ている。

C-2-4-3 ガイドラインの準備状況

コンセプトペーパーであるEMA/CHMP/CPWP/708420/2009のパブリックコメント募集は2010年3月に終了している。本コンセプトペーパーの最終版は未だ発出されていないが、発出後12-18カ月でガイドラインのドラフト版が公表されることになっている。その後6カ月のパブリックコメント募集期間を経てガイドライン最終版は更なる6カ月後に発出される予定である。

ATMPに関するリスクベースアプローチのガイドラインの策定はEMAのCHMP（ヒト用医薬品委員会 Committee for Human Medicinal Products）において、CPWP（細胞由来製品ワーキングパーティーCell-based Products Working Party）とGTWP（遺伝子治療ワーキングパーティーGene Therapy Working Party）とが主導し、CAT（先端医療委員会 Committee for Advanced Therapy）の指導に従って行われる予定である。BWP（生物製剤ワーキングパーティーBiologics Working Party）は品質面、SWP（安全性ワーキングパーティーSafety Working Party）が非臨床の側面、PhVWP（ファーマコビジランスワーキングパーティーPharmacovigilance Working Party）がリスクベースアプローチとリスク分析・リスクマネージメントシステムとの相補性について協力する予定となっている。必要に応じてその他の、PDCO（小児科委員会 Paediatric Committee）やCHMPなどの関連ワーキングパーティー・関連委員会、および外部団体とも協議することになると考えられる。

D. 考察

米国では早くも1997年の段階でHCT/Pに対する規制の方法としてのリスクベースアプローチが提唱されており、製品の特性や適用などの多様性が高いHCT/Pに対して合理的かつ包括的な枠組みが整備されている。プリンシプルに基づいた論理的・合理的な規制の枠組みをいち早く整備したことは、先端的なHCT/Pの実用化において米国が世界をリードしている今日の状況を作り出した大きな原因の一つではないかと考えられる。

EUでは2008年末からATMPの新たな規制の枠組みが敷かれるようになったと同時にリスクベースアプローチの具体的運用に関する議論が活発化し、EMAは現在そのガイドラインの策定の動きを見せている。リスクベースアプローチの細胞・組織利用製品への適用に関するガイドラインを策定することにより、製品の販売承認申請に必要となるデータの要件を決定する過程、すなわちリスクの同定・分析法、検証法、妥当性・合理性の説明方法等について、開発者（製薬企業、大学等）や関連団体（学会、患者団体等）の理解が促進されると期待される。開発者においては、科学的合理性のある開発戦略を立てることが可能となることにより、開発の合理化・能率化が期待される。また、ガイドラインは規制当局者の販売承認申請審査過程にも役立つと考えられ、開発者と規制当局者とがガイドラインを共有することにより、効率的な販売承認につながるとも期待される。

E．結論

再生医療・細胞治療および細胞・組織加工製品の開発は今日非常に速い速度で進んでおり、欧米のリスクベースアプローチに基づいた合理的かつ包括的な規制の枠組みは、この急速に進展する領域におけるイノベーションおよび製品開発を、不必要な規制の障壁により妨げられることなく推進することに役立つと考えられる。同時に、リスクベースアプローチは、医師や患者が期待する医薬品・医療機器の安全性を合理性をもって担保するために有用であると考えられる。わが国の細胞・組織加工製品の開発のためのミニマムコンセンサスパッケージの策定においても、欧米のリスクベースアプローチを意識し、参考とすることは非常に有意義であると考えられる。

F．健康危険情報

特記事項なし

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H．知的財産権の出願・登録状況

H-1. 特許取得 なし

H-2. 実用新案登録 なし

H-3.その他 特記事項なし

III. 研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別刷

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ORIGINAL ARTICLE

Positive and negative regulation of adenovirus infection by CAR-like soluble protein, CLSP

K Kawabata[1], K Tashiro[1,2], F Sakurai[1], N Osada[3], J Kusuda[3], T Hayakawa[4], K Yamanishi[2,5] and H Mizuguchi[1,2]

[1]*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan;* [2]*Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan;* [3]*Laboratory of Genetic Resources, National Institute of Biomedical Innovation, Osaka, Japan;* [4]*Pharmaceutics and Medical Devices Agency, Tokyo, Japan and* [5]*National Institute of Biomedical Innovation, Osaka, Japan*

Coxsackievirus and adenovirus receptor (CAR) is a member of the immunoglobulin (Ig) superfamily and a component of epithelial tight junction. CAR also functions as a primary receptor for coxsackievirus B and adenovirus (Ad) infection. In this study, we report the identification of a novel protein, CAR-like soluble protein (CLSP), which is closely related to CAR. Mouse CLSP (mCLSP) was composed of 390 amino acids, including three Ig domains, and showed strong homology to the IgV domain of CAR. Interestingly, mCLSP lacks a transmembrane domain, indicating that this is a soluble protein. mCLSP mRNA was detected primarily in the brain and ovary. When mCLSP cDNA was introduced into SK HEP-1 cells, which were known to be CAR positive and easily infected with Ad vector, the infection with Ad vector was severely inhibited. On the other hand, mCLSP promoted the infection with Ad vector in CAR-negative NIH3T3 cells. Furthermore, recombinant CLSP directly bound to Ad and inhibited the Ad vector-mediated transduction in SK HEP-1 cells. Computational analysis for a genome database showed that the CLSP gene is rodent-specific, and that human and bovine lack this gene. These results suggest that CLSP may play a role in the antiviral defense of the host in rodent animals.
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Keywords: *CAR; adenovirus; soluble protein*

Introduction

Coxsackievirus and adenovirus receptor (CAR) has been identified as the primary receptor for subgroups A and C–F adenovirus (Ad) and group B coxsackievirus (CBV).[1–3] CAR is a type I transmembrane protein with two extracellular immunoglobulin (Ig) domains, and also belongs to the CTX subfamily of the Ig superfamily, which consists of proteins containing one V and one C2 Ig domain.[4–6] CAR has many physiological functions, such as formation of a tight junction in epithelial cells,[7–9] growth-inhibitory activity in tumor cells,[10,11] and early embryonic cardiac development.[12,13]

One of the characteristics of members of the Ig superfamily is sequence homology among the members.[14] For example, CAR is structurally similar to CAR-like membrane protein (CLMP),[15] brain- and testis-specific Ig superfamily (BT-IgSF),[16] and endothelial cell-selective adhesion molecule (ESAM).[17] Another characteristic is that many of the Ig-superfamily genes produce soluble receptors that lack the transmembrane and cytoplasmic domains of full-length receptors.[18] Each of the soluble receptors is produced by proteolytic cleavage of the original membrane-bound receptor or *de novo* synthesis from alternatively spliced RNAs that are specific for the soluble receptor.[19] The soluble receptors are released from cells and appear in biological fluids or tissue culture supernatants.[20] They have been shown to play a significant role in various disorders, including viral infection. The soluble form of CAR is produced by alternative splicing in HeLa cells and shows inhibitory activity with regard to CVB infection.[21] It has been reported that soluble CAR in tumor effusions interferes with the Ad vector-mediated transduction in gene therapy.[22] However, much remains unknown regarding the physiological role of soluble receptors. In this study, we identified a novel protein, CAR-like soluble protein (CLSP), which is a soluble protein closely related to CAR. Mouse CLSP (mCLSP) has three IgV domains, each of which is homologous to the IgV domain of CAR. Overexpressed or recombinant mCLSP was found to inhibit the Ad vector-mediated transduction in CAR-expressing cells, supporting the notion that CLSP may play a role in the antiviral defense of the host.

Correspondence: Dr H Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka, Japan.
E-mail: mizuguch@nibio.go.jp
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Results

Identification of CLSP

A search of the expressed sequence tag (EST) database for the nucleotide sequences of the N-terminal IgV domain of mouse CAR (mCAR) led to the identification of an EST sequence potentially encoding a CAR-like

protein (GenBank accession number BB617533). To determine the full-length cDNA sequence, we carried out 5′- and 3′-RACE, as described in the Materials and methods. The full-length cDNA is 1567 bp in length and contains an open reading frame starting from the first methionine codon; it encodes a protein of 390 amino acids with a calculated molecular weight of 43 009 Da. The nucleotide sequence around the first methionine codon conforms well to the consensus sequence of the eukaryotic translational initiation site, and the 3′ non-coding region contains a typical AATAAA polyadenylation signal.[23,24] The deduced amino-acid sequence contains a highly hydrophobic N-terminal region characteristic of a signal peptide with a putative cleavage site between Ser-19 and Val-20.[25] Unlike CAR, this protein contains no transmembrane region. We performed a further search of the EST database, but could not identify the membrane-bound type of this protein. We therefore designated this protein as CLSP. mCLSP contained three IgV domains, IgV1, IgV2 and IgV3 (Figure 1a). The three IgV domains were homologous and showed significant homology to the IgV (D1) domain of mCAR (Figure 1b). A search of the mouse genome database showed that the mCLSP gene is on chromosome 9, while mCAR is situated on the distal portion of chromosome 16. Therefore, mCLSP is not a splice variant of mCAR. Rat CLSP was found in the GenBank database (accession number BC063181) as a CAR-like protein having unknown functions, and showed an amino-acid homology of 85% to mCLSP.

Expression of CLSP mRNA in mouse tissues

We determined the expression of CLSP and CAR mRNA in various female mouse tissues by reverse transcription (RT)-PCR analysis (Figure 2). While the CAR mRNA was ubiquitously expressed in all examined tissues, the CLSP mRNA was found to be expressed at high levels in the brain and ovary, and at low levels in the lung, heart, kidney and uterus. The CLSP expression was also examined by Western blotting, but the expression was not observed in any tissues (data not shown). Therefore, it seems that CLSP is expressed at low levels in mice.

Inhibition of Ad vector infection by mCLSP

Ad binds to CAR via its N-terminal IgV domain.[26] Because the mCLSP protein contains three IgV domains, each of which shows significant homology to the N-terminal IgV domain of CAR, we examined whether mCLSP could affect the infection with Ad vector. SK HEP-1 cells, human hepatic endothelial cell lines, are

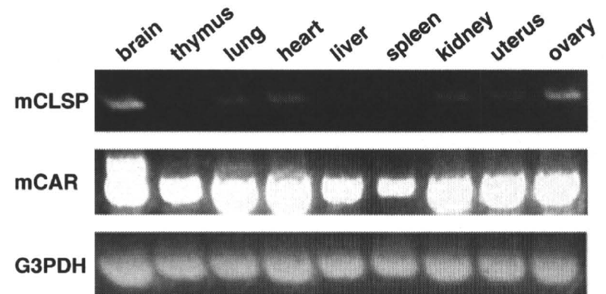

Figure 2 The expression of mCLSP and mCAR mRNA in adult female mouse. Total RNA from each tissue was reverse-transcribed, and PCR was performed as described in the Materials and methods. The expression of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a control. mCAR, mouse coxsackievirus and adenovirus receptor; mCLSP, mouse CAR-like soluble protein.

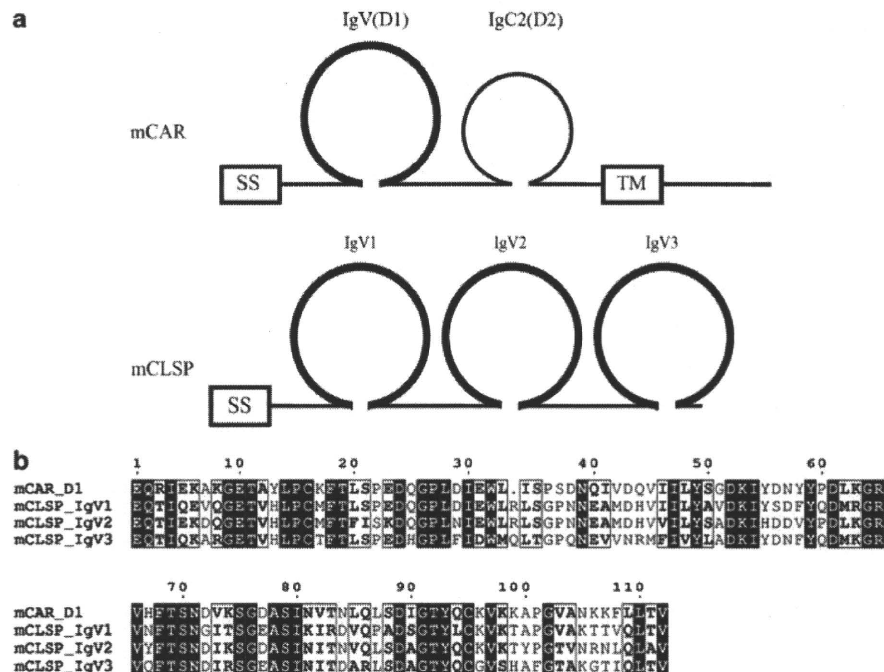

Figure 1 (**a**) The structure of mCAR and mCLSP. Both proteins have the signal sequence (SS) at the N-terminal. mCAR has an extracellular portion containing an Ig V- and C2-type domain (IgV and IgC2, respectively), a transmembrane region (TM), and a cytoplasmic tail. On the other hand, mCLSP has three IgV domains and no transmembrane region. Thick lines indicate the regions showing significant homology. (**b**) Sequence homology of the mCAR IgV (D1) domain and IgV1-3 domains of mCLSP. Ig, immunoglobulin; mCAR, mouse coxsackievirus and adenovirus receptor; mCLSP, mouse CAR-like soluble protein.

known to express high levels of CAR and are often used for experiments related to Ad vector infection.[27,28] The mCLSP-expressing plasmid, pcDNA-mCLSP, was introduced into SK HEP-1 cells, and G418-resistant colonies were picked up, expanded and examined for the expression of mCLSP mRNA. Both wild-type SK HEP-1 cells and a transfected clone, SK HEP-1-mCLSP cells, highly expressed human CAR (hCAR), and only SK HEP-1-mCLSP cells showed high levels of mCLSP expression (Figure 3a). These cells were infected with 100 VP/cell of luciferase-expressing Ad vector (Ad-L). As expected, the infection with Ad-L was significantly inhibited in SK HEP-1-mCLSP cells (Figure 3b). The levels of infection were approximately 40% of those observed in wild-type SK HEP-1 cells, suggesting that mCLSP could inhibit the infection with Ad vectors. Ad belonging to the subgroup B, such as Ad type 35, are known to infect the cells via not CAR but CD46.[29–31] We next examined whether the infection with Ad35 vectors was also inhibited by mCLSP. SK HEP-1 and human A549 cells, both of which could highly express CAR, were transiently transfected with pcDNA-mCLSP, and the luciferase activity in the cells was measured after the infection with Ad-L or Ad35-L. As shown in Figures 3c and d, mCLSP did not inhibit the infection with Ad35-L, although the infection of Ad-L was efficiently inhibited by mCLSP in both types of cells, suggesting that mCLSP might compete with CAR to bind the conventional type 5 Ad vector.

Inhibition of Ad vector infection by addition of the supernatants of mCLSP-expressing cells

mCLSP contains no transmembrane region and is a soluble protein. Therefore, we speculated that the culture supernatants of SK HEP-1-mCLSP cells, when added to the culture medium of wild-type SK HEP-1 cells, would inhibit subsequent Ad vector infection. When the supernatant of SK HEP-1-mCLSP cells was added to the culture medium of wild-type SK HEP-1 cells, Ad-L-mediated luciferase expression was decreased moderately in comparison with that by addition of the supernatant of wild-type SK HEP-1 cells (Figure 4a). However, the levels of inhibition caused by the mCLSP-containing supernatant were fairly low. To determine why the inhibition was limited, flow cytometry analysis was performed to measure the amount of mCLSP on the cell surface. A substantial amount of mCLSP was found on the cell surface (Figure 4b), suggesting that mCLSP is secreted from SK HEP-1-mCLSP cells and then binds to the cell surface by unknown mechanisms.

Recombinant mCLSP protects SK HEP-1 cells from Ad vector infection

We analyzed further whether mCLSP protein has an impact on viral infection when it is externally added to SK HEP-1 cells. For this purpose, mCLSP was bacterially expressed and purified as a NusA (*Escherichia coli* N-utilizing substance A)-mCLSP fusion protein (Figure 5a).

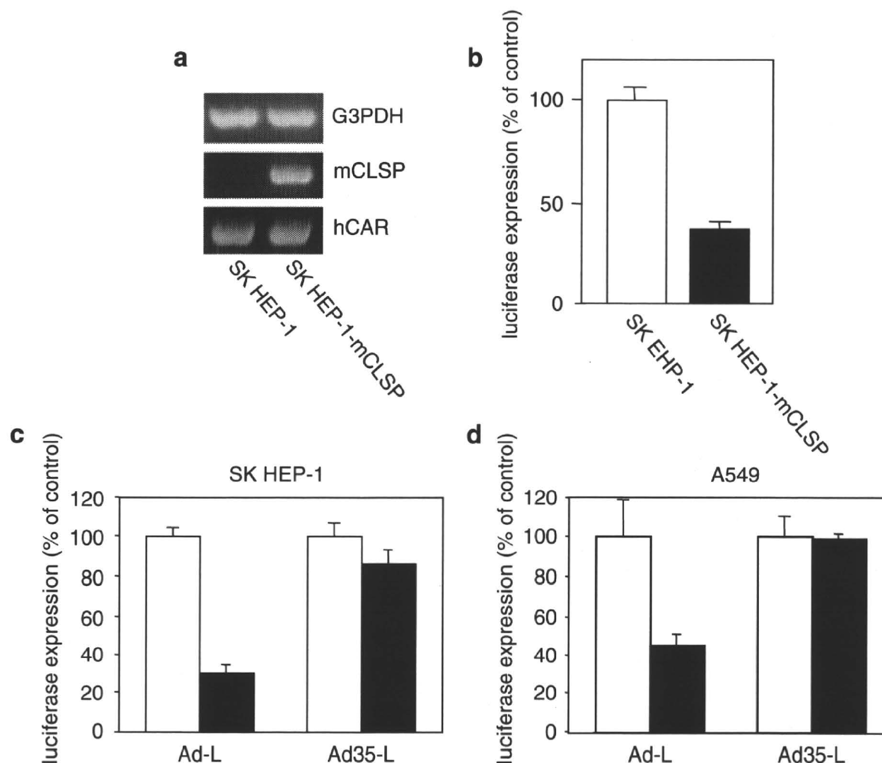

Figure 3 mCLSP inhibits the infection with Ad vector. (**a**) The expression of mCLSP and hCAR mRNA in SK HEP-1 cells and SK HEP-1-mCLSP cells was examined by RT-PCR. (**b**) SK HEP-1 cells and SK HEP-1-mCLSP cells were infected with 100 VP/cell of Ad-L. Twenty-four hours later, the luciferase activities in the cells were measured. The luciferase activity in SK HEP-1 cells infected with Ad-L was taken as 100%. SK HEP-1 (**c**) and human A549 (**d**) cells were transiently transfected with control plasmid pcDNA3 (white bar) or pcDNA-mCLSP (black bar), and the luciferase activity in the cells was measured after the infection of 100 VP/cell of Ad-L or Ad35-L. The luciferase activity in the cells transfected with pcDNA3 is indicated as 100%. The data are expressed as means ± s.d. ($n = 4$). hCAR, human coxsackievirus and adenovirus receptor; mCLSP, mouse CAR-like soluble protein.

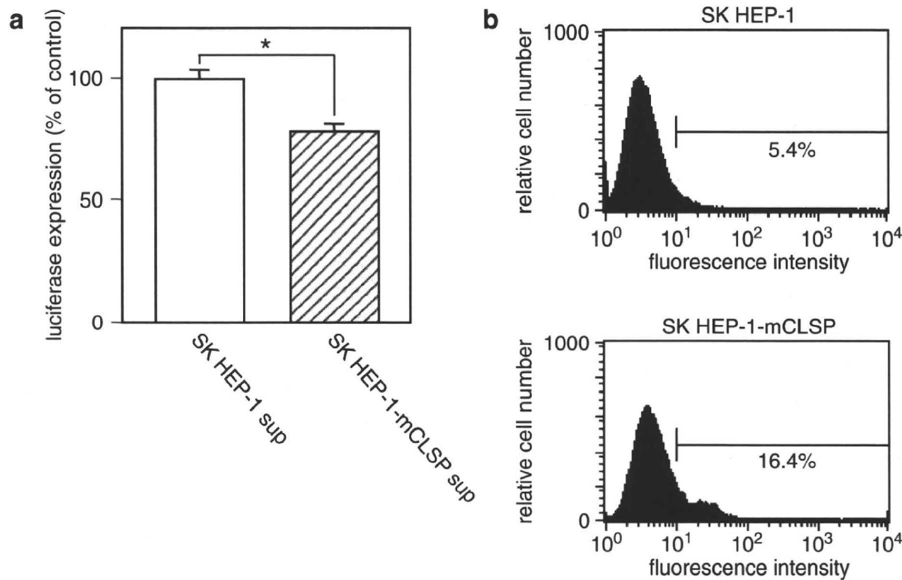

Figure 4 (**a**) The culture supernatant of SK HEP-1-mCLSP cells moderately inhibits the infection with Ad vector. The culture supernatant of wild-type SK HEP-1 cells (white bar) or SK HEP-1-mCLSP cells (hatched bar) was added to the culture medium of wild-type SK HEP-1 cells, which were later infected with 100 VP/cell of Ad-L. Two days later, luciferase activity in the cells was measured. The luciferase activity in the cells supplemented with the supernatant of wild-type SK HEP-1 cells and then infected with Ad-L was taken as 100% (white bar). The data are expressed as the means ± s.d. ($n = 4$). *$P < 0.05$. (**b**) Cell-surface binding of mCLSP in SK-HEP-1 cells. Cell-surface binding of mCLSP in SK HEP-1 and SK HEP-1-mCLSP cells was detected with anti-mCLSP antibody by flowcytometry. mCLSP, mouse CAR-like soluble protein.

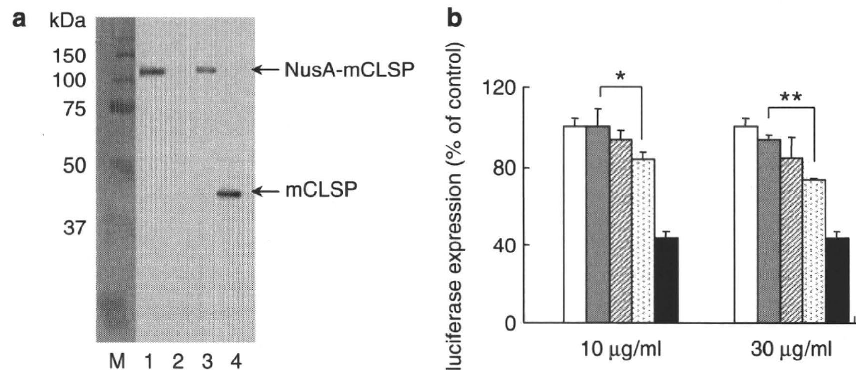

Figure 5 Bacterially synthesized recombinant mCLSP inhibits the infection with Ad vector. (**a**) *E. coli* lysate (lane 1), NusA fraction (lane 2), NusA-mCLSP fusion protein fraction (lane 3) and thrombin-digested NusA-mCLSP (lane 4) were loaded on the polyacrylamide gel and transferred to the membrane. Western blotting was performed with polyclonal anti-mCLSP antibody. M, molecular weight marker. (**b**) Recombinant proteins synthesized in *E. coli*, that is, NusA (gray bar), NusA-mCLSP fusion protein (hatched bar) and thrombin-digested NusA-mCLSP (dotted bar), were added to the medium of wild-type SK HEP-1 cells. The white bar indicates cell medium with no added recombinant proteins. The cells were then infected with 300 VP/cell of Ad-L, and the luciferase activity in the cells was measured 2 days later. The activity in the SK HEP-1-mCLSP cells infected with Ad-L is also shown (black bar). The data are expressed as the means ± s.d. ($n = 5$). *$P < 0.05$; **$P < 0.01$. mCLSP, mouse CAR-like soluble protein.

The major advantage of NusA, in addition to its good solubility characteristics, is its high expressivity.[32] Recombinant mCLSP was able to reduce moderately the infection with Ad vector in a dose-dependent manner when externally added to the culture medium of wild-type SK HEP-1 cells before and during Ad-L incubation (Figure 5b). The fusion protein formed of mCLSP and the adaptor protein NusA also slightly inhibited the infection with Ad-L. Thus, mCLSP indeed inhibits the infection with the Ad type 5.

Physical interaction of recombinant mCLSP with Ad

Because mCLSP contains three Ig domains, which show strong homology to the IgV domain of CAR, we hypothesized that mCLSP could bind to Ad. To test this hypothesis, we measured the binding activity between bacterially synthesized mCLSP and Ad by ELISA (Figure 6). mCLSP, but not the NusA control protein, could bind to the Ad-coated plate. This result clearly shows that mCLSP can directly bind to Ad.

mCLSP promotes the infection with Ad vector in CAR-negative cells

Certain types of soluble receptors are known to promote viral infection in receptor-deficient cells.[33–35] For example, the soluble isoform of the herpes simplex virus (HSV) receptor, nectin1, promotes the infection of

receptor-lacking cells with HSV.[35] We, therefore, wondered whether mCLSP might mediate the Ad vector infection of CAR-negative cells. NIH3T3 cells are known to be CAR-negative and resistant to Ad vector-mediated transduction.[36] pcDNA-mCLSP was introduced into NIH3T3 cells, and G418-resistant colonies were isolated, cultured and examined for the expression of mCLSP mRNA. In comparison with the wild-type NIH3T3 cells, cells of the transfected clone NIH3T3-mCLSP showed a high expression of mCLSP (Figure 7a). After infection with Ad-L, NIH3T3-mCLSP cells showed higher levels of luciferase expression than wild-type NIH3T3 cells (Figure 7b), and the amount of Ad DNA was also increased in NIH3T3-mCLSP cells (Figure 7c). These results suggest that mCLSP can support the infection of receptor-deficient cells with Ad vector.

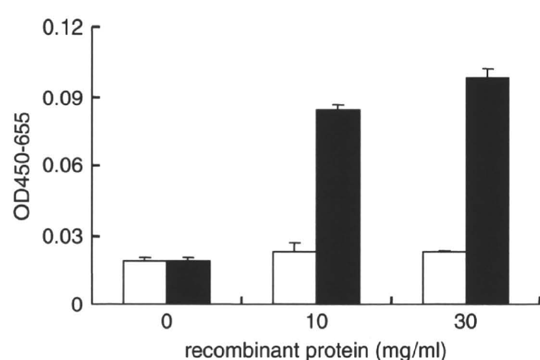

Figure 6 Binding of bacterially synthesized mCLSP to Ad. Bacterially synthesized NusA (white bar) or mCLSP (black bar) was added to Ad-L (1×10^9 VP)-coated plates. The plates were then reacted with rabbit anti-mCLSP antibodies followed by biotin-labeled goat anti-rabbit IgG secondary antibodies. Streptavidin-labeled HRP was then added to detect the binding activity. The data are expressed as the means ± s.d. ($n = 4$).

Evolutionary origin of the mCLSP gene

CAR belongs to the CTX family in the Ig superfamily.[15] We constructed a phylogenetic tree using amino-acid sequences of several vertebrate IgV domains in the CTX family. The results revealed that the closest relative to CLSP is CAR, followed by CLMP,[15] BT-IgSF[16] and ESAM.[17] Figure 8a shows a phylogenetic tree of the IgV domains of CAR and CLMP for the mouse, rat, human, bovine and chicken and three IgV domains of CLSP for the mouse and rat. The tree suggests that the segmental duplication including the first three exons of CAR occurred before the divergence of rodents and other mammals. Following the first duplication, the number of CLSP IgV domains increased before the divergence of the mouse and rat by two segmental duplication events, which harbored exons 2 and 3 of CLSP.

The gene encoding mCLSP was identified on the mouse chromosome 9E3.3 (Figure 8b). The phylogenetic tree indicates that rodent CLSP genes branched off before the Primates–Carnivoa–Rodentia divergence and were lost in the descendants except for the rodent lineage. We thoroughly examined syntenic regions of the human and bovine genome sequences and found that the human and bovine genomes completely lacked CLSP genes (Figure 8). The results indicated that the CLSP gene was convergently lost in mammals except for those of the rodent lineage, where the further segmental duplications of IgV domains occurred.

Discussion

In this study, we identified a novel CTX family protein, CLSP, which is related to CAR in terms of structure and function. The inclusion of CLSP in the CTX family in the Ig superfamily is based on studies of the structure of CLSP and its phylogenetic relationship with other CTX family proteins. The most distinctive feature of CLSP is that this protein lacks a transmembrane domain. The

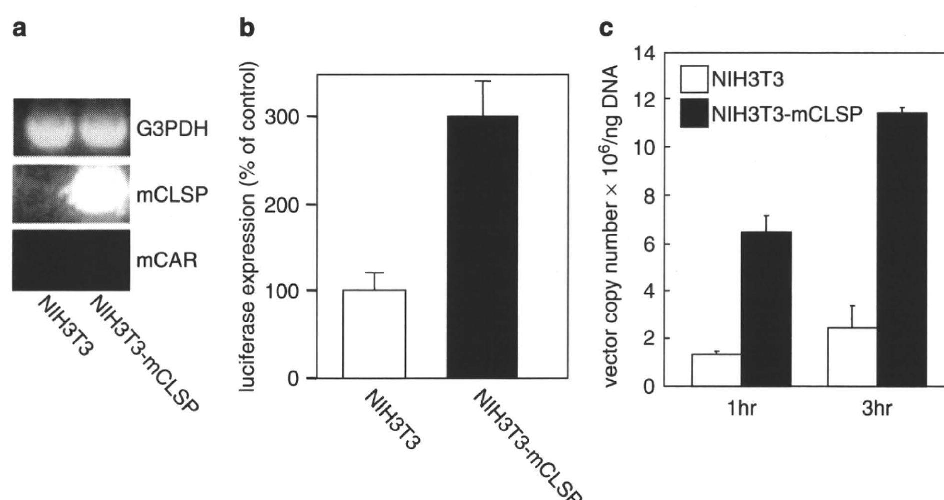

Figure 7 Stably expressed mCLSP promotes the infection with Ad vector in NIH3T3 cells. (**a**) The expression of mCLSP and mCAR mRNA in NIH3T3 cells and NIH3T3-mCLSP cells was examined by RT-PCR. (**b**) NIH3T3 cells and NIH3T3-mCLSP cells were infected with 300 VP/cell of Ad-L. Twenty-four hours later, the luciferase activities in the cells were measured. The luciferase activity in NIH3T3 cells infected with Ad-L is indicated as 100%. The data are expressed as the means ± s.d. ($n = 4$). (**c**) NIH3T3 cells and NIH3T3-mCLSP cells were infected with 3000 VP/cell of Ad-L. The amount of Ad vector DNA in cells was measured by the quantitative TaqMan PCR assay. The data are expressed as the means ± s.d. ($n = 4$). mCAR, mouse coxsackievirus and adenovirus receptor; mCLSP, mouse CAR-like soluble protein.

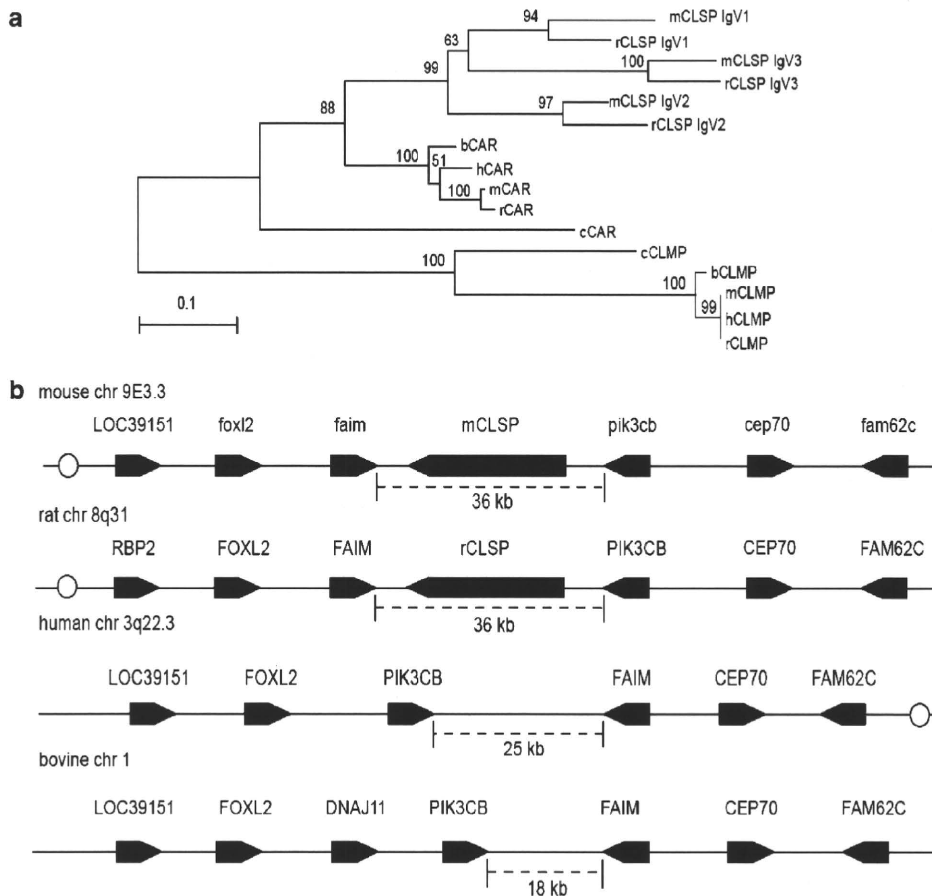

Figure 8 Phylogenetic tree of the CAR group and genomic structures of the mCLSP locus in mammals. (**a**) Amino-acid sequences of the mouse (m), rat (r), human (h), bovine (b) and chicken (c) IgV domains of CAR, CLSP and CLMP were used for the tree construction. The CLMP group was used as an outgroup. Accession numbers are presented in the Materials and methods. Bootstrap percentile values with 1000 replications are shown at each node. (**b**) Horizontal lines, arrow-shaped boxes and open circles indicate the intergenic regions, genic regions and centromeres of the mouse, rat, human and bovine genomes, respectively. The direction of the arrows corresponds to the direction of transcription. There were no gene-like sequences in the intergenic region of FAIM and PIK3CB of the human and bovine genomes. Note that the length of genic and intergenic regions in the figure is not proportional to the actual genomic distances. CAR, coxsackievirus and adenovirus receptor; CLMP, CAR-like membrane protein; CLSP, CAR-like soluble protein; Ig, immunoglobulin; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; rCLSP, rat CLSP (CAR-like soluble protein).

membrane-type of CLSP was not found either by a search of the EST database or by a 3′-RACE using the reverse-transcribed cDNA from the brain, kidney and ovary (data not shown). Therefore, we reached the conclusion that CLSP exists primarily as a soluble protein.

While CAR mRNA was ubiquitously expressed in many tissues, CLSP mRNA was expressed only in limited tissues in mice. CLSP mRNA was highly expressed in the brain and ovary, although CLSP protein expression was not detected in any organs. CLSP mRNA expression might be involved in several important functions, such as resistance to viral infection. Some members of the Ig superfamily have alternative splicing variants. For example, CAR has three variants that lack a transmembrane domain and are produced by alternative splicing events between exons IV and VII, exons III and VII, and exons II and VII.[21] Interestingly, these soluble forms of CAR have been found to inhibit CVB3 and Ad infection.[21,22] Because the Ad binds to the N-terminal IgV domain of CAR[26] and CLSP shows homology to its domain, we examined the effects of CLSP on Ad vector infection. As a result of the infection of CLSP-expressing SK HEP-1 cells with Ad vector, CLSP showed efficient inhibition of the Ad vector-mediated reporter gene expression. The culture supernatant of CLSP-expressing cells also inhibited Ad vector-mediated gene expression. ELISA experiments showed that CLSP could directly associate with Ad, suggesting that CLSP may compete with CAR for Ad fiber. As CLSP contains three IgV domains, it would be necessary to determine which domains bind to Ad. Bacterially synthesized recombinant CLSP could also inhibit the infection with Ad vectors, suggesting that CLSP competes with the IgV domain of CAR for Ad. However, the recombinant CLSP, which was collected from the supernatant of SK HEP-1 cells stably expressing mCLSP (SK HEP-1-mCLSP cells), showed only slight inhibition of Ad vector infection in comparison with the supernatant of wild-type SK HEP-1 cells. Flow cytometry analysis in SK HEP-1-mCLSP cells revealed that mCLSP was found on the cell surface. This is why the level of inhibition achieved by the addition of the mCLSP-containing supernatant was fairly low. Further investigations are needed to clarify the binding mechanism.

HSV is known to infect cells via nectin1, which is a member of the Ig superfamily.[37] It has been reported that

the soluble isoform of nectin1 positively and negatively modulates susceptibility to HSV infection.[35] The soluble nectin1 reduced HSV infectivity by competing with membrane-bound nectin1. On the other hand, forced expression of the soluble form of nectin1 cDNA in cells lacking membrane-bound nectin1 enabled HSV to enter the cells.[35] In this study, we showed that mCLSP inhibits the infectivity of Ad vectors to CAR-positive cells, while mCLSP promotes the entry of Ad vectors into CAR-negative cells. Although the exact mechanisms responsible for this difference remain unknown, this finding raises the possibility that mCLSP localizes to the plasma membrane to enable the entry of Ad vectors into CAR-negative cells. After Ad vector binds to CLSP in the plasma membrane, the Ad–CLSP complex might enter the cells in some fashion, such as via endocytosis, with low efficiency in the case of CAR-negative cells. On the other hand, in the case of CAR-positive cells, the formation of Ad–CAR complex would be inhibited by CLSP. Since the efficiency of entry of the Ad–CLSP complex into cells would be much lower than that of the Ad–CAR complex, CLSP negatively modulates susceptibility to Ad infection in CAR-positive cells, and positively modulates it in CAR-negative cells. Thus, it would be of interest to investigate how CLSP interacts with CAR.

Chromosome analysis suggests that the CLSP gene is rodent-specific, and that human and bovine lack this gene. The phylogenetic tree, however, indicates that rodent CLSP genes branched off before the radiation of mammals and were lost in all descendants except for those of the rodent lineage. Interestingly, the segmental duplications that tripled the IgV domain occurred after the first gene duplication, which duplication may increase the diversity of affinity to viruses. In future studies, the physiological function of CLSP will be further clarified by constructing and analyzing CLSP-deficient mice. CLSP, which is a soluble protein and the closest relative to CAR, may have antiviral potential in rodent animals.

Materials and methods

Molecular cloning of mCLSP

BLAST analysis of the mouse EST cDNA library with the sequence of the full-length mCAR cDNA led to identification of a cDNA fragment that is homologous to but different from mCAR. The EST accession number is BB617533. Total RNA was purified from 8 weeks of age of mouse ovary, and 5′- and 3′-RACE (rapid amplification of cDNA ends) was performed using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) to obtain the full-lengh cDNA of clone BB617533. The full-length cDNA was sequenced and designated as CLSP (GenBank accession number AB262662).

RT-PCR

Each tissue from adult female mouse was excised. Total RNA was extracted with ISOGEN (Wako, Osaka, Japan) and was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Synthesized cDNA was amplified by PCR using Ex Taq DNA Polymerase (Takara, Shiga, Japan). The sequences of the specific primers were as follows: G3PDH(F), 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH(R), 5′-TCCACCACCCTGTTGCTGTA-3′; mCAR(F), 5′-AATTCCTGCTGACCGTTCTT-3′; mCAR(R), 5′-TTTCTG CCAGCCATGGCGTA-3′; hCAR(F), 5′-ACCATGGCGCTC CTGCTGTGCT-3′; hCAR(R), 5′-AGGCTCTATACTATAGA CCCAT-3′; mCLSP(F), 5′-TGGACAGCCACCATGGTGTTT CTGCTGG-3′; mCLSP(R), 5′-GGGCAGATGGACAGTTTC CCCTTGGTCT-3′. The cycle conditions were 30 s at 94°C, followed by 30 cycles of 5 s at 94°C, 10 s at 60°C, and 1 min at 72°C and a final extension of 7 min at 72°C.

Transfection

The coding sequence of mCLSP was amplified by PCR with Ex Taq DNA Polymerase and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The cDNA was sequenced and cloned into the *Eco*RI site of the mammalian expression vector, pcDNA3 (Invitrogen), generating pcDNA-mCLSP. SK HEP-1 cells[27] and NIH3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, and were transfected with pcDNA-mCLSP using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For stable transfection, SK HEP-1 cells or NIH3T3 cells transfected with pcDNA-mCLSP were cultured for 10 days in DMEM containing 100 μg/ml G418 (Invitrogen). G418-resistant colonies were picked up, expanded, and used for further analysis.

Recombinant CLSP expression and purification

The pET43b-mCLSP plasmid was generated by inserting a PCR fragment, encoding mCLSP, into the *Sac*I–*Xho*I sites of pET43.1b(+) (Novagen, San Diego, CA, USA). The vector and insert sequence at the junctions was confirmed by sequencing. The pET43b-mCLSP-transformed Rosetta-gami B (DE3) cells (Novagen) were cultured with 0.5 mM IPTG at 25°C for 18 h. The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 0.1% Triton X-100, pH 7.9) mixed with 0.1 mg/ml of lysozyme (Wako) and incubated at 30°C for 15 min. The lysate was sonicated and centrifuged at 10 000 *g* for at 4°C for 20 min, and filtered with 0.45 μm filter. We chose NusA as a highly favorable solubility partner of mCLSP. The fusion protein NusA-mCLSP was purified with His-Bind resin (Novagen) by following the manufacturer's instructions, with slight modifications. Briefly, the lysate was loaded onto a Ni-NTA column before the purification of the fusion protein to remove free-NusA protein. The flow-through fraction containing fusion protein was collected and employed for purification. After the solvent was changed to phosphate-buffered saline (PBS) by dialysis, fusion protein NusA-mCLSP was digested by biotinylated-thrombin at 20°C for 2 h, and then biotinylated–thrombin was removed using streptavidin–agarose (Novagen). The purified protein was dialyzed in PBS, and the protein content was determined with the protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

Western blotting

A rabbit polyclonal antibody for mCLSP was prepared using peptide (FYQDMKGRVQFTSNDIR). *E. coli* lysate and each recombinant protein (2 μg) were mixed with the same amount of 2 $\times$ sodium dodecyl sulfate (SDS)

loading buffer. The samples were incubated at 95°C for 5 min, and they were loaded onto the 12.5% SDS-polyacrylamide gels. Proteins were separated at 20 mA and then blotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) in Tris-glycine transfer buffer at 100 V for 2 h. The membrane was blocked with 3% skim milk in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween20) at room temperature for 2 h. Polyclonal antibody against mCLSP (diluted with blocking solution to 1:2000) was added and incubated at 3 h, and the membrane was then incubated with anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h. The band was visualized by Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan). The signals were read using a LAS-3000 Image Analyzer (FUJIFILM, Tokyo, Japan).

Effects of CLSP on Ad vector infection

The luciferase-expressing conventional Ad vector (Ad-L), based on Ad type 5, and type 35 Ad vector (Ad35-L) had been generated previously.[27,38] Ad-L is identical to Ad-L2.[27] Both vectors contain the cytomegalovirus promoter-driven luciferase expression cassette in the E1 deletion region. SK HEP-1-mCLSP cells, which are SK HEP-1 cells stably expressing mCLSP, were infected with 100 virus particles (VP)/cell of Ad-L or Ad35-L at 37°C for 1.5 h. Cells were washed with PBS and further cultured. After 24 h, luciferase activity in SK HEP-1 cells was measured using a luciferase assay system Pica Gene LT2.0 (Toyo Inki, Tokyo, Japan).[27] NIH3T3-mCLSP cells, which are NIH3T3 cells stably expressing mCLSP, were infected with 300 VP/cell of Ad-L, and the luciferase activity was measured as described above. The Ad genome DNA in the cells was quantified with a TaqMan fluorogenic detection system (ABI PRISM 7700 sequence detector; Applied Biosystems, Foster City, CA, USA). Sample DNA was isolated with an automatic nucleic acid isolation system (NA-2000; Kurabo Industries, Osaka, Japan). Primers for amplification were located in the E4 region, with the sequences CACCACCTCCCGGTACC ATA (sense) and CCGCACCTGGTTTTGCTT (antisense). The fl detection probe had the sequence AACCTGCC CGCCGGCTATACACTG. Samples were amplified for 40 cycles in the ABI PRISM 7700 sequence detector with continuous fluorescence monitoring. Data were processed with ABI PRISM 7000 SD software (Applied Biosystems). For the inhibition assay with the recombinant mCLSP protein, the culture supernatant from SK HEP-1 cells, SK HEP-1-mCLSP cells or the bacterially synthesized mCLSP protein was added to the medium of wild-type SK HEP-1 cells. The cells were then infected with 100 VP/cell of Ad-L or Ad35-L, and the luciferase activity was measured as described above.

Flow cytometry analysis

SK HEP-1 and SK HEP-1-mCLSP cells were reacted with rabbit anti-mCLSP antibody and stained with fluorescein isothiocyanate-labeled goat anti-rabbit IgG. The cells were analyzed by using FACSCalibur and CellQuest software (BD Biosciences, Tokyo, Japan).

ELISA

Ad-L (1×10^9 VP) was coated on a 96-well plate by incubation at 4°C for 24 h. Bacterially synthesized NusA or thrombin-digested NusA-mCLSP was added to the plate at the indicated concentrations. The plate was incubated at room temperature for 2 h, washed with PBS and reacted with anti-mCLSP antibodies followed by biotin-conjugated anti-rabbit IgG. Binding was detected with streptavidin-conjugated HRP.

Computational analysis

The amino-acid sequences of CAR group IgV domains were aligned with Clustal W.[39] The Neighbor-Joining tree was constructed using the protein-Poisson distances,[40] and bootstrap percentile values were shown at each node (1000 replications). The amino-acid sequences were obtained from Genbank (mCAR, NP_001020363; rat CAR, NP_446022; hCAR, NP_001329; bovine CAR, NP_776723; chicken CAR, XP_416681; mouse CLMP, NP_598494; rat CLMP, NP_775177; human CLMP, NP_079045; bovine CLMP, XP_617487; chicken CLMP, XP_417886). The alignment and tree construction were performed using the MEGA 3.0 program package.[41] For genomic assignment of the CLSP locus, the mouse, rat, human and bovine genome sequences were examined using the UCSC genome browser (http://genome.ucsc.edu/).

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