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Development of Caged Diacylglycerol-Lactone Derivatives and Their Applications

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The central role of protein kinase C (PKC) in cellular signal transductions has established it as an important therapeutic target for cancer and other diseases. In order to elucidate PKC signaling mechanisms, it is useful to develop functional molecules which can control spatial-temporal activation of PKC. We developed caged diacylglycerol (DAG)-lactones which can control PKC activation by photoirradiation based on the application of a caged technology to the highly potent activating ligands of PKC, DAG-lactone.

Keywords: caging technology, diacylglycerol (DAG)-lactone, protein kinase C

Introduction

Protein kinase C (PKC), which is a serine/threonine-specific protein kinase, plays a pivotal role in cell signaling by functioning as a central signal transducing element [1]. These signals are generated by a broad range of ligands which produce the lipid second messenger, diacylglycerol (DAG). Marquez, V. E. *et al.* have developed DAG-lactone derivatives with a significant increase in binding affinity for PKC isozymes as possible templates for the construction of conformationally constrained analogues of DAG [2] (Fig. 1). Since the development of biomolecules, which are enabled to control PKC activation, might be useful, we attempted to apply the caging technique to DAG-lactones (Fig. 2). Caged compounds are designed so that their original biological activity will be temporarily disappeared and then reactivated by external triggers such as ultraviolet light [3]. In this study, caged DAG-lactones were synthesized for characterization of their photochemical properties.

Results and Discussion

Caged DAG-lactones were successfully synthesized. The hydroxyl group of DAG-lactones which is a critical pharmacophore moiety was protected by a 6-bromo-7-methoxycoumarin-4-ylmethoxycarbonyl (Bmcmoc) group. The Bmcmoc group is a suitable phototrigger for alchols with high photochemical efficiency.

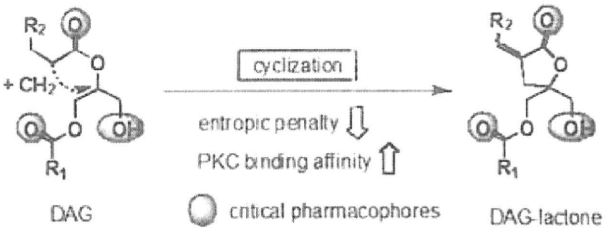

Fig. 1. Development of DAG-lactones by DAG cyclizaton. $R_1$and R_2 indicate alkyl groups.

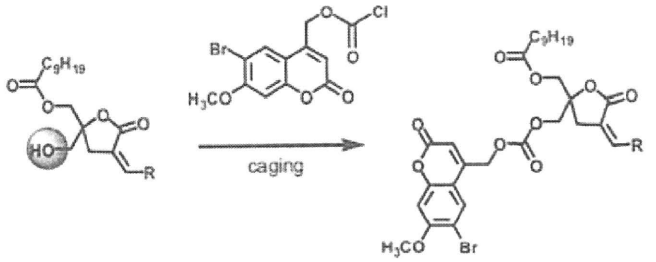

*Fig. 2. Caging technology was applied to DAG-lactones to regulate PKC activation by photoirradiation. R = alkyl groups; compound **1**, $(i\text{-}Pr)_2CHCH_2$; compound **2**, $CH(CH_3)_2$.*

Photolysis reaction of caged DAG-lactones by UV irradiation (350 nm) was monitored by HPLC analysis to find generation of DAG–lactones. The results revealed that the t_{90}s of compounds **1** and **2** are ca. 6 min and 5 min, respectively. In the kinase activity assays utilizing Pep Tag® (Promega), caged DAG–lactones didn't significantly activate PKC, which would correspond to loss of the binding activity of caged DAG–lactons to PKC. To further assess the properties of caged DAG–lactones, confocal laser microscopy analyses were utilized. Activation of PKC by exogenous ligands can be analyzed by its translocation in cells. The GFP–tagged PKC was expressed transiently in a CHO cell line. The caged DAG-lactones showed no change in location of PKC in cells. The results are compatible to the kinase activity of these compounds. On the contrary, the addition of DAG–lactones without a caging group showed clear translocation of PKC. These results indicate that photolysis of caged DAG–lactones could be a trigger of activation of PKC translocation.

It has been showed that caging technology is a powerful tool to investigate cellular functions. Our results indicate this technology could be applied to exogenous ligands of protein kinase C. The present results encourage us to further address the activity control of DAG–lactones with photo-removable protecting groups as useful tools for elucidation of activation mechanism of PKC in mammalian cells.

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Site-Selective Cytosine Methylation By A Split DNA Methylase

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Cytosine methylation plays pivotal roles in gene expression. Methylation pattern in genome is heritable, then the effect of methylation is largely influence across generation. Truly specific methylation has been a challenging problem because fusion methylases with zinc finger domain still methylate the native sites as background. To avoid this, split methylase domains were constructed. These domains were designed to assemble only on the zinc finger target site with high specificity. The split methylase showed it works as specific methylase to the target sites.

Keywords: DNA binding, methylation, zinc finger protein

Introduction

Covalent modification of DNA, such as cytosine methylation, can induce heritable gene silencing. If epigenetic modifications can be specifically targeted, new approaches to transcriptional therapy should result. Artificial zinc finger proteins have been studied as DNA binders with sequence specificity. To address this challenge, we sought to design methyltransferases that would act only at a desired site by adapting the sequence-enabled assembly strategy [1]. Methyltransferase HhaI (M.HhaI) was split into two domains [2], then the N-, and C-terminal domains were fused to HS2 and HS1 zinc finger domains, respectively.

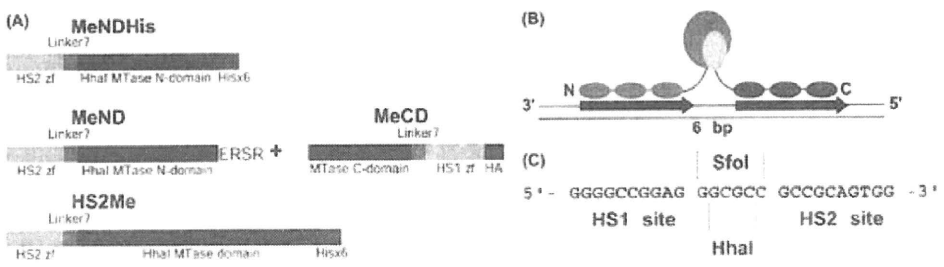

Fig.1. (A) Description of domains constructed in this study (B) An assembly model of split methylase. (C) Sequence of methylation site flanked by zinc finger target sites.

Results and Discussion

Protein expression of split methylase domains (N-terminal domain; MeND, and C-terminal domain; MeCD) were detected by western blotting. To evaluate methylation activity of split methylase, proteins were expressed for overnight. Target sequences were inserted to the plasmid coding split methylase domains before transformation. Detection of site-specific methylation was performed by using HhaI restriction enzyme digestion. HhaI restriction enzyme has the same target site with HhaI methyltransferase (5'-GCGC-3') and is sensitive to methylation at CpG site. Thus, methylation at the target sites is detected as uncleaved band after digestion. As the controls, MeND alone and fusion of full length HhaI methylase with a zinc finger domain (HS2Me) were prepared. After HhaI restriction enzyme reaction, plasmids were analysed by agarose gel electrophoresis. The results showed split methylase perform site-specific DNA methylation. The MeND alone didn't show any methylation on the plasmid. Additionally, HS2Me showed very high non-specific methylations. The result of HS2Me indicates that methylation activity of HhaI methyltransferase is high even with zinc finger domains as previously shown. This result indicates that the split domain methodology is effective to avoid the high-background methylation.

To confirm the site-specific methylation of split methylase, bisulfite sequencing analyses were performed. At the target sequence with zinc figner binding sites, the specific methylation was detected accordingly to the result of HhaI restriction enzyme digestion. To assess the non-specific methylation, three HhaI methylase target sites were selected for analyses. For split methylase, no methylation was observed on the off-target site. For HS2Me, methylation at all native GCGC sites were shown including one with zinc finger binding sites. Our split DNA methylase performed site-specific CpG methylation in living cells without any background methylation when appropriately assembled at the target site. This is the first successful application of the sequence-enable enzyme reassembly approach in vivo. This split domain re-assembly strategy will allow creation of programmable zinc finger methylases that act at any specific CpG site in the mammalian genome. Programmable methylases should orchestrate heritable gene silencing and should find application in DNA tagging approaches and in nanotechnology.

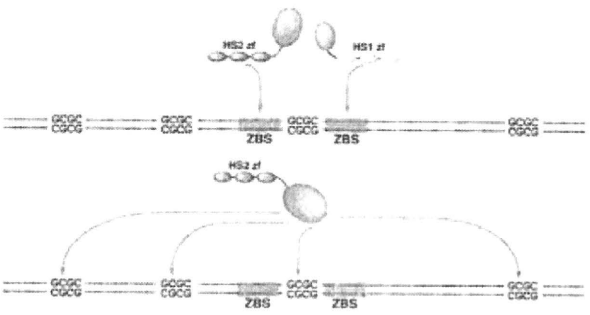

Fig. 2. Site-selective DNA methylation models. Split methylase are assembled on the target sites and Full length methylase fusion recognize native sites.

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O-24

標的遺伝子を特異的にメチル化する分割型酵素の開発

Development of Site-Specific Split DNA Methylase

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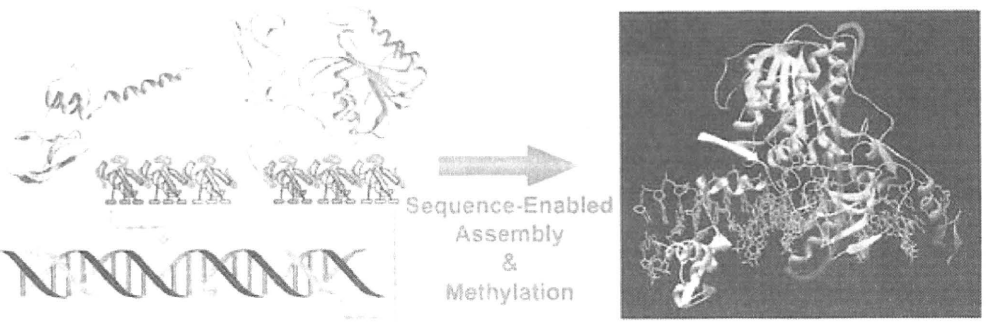
シトシン塩基のメチル化はヒストンの脱アセチル化を促進し、それによってクロマチン構造変化が誘起され、遺伝子発現の抑制を行う。DNA メチル化パターンは細胞の世代間をまたいで再生されるため、DNA のメチル化によって永久的な遺伝子発現の抑制が可能となる。そのため、特定の標的 DNA 配列上でのメチル化制御は、その標的遺伝子を抑制するために非常に有効な手段となり得る。これまで、DNA メチル化酵素と亜鉛フィンガーモチーフを融合することで、配列特異的な DNA メチル化を行う試みがなされてきた。しかし、DNA メチル化酵素に由来する DNA 認識能によって、非特異的なメチル化が高い頻度で観察されることが問題であった。

この問題を解決する試みとして、本研究では、DNA メチル化酵素ドメインを二分割し、それぞれを亜鉛フィンガードメインとの融合体とする酵素をデザインした。このデザイン酵素により、亜鉛フィンガードメインが DNA 結合し、近接効果によって標的遺伝子配列上で二分割型にされている酵素ドメインが再会合することでメチル化機能が発揮されると考えられた。これらの分割型酵素ドメインを大腸菌内で発現させ、そのメチル化機能を HhaI 制限酵素切断、Bisulfite シークエンス法などによって解析した。その結果、標的配列における CpG 配列のみがメチル化されていることが明らかになった。

本研究結果は、標的遺伝子配列に特異的な DNA メチル化反応、及び亜鉛フィンガーモチーフによる標的 DNA 上での酵素ドメインの再会合が in vivo で行われた初の例であり、今後の哺乳類細胞内でのメチル化反応への応用が期待される。



[Figure 1] 分割型メチル化酵素の標的遺伝子配列上での再会合モデル

[Reference]

Nomura, W. & Barbas, C. F., III (2007) *J. Am. Chem. Soc.* **129**, 8676-7.

P-07

蛍光標識したPKC C1B ドメインの化学合成とその応用

Chemical Synthesis and Application of Fluorescence-Labeled PKC C1B Domain Analogs

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Protein kinase C(PKC)は、細胞内シグナル伝達に関係するリン脂質依存性のセリン・スレオニン特異的リン酸化酵素であり、細胞の分化、増殖またはアポトーシスといった細胞機能の調節に関与している。またPKCは、腫瘍プロモーターであるphorbol esterと結合することやアミロイド沈着の主成分であるAβ生成に関与することから、がんやアルツハイマー病といった疾患に関わっているため、重要な創薬ターゲットの一つである。今回は、PKCのリガンドとなりうる合成化合物のスクリーニングツールや生体内のDAGセンサーへ応用するために、基礎研究としてPKCの調節領域であるC1Bドメインを化学合成し、蛍光色素の導入を試みた。

C1Bドメインを1) Fmoc solid-phase peptide synthesis (Fmoc-SPPS), 2) native chemical ligation (NCL)により合成した。まずFmoc-SPPSにより、２つのペプチド断片をそれぞれ合成し、NCLによりそれらを縮合した。そして、大腸菌を用いて発現したrecombinant C1Bとの構造や活性の比較およびPDBuとの結合活性評価を行って、合成品の機能を確認した。

蛍光基については蛍光色素をもったアミノ酸誘導体を合成し、Fmoc-SPPSによりリガンド結合部位近傍に導入した。これらの結果、蛍光基導入C1Bはフォールディングに伴い蛍光強度と波長に変化がみられたので、蛍光基の置かれる環境の違いが蛍光で検出できることがわかった。また、亜鉛滴定後の蛍光基導入C1BにPDBuを滴加することにより蛍光強度に変化がみられた。これにより、スクリーニングツールやバイオセンサーに応用できる可能性があると考えられる。

P-08

HIV 侵入の動的超分子機構をターゲットとした CD4 mimic 小分子の創製

CD4 Mimic Small Molecules Targeted for Dynamic Supramolecular Mechanism of HIV-1 Entry

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現在、AIDS および HIV 感染症の治療法としては一般に逆転写酵素阻害剤とプロテアーゼ阻害剤を 2,3 剤用いる highly active anti-retroviral therapy (HAART)が用いられており、多大な成功を収めている。しかし、耐性ウイルスが生じる、根治できない、副作用がある等の問題点があり、別の作用点の薬も 2 種ほど登場している。我々は以前から HIV 侵入段階に着目しており、コレセプターCXCR4 のアンタゴニストを中心に種々の阻害剤を創製してきた。今回は、低分子型 CD4 mimic である NBD-556 を取りあげ、その構造活性相関研究および HIV 侵入の動的超分子機構への影響や CXCR4 アンタゴニスト、中和抗体等との併用の効果を調べた。

NBD-556 の構造活性相関に関しては分子内に含まれる芳香環(CD4 の Phe43 に対応すると考えられる)部分が gp120 の cavity に相互作用し、さらに芳香環の p-位付近に比較的大きな空間が存在すると推定されるため、芳香環の p-位に種々の置換基を導入した誘導体を合成し、各種 HIV 株に対する抗ウイルス活性を評価した。高活性の誘導体が数個見つかり、また構造活性相関の情報が得られた。さらに、CD4 mimic の存在下での、CXCR4 アンタゴニスト(T140)や抗 V3 抗体、CD4 induced 抗体の gp120 への反応性を調べた。CD4 mimic の存在下では、非存在下よりも gp120 への反応性が上昇し、sCD4 の存在下と同様の効果が見られた。これにより NBD 誘導体が gp120 のコンフォメーション変化を誘起することが示唆された。また、CD4 mimic と T140 や抗 V3 抗体あるいは CD4 induced 抗体の併用は抗ウイルス活性を増強した。

これらの低分子型 CD4 mimic 誘導体は gp120 の構造変化を誘導し、中和抗体のエピトープを表面に露出させると考えられる。また、これらは HIV 侵入のダイナミックな超分子機構に作用する有用なツールになると示唆され、多剤併用療法を視野にいれた抗エイズ薬の創製研究に有用であると思われる。

P-24

新規ファルマコフォアを有するケモカインレセプターCXCR4 アンタゴニストの構造活性相関研究

Structure-Activity Relationship Study of CXCR4 Antagonists bearing a Novel Pharmacophore

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CXCR4 はケモカイン受容体の一つであり、7回膜貫通G蛋白質共役型受容体ファミリーに属する。CXCR4 は胎生期において血管新生や分化などの発生に重要な機能を果たす反面、成人においてはエイズ、がん転移、白血病、関節リウマチなど種々の難治性疾患の発症に関わることが知られている。このことから、CXCR4 アンタゴニストはこれらの疾患に対する有効な治療薬となる可能性を秘めていると言える。

著者の所属する研究室では、14 残基のペプチド性 CXCR4 アンタゴニスト T140 の創製に成功している。また、T140 の 4 つのファルマコフォア(Arg^{2}, 2-naphthylalanine(Nal)^{3}, Tyr^{5}, Arg^{14})を基に CXCR4 アンタゴニスト活性を持つ環状ペンタペプチド FC131 を創出している。

今回、この FC131 をリード化合物としてフォーカスドライブラリーを設計し、より高い CXCR4 結合活性を持ったリード化合物の創出を目指した。Nal^{1}, Arg^{6} は CXCR4 の結合に重要な部位であることがわかっているためこれらの部位を固定し、D-Tyr^{5}, Arg^{4} 部位に着目したライブラリーを設計した。これまでの研究から、D-Tyr^{5}, Arg^{4} 部位は主に疎水性相互作用と静電的相互作用により CXCR4 との結合に寄与していることが推測されるため、疎水性アミノ酸と塩基性アミノ酸を適切に空間配置することにより活性の向上が期待できると考え、疎水性アミノ酸として二環性芳香族アミノ酸 1-naphthylalanine, Nal, Trp、塩基性アミノ酸として Arg を選択し、それぞれの D/L 体を用いて、計 24 種の化合物を合成した。

それぞれの化合物について CXCR4 結合活性試験を行ったところ、高い CXCR4 結合活性を有する 2 種の新規リード化合物を見出した。これら 2 つの化合物は FC131 と異なる空間配置を有しており、それらの構造活性相関のデータから新たな pharmacophore を同定することができた。本研究の結果は今後の CXCR4 アンタゴニスト創製研究において有用な知見を与えるものであると考えられる。

1P-83

ケージドジアシルグリセロール-ラクトンの合成と機能評価

(東医歯大・生材研)

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Synthesis and Characterization of Caged Diacylglycerol-Lactone Derivaives. (*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University*) SERIZAWA, Yuki; NOMURA, Wataru; OHASHI, Nami; OKUDA, Yoshiaki; MATSUMOTO, Hironori; TSUTSUMI, Hiroshi; TAMAMURA, Hirokazu

【緒言】 細胞内におけるシグナル伝達は、タンパク質の翻訳後修飾やセカンドメッセンジャーによる標的タンパク質の活性化／不活性化および局在化によって制御されている。しかしながら、これらの制御機構は非常に複雑であり、また非常に速い過程を含むため、各タンパク質／セカンドメッセンジャーの機能を個別にかつリアルタイムに解析し、さらにコントロールしようとすることは困難を伴う。本研究では、当研究室で開発したセリン／スレオニン特異的リン酸化酵素プロテインキナーゼ Cδ（PKCδ）に特異的に結合するジアシルグリセロール（DAG）－ラクトン誘導体を基に PKC の活性化を光照射によってコントロールできる機能性物質の合成と機能評価を行った。

【方法】 DAG－ラクトン誘導体の PKC 活性発現に重要な OH 基を光分解性保護基によりケージングした化合物を合成し、特定波長の光照射により OH 基が脱保護され、DAG－ラクトンが生成するかどうかを HPLC によって確認した。保護基の有無によって DAG－ラクトン誘導体の添加による PKCδの細胞内局在にどのような影響を与えるかを検討した。この実験では、保護基の有無で DAG－ラクトン誘導体を個別に合成し、PKCδ-GFP 融合体を強制発現させた細胞培養液に化合物を添加し、細胞内局在の変化を共焦点顕微鏡によって観察した。また、脱保護された DAG－ラクトン誘導体が PKCδに対する結合活性および PKCδ活性化能を保持しているかどうかをバインディングアッセイおよび Pep Tag® Kinase Assay によって検討した。

【結果】 光分解性保護基によりケージングした DAG－ラクトンは紫外光照射によってアンケージングされることが HPLC による反応追跡で明らかになった。Kinase Assay の結果、脱保護した DAG－ラクトン誘導体は PKC を活性化することが明らかになった。本研究によって、光分解性保護基を用いたリガンドの活性制御はリン酸化酵素の活性化機構の解明に有用な方法であることが示された。

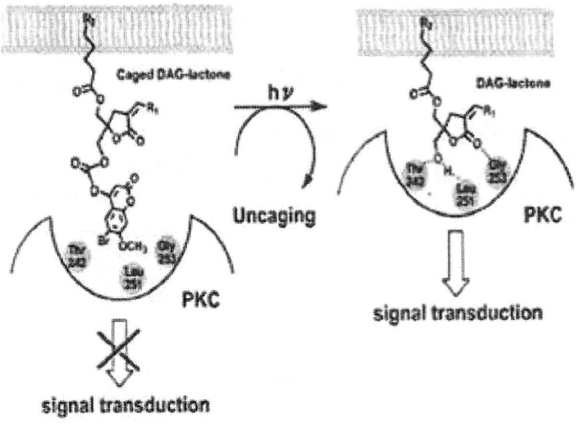

Figure. Schematic Drawing of photochemical release of DAG-lactone and activation of Protein Kinase C

3P-22

分子進化法による配列特異的 DNA 組換え酵素の機能最適化

（東医歯大・生材研）
○野村 渉・加藤 舞・増田 朱美・堤 浩・玉村 啓和

Development of A Target-Specific DNA Recombinase By SLiPE (Substrate-Linked Protein Evolution). (*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University*) NOMURA, Wataru; KATO, Mai; MASUDA, Akemi; TSUTSUMI, Hiroshi; TAMAMURA, Hirokazu

【緒言】 DNA 二重鎖切断、DNA 組換え、メチル化などを行う DNA 修飾酵素の働きを人為的に制御することで、遺伝子機能の活性化、抑制などを自在に操ることが可能になる。DNA 結合ドメインと酵素活性ドメインが独立した構造を有するタンパク質においては、DNA 結合ドメインを置換することによる活性への影響が少なく、比較的容易なアプローチが可能である。また、分子進化法を用いることで、酵素活性を最適化することが可能である。亜鉛フィンガータンパク質は DNA 結合特異性を制御することが可能であり、これとの融合体である酵素は部位特異的に働く新規な酵素となる。本研究では DNA 組換え酵素の機能改変に取り組み、分子進化法によって特定の標的配列に対して高い活性を持つ酵素を創製した。

【方法】 亜鉛フィンガードメインを原核細胞由来の DNA 組換え酵素である Gin または Tn3 の酵素活性ドメインに融合させた DNA 組換えタンパク質を作製した。ドメイン間をつなぐリンカー配列の最適な配列を決定するために様々な長さのリンカー配列にランダム化した配列を導入し、SLiPE 法によって分子進化を行った。SLiPE 法の簡易な説明は図に示す。この方法では、組換え酵素の酵素活性とそれをコードする遺伝子配列がリンクしているため、酵素活性を PCR 産物で確認し、それと同時に活性をもつ酵素ドメインを回収することが可能である。また、特定のスペーサー配列に対する酵素活性を最適化するために、同様に SLiPE 法を用いて、酵素ドメインに対してエラープローン PCR 法によってランダム配列を導入し、活性の高い酵素を選択する方法をとった。

【結果・考察】 分子進化法を繰り返し、短いリンカー配列のときに保存されたアミノ酸配列を見出すことができた。これは、短いリンカー配列によって各ドメインの挙動における自由度が小さくなったためであると考えられる。さらに短いリンカー配列では劇的に活性が減少したので、ドメイン間をつなぐリンカー配列の重要性が示された。フレキシブルな長いリンカー配列を用いるよりも、ある程度の制限があるリンカー配列を用いることで、活性が一定な酵素を創製することができ、また、標的配列外での反応(off-target 効果)も抑制できると考えられる。この方法を異なる標的配列においても応用し、同様に高い活性の DNA 組換え酵素が得られたことも発表において同時に報告する。このように一定の機能を有する酵素を用いた遺伝子機能の制御はノックアウト法として様々な疾病遺伝子に応用できる可能性が示された。

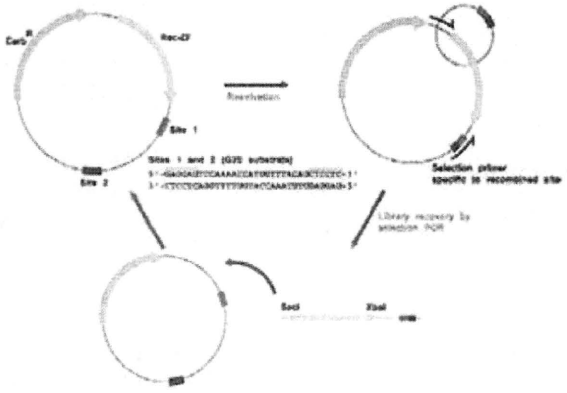

Figure. Schematic representation of SLiPE.

Y-20 FLUORESCENT LABELING FOR PKC DELTA C1b DOMAIN AND ITS APPLICATION TO SENSING BIOLOGY

Nami Ohashi[1], Wataru Nomura[1], Mai Kato[1,2], Hiroshi Tsutsumi[1], Kyoko Itotani[1], Teikichi Ikura[2], Nobutoshi Ito[2], Kiyotsugu Yoshida[3], Nancy Lewin[4], Peter M. Blumberg[4], and Hirokazu Tamamura[1,2]

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Protein kinase C (PKC), which is included in serin/threonine protein kinases and has 11 isozymes, is an important protein in the signal transductions related to cell growth, differentiation, apoptosis, etc. The PKC activation is mediated by binding of ligands such as diacylglycerol(DAG)/phorbol esters (tumor promoter) to C1 domains that are constructed by conserved sequences, His-X12-Cys-X2-Cys-Xn-Cys-X2-Cys-X4-His-Cys-X7-Cys (n = 13 or 14, X is an arbitrary amino acid) and form two Cys3His type zinc finger motifs. PKC is also a receptor for ligans such as phrbol esters that are known as tumor promoters. Thus, PKC is one of important targets of drug discovery. To date, several different ligands targeted to PKC C1 domains, which are ligand binding domains, have been synthesized.

PKCδ has been widely implicated as a pro-apoptotic and/or growth isozyme in tumor cells. For example, the levels of PKCδ were shown to be increased in human hepatocellular carcinomas compared to adjacent normal hepatic tissue. On the other hand, the PKCδ levels were shown to be reduced in colon carcinomas. This apparent discrepancy might be explained by differences in the regulation of PKCδ activity in these different tissues, although this has not been directly investigated. Thus, in this study, we have synthesized PKCδ regulatory domain (C1B domain) peptides and their analogs having fluorescent groups. Development of C1B domain analogs containing fluorescent groups that can be used as screening-tools for PKCδ-specific ligands would be desirable. Use of an environment responsive fluorescent dye led to detection of fluorescent intensity change or wavelength shift based on folding or conformational change of domains and binding of ligands to domains. The folding by coordination of zinc ions or binding of the λιγανδ το α δC1b with fluorescent change (wavelength shift and/or increase in fluorescent intensity) might suggest its possibility of application to ligand screening.

P-056

STRUCTEUR-ACTIVITY RELATIONSHIP STUDY OF CXCR4 ANTAGONISTS BASED ON THE CYCLIC PENTAPEPTIDE SCAFFOLD: IDENTIFICATION OF NEW PHARMACOPHORE MOIETIES

Tomohiro Tanaka[1], Hiroshi Tsutsumi[1], Wataru Nomura[1], Yasuaki Tanabe[1,2], Nami Ohashi[1], Ai Esaka[3], Chihiro Ochiai[1], Jun Sato[1], Kyoko Itotani[1], Tsutomu Murakami[4], Kenji Ohba[4], Naoki Yamamoto[4], Nobutaka Fujii[3], and Hirokazu Tamamura[1,2]

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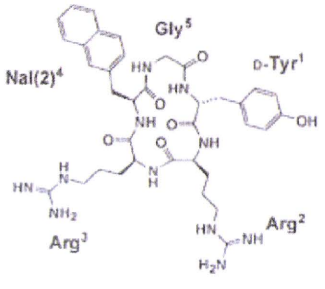
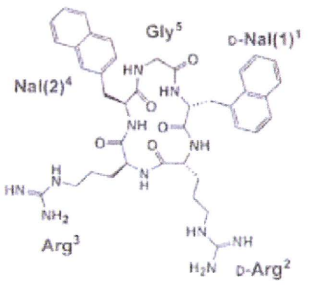
The chemokine receptor CXCR4 is a membrane-protein, which belongs to the G-protein coupled receptor family. While CXCR4 and its endogenous ligand SDF-1α/CXCL12 have various physiological effects in the embryonic stage, CXCR4 has also been proven to be related to HIV infection, cancer metastasis, progress of leukemia and rheumatoid arthritis. Thus, CXCR4 is expected as an attractive drug target. Previously, we developed a cyclic pentapeptide FC131 as a CXCR4 antagonist. In the present study, we tried to design novel cyclic pentapeptide libraries based on the FC131 structure for structure-activity relationship studies on CXCR4 antagonists.

Based on our previous data, we focused on the D-Tyr[1] and Arg[2] moieties of FC131. Cyclic pentapeptides, in which D-Tyr[1] and Arg[2] of FC131 were replaced with bicyclic aromatic and cationic amino acids, were synthesized, and their activity was evaluated towards CXCR4. As a result, novel cyclic pentapeptides **2** and **3** were found as novel CXCR4 antagonist. These cyclic pentapeptides have chiralities and pharmacophore defferent from those of FC131. Taken together, the information obtained from these peptides would be useful for the development of CXCR4 antagonist.



FC131 (1)



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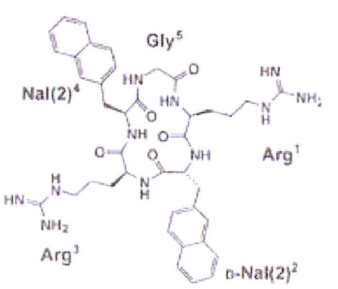

3

P-067 SMALL-SIZED CD4 MIMICS TARGETED FOR DYNAMIC SUPRAMOLECULAR MECHANISM OF HIV-1 ENTRY

Chihiro Ochiai[1], Yuko Yamada[1], Kazuhisa Yoshimura[2], Tomohiro Tanaka[1], Hiroshi Tsutsumi[1], Wataru Nomura[1], Hiroyuki Masuno[1], Kyoko Itotani[1], Junji Shibata[2], Makiko Hatada[2], Shuzo Matsushita[2], and Hirokazu Tamamura[1,3]

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Highly active anti-retroviral therapy (HAART), which utilizes a combination of HIV protease inhibitors and reverse transcriptase inhibitors, has brought us a great success and hope in the clinical treatment of HIV-infected patients to date. However, HAART involves serious clinical problems such as the emergence of multi-drug resistant (MDR) HIV-1 strains, significant side effects, high costs, etc. These drawbacks encouraged us to develop brand-new drugs with novel action mechanisms, such as entry and fusion inhibitors. Recently, a molecular mechanism involved in HIV-entry and -fusion into host cells has been disclosed in detail. The elucidation of the dynamic supramolecular mechanism in HIV-entry and -fusion has provided insights into new type of drugs that can block HIV infection. Based on this finding, we have developed entry inhibitors such as coreceptor CXCR4 antagonists, T22 and T140. In this study, we focused on CD4 mimic small compounds, such as NBD-556, and performed their structure-activity relationship study. In addition, effects on the dynamic supramolecular mechanism of HIV entry and a combinational use with CXCR4 antagonists or neutralizing antibodies were investigated.

It is speculated that there is a large space around the para-position of the phenyl ring of NBD analogues, which might interact with a cavity of a viral surface protein gp120. Thus, NBD analogues having various substituents on the phenyl ring were synthesized. Some compounds showed potent anti-HIV activity. In addition, NBD analogues showed highly synergic effects with an anti-gp120 antibody and with T140. In the FACS analysis, in the presence of NBD analogues, the anti-gp120 antibody strongly binds to the HIV envelope.

CD4 mimic compounds might expose the epitope region of gp120, which was hidden inside. These compounds would be important probes directed to the dynamic supramolecular mechanism of HIV Entry, and useful lead compounds for cocktail therapy of AIDS.

P-072 DEVELOPMENT OF INHIBTORY PEPTIDES AGAINST HIV-1 INTEGRASE

Yuta Nakanishi[1,2], Atsushi Komano[3], Hiroshi Tsutsumi[1], Toru Nakahara[1,2], Takayuki Yanagisawa[1,2], Shintaro Suzuki[1], Nami Ohashi[1], Tomohiro Tanaka[1], Wataru Nomura[1], Emiko Urano[3,4], Yan Han[3], Hideyoshi Fuji[5], Makiko Hamatake[3], Kosuke Miyauchi[1], Yoko Morikawa[1], Tyuji Hoshino[5], Wataru Sugiura[3], Naoki Yamamoto[3], and Hirokazu Tamamura[1,2]

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Highly active anti-retroviral therapy (HAART) is frequently adopted as clinical treatment for AIDS or HIV-1-infected patients. HAART has brought us a great success in the chemotherapy of patients by a combination of two or three different agents among reverse transcriptase inhibitors and protease inhibitors. However, there still remain several serious problems such as the emergence of the viral strains with multi-drug resistance (MDR), considerable adverse effects and high costs. Thus, development of novel drugs possessing action mechanisms different from the above inhibitors would be currently desirable. As such, we focus on HIV-1 integrase and explore finding of effective compounds with inhibitory activity against integrase.

Previously, we have successfully found inhibitory peptides against HIV-1 integrase through screening from peptide libraries, which are composed of fragments (overlapping peptides) of HIV-1 gene products, by a strand transfer assay using purified integrase. An essential hexapeptidic motif (Vpr 64-69) for the inhibition of the integrase was identified from the Vpr-derived peptide library by an *in vitro* screening experiment. Then, an octa-arginine sequence was added to the above Vpr-derived peptides with integrase inhibitory activity to introduce these peptidic inhibitors into cells. These cell-membrane permeable inhibitors exhibited clear inhibitory activity of HIV replication *in vitro*. In this study, we have developed higher active peptidic inhibitors against the integrase through experiments involving the systematic replacement of amino acids for structure-activity relationship studies, suggesting that these peptides are novel lead compounds of anti-HIV agents. The details of these results will be presented in this symposium.

P-117 DEVELOPMENT OF FLUORESCENT LABELED CXCR4 SPECIFIC LIGAND FOR IMAGING AND FLUORESCENCE-BASED SCREENING

Tomohiro Tanaka[1], Wataru Nomura[1], Yasuaki Tanabe[1,2], Hiroshi Tsutsumi[1], Chihiro Ochiai[1], Jun Sato[1], Kyoko Itotani[1], Kenji Ohba[3], Naoki Yamamoto[3], and Hirokazu Tamamura[1,2]

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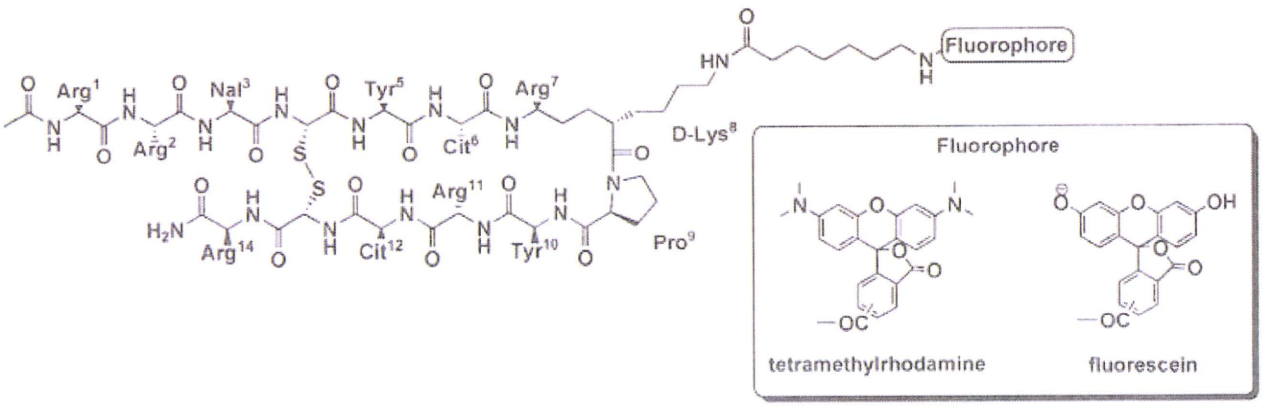
Development of CXCR4-specific ligands is an important issue in chemotherapy of HIV infection, cancer metastasis and rheumatoid arthritis, and numerous potential ligands have been developed to date to discover drugs against intractable diseases described above. However, it is difficult to screen numerous compounds for CXCR4 antagonists because of cost and risk in the previous method based on radioisotope(RI)-labeled ligands. To address this problem, fluorophore labeled Ac-TZ14011, which is derived from a powerful CXCR4 antagonist T140, has been synthesized (Figure).

Interaction of Ac-TZ14011 to CXCR4 on the cell membrane was observed by fluorescence microscope, and analysis of the binding data yielded IC_{50} values of several ligands comparable to those obtained in RI-based assays. This fluorescence-based assay is applicable to explore new pharmacophores of CXCR4-specific ligands with high-throughput screening and also to screening of the other GPCR binding ligands.



P-128 DEVELOPMENT OF A NOVEL TAG-PROBE SYSTEM FOR FLUORESCENT IMAGING OF PROTEINS IN LIVING CELLS

Seiichiro Abe[1,2], Hiroshi Tsutsumi[1], Tomoaki Mino[1,2], Masaki Haseyama[1], Nami Ohashi[1], Tomohiro Tanaka[1], Wataru Nomura[1], and Hirokazu Tamamura[1,2]

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Real time imaging of proteins in living cells is significantly useful for elucidation of the functions and behavior of proteins in detail. Fluorescent labeling of proteins has been widely used as one of the most powerful methods for bio-imaging of proteins. However, it is difficult to label proteins sequentially in different colors (pulse chase labeling) by using conventional labeling methods such as the fusion of target proteins with fluorescent proteins. A tag/probe system is a helpful imaging tool, which can label proteins by fluorescent probes bound specifically to tag peptides incorporated genetically into target proteins. In many cases, however, removal of excess free probes is required to eliminate the background fluorescence derived from the free probes and to detect selectively target proteins. Thus, we have developed fluorogenic tag/probe pairs based on leucine zipper peptides so that we can distinguish easily the labeled proteins from the free probes. A probe peptide was designed as an α-helix peptide, which had an solvatochromic fluorescent dye, 4-nitrobenzo-2-oxa-1,3-diazole (NBD). Tag peptides was designed as an antiparallel 2α-helix peptide, and the leucine residues, which were located on the complementary position against NBD of the probe peptide, were replaced replaced by alanines to accommodate the NBD unit in the hydrophobic area when the tag and probe form the 3α-helical bundle structure. The fluorescent titration analysis revealed that the probe peptide showed the drastic wavelength shift from 536 nm to 505 nm and approximately an 18-fold increase of the fluorescent intensity at 505 nm in the presence of the tag peptide. The fluorescent imaging of a tag-fused cell surface protein was successfully achieved using the probe peptide, clearly demonstrating that our tag/probe system is a valuable bio-imaging tool of proteins. Furthermore, the available fluorescent color of probe peptides was extended using the FRET system. The tetramethylrhodamine unit was introduced into the NBD-probe peptide as an acceptor fluorophore, in which the NBD unit works as a donor fluorophore. The FRET probe peptide also showed the fluorogenic activity in the presence of the tag peptide, indicating that our tag/probe pairs might be applicable for the fluorescent pulse chase imaging of proteins.

P-142 DEVELOPMENT OF CAGED DIACYLGLYCEROL-LACTONE DERIVATIVES AND THEIR APPLICATIONS

Yuki Serizawa[1], Wataru Nomura[1], Nami Ohashi[1], Yoshiaki Okuda[1,2], Hironori Matsumoto[1,2], Hiroshi Tsutsumi[1], Toshiaki Furuta[1,3], and Hirokazu Tamamura[1,2]

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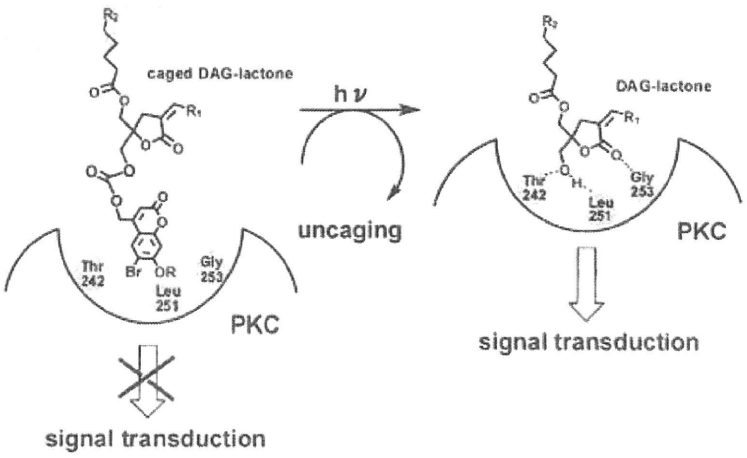
Caged compounds are molecules designed so that their biological activity will be temporary disappered and then reactivated by applying external triggers such as ultraviolet light. These compounds receive widespread attention as temporary and spatially controlled probes of cell-based processes. Protein kinase C (PKC), which is a serine/threonine-specific protein kinase, plays a pivotal role in cell signaling by functioning as a central signal transducing element. These signals are generated by a broad range of ligands which produce the lipid second messenger, diacylglycerol (DAG). Marquez et al have developed DAG-lactone derivatives with a significant increase in binding affinity for PKC as possible templates for the construction of conformationally constrained analogues of DAG. In the present study, caged DAG lactones were synthesized and their photochemically properties were characterized. We confirmed that caged DAG-lactones are photolysed by ultraviolet light irradiation with HPLC analysis and that uncaged DAG-lactones activate PKC by imaging analyses using a confocal microscope system. These results indicate that activity control of DAG-lactones with photo-removable protecting groups could be a useful tool for elucidation of activation mechanism of PKC in mammalian cells.



P-146 SITE-SELECTIVE CYTOSINE METHYLATION BY A SPLIT DNA METHYLASE

Wataru Nomura[1], Hirokazu Tamamura[1], and Carlos F. Barbas, III[2]

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Covalent modification of DNA, such as cytosine methylation, can induce heritable gene silencing. If epigenetic modifications can be specifically targeted, new approaches to transcriptional therapy should result. To address this challenge, we sought to design methyltransferases that would act only at a desired site by adapting the sequence-enabled assembly strategy. Methyltransferase HhaI (M.HhaI) was split into two domains and N-, C-terminal domains were fused to HS2 and HS1 zinc finger domains, respectively. Detection of site-specific methylation was performed by using HhaI restriction enzyme digestion. Moreover, the site-specific methylation was determined by bisulfite sequencings. Our split DNA methylase performed site-specific CpG methylation in living cells without any background methylation when appropriately assembled at the target site. This is the first successful application of the sequence-enable enzyme reassembly approach in vivo. This split domain re-assembly strategy will allow creation of programmable zinc finger methylases that act at any specific CpG site in the mammalian genome. Programmable methylases should orchestrate heritable gene silencing and should find application in DNA tagging approaches and in nanotechnology.

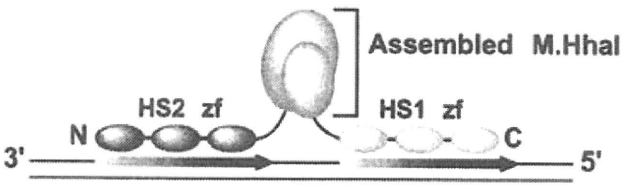

Figure. Schematic representation of split-methylase binding to the target DNA.

HIV 侵入の動的超分子機構を標的とした CD4 mimic

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【背景】

AIDS および HIV 感染症の治療法としては一般に逆転写酵素阻害剤とプロテアーゼ阻害剤を 2,3 剤用いる highly active anti-retroviral therapy (HAART)が用いられており、多大な成功を収めている。しかし、耐性ウイルスが生じる、根治できない、副作用がある等の問題点があり、別の作用点の薬も 3 種ほど登場している。我々は以前から HIV 侵入段階に着目しており、コレセプターCXCR4 のアンタゴニストを中心に種々の阻害剤を創製してきた。今回は、低分子型 CD4 mimic である NBD-556 を取りあげ [1]、その構造活性相関研究および HIV 侵入の動的超分子機構への影響や CXCR4 アンタゴニスト、中和抗体等との併用の効果を調べた。

【方法】

NBD-556 の構造活性相関に関しては分子内に含まれる芳香環(CD4 の Phe43 に対応すると考えられる)部分が gp120 の cavity に相互作用し、芳香環の p-位付近に比較的大きな空間が存在すると推定されるため、芳香環の p-位に種々の置換基を導入した誘導体を合成し、各種 HIV 株に対する抗ウイルス活性を評価した。さらに、CD4 mimic の存在下での、CXCR4 アンタゴニスト(T140)や抗 V3 抗体、CD4 induced 抗体の gp120 への反応性を調べた。

【結果・考察】

高活性の誘導体が数個見つかり、また構造活性相関の情報が得られた。また、CD4 mimic の存在下では、非存在下よりも gp120 への反応性が上昇し、sCD4 の存在下と同様の効果が見られた。これにより NBD 誘導体が gp120 のコンフォメーション変化を誘起することが示唆された。また、CD4 mimic と T140 や抗 V3 抗体あるいは CD4 induced 抗体の併用は抗ウイルス活性を増強した。

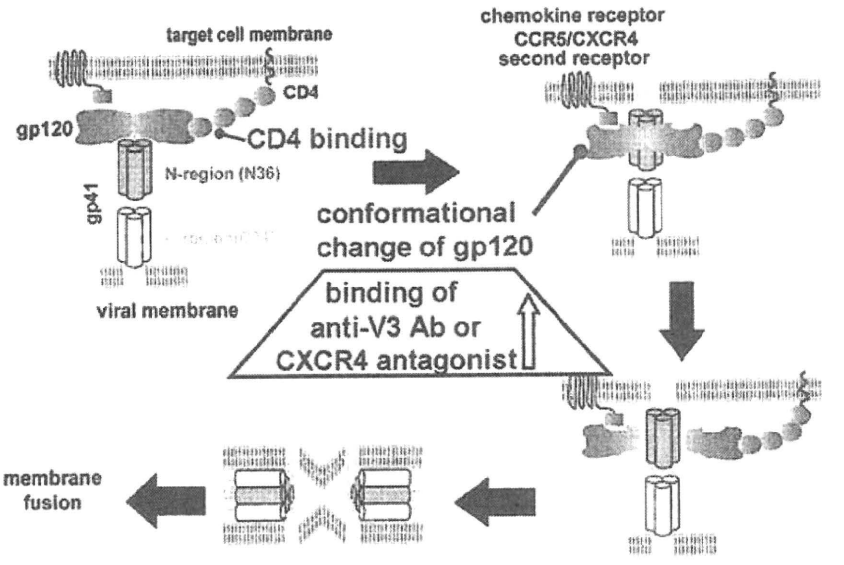

Fig. 1 Induction of change of the gp120 conformation by CD4 binding in HIV-1 entry

ここで得られた低分子型CD4 mimic誘導体はgp120の構造変化を誘導し、中和抗体のエピトープを表面に露出させると考えられる。また、これらはHIV侵入のダイナミックな超分子機構に作用する有用なツールになると示唆され、多剤併用療法を視野にいれた抗エイズ薬の創製研究に有用であると思われる。

【参考文献】

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CD4 mimic small molecules targeted for dynamic supramolecular mechanism of HIV entry

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To date, highly active anti-retroviral therapy (HAART), which utilizes a combination of HIV protease inhibitors and reverse transcriptase inhibitors, has brought us a great success and hope in the clinical treatment of HIV-infected patients. However, HAART involves serious clinical problems such as the emergence of multi-drug resistant strains. These drawbacks encouraged us to develop brand-new drugs with novel action mechanisms. Recently, a molecular mechanism involved in HIV-entry and -fusion into host cells has been disclosed in detail. The elucidation of the dynamic supramolecular mechanism in HIV-entry and -fusion has provided insights into new type of drugs that can block HIV infection. Based on this finding, we have developed entry inhibitors such as coreceptor CXCR4 antagonists, T22 and T140. In this study, we focused on CD4 mimic small compounds, such as NBD-556[1)], and performed their structure-activity relationship study. In addition, effects on the dynamic supramolecular mechanism of HIV entry and a combinational use with CXCR4 antagonists or neutralizing antibodies were investigated.

NBD analogues having various substituents on the phenyl ring were synthesized, and their anti-HIV activity was evaluated. Some compounds showed potent anti-HIV activity. In addition, NBD analogues showed highly synergic effects with an anti-gp120 antibody and with T140. In the FACS analysis, in the presence of NBD analogues, the anti-gp120 antibody strongly binds to the HIV envelope. It suggests that CD4 mimic compounds induce a conformational change of gp120.

CD4 mimic compounds might expose the epitope region of gp120, which was hidden inside. These compounds would be important probes directed to the dynamic supramolecular mechanism of HIV Entry, and useful lead compounds for cocktail therapy of AIDS.