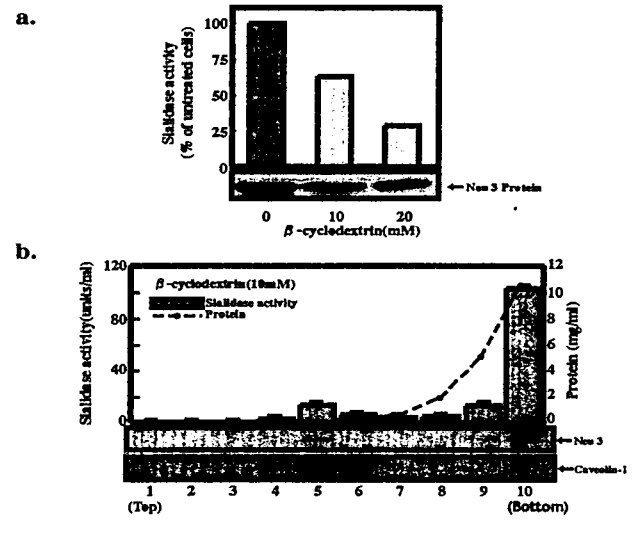

Fig.5