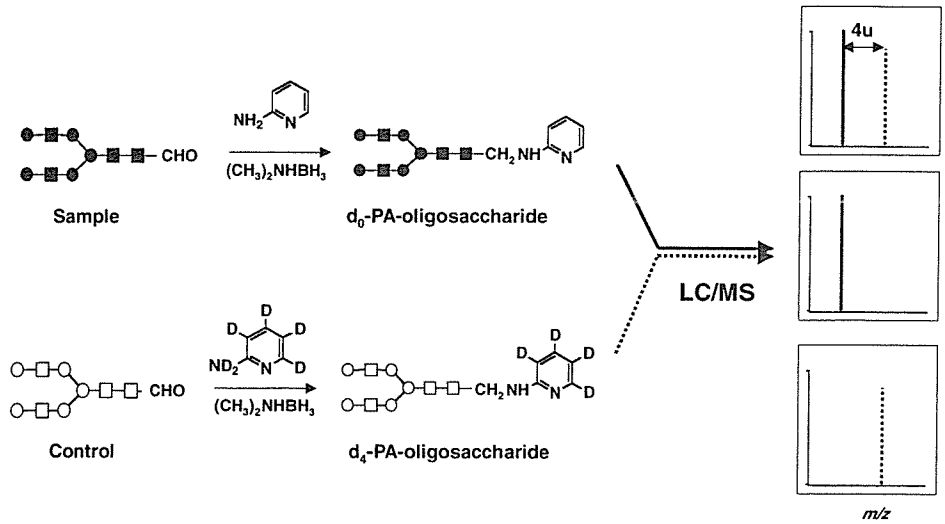

Fig. 4. Quantitative oligosaccharide profiling using LC/MS and an isotope tagging method.

profiling using isotope-labeled 2-aminopyridine (AP) and LC/MS (Fig. 4) (41). In this procedure, oligosaccharides released from an analyte and standard glycoprotein are tagged with d_0-AP and d_6-AP, respectively, and an equal amount of d_0-PA and d_4-PA oligosaccharides is analyzed by LC/MS. Oligosaccharides existing in either the analyte or standard glycoprotein appear as single ions, and oligosaccharides that exist in both analyte and standard glycoprotein are detected as paired ions with a difference of 4 u. The relative amount of analyte oligosaccharides can be determined on the basis of the analyte/ internal standard ion pair intensity ratio. This method can improve the precision of the mass spectrometric quantification and be used for glycan differential analysis among multiple samples.

D. MS of Glycopeptides

When glycans are released from protein, all information about their attachment to the protein is lost. Protein identification, determination of glycosylation sites and site-specific glycosylation analyses are generally achieved by the mass spectrometric analysis of glycopeptides. ESI and MALDI coupled with several analyzers are employed for glycopeptide analysis. ESI allows the accurate mass measurement of relatively large glycopeptide/protein because of the generation of multiple charged ions, whereas ions of large glycopeptide molecule ions are sometimes missed by MALDI-MS due to PSD and their poor ionization efficiency (42).

Fig. 5 illustrates three possible cleavages of the peptide backbone. The most common C-terminal and N-terminal fragment ions are b-ion and y-ion, respectively (37). In many cases, CID induces glycosidic bond cleavages rather than peptide backbones. Electron capture dissociation with FT-ICRMS is reported as a means for preferential cleavage of the

イリングを開発した(図4)(41)。この方法は、標準糖タンパク質及び検体糖タンパク質から切り出した糖鎖をそれぞれ6重水素置換AP(d_6-AP)、及び未置換AP (d_0-AP)で標識し、得られたd_4-PA糖鎖及びd_0-PA糖鎖を1対1の混合物としてLC/MSで分析するものである。標準糖タンパク質または検体糖タンパク質のどちらか一方にしか結合していない糖鎖は、d_4-PA糖鎖またはd_0-PA糖鎖どちらかの単独イオンとして検出される。標準糖タンパク質及び検体糖タンパク質に共通して存在する糖鎖は4u異なる1対のイオンとして検出され、d_4-PA糖鎖及びd_0-PA糖鎖のイオン強度比から相対糖鎖結合量を求めることができる。同位体標識糖鎖を内部標準物質として用いることによって、MSを用いた定量解析における再現性が改良されるので、定量的糖鎖プロファイリングは、複数のサンプル間の糖鎖の差異を質的量的に比較する場合に有用である。

D. 糖ペプチドのMS

タンパク質から糖鎖を切り離すと、糖鎖とタンパク質間の結合に関する情報が失われてしまうので、糖鎖含有タンパク質の同定、糖鎖結合位置の決定、及び部位特異的糖鎖不均一性の解析などには糖ペプチドのMSが適している。糖ペプチドの分析においても、ESIあるいはMALDIに様々なアナライザーを組み合わせた装置が利用されている。MALDI-MSでは、ほぼ一価イオンが生成するが、ESI-MSでは多価イオンが生成するため比較的高分子量の糖ペプチドの質量を正確に測定することが可能である。また、MALDI-MSを用いて糖鎖の割合が高い糖ペプチドを分析する場合、イオン化の抑制やPSDによって、分子関連イオンが測定されにくいとする報告がある(42)。

図5はペプチド骨格の開裂を示している。ペプチド結合の開裂によって生じたN末端側はbイオン、C末端側はyイオンと呼ばれる(43)。通常、糖ペプチドのCID-MS/MSでは、ペプチドよりも糖鎖の開裂が優先される。ペプチド部分が優先的に開裂する方法としてFT-ICRMS装置を用いた電子捕獲解

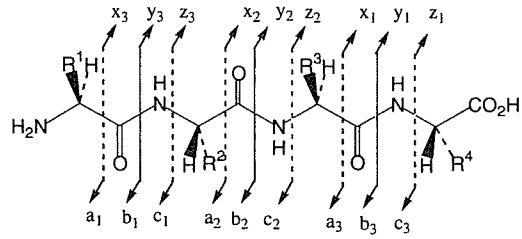

Fig. 5. Types of peptide fragmentation.

peptide backbone (44,45).

For the cleavages of both glycosidic and peptide bonds CID-MS/MS is carried out with relatively high energy (approx. 50V/1000 u) (46–51). Hence, it allows both peptide sequencing and estimation of the carbohydrate structure on the basis of b/y-ions and B/Y-ions. Fig. 6A presents the CID-MS/MS spectrum of a glycopeptide derived from human α-fetoprotein using the ESI-QqTOFMS instrument. Carbohydrate-specific B-ions, such as $HexNAc^+$ and $NeuAc^+$ are observed at *m/z* 204, 292 as well as y-series ions. Based on the y-series ions and peptide ion, this peptide is identified

糖鎖とペプチド部分を同時に開裂させるためには比較的高いエネルギー(約50V/1000 u)を与えてCID-MS/MSを行う(46–51)。生成したb、yイオン、及びB、Yイオンから、ペプチド部分のアミノ酸配列や、糖鎖構造を解析することができる。図6Aは、ESI-四重極飛行時間型MSを用いたCID-MS/MSによって得られたヒトアルファフェトプロテイン由来糖ペプチドのMS/MSスペクトルである。糖鎖Bイオンである*m/z* 204 ($HexNAc^+$)、及び292 ($NeuAc^+$)等と一緒に、ペプチドに由来する一連のyイオンが検出されていることがわかる。これら一

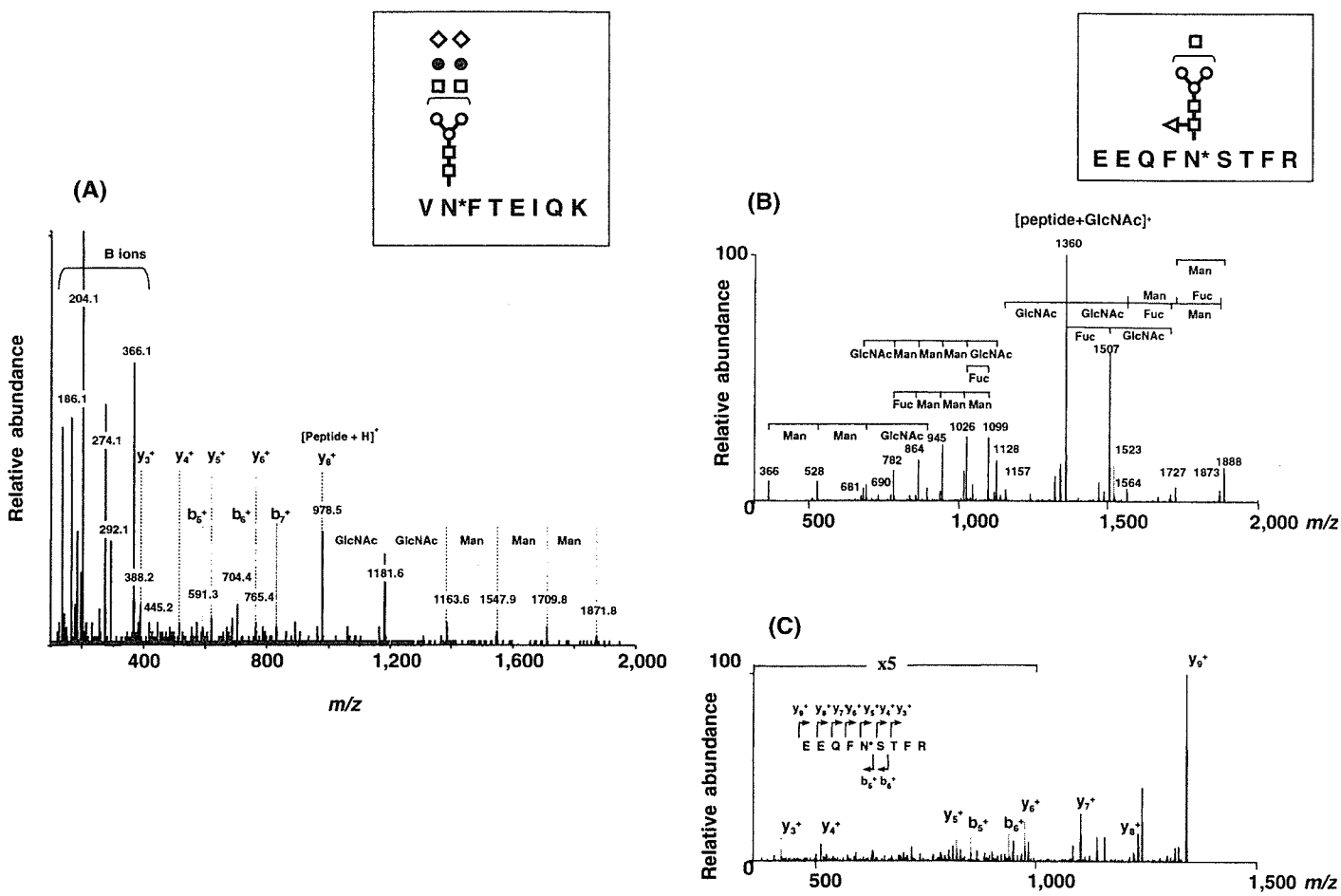

Fig. 6. CID-MS^n spectra of glycopeptides. (A) CID-MS/MS with relatively high energy (precursor ion, *m/z* 1,061.8), Sample: glycopeptide from human alpha-fetoprotein, MS: QSTAR (Applied Biosystems). (B) Low-energy CID-MS/MS (precursor ion, *m/z* 1,200). (C) Low-energy CID-MS^3 (precursor ion, *m/z* 1,360), Sample: glycopeptide from mouse IgG1, MS: LTQ (Thermo Electron).

as VNFTEIQK. The oligosaccharide structure can be deduced as disialylated biantennary from the molecular mass of the carbohydrate moiety together with B-ions in the MS/MS spectrum.

B- and Y-series ions are produced by the low-energy CID-MS/MS of glycopeptides (12, 52–55). Fig. 6B shows the low energy (approx. 5–20V/1000 u) CID-MS/MS spectrum of glycopeptide derived from mouse IgG1. Based on the carbohydrate related-ions, the carbohydrate structure could be determined as fucosylated biantennary. When using an ITMS instrument, MS^3 is automatically carried out for intense ions. In this experiment, [peptide +HexNAc +H]$^+$, which is generally detected as intense ion, was subjected to further product ion scan, and b- and y-series ions appeared in the MS^3 spectrum (Fig. 6C). Peptides can be identified by comparing experimental fragment ions with predictable fragment ions derived from proteins in a database. Moreover, the database analysis with the possibility of glycosylation at Asn and Ser/Thr with HexNAc, Hex, and dHex allows the identification of glycopeptides and glycosylation sites (56). For instance, this peptide was identified as EEQFN*STFR glycosylated with HexNAc at N*. This method would enable the glycosylation analysis of unknown glycoproteins and a mixture of glycoproteins.

E. Site-Specific Glycosylation Analysis of Glycoproteins

Fig. 7 illustrates the strategy for the site-specific glycosylation analysis of glycoproteins. First, a glycoprotein is digested with an appropriate proteinase, which provides

連のｙイオン、及びペプチドイオンの質量から、このペプチド部分は VNFTEIQK と同定することができる。また、糖鎖構造は、糖鎖部分の分子量と B イオンからジシアロ２本鎖糖鎖と推定される。

低エネルギー CID-MS/MS では、グリコシド結合が優先して開裂し、B、及び Y イオンが検出される (12、52–55)。図 6B はマウス IgG1 から得られた糖ペプチドの低エネルギー CID-MS/MS (約 5-20V/1000 u) スペクトルで、糖鎖関連イオンから、糖鎖構造を推定することができる。糖ペプチドの MS/MS では一般に、[ペプチド + HexNAc + H]$^+$ が比較的強く検出される。そこで、ITMS 装置を用いて [ペプチド +HexNAc + H]$^+$ を前駆イオンとして選択し、さらにプロダクトイオンスキャンを行うと、MS^3 スペクトル上に b 及びｙイオンが検出される (図 6C)。これらの b 及びｙイオンの実測値をタンパク質データベースに登録されているタンパク質の予測プロダクトイオンの理論質量と比較することによって、ペプチドを同定することができる．さらに、Asn や Ser/Thr に HexNAc、Hex、あるいは dHex 等による糖鎖修飾の可能性を追加してデータベース検索をすることによって、糖鎖結合位置を決定できる場合がある (56)。例えば、ここで分析された糖ペプチドはデータベース検索エンジンを用いて、EEQFN*STFR(N* は HexNAc 修飾 Asn) と同定された。この方法を利用することによって、混合物中の糖タンパク質や、未知の糖タンパク質の部位特異的な糖鎖解析が可能となるものと期待される。

E. 糖タンパク質の部位特異的糖鎖解析

糖タンパク質の部位特異的糖鎖解析の流れを図 7 にまと

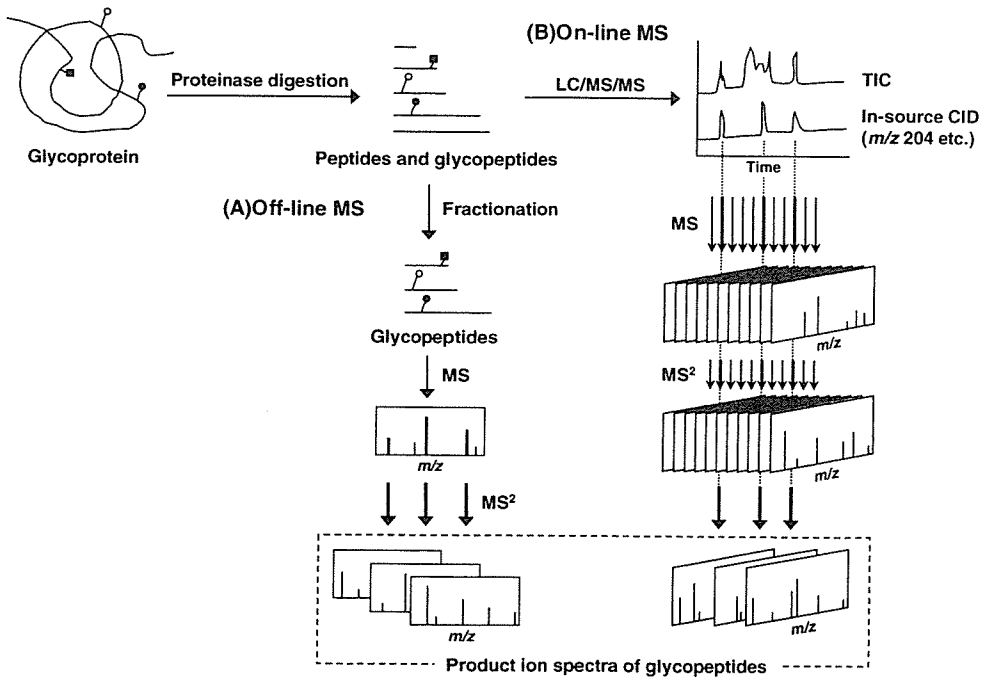

Fig. 7. Strategy for site-specific glycosylation analysis by LC off-line MS (A) and on-line LC/MS (B).

glycopeptides, including a single glycosylation site. Trypsin, Lys-C, Glu-C and Asp-N are commonly used for glycosylation analysis. Since glycopeptide ions are sometimes missed by MS in the presence of excess peptides due to their lower ionization efficiency, several methods have been proposed for the fractionation of glycopeptides, such as HPLC and affinity chromatography (57,58), followed by off-line MALDI-MS. LC on-line ESI/MS is an efficient method for the direct elucidation of glycopeptides in a complex mixture. Although LC/MS provide a complicated chromatogram, glycopeptides in a choromatographic position can be localized by the appearance of marker ions, such as $HexNAc^+$ and $HexHexNAc^+$, resulting from precursor ion scanning and in-source CID (59–66). However, for peptide identification these two means require additional CID-MS/MS scan for some intense ions (data-dependent CID-MS/MS scan). Recently we presented an alternative method, with which product ion spectra of glycopeptides can be selected directly using marker ions arising from glycopeptides by data-dependent CID-MS/MS scan with relatively high energy (49).

めける。まず、糖タンパク質を適切な酵素で糖ペプチドに断片化する。この際、同一ペプチドに複数の糖鎖結合部位が含まれないように酵素を選択する。基質特異性の高いトリプシン、Lys-C、Glu-C、及びAsp-N等がよく用いられている。糖ペプチドはペプチドに比べてイオン化効率が悪く、ペプチドが混在するとマススペクトルが得られにくい。そのため、アフィニティークロマトグラフィーや、HPLC等で予め糖ペプチドを分画してから(57,58)、マススペクトルを測定するオフライン法(図7A)や、C18カラム等を用いたLC/MSでペプチド・糖ペプチドを分離しながら直接マススペクトルを測定するオンライン法がよく用いられている(図7B)。オンラインLC/MSでは複雑なクロマトグラムが得られるが、プリカーサーイオンスキャンやインソースCIDによって生じた糖鎖に特徴的なBイオン(例えば、$HexNAc^+$、*m/z* 204や$HexHexNAc^+$、*m/z* 366など)を利用することによって、糖ペプチドの溶出位置を推定することができる(59–66)。ただしこれらの方法は、ペプチドを同定するために、別途、強度の高いイオンに対する自動的CID-MS/MSスキャン(データ依存的CID-MS/MSスキャン)を行う必要がある。そこで、我々は別法として、データ依存的なCID-MS/MSによって生成したBイオンを利用して糖ペ

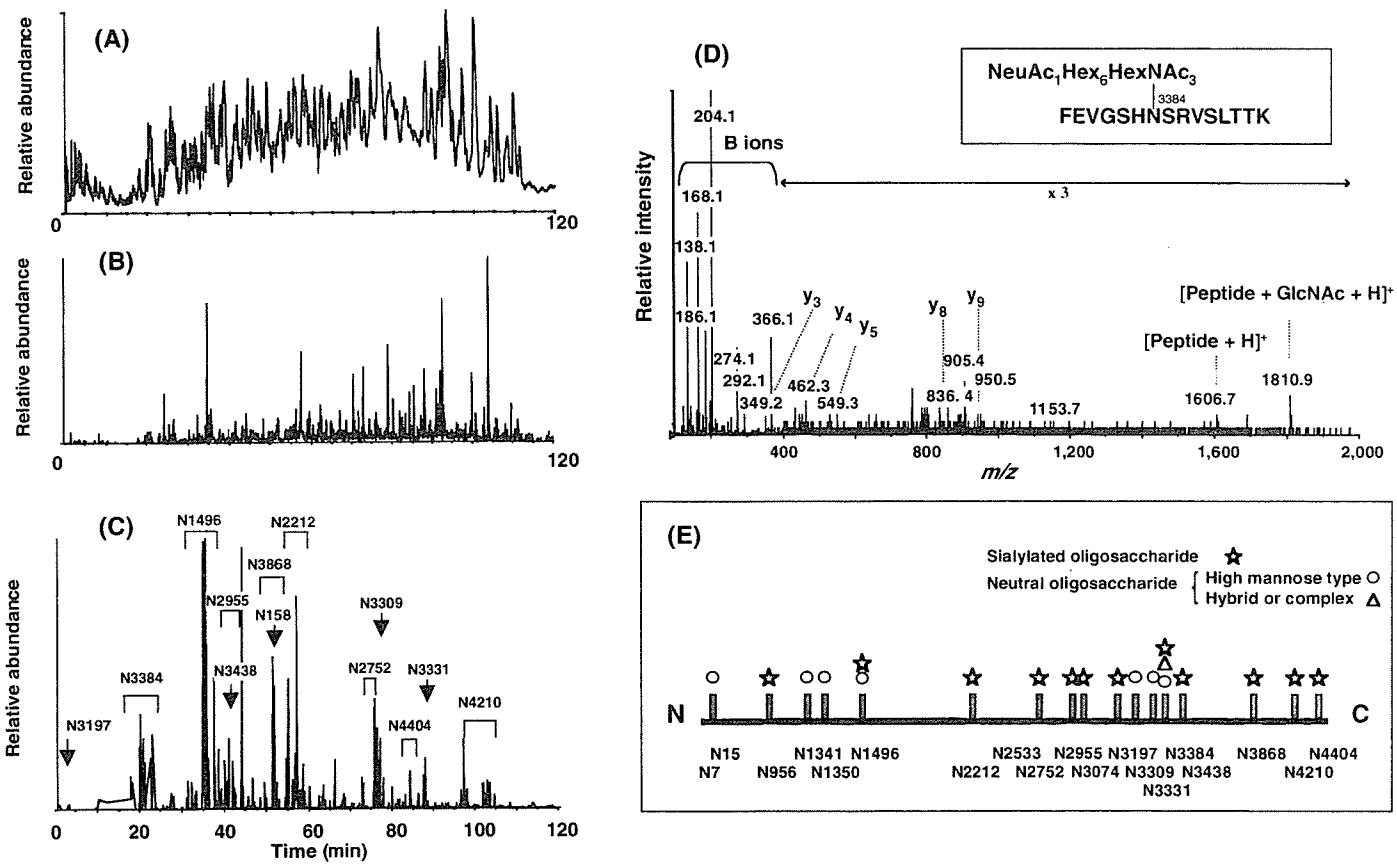

Fig. 8. Site-specific glycosylation analysis of apoB100 by LC/ESI-MS/MS. (A) TIC of full MS scan (*m/z* 400-2,000). (B) TIC of data-dependent CID-MS/MS scan (*m/z* 100-2,000). (C) Mass chromatogram at *m/z* 204 in data-dependent CID-MS/MS scan. (D) MS/MS spectrum of glycopeptide (precursor ion: *m/z* 1,160.4). (E) *N*-glycosylation of apoB100. Sample: tryptic digest of apoB100 (4 μg), LC: Paradigm (Michrome BioResources), Column: Magic C18 (0.2 × 50 mm, Michrome BioResources), MS: QSTAR (Applied Biosystems).

Here we demonstrate the site-specific glycosylation analysis of human apolipoprotein B100 (apoB100). Tryptic digest of apoB (4 μg) was injected into an LC/ESI-QqTOFMS instrument equipped with a C18 column. Figs. 8A and B are total ion chromatograms (TIC) obtained by full MS^1 scan and data-dependent CID-MS/MS scan, respectively (49). These chromatograms appear complicated due to a number of peptide-related ions derived from a large glycoprotein molecule of 500,000 Da. In order to localize glycopeptides in the peptide map, carbohydrate marker ion, $HexNAc^+$ at *m/z* 204, was extracted from the TIC of data-dependent CID-MS/MS scan (Fig. 8C). The MS/MS spectra of glycopeptides were then sorted from the peaks appearing in the mass chromatogram (Fig. 8D). We successfully identified 17 *N*-glycosylation sites among 19 potential *N*-glycosylation sites and deduced glycosylation at each glycosylation site from the mass of carbohydrate moieties (Fig. 8E).

F. Application in Proteomics and Glycomics

MS enables us to elucidate a small number of glycoproteins isolated by electrophoresis (67, 68) and micro HPLC (69). This method could be applied in comprehensive or carbohydrate structure-specific glycoprotein analysis by a combination with proteomic approaches such as 2-dimensional (2D) gel electrophoresis (GE) and 2D-LC followed by MS. Some applications have been already demonstrated, for instance, glycoprotein expression analysis using 2D-GE coupled with carbohydrate-specific dyeing or immunoblotting (70), and LC/MS combined with lectin affinity chromatography (71,72). These glycomic/proteomic technologies are expected to be a powerful tool for the functional study of glycoproteins, finding disease-related glycoproteins and identifying proteins attached to some glyco-epitopes.

プチドの MS/MS スペクトルを選び出す方法を見出した (49)。

図 8 に、我々が最近行ったオンライン法によるヒトアポリポプロテイン B100 (apoB100) の部位特異的な糖鎖解析例を示す (49)。apoB100 のトリプシン消化物 (4 μg) を C18 カラムを用いた LC/ESI-QqTOFMS 装置で分析した。図 8A 及び B はフル MS^1 スキャン (*m/z* 1,000-2,000) 及びデータ依存的 CID-MS/MS スキャンによって得られたトータルイオンクロマトグラム (TIC) である。apoB100 は分子量約 500,000 Da の大きな糖タンパク質なので、非常に多くのペプチドイオンが検出されている。そこで、糖ペプチドの溶出位置を推定するために、データ依存的 CID-MS/MS スキャンによって生じた *m/z* 204 イオンのみを抜き出した (図 8C)。出現したピーク周辺から B イオンを指標に糖ペプチドの MS/MS スペクトルを探し出し、それらのスペクトル上のプロダクトイオンを帰属した (図 8D)。その結果、19 カ所の推定 *N* 結合型糖鎖結合部位のうち 17 カ所に糖鎖が結合していることを明らかにするとともに、それぞれの部位に結合している糖鎖を推定することができた (図 8E)。

F. グライコミクス・プロテオミクスへの応用

現在では、電気泳動 (67,68) やミクロ液体クロマトグラフィー (69) で分離された僅かな糖タンパク質からでも、MS によって、多くの糖鎖構造情報を得ることができるようになった。これらの糖鎖解析技術とプロテオミクス的アプローチ、すなわち、2 次元電気泳動や 2 次元クロマトグラフィーによるタンパク質発現解析と MS を組み合わせることによって、糖タンパク質の網羅的解析や、任意の糖鎖構造を持つタンパク質の解析が可能になるものと期待されている。すでに、2 次元電気泳動と糖タンパク質特異的染色法や免疫ブロットを組み合わせた糖タンパク質発現解析や (70)、レクチンアフィニティークロマトグラフィーと各種 LC/MS を組み合わせた糖タンパク質の網羅的解析の例が報告されている (71,72)。今後、これらのグライコミクス・プロテオミクス解析技術が、糖鎖の機能研究や、疾患等に関与する糖鎖・糖タンパク質の探索、並びに様々な糖鎖エピトープ結合タンパク質の特定に貢献できるものと期待される。

References

1. Karas, M., and Hillenkamp, F. (1988) Anal. Chem. **60**, 2299–2301
2. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Science **246**, 64–71
3. Lemoine, J., Chirat, F., and Domon, B. (1996) J. Mass Spectrom. **31**, 908–912
4. Bahr, U., Pfenninger, A., Karas, M., and Stahl, B. (1997) Anal. Chem. **69**, 4530–4535
5. Hunt, D. F., Yates, J. R., 3rd, Shabanowitz, J., Winston, S., and Hauer, C. R. (1986) Proc. Natl. Acad. Sci. U S A. **83**, 6233–6237
6. Weiskopf, A. S., Vouros, P., and Harvey, D. J. (1998) Anal. Chem. **70**, 4441–4447
7. Naven, T. J., Harvey, D. J., Brown, J., and Critchley, G. (1997) Rapid Commun. Mass Spectrom. **11**, 1681–1686
8. Solouki, T., Reinhold, B. B., Costello, C. E., O'Malley, M., Guan, S., and Marshall, A. G. (1998) Anal. Chem. **70**, 857–864
9. Chai, W., Piskarev, V., and Lawson, A. M. (2001) Anal. Chem. **73**, 651–657
10. Harvey, D. J., Bateman, R. H., Bordoli, R. S., and Tyldesley, R. (2000) Rapid Commun. Mass Spectrom. **14**, 2135–2142
11. Sagi, D., Peter-Katalinic, J., Conradt, H. S., and Nimtz, M. (2002) J. Am. Soc. Mass Spectrom. **13**, 1138–1148
12. Zhang, S., and Chelius, D. (2004) J. Biomol. Tech. **15**, 120–133
13. Karlsson, N. G., Schulz, B. L., and Packer, N. H. (2004) J. Am. Soc. Mass Spectrom. **15**, 659–672
14. Domon, B., and Costello, C. E. (1988) Glycoconj. J. **5**, 397–409
15. Meisen, I., Peter-Katalinic, J., and Muthing, J. (2003) Anal. Chem. **75**, 5719–5725
16. Xue, J., Song, L., Khaja, S. D., Locke, R. D., West, C. M., Laine, R. A., and Matta, K. L. (2004) Rapid Commun. Mass Spectrom. **18**, 1947–1955

17. Takegawa, Y., Deguchi, K., Ito, S., Yoshioka, S., Sano, A., Yoshinari, K., Kobayashi, K., Nakagawa, H., Monde, K., and Nishimura, S. (2004) Anal Chem. **76**, 7294–7303
18. Takegawa, Y., Deguchi, K., Ito, S., Yoshioka, S., Nakagawa, H., and Nishimura, S. (2005) Rapid Commun. Mass Spectrom. **19**, 937–946
19. Harvey, D. J. (1999) Mass Spectrom. Rev. **18**, 349–450
20. Harvey, D. J., Bateman, R. H., and Green, M. R. (1997) J. Mass Spectrom. **32**, 167–187
21. Kawasaki, N., Ohta, M., Hyuga, S., Hashimoto, O., and Hayakawa, T. (1999) Anal. Biochem. **269**, 297–303
22. Kawasaki, N., Haishima, Y., Ohta, M., Itoh, S., Hyuga, M., Hyuga, S., and Hayakawa, T. (2001) Glycobiology **11**, 1043–1049
23. Sato, Y., Suzuki, M., Nirasawa, T., Suzuki, A., and Endo, T. (2000) Anal. Chem. **72**, 1207–1216
24. Takegawa, Y., Deguchi, K., Ito, S., Yoshioka, S., Nakagawa, H., and Nishimura, S. (2005) Anal. Chem. **77**, 2097–2106
25. Royle, L., Mattu, T. S., Hart, E., Langridge, J. I., Merry, A. H., Murphy, N., Harvey, D. J., Dwek, R. A., and Rudd, P. M. (2002) Anal. Biochem. **304**, 70–90
26. Hase, S., Ikenaka, T., and Matsushima, Y. (1981) J. Biochem. (Tokyo). **90**, 407–414
27. Suzuki-Sawada, J., Umeda, Y., Kondo, A., and Kato, I. (1992) Anal. Biochem. **207**, 203–207
28. Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., and Parekh, R. B. (1995) Anal. Biochem. **230**, 229–238
29. Ohta, M., Hamako, J., Yamamoto, S., Hatta, H., Kim, M., Yamamoto, T., Oka, S., Mizuochi, T., and Matsuura, F. (1991) Glycoconj. J. **8**, 400–413
30. Yoshimi, Y., Yamazaki, S., and Ikekita, M. (1999) Biochem. Biophys. Acta. **1426**, 69–79
31. Suzuki, S., Kakehi, K., and Honda, S. (1996) Anal. Chem. **68**, 2073–2083
32. Okamoto, M., Takahashi, K., and Doi, T. (1995) Rapid Commun. Mass Spectrom. **9**, 641–643
33. Lattova, E., and Perreault, H. (2003) J. Chromatogr. A. **1016**, 71–87
34. Morelle, W., Faid, V., and Michalski, J. C. (2004) Rapid Commun. Mass Spectrom. **18**, 2451–2464
35. Yoshino, K., Takao, T., Murata, H., and Shimonishi, Y. (1995) Anal. Chem. **67**, 4028–4031
36. Kawasaki, N., Ohta, M., Itoh, S., Hyuga, M., Hyuga, S., and Hayakawa, T. (2002) Biologicals. **30**, 113–123
37. Karlsson, N. G., Wilson, N. L., Wirth, H. J., Dawes, P., Joshi, H., and Packer, N. H. (2004) Rapid Commun. Mass Spectrom. **18**, 2282–2292
38. Zamfir, A., Seidler, D. G., Schonherr, E., Kresse, H., and Peter-Katalinic, J. (2004) Electrophoresis **25**, 2010–2016
39. Kremmer, T., Szollosi, E., Boldizsar, M., Vincze, B., Ludanyi, K., Imre, T., Schlosser, G., and Vekey, K. (2004) Biomed. Chromatogr. **18**, 323–329
40. Hashii, N., Kawasaki, N., Itoh, S., Hyuga, M., Kawanishi, T., and Hayakawa, T. (2005) Proteomics (in press)
41. Yuan, J., Hashii, N., Kawasaki, N., Itoh, S., Kawanishi, T., and Hayakawa, T. (2005) J. Chromatogr. A. **1067**, 145–152
42. Demelbauer, U. M., Zehl, M., Plematl, A., Allmaier, G., and Rizzi, A. (2004) Rapid Commun. Mass Spectrom. **18**, 1575–1582
43. Roepstorff, P., and Fohlman, J. (1984) Biomed. Mass Spectrom. **11**, 601
44. Hakansson, K., Chalmers, M. J., Quinn, J. P., McFarland, M. A., Hendrickson, C. L., and Marshall, A. G. (2003) Anal. Chem. **75**, 3256–3262
45. Hakansson, K., Cooper, H. J., Emmett, M. R., Costello, C. E., Marshall, A. G., and Nilsson, C. L. (2001) Anal. Chem. **73**, 4530–4536
46. Kurogochi, M., and Nishimura, S. (2004) Anal. Chem. **76**, 6097–6101
47. Krokhin, O., Ens, W., Standing, K. G., Wilkins, J., and Perreault, H. (2004) Rapid Commun. Mass Spectrom. **18**, 2020–2030
48. Wuhrer, M., Hokke, C. H., and Deelder, A. M. (2004) Rapid Commun. Mass Spectrom. **18**, 1741–1748
49. Harazono, A., Kawasaki, N., Kawanishi, T., and Hayakawa, T. (2005) Glycobiology **15**, 447–462
50. Nemeth, J. F., Hochgesang, G. P., Jr., Marnett, L. J., and Caprioli, R. M. (2001) Biochemistry **40**, 3109–3116
51. Hui, J. P., White, T. C., and Thibault, P. (2002) Glycobiology **12**, 837–849
52. Sandra, K., Devreese, B., Van Beeumen, J., Stals, I., and Claeyssens, M. (2004) J. Am. Soc. Mass Spectrom. **15**, 413–423
53. Sandra, K., Stals, I., Sandra, P., Claeyssens, M., Van Beeumen, J., and Devreese, B. (2004) J. Chromatogr. A. **1058**, 263–272
54. Satomi, Y., Shimonishi, Y., and Takao, T. (2004) FEBS Lett. **576**, 51–56
55. Wada, Y., Tajiri, M., and Yoshida, S. (2004) Anal. Chem. **76**, 6560–6565
56. Itoh, S., Kawasaki, N., Hashii, N., Harazono, A., Matsuishi, Y., Kawanishi, T., and Hayakawa, T. (2005) J. Chromatogr. A. Submitted
57. Garcia, R., Rodriguez, R., Montesino, R., Besada, V., Gonzalez, J., and Cremata, J. A. (1995) Anal. Biochem. **231**, 342–348
58. Fu, D., and van Halbeek, H. (1992) Anal. Biochem. **206**, 53–63
59. Carr, S. A., Huddleston, M. J., and Bean, M. F. (1993) Protein Sci. **2**, 183–196
60. Ethier, M., Saba, J. A., Spearman, M., Krokhin, O., Butler, M., Ens, W., Standing, K. G., and Perreault, H. (2003) Rapid Commun. Mass Spectrom. **17**, 2713–2720
61. Huddleston, M. J., Bean, M. F., and Carr, S. A. (1993) Anal. Chem. **65**, 877–884
62. Kapron, J. T., Hilliard, G. M., Lakins, J. N., Tenniswood, M. P., West, K. A., Carr, S. A., and Crabb, J. W. (1997) Protein Sci. **6**, 2120–2133
63. Medzihradszky, K. F., Besman, M. J., and Burlingame, A. L. (1997) Anal. Chem. **69**, 3986–3994
64. Ritchie, M. A., Gill, A. C., Deery, M. J., and Lilley, K. (2002) J. Am. Soc. Mass Spectrom. **13**, 1065–1077
65. Schindler, P. A., Settineri, C. A., Collet, X., Fielding, C. J., and Burlingame, A. L. (1995) Protein Sci. **4**, 791–803
66. Wang, F., Nakouzi, A., Angeletti, R. H., and Casadevall, A. (2003) Anal. Biochem. **314**, 266–280
67. Wilson, N. L., Schulz, B. L., Karlsson, N. G., and Packer, N. H. (2002) J. Proteome Res. **1**, 521–529
68. Itoh, S., Harazono, A., Kawasaki, N., Hashii, N., Matsuishi, Y., Kawanishi, T., and Hayakawa, T. (2004) J. Electrophoresis **48**, 163–168
69. Fan, X., She, Y. M., Bagshaw, R. D., Callahan, J. W., Schachter, H., and Mahuran, D. J. (2004) Anal. Biochem. **332**, 178–186
70. Higai, K., Shibukawa, K., Muto, S., and Matsumoto, K. (2003) Anal. Sci. **19**, 85–92
71. Kaji, H., Saito, H., Yamauchi, Y., Shinkawa, T., Taoka, M., Hirabayashi, J., Kasai, K., Takahashi, N., and Isobe, T. (2003) Nat. Biotechnol. **21**, 667–672
72. Qiu, R., and Regnier, F. E. (2005) Anal. Chem. **77**, 2802–2809

Received on June 3, 2005, accepted on July 19, 2005

Trends in Glycoscience and Glycotechnology
Vol.17 No.97 (September 2005) pp.193–203

Profile of the Author

Nana Kawasaki studied organic chemistry and received MS degree from Faculty of Pharmaceutical Sciences, Hokkaido University in 1986. She joined the Division of Biological Chemistry & Biologicals, the National Institute of Health Sciences in 1986. She obtained her PhD from Hokkaido University in 1996. Her current research focuses on a development of evaluation methods of glycoprotein products by mass spectrometry.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytomedicine 13 (2006) 401–411
Phytomedicine
www.elsevier.de/phymed

Screening of novel nuclear receptor agonists by a convenient reporter gene assay system using green fluorescent protein derivatives

T. Suzuki[a,b], T. Nishimaki-Mogami[a], H. Kawai[a], T. Kobayashi[a], Y. Shinozaki[a], Y. Sato[a], T. Hashimoto[c], Y. Asakawa[c], K. Inoue[a], Y. Ohno[a], T. Hayakawa[a], T. Kawanishi[a,*]

[a]*National Institute of Health Sciences, Tokyo, Japan*
[b]*Pharmaceuticals and Medical Device Agency, Tokyo, Japan*
[c]*Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan*

Received 27 September 2004; accepted 6 April 2005

Abstract

Nuclear receptors represent a very good family of protein targets for the prevention and treatment of diverse diseases. In this study, we screened natural compounds and their derivatives, and discovered ligands for the retinoic acid receptors (RARs) and the farnesoid X receptor (FXR). In the reporter assay systems of nuclear receptors presented here, two fluorescent proteins, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP), were used for detection of a ligand-based induction and as an internal control, respectively. By optimizing the conditions (e.g., of hormone response elements and promoter genes for reporter plasmids), we established a battery of assay systems for ligands of RARs, retinoid X receptor (RXR) and FXR. The screening using the reporter assay system can be carried out without the addition of co-factors or substrates. As a result of screening of more than 140 compounds, several compounds were detected which activate RARs and/or FXR. Caffeic acid phenylethyl ester (CAPE), known as a component of propolis from honeybee hives, and other derivatives of caffeic acid up-regulated the expression of reporter gene for RARs. Grifolin and ginkgolic acids, which are non-steroidal skeleton compounds purified from mushroom or ginkgo leaves, up-regulated the expression of the reporter gene for FXR.

Keywords: FXR; RAR; Reporter assay; Fluorescence; GFP; Caffeic acid; Ginkgolic acid; Grifolin

Introduction

Nuclear hormone receptors are ligand-activated transcription factors that are involved in a variety of physiological, developmental, and toxicological processes. The nuclear hormone receptor superfamily includes receptors for thyroid and steroid hormones, retinoids and vitamin D, as well as receptors for unknown ligands. These receptors share a highly conserved DNA-binding domain and a discrete ligand-binding domain, and bind to hormone response elements (HREs) on the DNA during the formation of homodimers, heterodimers, or monomers. This ligand binding to nuclear receptors leads to conformational

*Corresponding author. Tel.: +81 3 3700 9064; fax: +81 3 3700 9084.
E-mail address: kawanish@nihs.go.jp (T. Kawanishi).

0944-7113/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2005.09.003

change of these receptors and the recruitment of coactivator complexes, resulting in transcriptional activation (Khorasanizadeh and Rastinejad, 2001). Their ligand-dependent activity makes nuclear receptors good pharmacological targets.

Nuclear receptors form a superfamily of phylogenetically related proteins encoded by 48 genes in the human genome. Three isotypes of retinoic acid receptors (RARs: RARα, RARβ and RARγ) are receptors for retinoids such as all-*trans*-retinoic acid (ATRA) (Petkovich et al., 1987; Brand et al., 1988; Krust et al., 1989). RARα is associated with differentiation therapy for human acute promyelocytic leukemia (Hansen et al., 2000). RARβ plays a central role in limiting the growth of different cell types (reviewed in Hansen et al., 2000), and is thus a possible target for the treatment of breast and other cancers. RARγ is also primarily expressed in the skin and is involved in skin photoaging and carcinogenesis, and in skin diseases such as psoriasis and acne (Fisher et al., 1996).

The farnesoid X receptor (FXR) is a receptor for bile acids such as chenodeoxycholic acid (CDCA), deoxycholic acid, cholic acid, and their conjugates. Bile acids are synthesized in the liver and secreted into the intestine, where their physical properties facilitate the absorption of fats and vitamins through micelle formation. Cholesterol disposal from the liver is also dependent on the bile acid composition of the secreted bile. Bile acids bind to FXR to activate and regulate the transcription of FXR target genes. FXR controls the expression of critical genes in bile acid and cholesterol homeostasis (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). FXR-null mice show elevated serum cholesterol and triglyceride levels (Sinal et al., 2000), and an FXR agonist has been shown to reduce serum triglyceride levels (Maloney et al., 2000). FXR is thus an attractive pharmacological target for the treatment of hyperlipidemia. Moreover, an FXR agonist has been reported to confer hepatoprotection in a rat model of cholestasis (Liu et al., 2003).

The retinoid X receptor (RXR) is a common heterodimeric partner for many receptors, including thyroid hormone receptor (TR), RAR, vitamin D_3 receptor (VDR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and FXR, in addition to functioning as a receptor for 9-*cis*-retinoic acid (9CRA) during formation of a homodimer.

To determine ligands for these nuclear receptors, we developed a reporter assay system using GFP derivatives. To study the promoter and enhancer control of gene expression, firefly luciferase is widely used as a reporter protein because it has high sensitivity and a broad linear range. In the commonly used reporter assay, β-galactosidase, a well-characterized bacterial enzyme, or renilla luciferase is usually used in conjunction with firefly luciferase to normalize the transfection efficiency of the reporter gene (Sherf et al., 1996; Martin et al., 1996). In such cases, the activity of the two reporter proteins must be measured in different ways (e.g., absorptiometry and luminescence photometry) or by using two substrates. In the reporter assay presented here, we used two species derived from green fluorescent protein (GFP), one (enhanced yellow fluorescent protein, (EYFP)) to measure the promotion and enhancement of gene expression, and the other (enhanced cyan fluorescent protein, (ECFP)) to normalize the transfection, and were thus able to measure the fluorescent protein signals simultaneously without any co-factor or substrates. As a result of screening of more than 140 compounds, it was found that several compounds activate RARs and/or FXR.

Materials and methods

Chemicals

Chenodeoxycholic acid was purchased from Sigma-Aldrich (St. Louis, MI, USA), and ATRA and 9CRA were from Wako (Osaka, Japan). Ginkgolic acid 17:1, 15:0, and 13:0 were purchased from Nagara Science (Gifu, Japan).

Purification and synthesis of test compounds

Ginkgolic acid 15:1 was purified from *Ginkgo biloba L.* var. *diptera* according to Morimoto et al. (1968). 2-Methyl ginkgolic acid methyl ester was prepared by methylation of the ginkgolic acid with methyl iodide and K_2CO_3 (Paul and Yeddanapalli, 1956; Begum et al., 2002). Grifolin was purified from *Albatrellus confluens* and *Albatrellus ovinus* (Ishii et al., 1988; Nukata et al., 2002). We isolated bazzanenyl caffeate from the liverwort *Bazzania fauriana* (Toyota and Asakawa, 1988). We synthesized caffeic acid phenethyl ester (CAPE), farnesyl caffeate and geranyl caffeate for acquirement in quantity. The synthesis of CAPE by coupling reactions of caffeic acid and β-phenylethyl bromide was reported by Hashimoto et al. (1988), and the details of the synthesis of farnesyl and geranyl caffeates are described below. The purity of the compounds for the bioactivation test was shown to be over 95% by ^1H and ^{13}C NMR spectra.

Synthesis of farnesyl caffeate

Twenty-five percent NaOH (2.5 ml) was added to a solution of caffeic acid (3,4-dihydroxycinnamic acid) (2.10 g) in HMPA (hexamethylphosphoric triamide) (150 ml), and the mixture was stirred for 1 h under N_2 at room temperature. A solution of farnesyl bromide (4.98 g) in HMPA (20 ml) was added dropwise for

10 min to the reaction mixture. The reaction mixture was stirred for 24 h at room temperature, and poured in ice cold H_2O (300 ml). The organic layer, which was extracted with Et_2O (200 ml × 2), was washed with brine (300 ml), dried ($MgSO_4$) and evaporated under reduced pressure to an oil (6.75 g). The oil was chromatographed on silica gel (200 g) with a gradient solvent system of $CHCl_3$–EtOAc, increasing the amount of 2% portions EtOAc stepwise to give 32 fractions. Farnesyl caffeate (1.435 g; Y. 43.2%) was obtained from 10% EtOAc-*n*-hexane eluate (Fr. 12-18) as a pure white powder. Caffeic acid (1.025 g; Y. 48.8%), the starting material, was recovered from 20% EtOAc-*n*-hexane eluate (Fr. 25-31).

Farnesyl caffeate: EI-MS: *m/z* 384 (M^+,5%), 315, 204, 180, 163 (100%), 135, 93, 69; HR-MS: *m/z* 384.2307, $C_{24}H_{32}O_4$ requires 384.2300; anal. calcd. for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39. Found: C, 74.85; H, 8.30; FT-IR (KBr) cm^{-1}: 3480 (OH), 3301 (OH), 1678 (C=O), 1600, 1278, 1183; UV (EtOH) λ_{max} nm (log ε): 333 (4.15), 303 (4.00), 248 (3.90), 220 (4.03); ^{1}H NMR (acetone-d_6): δ1.56 (3H, *s*, CH_3), 1.62 (3H, *s*, CH_3), 1.65 (3H, *s*, CH_3), 1.76 (3H, *s*, CH_3), 4.68 (1H, *d*, *J* = 7.0 Hz, H-1′), 5.12 (2H, *m*, H-6′ and H-10′), 5.41 (1H, *t*, *J* = 7.0 Hz, H-2′), 6.26 (1H, *d*, *J* = 15.9 Hz, H-β), 6.87 (1H, *d*, *J* = 8.2 Hz, H-5), 7.03 (1H, *dd*, *J* = 1.8, 8.2 Hz, H-6), 7.15 (1H, *d*, *J* = 1.8 Hz, H-2), 7.53 (1H, *d*, *J* = 15.9 Hz, H-α), 8.26 (1H, *br.s*, –OH), 8.49 (1H, *br.s*, –OH); ^{13}C NMR ((acetone-d_6): δ16.1 (*q*, CH_3), 16.4 (*q*, CH_3), 17.7 (*q*, CH_3), 25.8 (*q*, CH_3), 26.8 (*t*, CH_2), 27.4 (*t*, CH_2), 40.1 (*t*, CH_2), 40.4 (*t*, CH_2), 61.3 (*t*, CH_2), 115.1 (*d*, CH), 115.7 (*d*, CH), 116.3 (*d*, CH), 120.1 (*d*, CH), 122.4 (*d*, CH), 124.6 (*d*, CH), 125.1 (*d*, CH), 127.6 (*s*, C), 131.6 (*s*, C), 135.9 (*s*, C), 142.1 (*s*, C), 145.6 (*d*, CH), 146.3 (*s*, C), 148.7 (*s*, C), 167.3 (*s*, –COO)).

Synthesis of geranyl caffeate

Twenty-five percent NaOH (2.1 ml) was added to a solution of caffeic acid (2.00 g) in HMPA (150 ml), and the mixture was stirred for 1 h under N_2 at room temperature. A solution of geranyl bromide (3.10 g) in HMPA (20 ml) was added dropwise for 10 min to the reaction mixture. The reaction mixture was treated further as described above to afford geranyl caffeate (1.48 g; Y. 61.4%) as a white powder, and caffeic acid (0.56 g; Y. 28.0%).

Geranyl caffeate: EI-MS: *m/z* 316 (M^+, 10%), 247, 180, 163 (100%), 136, 69; HR-MS: *m/z* 316.1682, $C_{19}H_{24}O_4$ requires 316.1674; anal. calcd. for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 72.01; H, 7.68; FT-IR (KBr) cm^{-1}: 3483 (OH), 3295 (OH), 1678 (C=O), 1599, 1278, 1183; UV (EtOH) λ_{max} nm (log ε): 334 (4.16), 302 (4.05), 249 (3.93), 222 (4.01); ^{1}H NMR (acetone-d_6): δ1.60 (3H, *s*, CH_3), 1.66 (3H, *s*, CH_3), 1.75 (3H, *s*, CH_3), 4.68 (1H, *d*, *J* = 7.0 Hz, H-1′), 5.12 (1H, *t*, *J* = 7.0 Hz, H-6′), 5.40 (1H, *t*, *J* = 7.0 Hz, H-2′), 6.27 (1H, *d*, *J* = 15.9 Hz, H-β), 6.87 (1H, *d*, *J* = 8.2 Hz, H-5), 7.03 (1H, *dd*, *J* = 2.0, 8.2 Hz, H-6), 7.16 (1H, *d*, *J* = 2.0 Hz, H-2), 7.55 (1H, *d*, *J* = 15.9 Hz, H-α), 8.28 (1H, *br.s*, –OH), 8.50 (1H, *br.s*, –OH); ^{13}C NMR ((acetone-d_6): δ16.4 (*q*, CH_3), 17.7(*q*, CH_3), 25.8 (*q*, CH_3), 27.0 (*t*, CH_2), 40.1 (*t*, CH_2), 61.3 (*t*, CH_2), 115.1 (*d*, CH), 115.6 (*d*, CH), 116.3 (*d*, CH), 120.0 (*d*, CH), 122.4 (*d*, CH), 124.6 (*d*, CH), 127.6 (*s*, C), 132.0 (*s*, C), 142.1 (*s*, C), 145.6 (*d*, CH), 146.3 (*s*, C), 148.7 (*s*, C), 167.3 (*s*, –COO)).

Plasmid construction

Plasmids were constructed for the expression of RXRα, FXR and RARs. The ORF regions of human RXRα, human FXR, mouse RARα1, mouse RARβ2, and mouse RARγ1 (accession numbers X52773, U68233, X57528, S56660, X15848) were amplified by PCR and inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), respectively. For reporter plasmids, the luciferase region of the pGL3-Control Vector (Promega, Madison, WI, USA) was replaced with the EYFP fragment of pEYFP-N1 or the ECFP fragment of pECFP-N1 (Clontech, Palo Alto, CA, USA) using *Nco*I and *Xba*I sites. Subsequently, the simian virus 40 (SV40) early promoter was cut out with *Bgl*II and *Hin*dIII, and replaced with the thymidine kinase (TK) promoter of the pRL-TK vector (Promega) or one of several other promoters (the 3′ region of the TK promoter, the cytomegalovirus (CMV) promoter, or the minimal CMV promoter and the 3′ region of the CMV promoter (201 and 265 bp)) amplified using the following PCR primers:

5′-ggagatctggccccgcccagcgtcttgtc-3′ and 5′-ggaagcttgcggcacgctgttgacgctgttaagcgggtcgctgcaggg-3′ (3′ region of the TK promoter);
5′-ccagatcttagttattaatagtaatcaattacggggtc-3′ and 5′-ccaagcttgatctgacggttcactaaaccagc-3′ (CMV promoter);
5′-ccagatcttaggcgtgtacggtgggagg-3′ and 5′-ccaagcttaggctggatcggtcccggtg-3′ (minimal CMV promoter);
5′-ccagatcttgggagtttgttttggcacc-3′ and reverse primer of CMV promoter (CMV 201); and
5′-ccagatcttcaatgggcgtggatagcgg-3′ and reverse primer of CMV promoter (CMV265).

Double-stranded oligonucleotides containing HREs (RXRE, RARE and FXRE; shown in Fig. 1B) were ligated into the upstream region of these promoters using *Mlu*I and *Bgl*II sites. The sequences of the constructed plasmids were confirmed by sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

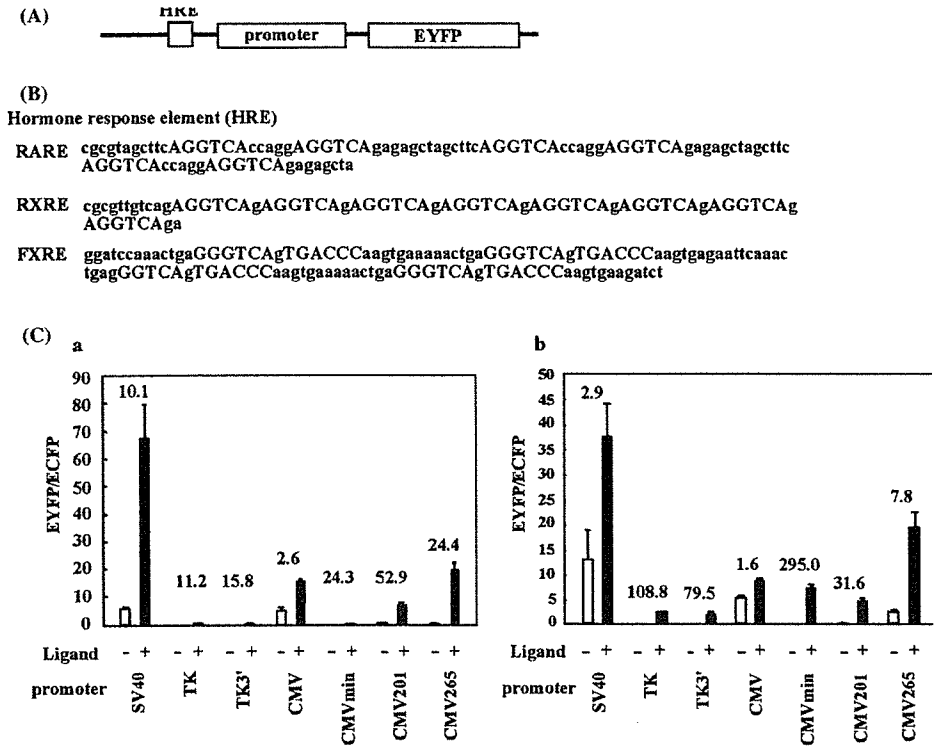

Fig. 1. Reporter plasmids for the assay of nuclear receptors. (A) Model of the constructed reporter plasmids. (B) The sequences for HREs of RAR, RXR and FXR (RARE, RXRE, and FXRE). (C) Effect of different promoters on the reporter assay. Seven species of promoter were employed in the reporter plasmid containing the HRE and EYFP genes. The activations of RARα (a) and FXR (b) are shown. The transfected cells were treated with ligands (black bar), 1 μM of ATRA for the RAR reporter assay or 100 μM of CDCA for FXR, or DMSO as a vehicle (white bar). The vertical axis indicates the ratio of fluorescence of EYFP (signal) to ECFP (internal control). The fold response relative to vehicle-treated cells is shown above the bars. Data are shown as the means + SD derived from six experiments.

Cotransfection and reporter assay

A monkey kidney cell line, COS-7, was kept in DMEM with 10% FBS. Transfections were performed using an Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. The ratio of the reporter plasmid, receptor expression plasmids (for example, the RARα and RXRα expression plasmids for assay of RARα ligands) and the internal control plasmid was 4:1:1:1. The culture medium was replaced with DMEM without phenol red (Gibco BRL, Gaithersburg, MD) supplemented with 10% charcoal-treated FBS (Hyclone, Logan, UT) when the transfections were performed. At 15 h after transfections, the cells were treated with trypsin/penicillin reagent and divided among wells of a black, 96-well plate with 100 μl of the culture medium. At 6 h after division among wells, the cells were treated with chemicals. After a 40-h incubation, the medium was eliminated by decantation, the cells were washed twice with PBS, and the wells were filled with 200 μl PBS. Fluorescence was detected using a microplate reader (ARVO; Perkin Elmer, Fremont, CA, USA). The fluorescence of EYFP was detected with an excitation filter of 485 nm and an emission filter of 545 nm, and that of ECFP was detected with filters of 420 and 486 nm (Perkin Elmer), respectively. The autofluorescence in COS-7 cells was subtracted from each of the detected fluorescences, and the EYFP/ECFP ratio was calculated using the resulting values.

Results

Reporter assay system

In the present reporter assay, EYFP and ECFP were selected as a reporter protein and an internal control for normalization of transfection, respectively. These two fluorescent proteins were chosen, because the peaks of their excitation and emission wavelengths are sufficiently different (a difference of 80 and 50 nm,

respectively) so that they can be detected simultaneously without cross-detection. The considerable cross-detection between EYFP and ECFP could be prevented using a set of optical filters (see Materials and methods). The EYFP/ECFP ratio was calculated after the autofluorescence of COS-7 cells was subtracted from the fluorescence intensities of EYFP and ECFP, because the autofluorescence was not negligible.

The reporter plasmids were constructed as shown in Fig. 1A. As HREs for FXR (FXR-RXR heterodimer), RAR (RAR-RXR heterodimer) and RXR (RXR homodimer), the fragments shown in Fig. 1B were used. In order to amplify signals, we employed three copies of DR5 (direct repeat with 5 bp of spacing) and four copies of DR1 as RAR and RXR response elements (RARE and RXRE). For the FXR response element (FXRE), four copies of the response element (inverted repeat) existing in the upstream region of the phospholipid transfer protein (PLTP) gene were employed. The tandem repeats in HREs elevated the response to a sufficient degree to detect the chemicals that activated the receptor. Then, an appropriate promoter for enhancing the fluorescent signal while retaining the response to the chemicals was selected from among seven promoters (Fig. 1C). Since the SV40 or CMV promoter caused a high fluorescence intensity with or without ligands, the responses to the ligands were not strong. The response of the RAR reporter plasmid with the SV40 promoter was about ten-fold. However, the apparent rate of the response was enhanced by interference of the expression of ECFP by the expression of EYFP, because the same promoter was employed for the reporter plasmid and the internal control plasmid. Therefore, the rate did not reflect a real response, and had a large SD. The TK promoter, the 3′ region of the TK promoter and the minimal CMV promoter caused strong responses, but the expression in the control plasmid was too low for quantitative measurement. The expression of reporter proteins with the 3′ region of the CMV promoter was higher than that with TK or the minimal CMV promoter, maintaining the induction rate by the ligands. Based on a comparison between the 3′ regions of the CMV promoters, we selected the CMV201 (201 bp of the CMV promoter) promoter for use in the experiments below, since the response of CMV201 was stronger than that of CMV265.

In addition to the promoter for reporter plasmids, the promoter for the internal control plasmid and the expression plasmids of nuclear receptors were examined in order to establish an appropriate assay system of the nuclear receptor ligands. When the SV40 promoter was employed for the expression of ECFP in the internal control plasmid, the SV40 promoter for nuclear receptor expression interfered with the expression of ECFP (data not shown). Therefore, the CMV promoter was employed for nuclear receptor expression plasmids. Finally, we established the following plasmid set as the reporter assay system: a reporter plasmid containing the EYFP gene, whose expression was regulated by the HRE and CMV201 promoter; an internal control plasmid containing the ECFP gene expressed by the SV40 promoter; and the expression plasmid of the nuclear receptor containing each nuclear receptor gene expressed by the CMV promoter.

Fig. 2A shows the response to typical agonists for FXR, RARs and RXRα in the screening system. For screening of RAR ligands, three subtypes of RARs (RARα1, RARβ2, RARγ1) were expressed in the cells independently. Although endogenous RARs co-exists in the cell, the preference for the subtype of compounds could be detected. Fig. 2B and C show the dose-dependence of the assay system of FXR and RAR ligands, respectively. RARs were activated by 100 pM of ATRA. ED_{50} values were estimated to be about 1–10 nM for RARα and 0.1–1 nM for RARβ and RARγ (only the result of RARα is shown in Fig. 2B). On the other hand, activation of FXR was seen in 3–10 μM CDCA and greater activation was observed at 100 μM CDCA (Fig. 2C). These dose-dependent response patterns were comparable to those reported previously (Brand et al., 1988; Parks et al., 1999), indicating that these assays could be used for quantitative measurement of the activation by ligands. The established method of the reporter assay was described in Materials and methods.

Screening of a novel ligand for nuclear receptors

Using the established screening system, we found some natural compounds and their derivatives which acted as agonists for RARs and FXR. In the screening, there was a possibility that unexpected factors may have changed the signal responses (in the present assay system, the transcriptional efficiency may be changed irrespective of the nuclear receptor, the tested chemicals may have their own fluorescence, and so forth). Therefore, another reporter plasmid without HRE was also constructed to eliminate these unexpected factors. As this plasmid was used in place of the reporter plasmid, the compounds that regulated the expression of EYFP without HRE were eliminated. Some results of the response for each nuclear receptor are shown in Fig. 3 (RAR, upper panel; FXR, middle panel; control, lower panel). The results for RARβ are presented as representative of those for RARs. Ten millimolar of each compound referring to the stock solution in DMSO was added to the culture medium of the transfected COS-7 cells at a final concentration of 30 μM (Fig. 3, Nos. 1–26). Compound Nos. 27, 28, and 29 were 3 μM ATRA, 30 μM CDCA, and vehicle, respectively. ATRA also slightly activated the FXR-RXR heterodimer, due

to the activation of RXR. Although, for example, Nos. 16, 18, 19, and 25 enhanced the relative EYFP/ECFP ratio, these compounds also enhanced the control that was used with the reporter plasmid without HRE. Thus it was concluded that these compounds were not ligands for the nuclear receptors.

As a result of screening more than 140 compounds (a part of the results is shown in Fig. 3), five compounds were found as ligands for the nuclear receptors. CAPE (compound No. 20 in Fig. 3), geranyl caffeate (No. 21), and farnesyl caffeate (not shown in Fig. 3) were found to be RAR agonists. Ginkgolic acid 15:1 (No. 12), geranyl caffeate (No. 21), and grifolin (No. 26) were found to be FXR agonists.

The structures of the caffeic acid derivatives tested in the screening are shown in Fig. 4A. CAPE, known as an active compound of propolis from honeybee hives, was synthesized from caffeic acid and β-phenylethyl bromide and other caffeic acids were purified and synthesized as described in Materials and methods. Three of these compounds (i.e., all of those tested except for bazzanenyl caffeate) activated RARs (Fig. 4B). The cells treated with over 30 μM of these compounds were removed from wells by washing of the reporter assay, because these compounds were toxic to the cell. Therefore, the results shown are for a reporter assay conducted using lower concentrations. Although the activation of RARs could be hardly detected by a low concentration of caffeic acid-derivatives, the activation by the compounds 10–30 μM was comparable to maximum activation by ATRA. As shown in Fig. 4B, CAPE activated RARβ to a greater degree than RARα or RARγ.

As FXR agonists, geranyl caffeate, ginkgolic acid 15:1 and grifolin were found. Geranyl caffeate, the RAR agonist, highly activated FXR (Fig. 3, No. 21), but the activation of the RXR homodimer was not detected (data not shown). It could not be determined whether or not farnesyl caffeate, a compound similar to geranyl caffeate, activated FXR, because 30 μM of these compounds showed toxicity for cells. The structures of ginkgolic acids and grifolin are shown in Fig. 5A. It has been reported that ginkgolic acid 15:1 was present in ginkgolic leaves (Ahlemeyer et al., 2001), and grifolin in mushrooms (Hirata and Nakanishi, 1949; Sugiyama et al., 1992). The activations of FXR by ginkgolic acid 15:1 and geranyl caffeate were comparable to that by CDCA,

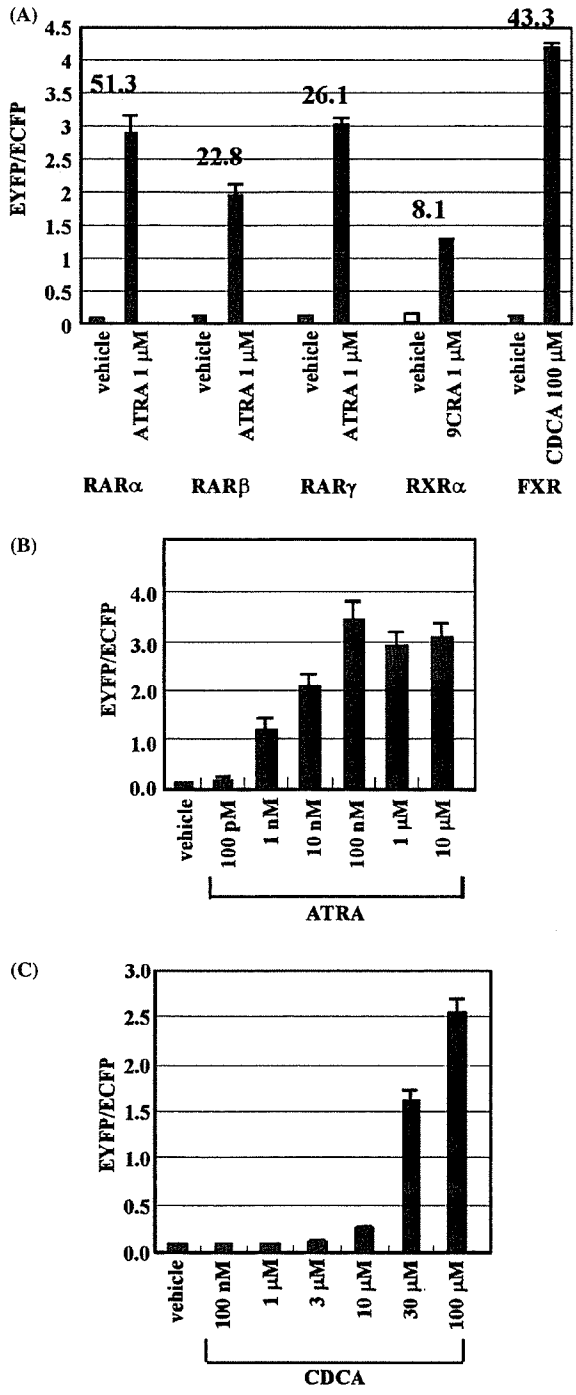

Fig. 2. Response in the reporter expression. (A) The responses in the reporter assay system by typical agonists for RAR, RXR, and FXR. COS-7 cells were transfected with an appropriate set of the plasmids (e.g. for assay of RARα ligand, the reporter plasmid containing RARE, the expression plasmids of RARα and RXRα and the internal control plasmid; for assay of RXRα ligand, the reporter plasmid containing RXRE, the RXRα expression plasmid, and the internal control plasmid). The transfected cells were treated with 1 μM of ATRA, 1 μM of 9CRA, or 100 μM of CDCA as ligands (black bar), or DMSO as a vehicle (white bar). The response rate is shown above the bars. Data are shown as the means + SD derived from three experiments. (B), (C) Dose–response analyses of ATRA and CDCA on the reporter assay of RAR and FXR. Data are shown as the means + SD derived from four experiments.

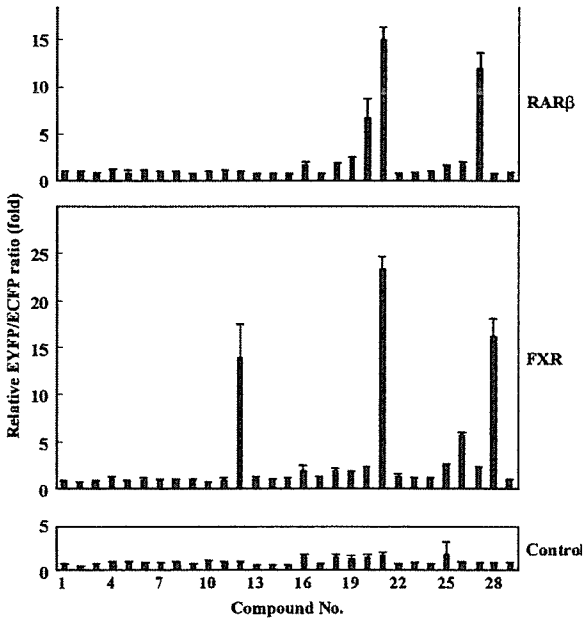

Fig. 3. Screening of ligands for RAR and FXR. COS-7 cells were transfected with the reporter plasmid, the receptor expression plasmid, and the internal control plasmid as shown in Fig. 2. The cells were treated with 30 μM of each compound. The results of the screening for RAR are shown in the upper panel, those for FXR in the middle panel, and those for the control (no HRE) in the lower panel. The results for RARβ are presented as representative of those for RARs (No. 1, hydrangeic acid; No. 2, ethyl 4′-ethylhydrangenate; No. 3, hydrangenol; No. 4, 8,3′-dimethoxyphylodulcin; No. 5, macrophyllaside A; No. 6, yashabashiletodiol A; No. 7, lycogarbin C; No. 8, lycogarbin A; No. 9, polygodial; No. 10, sacculatal; No. 11, ptychantin A; No. 12, ginkgolic acid 15:1; No. 13, 2-methyl ginkgolic acid methyl ester; No. 14, bilobal dimethyl ether; No. 15, 3-tridecanyl-*m*-cresol; No. 16, [11]-cytochalasa-6(12),13-diene-1,21-dione-7,18-dihydroxy-16,18-dimethyl-19-methoxy-10-phenyl-(7*S**,13*E*,16*S**,18*S**,19*R**); No. 17, hispidin; No. 18, costunolide, No. 19, beta-cyclocostanolide; No. 20, caffeic acid phenethyl ester; No. 21, geranyl caffeate; No. 22, atroctylon).

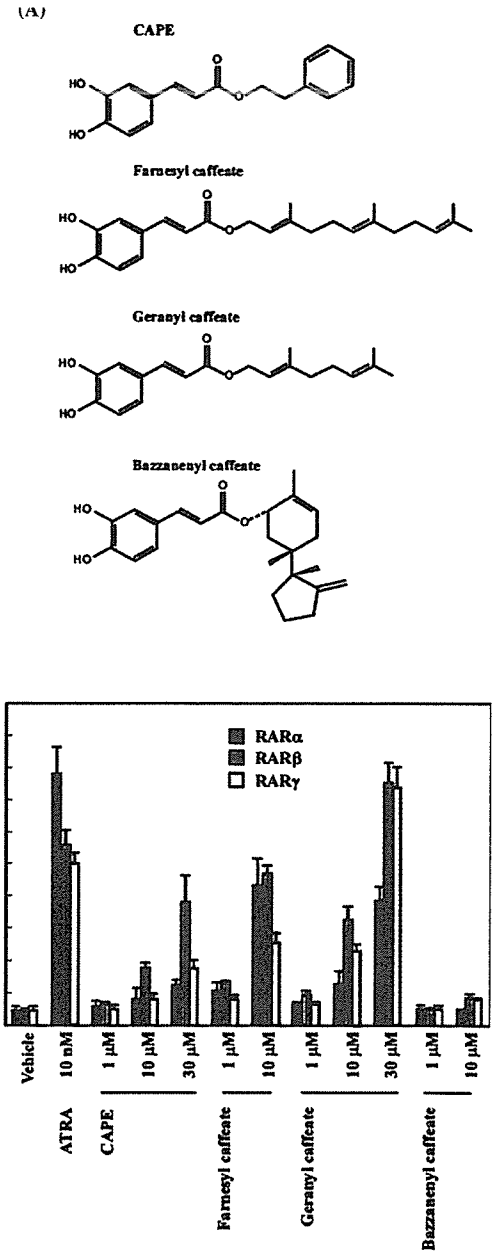

Fig. 4. Ligands for RARs. (A) The structures of caffeic acid derivatives tested in the screening. (B) Response in the RAR reporter assay. The responses in the COS-7 cells expressing RARα, RARβ or RARγ are indicated by black, gray, and white bars, respectively. Data are expressed as the fold response relative to vehicle (0.1% DMSO)-treated cells and are shown as the means + SD derived from four experiments.

the most potent endogenous bile acid. Ginkgolic acids 17:1, 15:0 and 13:0 (described in Fig. 5A) were also investigated as the other ginkgolic acids of ginkgo leaves (Fig. 5B). Ginkgolic acid 17:1 activated FXR more strongly than did 15:1, and ginkgolic acids with an alkyl chain (13:0, 15:0) activated FXR at concentrations of more than 20 μM. It seemed that the double bond and length of the carbon chain had an influence on FXR activation. Moreover, the structures except for the carbon chain were also important for FXR activation, because the methylated compound of ginkgolic acid 15:1 (2-methyl ginkgolic acid methyl ester, Fig. 5A) had no potency for FXR activation (Fig. 3, No. 13).

Discussion

To discover ligands for the nuclear receptors, we developed a battery of reporter assay systems

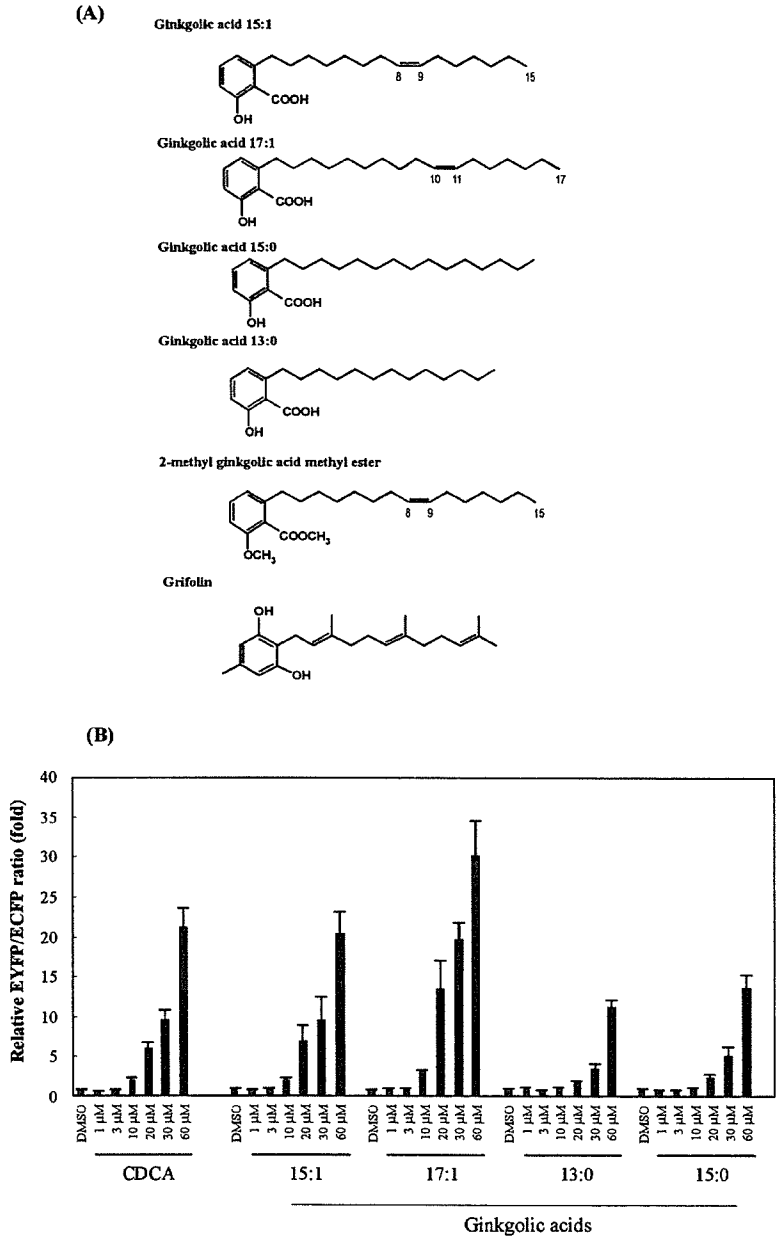

Fig. 5. Ligands for FXR. (A) The structure of candidates for FXR agonists and their related compounds (2-methyl ginkgolic acid methyl ester). (B) The activation of FXR by ginkgolic acids. COS-7 cells were transfected with the reporter plasmid containing FXRE, the expression plasmids of FXR and RXRα and the internal control plasmid. The transfected cells were treated with each compound. Data are shown as the means + SD derived from four experiments.

incorporating the advantages of fluorescent proteins. The disadvantage of GFP (low sensitivity) could be overcome by modifications. The present screening system using fluorescent proteins has clear merits of a high efficiency, convenience and low cost, because the two fluorescent signals can be measured simultaneously without addition of any co-factors. Moreover, the fluorescent signal was stable for more than 2 h after the wash. Considering these merits, this reporter assay system with fluorescent proteins might be advantageous for automatic high-throughput screening. If the expression of the fluorescent protein can be increased, the measurement of fluorescence can be carried out in culture medium, and the signal can be measured by time-course without any treatment. Moreover, the use of three fluorescent proteins (for example, DsRed with EYFP and ECFP) would enable us to carry out more efficient measurement.

Using this assay system, several compounds that induce expression of the reporter gene for RARs and/or FXR were identified. These compounds were described as ligands in this report, although there is a possibility that these compounds are metabolized and their metabolites bind to the receptors as ligands.

Three new ligands for RARs were identified: CAPE, geranyl caffeate, and farnesyl caffeate. The whole structure of these compounds may be needed for RAR-activation, because caffeic acid, a constituent compound of the compounds, did not activate RARs (data not shown). CAPE has been reported to have antioxidant, antiviral, anti-inflammatory and immunomodulatory activities (Grunberger et al., 1988), and has also been shown to inhibit the growth of different types of oncogene-transformed cells and to induce apoptosis (Grunberger et al., 1988; Burke et al., 1995; Su et al., 1994; Watabe et al., 2004). Since RARs have been reported to mediate many biological processes, it is possible that some of the diverse activities are due to their binding to RARs. Since geranyl and farnesyl caffeate have also been reported to exert antioxidant effects and to inhibit the growth of cancer cells (Inoue et al., 2004), the three compounds may suppress the growth of cancer by at least two pathways: induction of RAR and antioxidant effects. Considering its preferential activation of RARβ (Fig. 4B), CAPE may inhibit cancer (e.g., lung cancer) growth more selectively without substantial toxicity, such as the triglyceride elevation associated with RARα, and the skin, bone and teratogenic toxicity associated with RARγ Thus, especially CAPE could be assumed to be a seed for the development of an anti-cancer drug.

We also found that two natural compounds, ginkgolic acids and grifolin, activated FXR. Grifolin was first isolated as an antibiotic constituent of a mushroom, *Grifola confluens* (Hirata and Nakanishi, 1949). In 1992, it was reported that grifolin decreased liver cholesterol content, plasma total cholesterol levels, and plasma (very low-density lipoprotein (VLDL) + low-density lipoprotein (LDL)) cholesterol levels, and increased plasma high-density lipoprotein (HDL) cholesterol and plasma triglyceride levels (Sugiyama et al., 1992). It has been suggested that the effect of grifolin might be elicited, at least in part, by the augmented excretion of cholesterol into the faces (Sugiyama et al., 1994). On the other hand, FXR controls the expression of critical genes in bile acid and cholesterol homeostasis. In fact, FXR-null mice show elevated serum cholesterol and triglyceride levels (Sinal et al., 2000), and an FXR agonist has been shown to reduce serum triglyceride levels (Maloney et al., 2000). Moreover, FXR induces the expression of the gene of PLTP, which plays a role in HDL metabolism (Urizar et al., 2000). It seems that the cholesterol-lowering and HDL-cholesterol-increasing effects of grifolin are related to FXR activation, although grifolin's enhancement of triglyceride production was not consistent with its down-regulation of FXR agonists.

The FXR agonists found in this study are all non-steroidal compounds, whereas the well-known ligand of FXR, bile acid, is a steroidal one. The common characteristic of the structure of the ligands is their long carbon chains (i.e., geranyl, farnesyl and pentadecenyl), and farnesol has been shown to be a FXR ligand (Forman et al., 1995). However, aspects of the structures other than the carbon chains also appear to be important for FXR activation, because geraniol, a constituent compound of geranyl caffeate, has been reported not to activate FXR (Forman et al., 1995), and the methylated compound of ginkgolic acid 15:1 had no potency for FXR activation in the present study.

Several compounds, such as TTNPB, GW4064, Farnesoid, Forskolin, Fexaramine, AGN29 and AGN31, have been reported as non-steroidal agonists (Maloney et al., 2000; Howard et al., 2000; Downes et al., 2003; Dussault et al., 2003). The non-steroidal ligands may be important tools for studying the pharmacology of the receptor, because they may not have the property of bile acids and are not metabolized to form harmful lithocholic acid (Fischer et al., 1996; Javitt, 1966). In the present study, ginkgolic acids and geranyl caffeate strongly activated FXR, and both had structures quite different from bile acids, so that they could be good tools in this sense. Moreover, the importance of identifying gene-selective modulators that regulate a subset of FXR-specific genes as therapeutic agents has been recognized (Cui et al., 2003; Dussault et al., 2003). The gene-selective modulators of estrogen receptor, selective estrogen receptor modulators (SERMs), have been well studied (reviewed in McDonnell et al., 2002), and some compounds with a structure divergent from that of estrogen have been identified and applied to therapies of breast cancer and osteoporosis. The non-steroidal compounds could also be good tools for studying the selective response of FXR target genes.

In this report, we developed a new method for screening novel nuclear receptor agonists, and used it to identify new candidate ligands for FXR and RARs. We expect that these new ligands will be good pharmacological tools. Since the compound whose structure is much different from bile acids is expected to possess a specific effect as a ligand, we continue to screen various ligands from natural compounds with a wide variety of structures.

Acknowledgements

This work was supported by a grant-in-aid (MF-16) from the Pharmaceuticals and Medical Device Agency, a grant-in-aid for Research on Health Sciences Focusing

on Drug Innovation from the Japan Health Science Foundation, and a grant-in-aid for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.

References

Ahlemeyer, B., Selke, D., Schaper, C., Klumpp, S., Krieglstein, J., 2001. Ginkgolic acids induce neuronal death and activate protein phosphatase type-2C. Eur. J. Pharmacol. 430, 1–7.

Begum, P., Hashidoko, Y., Islam, M.T., Ogawa, Y., Tahara, S., 2002. Zoosporicidal activities of anacardic acids against *Aphanomyces cochlioides*. Z. Naturforsch. [C] 57, 874–882.

Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P., Dejean, A., 1988. Identification of a second human retinoic acid receptor. Nature 332, 850–853.

Burke Jr., T.R., Fesen, M.R., Mazumder, A., Wang, J., Carothers, A.M., Grunberger, D., Driscoll, J., Kohn, K., Pommier, Y., 1995. Hydroxylated aromatic inhibitors of HIV-1 intergrase. J. Med. Chem. 38, 4171–4178.

Cui, J., Huang, L., Zhao, A., Lew, J.-L., Yu, J., Sahoo, S., Meinke, P.T., Royo, I., Peláez, F., Wright, S.D., 2003. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. J. Biol. Chem. 278, 10214–10220.

Downes, M., Verdecia, M.A., Roecker, A.J., Hughes, R., Hogenesch, J.B., Kast-Woelbern, H.R., Bowman, M.E., Ferrer, J.-L., Anisfeld, A.M., Edwards, P.A., Rosenfeld, J.M., Alvarez, J.G.A., Noel, J.P., Nicolaou, K.C., Evans, R.M., 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. Mol. Cell 11, 1079–1092.

Dussault, I., Beard, R., Lin, M., Hollister, K., Chen, J., Xiao, J.H., Chandraratna, R., Forman, B.M., 2003. Identification of gene-selective modulators of the bile acid receptor FXR. J. Biol. Chem. 278, 7027–7033.

Fischer, S., Beuers, U., Spengler, U., Zwiebel, F.M., Koebe, H.G., 1996. Hepatic levels of bile acids in end-stage chronic cholestatic liver disease. Clin. Chim. Acta 251, 173–186.

Fisher, G.J., Voorhees, J.J., 1996. Molecular mechanisms of retinoid actions in skin. FASEB J. 10, 1002–1013.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T., Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M., Weinberger, C., 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. Cell 81, 687–693.

Grunberger, D., Banerjee, R., Eisinger, K., Oltz, E.M., Efros, L., Caldwell, M., Estevez, V., Nakanishi, K., 1988. Preferential cytotoxity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 44, 230–232.

Hansen, L.A., Sigman, C.C., Andreola, F., Ross, S.A., Kelloff, G.J., De Luca, L.M., 2000. Retinoids in chemoprevention and differentiation therapy. Carcinogenesis 21, 1271–1279.

Hashimoto, T., Tori, M., Asakawa, Y., Wollenweber, E., 1988. Synthesis of two allergenic constituents of propolis and poplar bud excretion. Z. Naturforsch. [C] 43, 470–472.

Hirata, Y., Nakanishi, K., 1949. Grifolin, an antibiotic from a basidiomycete. J. Biol. Chem. 184, 135–143.

Howard, W.R., Pospisil, J.A., Njolito, E., Noonan, D.J., 2000. Catabolites of cholesterol synthesis pathways and forskolin as activators of the farnesoid X-activated nuclear receptor. Toxicol. Appl. Pharmacol. 163, 195–202.

Inoue, K., Nishitani, N., Tanabe, A., Yamanaka, H., Hashimoto, T., Asakawa, Y., Fujiki, H., 2004. Significance of structural specificity in the antioxidant activity of hydrangenol and caffeic acid-derivatives. Abstract for 124th Congress on Nihon yakugakukai, Osaka, Japan, vol. 2, p. 173.

Ishii, N., Takahashi, A., Kusano, G., Nozoe, S., 1988. Studies on the constituents of *Polyporus dispansus* and *P. confluens*. Chem. Pharm. Bull. 36, 2918–2924.

Javitt, N.B., 1966. Cholestasis in rats induced by taurolithocholate. Nature 210, 1262–1263.

Khorasanizadeh, S., Rastinejad, F., 2001. Nuclear–receptor interactions on DNA-response elements. Trends Biochem. Sci. 26, 384–390.

Krust, A., Kastner, P.H., Petkovich, M., Zelent, A., Chambon, P., 1989. A third human retinoic acid receptor, hRAR-γ. Proc. Natl. Acad. Sci. USA 86, 5310–5314.

Liu, Y., Binz, J., Numerick, M.J., Dennis, S., Luo, G., Desai, B., MacKenzie, K.I., Mansfield, T.A., Kliewer, S.A., Goodwin, B., Jones, S.A., 2003. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J. Clin. Invest. 112, 1678–1687.

Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J., Shan, B., 1999. Identification of a nuclear receptor for bile acids. Science 284, 1362–1365.

Maloney, P.R., Parks, D.J., Haffner, C.D., Fivush, A.M., Chandra, G., Plunket, K.D., Creech, K.L., Moore, L.B., Wilson, J.G., Lewis, M.C., Jones, S.A., Willson, T.M., 2000. Identification of a chemical tool for the orphan nuclear receptor FXR. J. Med. Chem. 43, 2971–2974.

Martin, C.S., Wight, P.A., Dobretsova, A., Bronstein, I., 1996. Dual luminescence-based reporter gene assay for luciferase and β-galactosidase. Biotechniques 21, 520–524.

McDonnell, D.P., Connor, C.E., Wijayaratne, A., Chang, C.Y., Norris, J.D., 2002. Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. Recent Prog. Horm. Res. 57, 295–316.

Morimoto, H., Kawamatsu, Y., Sugihara, H., 1968. Stereostructure of toxin from the fruit of *Ginkgo biloba L.* Chem. Pharm. Bull. 16, 2282–2286.

Nukata, M., Hashimoto, T., Yamamoto, I., Iwasaki, N., Tanaka, M., Asakawa, Y., 2002. Neogrifolin derivatives possessing anti-oxidative activity from the mushroom *Albatrellus ovinus*. Phytochemistry 59, 731–737.

Parks, D.J., Blanchard, S.G., Bledsoe, R.K., Chandra, G., Consler, T.G., Kliewer, S.A., Stimmel, J.B., Willson, T.M., Zavacki, A.M., Moore, D.D., Lehmann, J.M., 1999. Bile acids: natural ligands for an orphan nuclear receptor. Science 284, 1365–1368.

Paul, V.J., Yeddanapalli, L.M., 1956. On the olefinic nature of anacardic acid from Indian cashew nut shell liquid. J. Am. Chem. Soc. 78, 5675–5678.

Petkovich, M., Brand, N.J., Krust, A., Chambon, P., 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330, 444–450.

Sherf, B.A., Navarro, S.L., Hannah, R.R., Wood, K.V., 1996. Dual-luciferase™ reporter assay: an advanced co-reporter technology integrating firefly and *Renilla* luciferase assays. Promega Notes 57, 2.

Sinal, C.J., Tohkin, M., Miyata, M., Ward, J.M., Lambert, G., Gonzalez, F.J., 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 102, 731–744.

Su, Z.-Z., Lin, J., Grunberger, D., Fisher, P.B., 1994. Growth suppression and toxicity induced by caffeic acid phenethyl ester (CAPE) in type 5 adenovirus-transformed rat embryo cells correlate directly with transformation progression. Cancer Res. 54, 1865–1870.

Sugiyama, K., Kawagishi, H., Tanaka, A., Saeki, S., Yoshida, S., Sakamoto, H., Ishiguro, Y., 1992. Isolation of plasma cholesterol-lowering components from Ningyotake (*Polyporus confluens*) mushroom. J. Nutr. Sci. Vitaminol. 38, 335–342.

Sugiyama, K., Tanaka, A., Kawagishi, H., Ojima, F., Sakamoto, H., Ishiguro, Y., 1994. Hypocholesterolemic action of dietary grifolin on rats fed with a high-cholesterol diet. Biosci. Biotechnol. Biochem. 58, 211–212.

Toyota, M., Asakawa, Y., 1988. Sesquiterpenoids from the liverwort *Bazzania fauriana*. Phytochemistry 27, 2155–2159.

Urizar, N.L., Dowhan, D.H., Moore, D.D., 2000. The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. J. Biol. Chem. 275, 39313–39317.

Wang, H., Chen, J., Hollister, K., Sowers, L.C., Forman, B.M., 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol. Cell 3, 543–553.

Watabe, M., Hishikawa, K., Takayanagi, A., Shimizu, N., Nakaki, T., 2004. Caffeic acid phenethyl ester induces apoptosis by inhibition of NFκB and activation of Fas in human breast cancer MCF-7 cells. J. Biol. Chem. 279, 6017–6026.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1103 (2006) 296–306
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

N-linked oligosaccharide analysis of rat brain Thy-1 by liquid chromatography with graphitized carbon column/ion trap-Fourier transform ion cyclotron resonance mass spectrometry in positive and negative ion modes

Satsuki Itoh [a], Nana Kawasaki [a,b,*], Noritaka Hashii [b], Akira Harazono [a], Yukari Matsuishi [a], Takao Hayakawa [c], Toru Kawanishi [a]

[a] *Division of Biological Chemistry and Biologicals, National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *CREST, Japan Science and Technology Agency (JST), Japan*
[c] *Pharmaceutical and Medical Devices Agency, 3-3-2 Kasumigaseki, Chiyoda-ku, Tokyo 100-0013, Japan*

Received 25 July 2005; received in revised form 10 November 2005; accepted 14 November 2005
Available online 20 December 2005

Abstract

We have previously described the site-specific glycosylation analysis of rat brain Thy-1 by LC/multistage tandem mass spectrometry (MS^n) using proteinase-digested Thy-1. In the present study, detailed structures of oligosaccharides released from Thy-1 were elucidated by mass spectrometric oligosaccharide profiling using LC/MS with a graphitized carbon column (GCC–LC/MS). First, using model oligosaccharides, we improved the oligosaccharide profiling by ion trap mass spectrometry (IT–MS) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR–MS). Sequential scanning of a full MS^1 scan with FT–ICR–MS followed by data-dependent MS^n with IT–MS in positive ion mode, and a subsequent full MS^1 scan with FT–ICR–MS followed by data-dependent MS^n with IT–MS in negative ion mode enabled the monosaccharide composition analysis as well as profiling and sequencing of both neutral and acidic oligosaccharides in a single analysis. The improved oligosaccharide profiling was applied to elucidation of N-linked oligosaccharides from Thy-1 isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was demonstrated that Thy-1 possesses a significant variety of N-linked oligosaccharides, including Lewis a/x, Lewis b/y, and disialylated structure as a partial structure. Our method could be applicable to analysis of a small abundance of glycoproteins, and could become a powerful tool for glycoproteomics.

Keywords: Mass spectrometric oligosaccharide profiling; Graphitized carbon column; Ion trap mass spectrometry; Fourier transform ion cyclotron resonance mass spectrometry; Data-dependent MS^n; Thy-1

1. Introduction

Glycosylation is one of the most abundant post-translational modifications of proteins [1]. It is already known that glycosylation influences the biological functions as well as the physicochemical properties of proteins, i.e., folding, solubility, aggregation, and stability. A number of reports have noted a positive relationship between a change in glycosylation and development, aging, and certain diseases [2–4]. Elucidation of structural detail in oligosaccharides is necessary to clarify the biological properties of glycoproteins.

MS is now a powerful tool for structural analysis of glycoproteins. There are two major mass spectrometric approaches to the structural analysis of glycoproteins, i.e., MS of glycopeptides [5–7] and of oligosaccharides [8–13]. For oligosaccharide sequencing, tandem mass spectrometry as well as exoglycosidase digestions in conjuction with MS is recognized as an effective means of oligosaccharide sequencing [14–16]. Mass spectrometric peptide/glycopeptide mapping by LC coupled with tandem mass spectrometry (LC/MS/MS) is effective for the determination of glycosylation sites and the analysis of site-specific heterogeneity [17–22]. However, structural detail in

* Corresponding author. Division of Biological Chemistry and Biologicals, National Institute of Health Science, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 3 3700 1141; fax: +81 3 3707 6950.
E-mail address: nana@nihs.go.jp (N. Kawasaki).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.11.043

oligosaccharides is not always available by product ion spectra of glycopeptides, as many of the precursor ions consist of uniform peptides carrying different oligosaccharides with identical *m/z* values. LC/MS/MS of glycopeptides has limitations for the structural analysis of carbohydrates due to the difficulty of isolating of glycopeptide isomers. Mass spectrometric oligosaccharide profiling through the separation of isomers by LC can supply the structural detail of each oligosaccharide although it cannot provide information regarding glycosylation sites and site-specific glycosylation [23–29]. MS of both glycopeptides and oligosaccharides is needed for glycosylation analysis of a glycoprotein [30].

Thy-1 is a cell adhesion molecule that belongs to the immunoglobulin superfamily and is attached to the cell membrane via a glycosylphosphatidylinositol (GPI)-anchor. We recently, studied the glycosylation of Thy-1 in rat brain by mass spectrometric peptide/glycopeptide mapping, and demonstrated that Thy-1 possesses various *N*-glycans at Asn23, 74, and 98 [31]. The monosaccharide composition of *N*-glycan at each glycosylation site was estimated by masses of molecular ions; however, structural detail regarding some of the oligosaccharides could not be elucidated by MS^n since many glycopeptides with identical *m/z* values contained several oligosaccharide isomers and yielded product ions from a mixture of these glycopeptide isomers. Mass spectrometric oligosaccharide profiling is necessary for detailed structural analysis of oligosaccharides.

We have previously demonstrated a simple means of oligosaccharide profiling using liquid chromatography/electrospray ionization mass spectrometry with a graphitized carbon column (GCC–LC/MS) [32–34], in which oligosaccharides can be separated on the basis of their branching, sequence, and linkage, and can be characterized based on their monosaccharide compositions estimated from their calculated molecular masses. Here, we study the glycosylation of Thy-1 by oligosaccharide profiling with GCC–LC/MS. First, we improved our oligosaccharide profiling by ion trap mass spectrometry (IT–MS) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR–MS). This instrument is capable of both monosaccharide composition analysis by acquisition of accurate masses and data-dependent multistage tandem MS (MS^n) for sequencing with fast switching between positive and negative ion modes. Using a mixture of typical oligosaccharides, including high-mannose-type, and asialo-, trisialylated, and tetrasialylated complex-types, we confirmed that the improved method can be used for monosaccharide composition analysis and detailed structural analysis of both neutral and acidic oligosaccharides. The method was then applied to N-linked oligosaccharide analysis of rat brain Thy-1.

2. Experimental

2.1. Materials

Man7/D1, Man7/D3, and asialo-triantennary (Tri) were obtained from Oxford Glycosystems (Abingdon, UK). Trisialylated triantennary ($TriNA_3$) and tetrasialylated tetraantennary ($TetraNA_4$) were purchased from Dionex (Sunnyvale, CA, USA). Rat brain was purchased from Nippon SLC (Hamamatsu, Japan). Phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus cereus* was purchased from Molecular Probes (Eugene, OR, USA). Peptide-*N*-glycosidase F (PNGase F) was purchased from Roche Diagnostics (Mannheim, Germany). SimplyBlue SafeStain was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Release of N-linked oligosaccharides from rat brain Thy-1 by in-gel PNGase F digestion

PIPLC-treated GPI-anchored proteins were prepared from rat brain as reported previously [31]. Briefly, the homogenate of rat brain was defatted and solubilized with 2% Triton X-114 at 4 °C overnight [35,36]. After centrifugation, the supernatant was subjected to Triton X-114 phase-partitioning at 37 °C. Solubilized membrane proteins in the detergent phase were precipitated with cold acetone, and the precipitates were digested with PIPLC. After resubjecting the digest mixture to Triton X-114 phase-partitioning, PIPLC-treated soluble GPI-anchored proteins in aqueous phase were precipitated by adding cold acetone. These proteins were carboxyamidomethylated [30], and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5%) followed by staining with SimplyBlue SafeStain.

In-gel PNGase F digestion of Thy-1 and extraction of N-linked oligosaccharides were performed as previously described

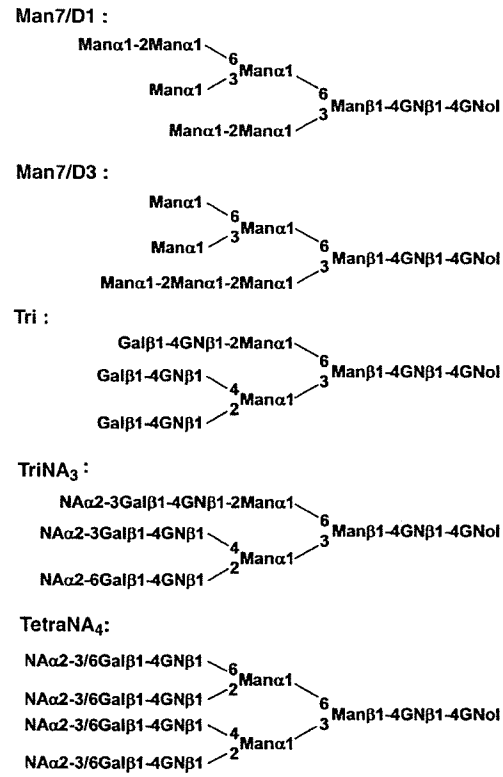

Fig. 1. Structures of major oligosaccharides and their abbreviations. Man: mannose, Gal: galactose, GN; *N*-acetylglucosamine, GNol; *N*-acetylglucosaminitol, NA; *N*-acetylneuramic acid.