Fluorescent and radiolabelling of pepsin-digested human glomerular basement membrane with a newly developed hydroxy-coumarin derivative (CASE)

Mariann Rand-Weaver, Ramadan A. Abuknesha and Robert G. Price*

Department of Biochemistry, Queen Elizabeth College (University of London), Campden Hill Road, London W8 7AH, England

Received 11 December 1984

The labelling of pepsin-digested human glomerular basement membrane (pHGBM) with a newly developed fluorescent iodine acceptor 7-hydroxy-coumarin-3-acetic acid *N*-hydroxysucciniimydyl ester (CASE) is described. The binding of a monoclonal antibody to pHGBM was assessed by radiobinding assays, and when directly iodinated pHGBM was used there was no apparent binding. When CASE was conjugated to pHGBM prior to iodination 11% binding was achieved. CASE acting as an iodine acceptor may be useful for proteins containing few or inaccessible tyrosine residues or which are destroyed by introduction of ^{125}I. Since CASE is fluorescent, small amounts of material can be detected during isolation prior to iodination.

Iodination *Protein labelling* *Human glomerular basement membrane*
7-Hydroxy-coumarin-3-acetic acid N-*hydroxysucciniimydyl ester* *Fluorescence*

1. INTRODUCTION

Glomerular basement membrane is a complex extracellular matrix consisting of collagenous and noncollagenous components which can be solubilised by proteolytic digestion. Pepsin-digested human glomerular basement membrane (pHGBM) is a heterogenous mixture of proteins [1]. Studies using a monoclonal antibody MBM4, to characterise components of these digests, proved difficult because of the small amounts of material available. The incorporation of a radiolabel was therefore essential. In order that radiobinding assays could be performed, it was necessary to label the pHGBM fractions to a high specific activity, and iodine was chosen for this purpose. However, to our knowledge there are no reports on the successful iodine labelling of basement membranes as most studies report the labelling of purified proteins [2–4], the metabolic labelling of cells [5,6] or EHS tumour tissue [7].

Direct iodination of pHGBM resulted in very little incorporation of iodine into the MBM4-bound component as shown by radiolabelling studies, even though the total iodine incorporation was often high. Attempts were therefore made to insert into the component a compound which could readily be iodinated but which would not affect its antigenicity. For this purpose, 7-hydroxy-coumarin-3-acetic acid-*N*-hydroxysucciniimidyl ester (CASE) proved to be particularly satisfactory. This compound has the added advantage that it is fluorescent and therefore allows detection and characterization of the antigen prior to iodination.

2. EXPERIMENTAL

Glomerular basement membrane (HGBM) was prepared from normal human kidneys obtained within 8 h of death. The cortex was dissected from the medulla, glomeruli prepared by sieving using a

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

modification of the method described by Spiro [8] and GBM isolated following sonication. Collagenous components were solubilised by digestion with pepsin (1 mg/10 mg GBM) in 0.5 M acetic acid at 10°C for 48 h [9].

7-Hydroxy-coumarin-3-acetic acid was prepared by the method of Banerjee [10]. The coumarin active ester, CASE, was prepared by reacting the 7-hydroxy-coumarin-3-acetic acid (233 mg, 1 mmol) with dicyclohexylcarbodiimide (220 mg, 1 mmol) and *N*-hydroxy-succiniimide (130 mg, 1.18 mmol) in tetrahydrofuran (10 ml) at 4°C for 16 h and then for 1 h at room temperature. The dicyclohexylurea formed was removed by filtration and the filtrate evaporated to dryness under vacuum. The residue was taken up into cold ethyl acetate (25 ml), filtered and the filtrate evaporated to dryness. The product was stored desiccated in the dark at 4°C. Prior to use CASE was dissolved (9 mg/ml) and diluted (1:100) in 1,4-dioxan.

Pepsin-digested GBM (pGBM) in acetic acid was adjusted to pH 8.3 with 3 M NaOH prior to labelling with CASE. CASE (100 μl) was added over 30 min with continuous stirring and the reaction allowed to proceed for a further 30 min. Extensive dialysis against 0.01 M phosphate-buffered saline (pH 7.4) (PBS) was carried out at 4°C to remove excess unbound CASE.

The CASE-labelled pHGBM and untreated pHGBM were fractionated on Sephadex G-75 (fig.1) prior to iodination. Selected fractions (a) and (b) were iodinated using Iodogen™ reagent (Pierce and Warriner) as described by Salacinski et al. [11] (fig.2).

The iodinated proteins were then used as antigens in radiobinding assays. Dilutions of ^{125}I-antigens (50 μl) were added to PBS/0.4% bovine serum albumin (50 μl) and monoclonal antibody MBM4 (50 μl, 1:20 dilution). The tubes were incubated at 37°C for 1 h and cooled on ice for 5 min prior to addition of sheep anti-mouse serum (50 μl, 1:22 dilution) and mouse serum (50 μl, 1:200 dilution). The tubes were further incubated at 37°C for 30 min and at 4°C overnight. The precipitates were washed twice by adding ice cold buffer (900 μl). They were then centrifuged at 2000 $\times$ *g* for 30 min, after which the radioactivity present in the precipitate was counted in a gamma counter. The radioactivity present in the precipitate was the amount of labelled antigen bound to excess MBM4 under the conditions stated.

3. RESULTS

Previous studies (unpublished) have indicated that MBM4 recognises a 350 kDa component in the pepsin-solubilised pHGBM fraction. Since this fraction is heterogeneous, gel filtration was performed in order to remove smaller proteins. The elution profile is shown in fig.1 and the CASE-

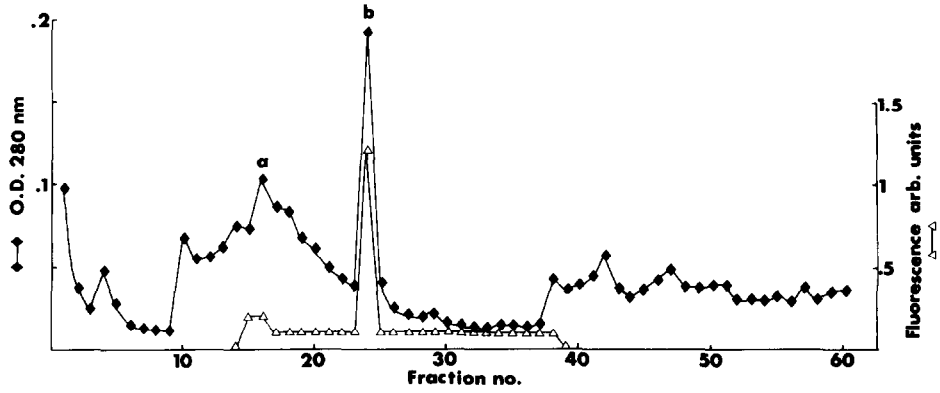

Fig.1. Fractionation of pHGBM on Sephadex G-75. The column (1 $\times$ 50 cm) was equilibrated and eluted with PBS. Fractions (1.5 ml) were collected at a flow rate of 45 ml/h. Fluorescence was measured at excitation and emission wavelengths of 365 and 455 nm, respectively, and protein absorbance at 280 nm. The fractions used for iodination are indicated by (a) and (b).

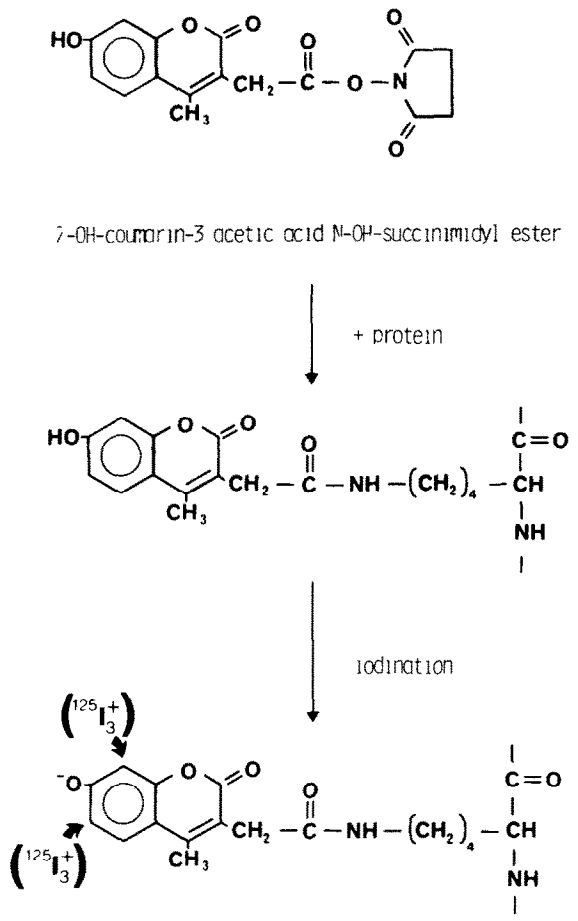

Fig.2. Iodination of coumarin-labelled protein.

containing fractions (a) and (b) were selected for iodination.

There was no binding to the antibody when directly labelled ^{125}I-pHGBM was used as antigen. This could be improved slightly by fractionating the pHGBM on a gel filtration column prior to iodination but the binding of peak (a) was still less than 1% and there was no binding of MBM4 to peak (b). However, there was a great increase in the percentage binding when ^{125}I-CASE-pHGBM was used as the antigen and binding between 5–11% of peak (a) to MBM4 was achieved. Binding of peak (b) remained lower, although that too was increased (0.3 to 2.4%). Some of the variation in the extent of labelled pHGBM bound to MBM4 may be as a consequence of less antigen being present. This is governed by such factors as the sonication procedure and the extent of breakdown of the HGBM achieved during pepsin digestion. Introduction of CASE into pHGBM prior to iodination results in a greater capacity to accept iodine and typically 55–65% of the total ^{125}I would be incorporated into the protein using the iodogen method.

4. DISCUSSION

Tritium labelling of collagenous proteins has been used successfully for the analysis of proteins by SDS-polyacrylamide gel electrophoresis by several investigators [5,12]. However, high specific activity of labelled proteins is required for radiobinding assays and therefore, ^{125}I labelling was essential.

When direct labelling of pHGBM with iodine was attempted, insufficient label was incorporated into the antigen to distinguish binding of ^{125}I-pHGBM to MBM4 above background levels. Since pHGBM contains low levels of tyrosine residues [1] and some of these may be buried in the core of the α-helix (i.e., inaccessible for efficient iodination), it is probable that this resulted in the low specific activity of the labelled material. When the low M_r non-antigenic components were removed by gel filtration a slight improvement in binding was observed, although it was still too low to be of practical value. The introduction of CASE as the iodine acceptor greatly increased the radioactivity present in the immune precipitates, which is directly proportional to the extent of antigen binding. This is presumably because a more even distribution of ^{125}I molecules throughout the molecule was achieved. It is possible that direct iodine labelling destroyed the antigenicity of the protein, implicating a tyrosine residue(s) in the epitope. Since the ^{125}I is on a spacer arm in the CASE-conjugated protein such damage may have been prevented allowing recognition of the pHGBM by the antibody. CASE, like the Bolton and Hunter reagent [13] couples specifically to free amino groups under mild conditions and has the additional advantage of being fluorescent. This latter property of CASE is very useful as it allows detection of proteins before iodination and small amounts of protein may be followed during a purification procedure. The low fluorescence values recorded in this study are probably because of the small number of lysine residues in pepsin-digested base-

ment membranes [14]. As CASE is a stable compound, the resulting CASE-protein conjugate can be stored at −20°C and aliquots iodinated as required.

The novel approach to the iodination of the pHGBM described here might be of more general applicability to the labelling of proteins with a low number of oxidisable or clustered tyrosine residues. In a parallel study the conjugation of CASE to ovalbumin has been described [15]. CASE is easy to prepare, is stable when stored desiccated below 4°C and its fluorigenic property is useful in characterising the protein prior to iodination. This procedure should prove a valuable addition to the protein labelling methods currently available.

ACKNOWLEDGEMENTS

Monoclonal antibody MBM4 was a gift from Professors A.J. Fish and A.F. Michael, University of Minnesota, Minneapolis. Sheep anti-mouse serum was kindly donated by Professor J. Landon, St. Bartholomew's Hospital, London. The authors are grateful for financial support received from the National Kidney Research Fund (UK) and the University of London Central Research Fund.

REFERENCES

[1] Tryggvason, K. and Kivirkikko, K. (1978) Nephron 21, 230–235.
[2] Sundarraj, N. and Willson, J. (1982) Immunology 47, 133–140.
[3] Foellmer, H.G., Madri, J.A. and Furthmayr, H. (1983) Lab. Invest. 48, 639–649.
[4] Yurchenco, P.D. and Furthmayr, H. (1984) Biochemistry 23, 1839–1850.
[5] Duncan, K.G., Fessler, L.I., Bachinger, H.P. and Fessler, J.H. (1983) J. Biol. Chem. 258, 5869–5877.
[6] Fessler, L.I., Duncan, K.G., Fessler, J.H., Salo, T. and Tryggvason, K. (1984) J. Biol. Chem. 259, 9783–9789.
[7] Tryggvason, K., Gehron Robey, P. and Martin, G.R. (1980) Biochemistry 19, 1284–1289.
[8] Spiro, R.G. (1967) J. Biol. Chem. 242, 1915–1922.
[9] Price, R.G., Taylor, S.A. and Khalil-Manesh, F. (1980) Renal Physiol. (Basel) 3, 41–48.
[10] Banerjee, S.K. (1931) J. Ind. Chem. Soc. 8, 777–782.
[11] Salacininski, P., Hope, J., McClean, C., Clement-Jones, V., Sykes, J., Price, J. and Lowry, P.J. (1979) J. Endocrinol. 81, 131.
[12] Carey, D.J., Eldridge, C.F., Cornbrooks, C.J., Timpl, R. and Bunge, R.P. (1983) J. Cell Biol. 97, 473–479.
[13] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
[14] Babel, W. and Glanville, R.W. (1984) Eur. J. Biochem. 143, 545–556.
[15] Samuel, D., Amlot, P.L. and Abuknesha, R.A.J. (1985) Immunol. Meth., submitted.