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1．研究テーマ

視細胞外マトリックス分子 SPACR のこつのドメインと糖鎖結合活性の違い

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14回愛知眼科フォーラム

SPACR のこつの分子ドメインと糖鎖結合活性

(2) 学会誌等に発表した論文 有・(無) （雑誌名・論文名）

3．今後の研究計画

視細胞外マトリックス分子 SPACR は b-HA と b-Heparin 反応する
ことはもう証明されました。SPACR には，b-HA，b-Heparin と反応する
部位を証明することはこれからの目標です。
反応する可能性が高い部位を template にして，fusion protein を
融合して，反応部位を証明すること。

4．指導責任者の意見

眼球内のプロテオグリカンの機能と局在，相互関係に
ついて，研究を続行している。ここつと実験するタイプで
結果を出して，論文にまとめる日も近いと信じております

指導責任者氏名 岩成正浩 印

5．研究報告書

別紙「研究報告書の作成について」に倣い、指定の用紙で作成して下さい。

研究発表または研究状況を記録した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。

※発表に当っては、*日中医学協会助成金による*旨を明記して下さい。

-日中医学協会助成事業-

視細胞外マトリックス分子SPACRの二つのフォームと糖鎖結合性の違い

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Abstract

SPACR (sialoprotein associated with cones and rods) which has a molecular weight of 150KDa,is a glycoprotein around the photoreceptor in chick IPM(interphotoreceptor martrix).A new form of chick SPACR was identified by two antibodies O46-F and MY-174. O46-F is SPACR C-terminal peptide polyclonal antibody,MY-174 is a monoclonal antibody which can react to chick SPACR(1).The new form has a molecular weight of 100kDa. Both of 150kDa and 100kDa were labeled with biotin-hyaluronan and biotin-heparin.The activity binds to heparin of two forms were tested by heparin affinity chromatography, 100kDa binding to the heparin with low affinity, 150kDa binding to be of high affinity.Heparin inhibit hyaluronan binding to 150kDa,which suggests that heparin and hyaluronan binding site of SPACR are closely related.

Key Word　SPACR , form, interphotoreceptor martrix,chick

Introduction

The IPM is located between the neural retina and the retinal pigment epithelium in the vertebrate eye(2).A number of activities of fundamental importance for vision are thought to be mediated by the IPM,including retinal adhesion,visual pigment chromophore exchange,metabolite trafficking, photoreceptor aligment and membrane turnover(3).The IPM consists of aqueous-extractable molecules,and fixed matrix that resists aqueous extraction.Glycoproteins,proteoglycans and hyaluronan(HA) are the major components in the fixed matrix(3,4,5).

SPACR was isolated and characterized in the insoluble IPM of human.Immunocytochemistry localizes SPACR to the matrix surrounding rods and cones(6).SPACR is a glycoprotein in human,monkey and chick (1,6,7,8) and a proteoglycan in mouse,rat and bovine(8,9).Functional studies demonstrate that SPACR bind hyaluronan,suggesting that these molecules may help stabilize the hyaluronan scaffold that forms the framework of the insoluble IPM(1,5,7).

Except for SPACR bind to hyaluronan,other properties or functional roles is not clear. To clarify the other biological functions of SPACR, we isolated、purified and characterized SPACR from adult chick IPM by DEAE sephacel column.

In Western blots of IPM extracts, O46-F immunoreactive bands were present at approximately 150kDa and 100kDa,the 150kDa is SPACR which we have identified(1).In Western blots probed with MY-174, immunoreactive bands were present like O46-F,both of 150kDa and 100kDa were labeled,leading us to postulate that may be this 100kDa protein is a different form from SPACR.

Materials and methods

Protein Isolation

The retina of adult white leghorns were prepared according to reported procedures(6,7).The insoluble samples from retina were suspended in 10× volume of 50mM Tris-HCL PH 8.0, 0.15M NaCL,10mM EDTA,1mM PMSF,after for 4 hr at 4℃ with gentle agitation,the suspension was centrifuged at 8000g for 30 min at 4℃,the suspernatant was immediately loaded at 0.5ml/min onto a DEAE sephacel column in the above solution,after thorough washing,the band proteins were eluted with a linear gradient 0.15M~1M NaCL in above solution,the elution of 0.4M ~0.6M were dialyzed against 50mM Tris-HCL PH 8.0, 0.15M NaCL.

Two-dimensional gel electrophoresis and western blot analyses

Samples were solubilized at a protein concentration of ~1mg/ml in 1.6% Bio-lyte 5/7 ampholyte,0.4% Bio-lyte 3/10 ampholyte,9.5M urea,2.0% Triton X-100,5% β-mercaptoethanol. Isoelectric focusing of 20 μl sample took place in 0.9×57mm tube gels,the first-dimension isoelectric focusing was performed at 500V for 10 min and then at 750V for 4h.after the isoelectric focusing,the tube gels were extruded and placed on top of a 7.5% polyacrylamide gel,the second-dimension took place at a 75V for 20 min,120V for 1h.The protein separated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate(SDS-PAGE) was then electrotransferred on to a nitrocellulose membrane,the transferred membrane was developed with MY 174(1/5000) antibody.The membrane was incubated at room temperature for 30 min in stripping buffer,and then wash with PBS-Tween for removing the reaction solution,the stripping membrane was developed with o46F(1/1000) antibody ,and b-hyaluronan(1/5000),b-Hep(1/5000).

Affinity chromatography on heparin- HiTrap column

The heparin binding abilities were tested by heparin affinity chromatography.3 ml sample after dialyed were added 1mM PMSF and 10mM Ca2+ then applied to a heparin-HiTrap column (1ml) at a flow rate of 0.5ml/min,equilibrated with the same buffer and bond proteins were eluted with a 20ml linear gradient 0.15M~1M NaCL in above solution.The flow through and fraction were pooled and run on 7.5% SDS-PAGE gels,proteins were transferred to a nitrocellulose membrane,then developed with o46F antibody.

Inhibition of hyaluronan binding to SPACR by heparin

To test the effect of heparin on the b-hyaluronan binding ability of 150kDa, western blot analyses were performed,transferred membranes were blocked ,then incubated for 1h at room temperature with b-hyaluronan in the absence and presence of heparin(0.6mg/ml),after washing,membranes were further incubated with peroxidase conjugated streptavidin for 1h at room temperature.the membranes were washed and developed. The films were digitized with a image scanner then analysed using NIH image software.

Results

1.A new form of SPACR

Insoluble IPM extracts from the chick retina separated with two-dimensional gel electrophoresis,then developed with MY-174 antibody, the same membrane after stripping and developed with O46F antibody and b-HA respectively.Fig 1 show arrows located approximately at 150kDa

represent the location of SPACR(1).A new band appears at 100kDa(arrowheads) which react with MY-174,O46F and b-HA like 105kDa.

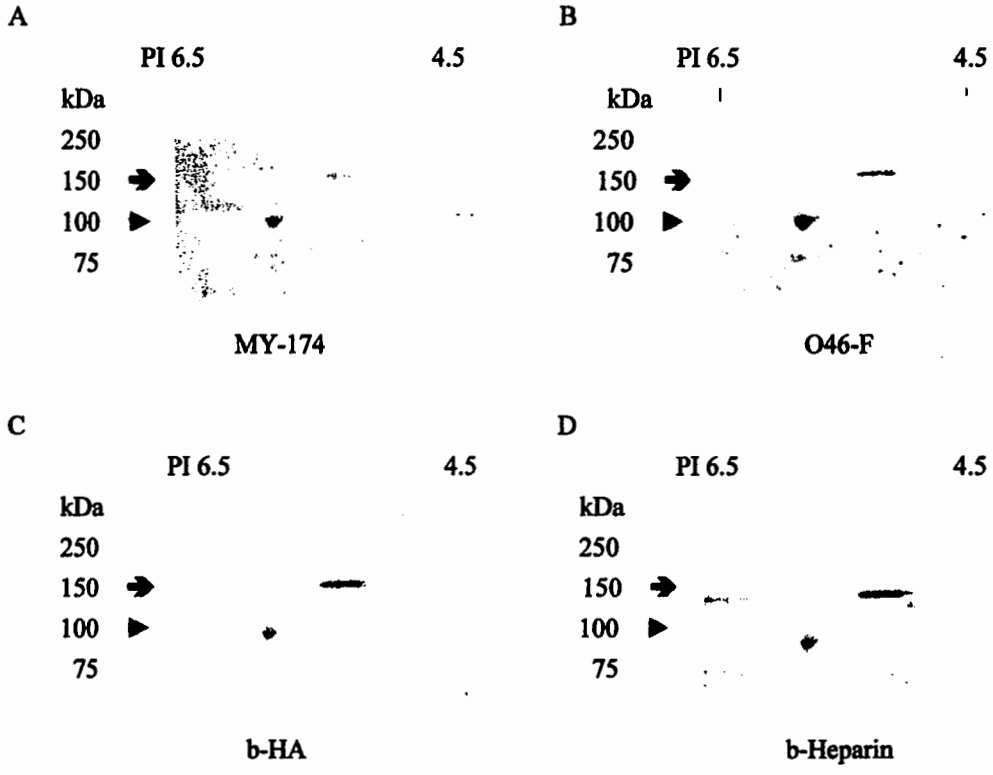

Fig 1 Insoluble IPM of chick retina extracts from the DEAE column separated with two-dimensional gel electrophoresis.Molecular mass marker positions are indicated on the left.PI is indicated at the top.Isoelectric focusing in the horizontal dimension with the anode is on the left. A, developed with MY-174 antibody diluted 1:5000.B,C,D the immunoblot of the same membrane in A after stripping and developed with O46F antibody(1:1000), b-HA (1:5000) ,b-Hep (1:5000) respectively.

2.SPACR binding heparin and compare the ability of two forms to bind heparin

Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions recently been shown(11), we queried whether SPACR functioned as a heparin binding protein.

Western blot analysis of purified SPACR that both the 150kDa and the 100kDa can reacted with b-heparin as can be seen in fig 1 D.

Elution of insoluble IPM from DEAE column were dialyzed,then applied to a heparin-column,after washing,proteins eluted with linear gradient 0.15M~1M NaCL were shown by SDS-PAGE(Fig 2),electrophoretically blotted to nitrocellulose,and developed with o46F antibody,100kDa(arrowhead) could not be bound to column at 0.15M NaCl,but 150kDa (arrows) can be bound and eluted with 0.6M NaCl,indicating two forms have different affinities for heparin.

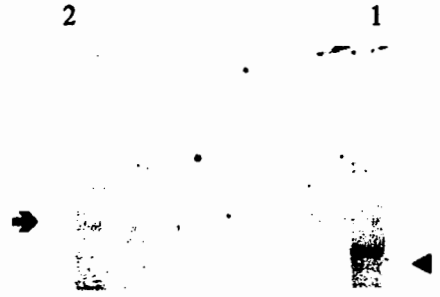

Fig 2.The immunoblot of fraction from heparin-column developed with o46F antibody. The elution from DEAE column were dialyzed,then applied to heparin-column,flow through (0.15M NaCl) (line 1),eluted with gradient NaCL(line 2,0.6M NaCl). Arrows represent 150kDa , arrowhead represent 100kDa.

3.inhibition of hyaluronan binding to SPACR by heparin

Insoluble IPM of chick retina extracts from the DEAE column separated with two-dimensional gel electrophoresis,the membrane developed with b-HA in the absence(A) and presence(B) of heparin(0.6mg/ml). Analysis the films using NIH image software,it was shown that the digital image retained 25%.

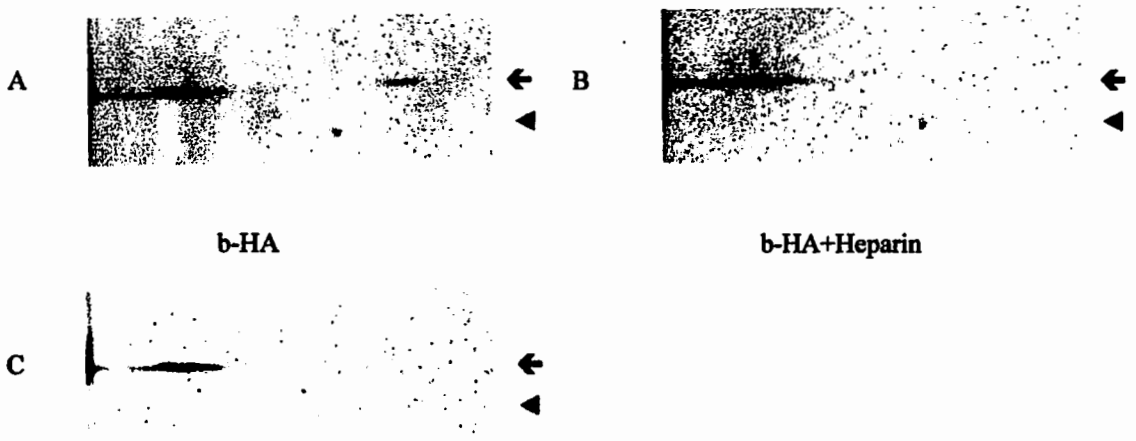

Fig 3.Effect of hyaluronan binding to SPACR by heparin. Insoluble IPM extracts from the chick retina separated with two-dimensional gel electrophoresis.A, developed with b-HA , arrows located 150kDa represent SPACR,arrowheads represent 100kDa.B, developed with b-HA in the presence of heparin shown decrease HA binding to 150kDa.C,developed with only streptavidin-horseradish peroxidase conjugate as control,no 150kDa and 100kDa band in the control.

Discussion

1.Two forms of SPACR

MY174 antigen in the chick IPM is identical to chick SPACR,the O46-F is a polyclonal antibody for

chick SPACR(1).Here we showed that there were two bands react with MY174, O46-F antibodies in the chick insoluble IPM.And one function of SPACR is to bind to hyaluronan in the IPM(1,7).The band was located about 150KDa which is SPACR(1), another located about 100KDa,the studies reported here that IPM in the chick retina express a protein having a molecular mass of 100KDa which immunostaining characterization similar to SPACR(150kDa).

SPACR may be consist of two different forms in chick IPM,the long form is 150KDa another short form which has a molecular weight of 100KDa,the short form may be resulted from the cleavage reaction of the long form of SPACR.

In many G-protein coupled receptors SEA modules are found proteolytic cleavage site,and were cleaved during them residence in the ER(10).SPACR contain two SEA modules,leading us to postulate that may be this is one cleavage site for SPACR.

The biological purpose of the cleavage is not understood,further studies will be required to establish whether SPACR undergoes a proteolytic cleavage.

2.Heparin binding assays

Heparin is a polysaccharide belonging to the GAG family,heparin has been implicated in modulating various biological processes,such as blood clotting,cell adhesion recognition migration ,growth factor signaling,and viral infection.The biological function of heparin is primarily mediated through its binding and regulation of various proteins,including enzymes growth factors,cytokines,and extracellular matrix proteins etc(12).

Chick SPACR is not a chondroitin-type proteoglycan(1),whereas there are some consensus sites for GAG in peptide sequences of chick SPACR.Our data showed chick SPACR can binding heprain.

Schubert indicated that retinal purpurin protein can interacts with heparin and heparan sulfate,plays a role in adhesive interactions of neural retina cells(13).

Because binding to heparin may serve to immobilize proteins in the extracellular matrix on cell surfaces or in the extracellular space,suggest may be this is ability to binding to retinal photoreceptor cells like hyaluronan.

3.Compare the ability of two forms to bind heparin

Two forms of SPACR can bind to heparin ,we next compared the ability of two forms to bind heparin.as is shown in fig 4.100kDa could not bind to this column when was carried out in the 0.15M NaCL in 50mM TrisHCL PH 8.0,1mM PMSF 10mMCa2+.150kDa was able to bind to this column and was eluted at about 0.6M NaCL in 50mM TrisHCL PH 8.0,1mM PMSF 10mMCa2+. Solubilized proteins may bind to heparin mainly electrostatically and could be dissociated by changing ionic strength.

The difference in NaCL concentration for elution from heparin column was indicated that 150kDa and 100kDa may have different binding abilities to heparin.100kDa binding to the heparin with low affinity,150kDa binding to be of high affinity.

4. Addressing the question of whether heparin effect the hyaluronan binding to 150kDa,we performed western blot analyses.It was shown that hyaluronan binding to 150kDa was decreased by added heparin,analysis the films using NIH image software,it was shown that the digital image retained 25%. heparin competes with hyaluronan for 150kDa binding.Chick SPACR has a 280KEIHVLGFK288 may be is a candidate for hyaluronan binding motif(1),and there are some consensus sites for GAG in peptide sequences of chick SPACR,and the nearest GAG binding site is 294 DGS296.may be this site involved in the binding of heparin.Our data imply that heparin and hyaluronan binding site of SPACR

are closely related.

In conclusion,the present data demonstrate

1. SPACR have two forms in chick retina,one have molecular mass of 150kDa another have 100kDa.
2. Two forms of SPACR can bind to heparin
3. The heparin binding ability of them is difference.
4. hyaluronan binding ability of SPACR can be inhibited by heparin.

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