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DETERMINATION OF THE PHOTOAFFINITY-LABELED SITE ON THE LIGAND-BINDING DOMAIN OF RETINOIC ACID RECEPTOR α

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SUMMARY: The ligand-binding domain of human retinoic acid receptor α (hRARα) was photoaffinity-labeled with a fluorescent retinoid, ADAM-3, by the use of a recombinant fused protein constructed from a maltose-binding protein and the E/F-domain of hRARα (MBP-RARα/E), which was expressed in E. coli. The labeled site was identified as Arg-589 (this corresponds to amino acid residue 385 of hRARα) or a residue in its vicinity.

Retinoids (retinoic acid and its biological isosters) control cell proliferation/differentiation by binding to retinoic acid receptor(s) (RARs).[1] Three subtypes of RAR (α, β and γ) are so far known, all of them being closely related in their structure and function. RARs are members of the steroid/thyroid nuclear receptor superfamily, and act as retinoid-dependent transcription factors by binding to their cognate response element, predominantly as a heterodimer with another nuclear receptor, RXR.[1]

RARs, as well as other nuclear receptors, can be functionally and structurally divided into six domains (A-F domains). DNA-binding activity and ligand-binding activity reside in the C- and the E-domain, respectively.[1] The E-domain is the largest, accounting for almost half of the size of RARs, and it has been proposed that four subdomains (τ_1, DIMERIZATION/HEPTAD REPEAT, $LIGAND_1$, and $LIGAND_2$ subdomains) exist within it.[2] Though the $LIGAND_1$ and $LIGAND_2$ subdomains were suggested to be the domains in direct contact with retinoid,[2] no chemical evidence to support this has been obtained so far. Because RARs are activated by ligand binding to the E-domain, precise knowledge of the nature of the ligand

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binding, i.e., determination of the binding site at the amino acid sequence level, would be mandatory for both elucidation of the RAR-activation mechanism and development of superior/selective retinoids.

During previous studies on structural development of synthetic retinoids (retinobenzoic acids) and on RARs,[3,4] we have obtained fluorescent and photolabile synthetic retinoids.[5-7] Among them, ADAM-3 (Fig. 1), which consists of a retinobenzoic acid moiety and an azido-dansyl moiety, has been revealed to be a useful probe for photoaffinity labeling of RARα .[6,7] We also established a method for mapping the photoaffinity-labeled site using the endoproteinase combination technique, and employed it to identify the rhizoxin-binding site on tubulin.[8] We have now applied this method for determination of the photoaffinity-labeled site with ADAM-3 on the recombinant E-domain of hRARα fused with maltose-binding protein (MBP-RARα /E).

MATERIALS AND METHODS

Materials:

A fluorescent probe for RAR-photoaffinity labeling, ADAM-3, and a synthetic retinoid, Am80, were prepared as described previously.[6,9] Endoproteinases Arg-C, Asp-N, Glu-C and Lys-C were purchased from Boehringer Mannheim. A μ -Bondasphere HPLC column (5 μ C18-100Å, 3.9 x 150 mm) was purchased from Waters Chromatography Ltd. An expression vector for full-size hRARα (pSG-RARα 0) was generously supplied by Prof. P. Chambon (INSERM, Strasburg, France). A plasmid for maltose-binding protein gene (pMAL-c2) was purchased from New England Biolabs.

Preparation of MBP-RARα /E:

The plasmid pMAL-c2-RARα (E) for expression of MBP-RARα /E in E. coli was constructed from pSG-RARα 0, pMAL-c2, and synthetic adaptor for Factor Xa recognition site as previously reported.[10] The gross structure coded in pMAL-c2-RARα (E), i.e., the structure of MBP-RARα /E, is shown in Fig. 3. Introduction of pMAL-c2-RARα (E) into E. coli, its expression, and extraction of MBP-RARα /E were performed as described previously.[10,11] Extracted MBP-RARα /E was purified by the usual method using an amylose-resin column.[10,11]

Photoaffinity Labeling:

Retinoid-binding activity of MBP-RARα /E was checked by filter binding assay using [^{3}H]Am80 as described previously.[6,10,11] Photoaffinity labeling of MBP-RARα /E with ADAM-3 was performed as described previously with slight modifications.[6] Brief-

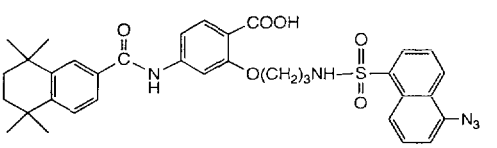

Fig. 1. Structure of ADAM-3.

ly, MBP-RARα /E in a reaction buffer (20 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 10 mM mercaptoethanol, 4% v/v ethanol) was incubated with ADAM-3 (2 eq.) in the presence or absence of Am80 (2 eq.) at 4 ℃ for 15 hr. Then the mixture was irradiated for 10 min. with a high-pressure mercury lamp (450 W) under ice cooling. It was treated with 0.1% SDS for 1 min at 100℃ and dialyzed against 0.1% SDS - 20 mM Tris-HCl (pH 8.0) - 10 mM mercaptoethanol to remove non-covalently included ADAM-3 (and/or its photo-decomposed products).

Endoproteinase Combination Technique:

Mapping of the photoaffinity-labeled site was performed by the endoproteinase combination method described previously.[8] Briefly, ADAM-3-labeled MBP-RARα /E was carboxymethylated and digested with an endoproteinase for the first-stage enzymatic digestion. The digestion conditions were chosen according to the supplier's recommendation. When the digestion was completed, trifluoroacetic acid was added at the concentration of 0.1%, and then the first digest was analyzed by HPLC on an ODS-column eluted with a linear gradient of 0-75% acetonitrile/isopropanol (3:7). An example of the chromatogram monitored by measuring OD_{215} and fluorescence intensity is shown in Fig. 2. The fluorescent peak fractions, containing ADAM-3-bound fragments, were isolated, evaporated, and subjected to the second-stage enzymatic digestion with another endoproteinase under the conditions recommended by the supplier. The second digest was analyzed by HPLC under the same conditions as the first. The retention times (T_R's) of the fluorescent peaks in the first and the second digests were compared (Table I), and from the difference of the T_R's, the presence/absence of amino acid residue(s) which should be recognized by the second-stage endoproteinase in the first-digest fluorescent peak was judged (Table I).

RESULTS

The usefulness of ADAM-3 as a specific photoaffinity labeling agent for RARs has already been reported.[5-7] The endoproteinase digests of ADAM-3-labeled MBP-RARα /E were analyzed by HPLC. As an example, a chromatogram of MBP-RARα /E digested with endoproteinase Lys-C as the first-stage digestion is shown in Fig. 2. Two major fluorescent peaks (peaks I and II), one larger than the other (approximately 3:2), were observed with several minor fluorescent peaks (Fig. 2b). The fluorescence intensities of these two peaks were decreased when the photoaffinity labeling was performed in the presence of Am80 (Fig. 2c), indicating that these peaks are derived from specific binding of ADAM-3 at the retinoid-binding pocket of MBP-RARα /E. Digestions with other endoproteinases as the first-stage digestion, i.e., Arg-C, Asp-N, and Glu-C, also gave two major fluorescent peaks with a similar intensity ratio (3:2), all of which were established to be derived from specific binding of ADAM-3 to MBP-RARα /E. Because both Am80 and ADAM-3 have been established to bind physically to only one site of MBP-RARα /E from Scatchard analysis,[12] the results indicate that; (i) ADAM-3 physically binds the specific cognate binding site of MBP-RARα /E, and then (ii) irradiation caused

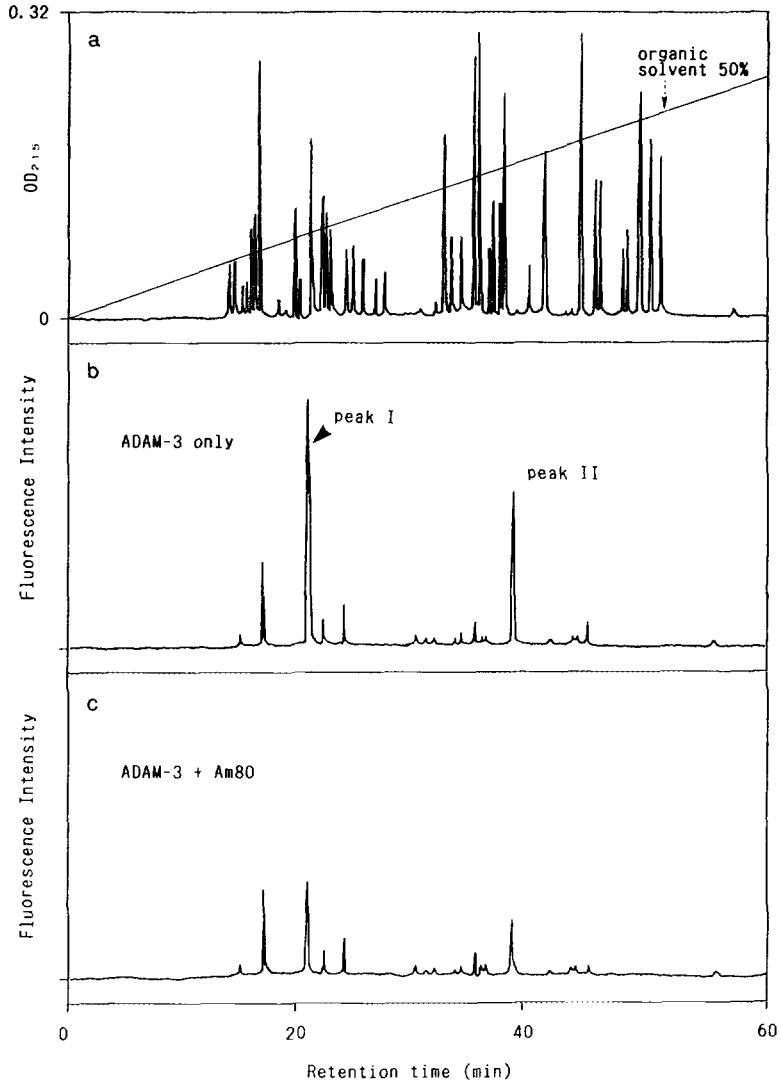

Fig. 2. HPLC Analysis of Endoproteinase Lys-C-Digest of MBP-RAR α /E Photolabeled with ADAM-3.

a: Monitored by measuring OD_{215}. b and c: Monitored by measuring fluorescence intensity. c: Photolabeling reaction was performed in the presence of Am80. Details are given in MATERIALS AND METHODS. A fluorescent peak indicated by an arrow (peak I) in the panel b was used for further digestion.

covalent binding of ADAM-3 exclusively at two amino acid residues located inside the ligand-binding pocket of MBP-RARα /E.

We focused on the largest fluorescent peak observed in the HPLC chromatogram of each first-stage digestion, and the isolated peak fractions were examined by using the endoproteinase combination technique.[8] This technique is based on the principle that if the fragment of T_R=X isolated by HPLC from the first-stage

digest contains a recognition site(s) for the second endoproteinase, then the analysis of the second digest on HPLC gives a fluorescent peak of T_R=Y where X is different from Y.

For example, the second digestion with endoproteinase Arg-C or Glu-C of the largest fluorescent peak isolated from the first-stage digest with Lys-C resulted in no change of T_R of the fluorescent peak on HPLC (Table I-③, ⑨), indicating that there exists no Arg or Glu residue between two Lys residues of the ADAM-3-labeled fragment. On the other hand, digestion of the same isolated Lys-C digest with Asp-N resulted in a shift of the T_R on HPLC (Table I-⑥), indicating that there is at least one Asp residue between two Lys residues of the labeled fragment. Applying this procedure to all twelve combinations of four endoproteinase (Table I) allows us to determine the context of the amino acid residues which should be recognized by the four endoproteinases, i.e., Lys, Arg, Asp and Glu residues. The results shown in Table I suggest that the amino acid residues around the ADAM-3-labeled site should satisfy the following relationships; (1) Arg---Asp---Arg (Table I-④), (2) Asp---Arg---Asp (①), (3) Asp---Glu---Asp (⑧), (4) Glu---Asp---Glu (⑤), (5) Glu---Lys---Lys---Glu (⑨, ⑫), (6) Arg---Lys---Lys---Arg (③, ⑩), (7) Arg---Glu---Arg (⑦), (8) Glu---Arg---Glu (②), (9) Asp---Lys---Asp (⑪), and (10) Lys---Asp---Lys (⑥). However, we could not find a partial sequence from the MBP-RARα/E sequence shown in Fig. 3 which satisfies all these criteria (1)-(10). We considered that this inconsistency resulted from covalent binding of ADAM-3 at or very near to one of the endoproteinase-recognition sites.

Table I. Summary of The Results of Endoproteinase Combination Experiments

2nd \ 1st	Arg-C	Asp-N	Glu-C	Lys-C
Arg-C	–	① R	② R	③ N
Asp-N	④ R	–	⑤ R	⑥ R
Glu-C	⑦ R	⑧ R	–	⑨ N
Lys-C	⑩ R	⑪ R	⑫ R	–

1st and 2nd: Endoproteinase used for the first-stage and second-stage digestions, respectively.

R and N: Recognized and not recognized by the second endoproteinase, judging from the T_R of the fluorescent peak on HPLC. For details, see MATERIALS AND METHODS and the text.

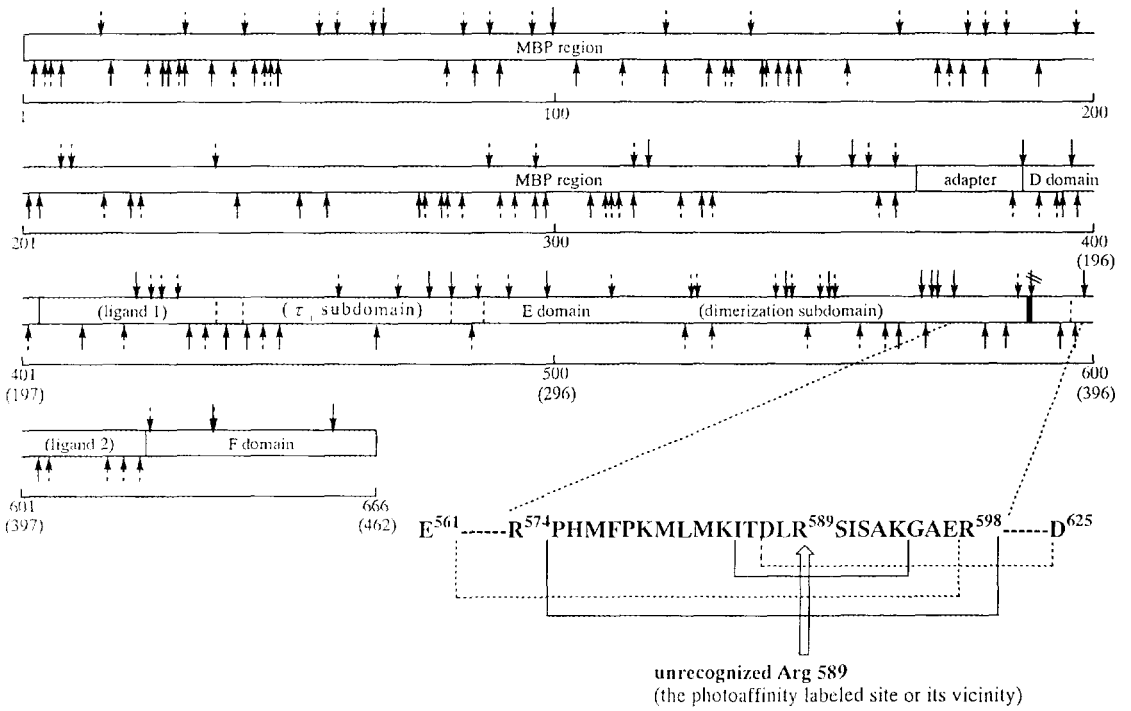

Fig. 3. Map of MBP-RARα /E.
Numbers and numbers in parentheses are the numbers of amino acid residues in MBP-RARα /E and hRAR, respectively. ↓ : Arg residue. ↓ :Asp residue. ↑ : Glu residue. ↑ : Lys residue. ↓ : Blocked Arg residue.

Therefore, we reanalyzed the results shown in Table I on the basis that one of the amino acid residues which should be recognized by any one of the four endoproteinases would be blocked by ADAM-3-labeling. When we assumed that a single Lys, Asp, or Glu residue was not recognized by the cognate endoproteinase, no sequence consistent with all the results shown in Table I could be found. However, when a single Arg residue was assumed to be blocked, a unique partial sequence of MBP-RARα /E was found as the only sequence consistent with the results shown in Table I. That is, the amino acid context deduced from the results shown in Table I with the assumption that one Arg residue is blocked should be the same as (1)-(10) described above, except that one Arg residue in (1)-(10) disappears. The unique partial sequence thus identified in the MBP-RARα /E sequence is Glu_{561}---Arg_{574}---Lys_{584}---Asp_{587}---Arg_{589}(blocked)---Lys_{594}---$Glu_{597}Arg_{598}$---Asp_{625}. Therefore, we concluded that ADAM-3 covalently binds to Arg_{589} or its vicinity.

DISCUSSION

The recombinant protein used, i.e., MBP/RARα (E), binds all-trans-retinoic acid (ATRA) and Am80 in a mutually competitive manner with the association constant (K_a) of approximately 2 x 10^{10} M^{-1}.[12] This value is close to the K_a's for binding of hRARα to ATRA (3.7 x 10^{10} M^{-1}) and Am80 (2.4 x 10^{10} M^{-1}).[3,4] Moreover, the ligand-binding selectivity of MBP/RARα (E) is the same as that of hRARα as far as examined,[3,4,10-12] i.e., the affinity to ATRA, Am80 and a potent synthetic retinoid, (E)-4-[3-(3,5-di-tert-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55) decreased in the order of Ch55 > ATRA > Am80 for both MBP/RARα (E) and hRARα. Binding affinity of various retinoids to MBP/RARα (E) correlates with differentiation-inducing activity on human leukemia HL-60 cells.[10] These facts suggest that MBP/RARα (E) produced in E. coli possesses identical (or similar) ligand-binding characteristics to those of hRARα produced in mammalian cells. Therefore, the results obtained by analysis of the ligand-binding site of MBP/RAR α (E) would be applicable to elucidation of the ligand-binding site of hRAR α.

We have identified the photoaffinity-labeled site in MBP-RAR α /E with ADAM-3 as Arg_{589} or its vicinity. The Arg_{589} residue in the recombinant RAR corresponds to Arg_{385} of hRARα, which is located between the last heptad repeat motif and the $LIGAND_2$-subdomain.[2] The covalent binding of ADAM-3 to this site is consistent with suggestions, based on various studies, including site-directed mutagenesis, analysis of amino acid sequences of nuclear receptors, and so on, that the $LIGAND_1$ and $LIGAND_2$ subdomains form the site at which the retinoid directly interacts.[2] Our data represent the first direct chemical evidence for this hypothesis.

We have previously reported that the dansyl moiety of ADAM-3 is located inside the RAR's binding pocket, with the tetrahydrotetramethylnaphthalene moiety of ADAM-3 being located at the bottom of the pocket in the ADAM-3/RAR complex.[5-7] Therefore, Arg_{385} is considered to be located near the entrance of the retinoid-binding pocket of hRARα. This idea is also consistent with a theoretical three-dimensional construct of the ligand-binding domain of nuclear receptors.[13,14]

In our study, other fluorescent peaks which are considered to be derived from specific labeling with ADAM-3 were observed (Fig. 2, and data not shown). These fragments are still under analysis, and determination of all of the labeled sites at the amino acid sequence level should afford further information concerning the three-dimensional structure of hRARα.

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REFERENCES

1) M.B.Sporn, A.B.Robert, eds. "The Retinoids", 2nd Ed., Vols. 1 and 2. Raven Press (New York), 1993.
2) B.M.Forman, H.H.Samuels, Mol.Endocrinol., 4: 1293-1301 (1990).
3) Y.Hashimoto, Cell Struct.Funct., 16: 113-123 (1991).
4) Y.Hashimoto, K.Shudo, Cell Biol.Rev., 25: 209-230 (1991).
5) R.Shimazawa, S.Hibino, H.Mizoguchi, Y.Hashimoto, S.Iwasaki, H.Kagechika, K.Shudo, Biochem.Biophys.Res.Comm., 180: 249-254 (1991).
6) R.Shimazawa, R.Sanda, H.Mizoguchi, Y.Hashimoto, S.Iwasaki, H.Tanaka, H.Kagechika, K.Shudo, Biochem.Biophys.Res.Comm., 179: 259-265 (1991).
7) Y.Hashimoto, R.Shimazawa, Clin.Dermatol., Vol. 5, p585-598, Marcel Dekker (New York), 1993.
8) T.Sawada, H.Kobayashi, Y.Hashimoto, S.Iwasaki, Biochem.Pharm., 45: 1387-1394 (1993).
9) H.Kagechika, E.Kawachi, Y.Hashimoto, T.Himi, K.Shudo, J.Med. Chem., 31: 2182-2192 (1989).
10) L.Eyrolles, H.Kagechika, E.Kawachi, H.Fukasawa, T.Iijima, Y.Matsushima, Y.Hashimoto, K.Shudo, J.Med.Chem., 37: 1508-1517 (1994).
11) H.Fukazawa, T.Iijima, H.Kagechika, Y.Hashimoto, K.Shudo, Biol.Pharm.Bull., 16: 343-348 (1993).
12) T.Iijima, Master's Thesis, University of Tokyo, 1994.
13) R.A.Goldstein, J.A.Katzenellenbogen, Z.A.Luthey-Schulten, D.A. Seielstad, P.G.Wolynes, Proc.Natl.Acad.Sci.USA., 90: 9949-9953 (1993).
14) M.E.Newcomer, R.S.Pappas, D.E.Ong, Proc.Natl.Acad.Sci.USA., 90: 9223-9227 (1993).