Journal of Chromatography, 597 (1992) 331–334
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 948

Affinity chromatographic purification of bovine lung endothelin receptor using biotinylated endothelin and avidin–agarose

Hiromi Hagiwara*, Toru Nagasawa, Khalid M. Lodhi, Masamichi Kozuka, Teizo Ito and Shigehisa Hirose

Department of Biological Sciences, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo 152 (Japan)

ABSTRACT

Endothelin receptor was purified from bovine lung by affinity chromatography using biotinylated endothelin and avidin–agarose. Endothelin was biotinylated with sulphosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate, a reactive form of biotin with a cleavable spacer arm containing a disulphide bond designed for a simple elution by thiols. Starting from 3.5 kg of bovine lung, about 200 μg of pure receptor were obtained.

INTRODUCTION

Endothelin, which consists of 21 amino acid residues containing two disulphide bridges, is synthesized by endothelial cells and secreted into the circulation. Its diverse biological actions, such as vascular and non-vascular smooth muscle contractions [1–4], the regulation of release of atrial natriuretic peptide [5] and steroid [6] and stimulation of mitogenesis in glial cells [7] and 3T3 fibroblasts [8], are mediated by specific receptors located on various target tissues (*e.g.* kidney, lung, brain, vascular smooth muscle and endothelial cells). The relative molecular mass (M_r) of the receptor has been estimated to be about 30 000 and 50 000 by affinity labelling analysis [9]. After many trials, we found that the combination of biotinylated endothelin and avidin–agarose was the most effective affinity chromatography for purification of endothelin receptors. Using this affinity chromatography, we isolated M_r 34 000 and 52 000 species of endothelin receptors from bovine lung and demonstrated that the 34 000 species is a proteolytic product of the native M_r 52 000 species.

Since biotin has a high affinity ($K_d = 10^{-15}$ *M*) for egg white avidin and streptavidin [10], biotinylated ligands were employed as a good tool for sensitive detection and purification of receptors. In fact, biotinylated peptides have been used as specific probes for localizing receptors for adrenocorticotropin [11], gonadotropin-releasing hormone [12], corticotropin-releasing hormone [13] and vasopressin [14] and for isolating angiotensin II receptors [15]. This paper reports a successful example of affinity chromatography using biotinylated ligand (endothelin) and avidin–agarose.

EXPERIMENTAL

Materials

Human endothelin-1 was purchased from the Peptide Institute (Osaka, Japan); sulphosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin) and immobilized avidin (avidin–agarose) were from Pierce; [^{125}I-Tyr^{13}]endothelin-1 (74 TBq/mmol) was from Amersham; digitonin and CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulphonate, were from Wako Pure

0021-9673/92/$05.00

Chemicals (Osaka, Japan); the μBondasphere 5-μm C_{18}-100 Å high-performance liquid chromatography (HPLC) column (3.9 × 150 mm) was from Waters.

Biotinylation of endothelin-1

Biotinylation was initiated by addition of the biotinylation reagent NHS-SS-biotin (430 nmol) in 25 μl of dry dimethylformamide to endothelin-1 (0.11 mg, 43 nmol) in 400 μl of 50 m*M* sodium borate, pH 8.5, containing 0.015% Triton X-305. After shaking for 3 h at 20°C, another 430 nmol of NHS-SS-biotin were added and the reaction mixture was incubated at 20°C overnight. Biotinylated endothelin-1 was separated from free endothelin and biotin by reversed-phase HPLC using an acetonitrile gradient after mixing with 0.5 ml of 0.1% trifluoroacetic acid. The peak containing biotinylated endothelin was identified by receptor binding assay.

Preparation of membranes

Bovine lung plasma membranes were prepared according to the previously described methods [16]. Fresh bovine lung (1.5 kg) was homogenized in a Waring blender for 2 min in three volumes of 20 m*M* Tris–HCl, pH 7.4, containing 50 m*M* EDTA, 0.2 m*M* phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml pepstatin and 10 μg/ml leupeptin (buffer A). The homogenates were centrifuged at 5000 *g* for 20 min at 4°C and the supernatants were discarded. The membranes were suspended in three volumes of buffer A and recentrifuged; this washing step was repeated three times. The pellets were further washed twice with 20 m*M* phosphate buffer, pH 7.4, containing 130 m*M* sodium chloride, 50 m*M* EDTA, 0.2 m*M* PMSF, 10 μg/ml pepstatin and 10 μg/ml leupeptin (buffer B). The washed membranes were stored at −40°C until use in 500-g aliquots.

Purification

The frozen membranes (500 g) were suspended in two volumes of buffer B containing 0.25% 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS) and 0.4% digitonin (buffer C). After gently stirring for 2 h at 4°C, the membrane suspensions were filtered through cheese cloth. The filtrates were centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant (1 l) containing solubilized endothelin receptors and biotinylated endothelin-1 (15 nmol) in 1.5 ml of buffer B containing 0.015% Triton X-305 were mixed and incubated for 1 h at 20°C with gentle shaking. To the mixture were then added 10 ml of avidin–agarose equilibrated with buffer C and the incubation was continued at 4°C overnight. The gel was collected by centrifugation at 1200 *g* for 10 min at 4°C and packed into a column (10 × 1.5 cm I.D.). The column was washed with 500 ml of buffer C and eluted with buffer C containing 0.2 *M* 2-mercaptoethanol at a flow-rate of 0.2 ml/min, collecting 0.5-ml fractions. Fractions containing the endothelin receptor were identified by the presence of the M_r 52 000 and 34 000 protein bands on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) which co-migrated with the affinity-labelled receptor, pooled, dialysed against four changes of 500 ml of 0.1 *M* ammonium bicarbonate, pH 7.8, and freeze-dried.

RESULTS AND DISCUSSION

We first prepared biotinylated endothelin-1 and characterized it. The biotinylation procedure is described in detail in the Experimental section. Biotinylated endothelin-1 was eluted as a major peak later than native endothelin-1 on reversed-phase HPLC. Overall yield was approximately 30%. The chemical structure of biotinylated endothelin-1, determined by amino acid sequencing, is illustrated in Fig. 1. Fortunately, binding analysis using [^{125}I]endothelin-1 showed that biotinylated endothelin-1 retained a high affinity ($3 \cdot 10^{-10}$ *M*) for the receptor solubilized from bovine lung plasma membranes. Using this active biotinylated endothelin-1, we isolated the bovine lung endothelin receptor. A typical elution profile of endothelin receptor from the avidin–agarose gel, examined by SDS-PAGE, showed the M_r 52 000 and 34 000 bands corresponding to intact receptor and proteolytic products, respectively (Fig. 2A). The receptor was finally

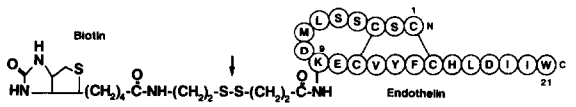

Fig. 1. Chemical structure of biotinylated endothelin. Amino acid sequencing of HPLC-purified biotinylated endothelin-1 revealed that biotin is incorporated into the Lys residue at the ninth position of endothelin-1. The arrow indicates the disulphide bond cleavable with thiol reagents.

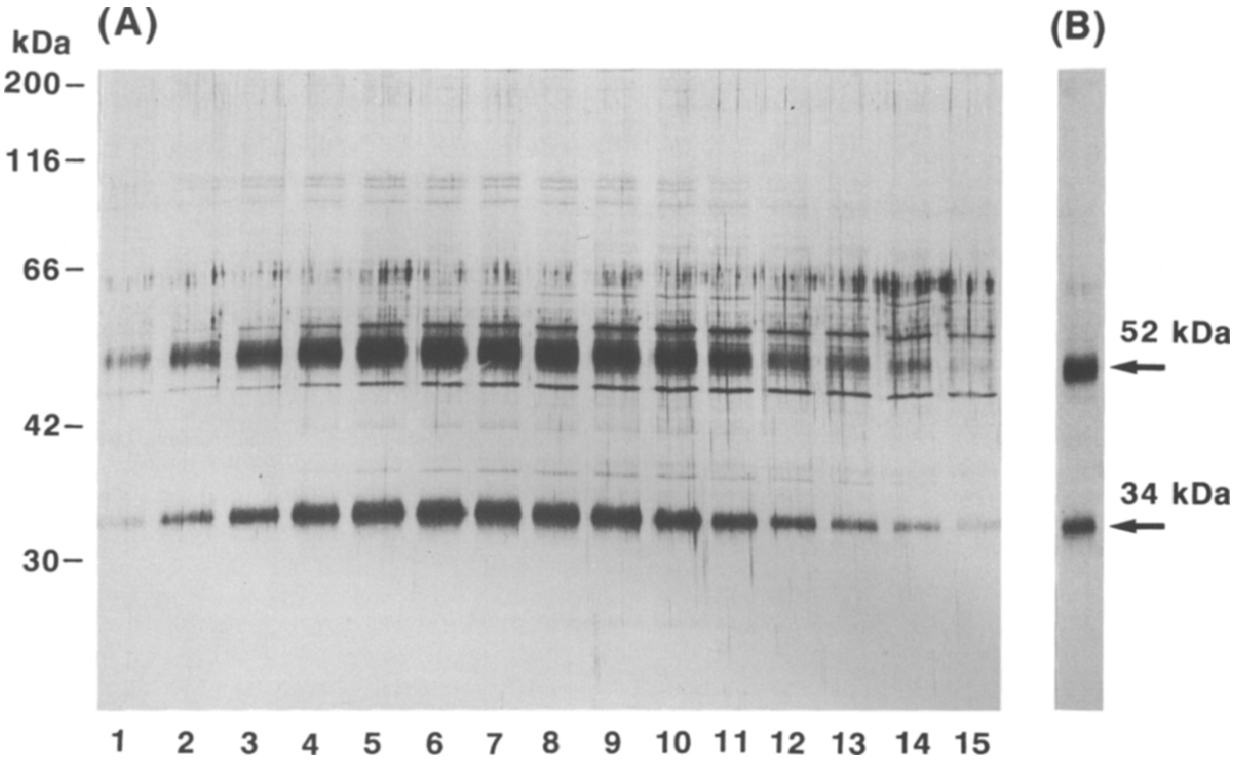

Fig. 2. SDS-PAGE profile of a typical elution of endothelin receptor (A) and the purified receptor (B). The eluates (12 μl from a 500-μl fraction) from the affinity column and the purified receptor were electrophoresed on SDS polyacrylamide gels under reducing conditions and stained with a Kanto silver stain kit. The arrows show M_r 52 000 and 34 000 bands representing the intact endothelin receptor and a proteolytic product, respectively. The detailed purification procedure is described in the Experimental section. kDa = kilodalton.

purified by eliminating the persistent contaminants as precipitates by dialysis against 0.1 *M* ammonium bicarbonate (see Fig. 2B). Starting from 7 l of the detergent extracts (16 g of protein), about 200 μg of the receptor were obtained. When preparative buffers containing 1 m*M* EDTA were used instead of 50 m*M* EDTA during the purification of endothelin receptor, we obtained only the M_r 34 000 species [16]. Sequence analysis of tryptic fragments of the purified protein revealed a high sequence similarity with the rat endothelin ET_B receptor that was cloned by expression cloning and shown to be non-selective in terms of the ligand specificity [17].

In summary, the endothelin ET_B receptor was purified from bovine lung in quantities sufficient for biochemical analysis. The crucial steps for the successful purification were: (1) solubilization of the membrane proteins with a detergent mixture of 0.25% CHAPS and 0.4% digitonin in a highly dispersed state [9], (2) preparation of biotinylated endothelin-1 that has a high affinitiy for the receptor and (3) affinity chromatography that utilizes the strong biotin–avidin interaction.

ACKNOWLEDGEMENTS

We thank Setsuko Satoh and Kazuko Tanaka for their secretarial and techhnical assistance. This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and grants from Chichibu Cement, the Japan Shipbuilding Industry Foundation, Kanagawa Academy of Science and Technology and the Mitsubishi Foundation.

REFERENCES

1 M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazai, K. Goto and T. Masaki, *Nature (London)*, 332 (1988) 411.
2 R. Borges, H. Von Grafenstein and D. E. Knight, *Eur. J. Pharmacol.*, 165 (1991) 223.
3 S. Eguchi, M. Kozuka, S. Hiroze, T. Ito and H. Hagiwara, *Biomed. Res.*, 12 (1991) 35.
4 H. Hagiwara, M. Kozuka, T. Ito, S. Eguchi and S. Hirose, *Biomed. Res.*, 11 (1990) 93.
5 Y. Fukuda, Y. Hirata, H. Yoshimi, T. Kojima, Y. Kobayashi, M. Yanagisawa and T. Masaki, *Biochem. Biophys. Res. Commun.*, 155 (1988) 167.
6 C. Delarue, I. Delton, F. Fiorini, F. Homo-Delarche, A. Fasolo, P. Braquet and H. Vaudry, *Endocrinology*, 127 (1990) 2001.
7 M. W. Maccumber, C. A. Ross and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 2359.
8 N. Takuwa, Y. Takuwa, M. Yanagisawa, K. Yamashita and T. Masaki, *J. Biol. Chem.*, 264 (1989) 7856.
9 H. Hagiwara, M. Kozuka, S.Eguchi, S. Shibabe, T. Ito and S. Hirose, *Biochem. Biophys. Res. Commun.*, 172 (1990) 576.
10 N. M. Green, *Biochem. J.*, 89 (1963) 599.
11 K. Hofmann and Y. Kiso, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 3516.
12 G. V. Child, Z. Noar, E. Hazum, R. Tibolt, K. N. Westlund and M. Hancock, *J. Histochem. Cytochem.*, 31 (1983) 1422.
13 K. N. Westlund, P. C. Wynn, S. Chmielowiec, T. J. Collins and G. V. Childs, *Peptides*, 5 (1984) 627.
14 D. A. Jans, L. Bergmann, R. Peters and F. Fahrenholz, *J. Biol. Chem.*, 265 (1990) 14599.
15 J. Marie, R. Seyer, C. Lombard, F. Desarnaud, A. Aumelas, S. Jard and J. C. Bonnafous, *Biochemistry*, 29 (1980) 8943.
16 M. Kozuka, T. Ito, S. Hirose, K. M. Lodhi and H. Hagiwara, *J. Biol. Chem.*, 266 (1991) 16892.
17 T. Sakurai, M. Yanagisawa, Y. Takuwa, H. Miyazaki, S. Kimura, K. Goto and T. Masaki, *Nature (London)*, 348 (1990) 732.