¹⁴C-Methylamine-glutaraldehyde conjugation as an alternative to iodination for protein labeling

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Radiolabeling of native proteins conventionally has required iodination using ¹²⁵Iodine (¹²⁵I). Although radioiodination can result in high specific activity, there are several drawbacks in the use of ¹²⁵I (e.g., radiological hazards and short half-life). ¹⁴C-Methylamine-glutaraldehyde conjugation to proteins offers an alternative for radiolabeling of proteins that is safer and longer-lived. α -2-Macroglobulin was radiolabeled by conjugation to a ¹⁴C-methylamine-glutaraldehyde conjugate. Analysis of the labeling procedure was performed using scintillation counting, gel filtration chromatography, and protein assays. The radiolabeled α -2-macroglobulin was activated using established protocols and tested for functional integrity using competitive binding assays in the presence of recombinant receptor associated protein, an alternative ligand for the α -2-macroglobulin cellular receptor. The function of α -2-macroglobulin was unaffected by the labeling procedure. Comparison of ¹⁴C-methylamine-labeling and iodination by Scatchard analysis yielded nonlinear plots that suggested the presence of two sets of receptors with different binding affinities but that do not show cooperativity. This technique offers an alternative to radioiodination for the sensitive labeling of proteins.

INTRODUCTION

Radiolabeling of proteins after their synthesis conventionally has required iodination using ¹²⁵Iodine (¹²⁵I). Although radioiodination can result in high specific activity, there are several drawbacks to the use of ¹²⁵I. The radiological hazards associated with this isotope are significant (e.g., the volatility of iodine results in a high risk of dermal or inhalation absorption) and necessitate high levels of staff training and specialized safety facilities (1). The half-life of ¹²⁵I is relatively short (59.6 days), requiring frequent replacement of the isotope and repeated labeling of proteins. We have developed an alternative procedure for the radiolabeling of proteins that is safer, more economical, and utilizes ¹⁴Carbon (¹⁴C), thereby eliminating problems associated with the hazards and short half-life of ¹²⁵I. We have compared the receptor binding of ¹⁴C-labeled protein with that of protein labeled using standard iodination techniques.

MATERIALS AND METHODS

Protein Labeling by Conjugation to a ¹⁴C-Methylamine-Glutaraldehyde Conjugate

α -2-Macroglobulin (Sigma, Poole, UK) was dissolved in PBS (pH 7.4) and stored in aliquots at -20°C. ¹⁴C-Methylamine hydrochloride (50 mCi/mmol) was obtained from Amersham Biosciences (Little Chalfont, UK) and dissolved in distilled water at a final concentration of 0.05 M. A glutaraldehyde-¹⁴C-methylamine hydrochloride conjugate (glut-¹⁴C-MA) was formed by incubating equimolar quantities (0.25 μ mol) of glutaraldehyde and ¹⁴C-methylamine hydrochloride together in PBS (pH 7.4) for 1 h at room temperature. The glut-¹⁴C-MA was then incubated with an excess of α -2-macroglobulin (100 μ g at a concentration of 1 μ g/ μ L) in PBS (pH 7.4), thereby allowing the free aldehyde group of glut-¹⁴C-MA to react with amine residues present on the α -2-

macroglobulin, resulting in glut- ^{14}C -MA- α -2-macroglobulin. Reductive amination using sodium cyanoborohydride (1 M in PBS, pH 7.4, freshly prepared and added to the protein solution to a final concentration of 0.1 M) was then carried out for 2 h at room temperature to stabilize the glut- ^{14}C -MA- α -2-macroglobulin conjugate. The radiolabeled protein (glut- ^{14}C -MA- α -2-macroglobulin) was washed eight times with 50 mM phosphate buffer (pH 8.0, containing 150 mM EDTA) using a Nanosep[®] 10K centrifugal filter (Pall, Portsmouth, Hampshire, UK). Centrifugation was performed at 5000 $\times$ g for 4 min.

Analysis of Labeling

Aliquots of the flow-through and material retained by the centrifugal filter during the washing procedure (150 μL) were added to 2 mL scintillant (OptiScint HiSafe[®]; PerkinElmer Life Sciences, Boston, MA, USA) and counted for 5 min/sample using a Model LS 6500 Multipurpose Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). The labeled protein was diluted to 1 mg/mL, and gel filtration chromatography was carried out using Bio-gel A-1.5M (Bio-Rad Laboratories, Hercules, CA, USA), which had been equilibrated in PBS (pH 7.4). The resin was packed into a 14 $\times$ 1 cm column, and the radiolabeled protein (100 μg at a concentration of 1 $\mu\text{g}/\mu\text{L}$) was loaded onto the column in 3% glycerol. Thirty fractions of 500 μL were collected and analyzed

for protein content using the bicinchoninic acid (BCA) protein assay (Pierce Chemical, Rockford, IL, USA; according to the manufacturer's instructions) and ^{14}C using scintillation counting.

Analysis of the Functional Integrity of ^{14}C - α -2-Macroglobulin

A plasmid encoding the glutathione-S-transferase-receptor associated protein (GST-RAP) fusion protein, pGEX-KG-RAP, was kindly provided by Prof. J. Herz (University of Texas Southwestern Medical Center, Dallas, TX, USA). Recombinant GST was also expressed from pGEX 4T 3 (Amersham Biosciences) for use as a negative control. Recombinant receptor associated protein (rRAP) and recombinant glutathione-S-transferase (rGST) were produced and purified as described previously (2). For comparative purposes, α -2-macroglobulin was iodinated using established techniques (3). Both ^{14}C - α -2-macroglobulin and ^{125}I - α -2-macroglobulin were activated in the presence of methylamine as described previously (4), resulting in ^{14}C - α -2-macroglobulin* and ^{125}I - α -2-macroglobulin*, respectively.

Competitive binding assays were carried out to confirm that radiolabeled ^{14}C - α -2-macroglobulin* was inhibited competitively from cell binding by recombinant RAP. Hepatocyte-derived Huh7 cells were seeded into 24-well plates at a density of 4×10^5 cells/well. Cultures were incubated overnight at 37°C in 5% CO_2 . The cell culture

medium was removed and replaced with 1 mL/well Dulbecco's minimal essential medium (no serum) containing increasing amounts of either rGST-RAP or rGST (negative control). The cells were incubated for 20 min before the addition of 10 μg ^{14}C - α -2-macroglobulin*. The cells were incubated for a further 4 h at 37°C in 5% CO_2 , and then the monolayers were washed three times with PBS. The cells were then solubilized in 200 μL /well of 0.1 M NaOH, and cell-associated radioactivity was determined by scintillation counting of washed and solubilized cells. For comparison, competitive binding assays were also performed using ^{125}I - α -2-macroglobulin*. Cell-associated ^{125}I was determined by γ counting using a COBRA II Automatic Gamma Counter (PerkinElmer Life Sciences).

For Scatchard analysis, Huh7 cells were seeded into 24-well plates at a density of 4×10^5 cells/well. Cultures were incubated overnight at 37°C in 5% CO_2 . The cell culture medium was removed and replaced with 1 mL/well Dulbecco's minimal essential medium (no serum) containing increasing amounts of either ^{14}C - α -2-macroglobulin* or ^{125}I - α -2-macroglobulin*. The cells were incubated for a further 4 h at 37°C in 5% CO_2 , and then the monolayers were washed three times with PBS and solubilized as described above. Bound radiolabeled proteins were quantified using scintillation counting (^{14}C) or γ counting (^{125}I), and the values were used in a Scatchard plot.

Figure 1. Analysis of ^{14}C - α -2-macroglobulin*. (A) Association of radioisotope with protein during sequential washes [wash number vs. counts per minute (cpm)]. Flow-through from sequential washes numbered 1–8 (line graph) and the material retained by the centrifugal filter (bar graph) were analyzed by scintillation counting. (B) Analytical gel filtration chromatography of radiolabeled α -2-macroglobulin* (fraction number versus cpm/ A_{570}). Fractions were assayed for protein content (by measurement of the absorbance at 570 nm using the BCA protein assay; black line) and ^{14}C by scintillation counting (cpm; gray line).

Figure 2. Competitive binding assay measuring the cell-associated ^{14}C - α -2-macroglobulin*. Binding assays [counts per minute (cpm) bound] were performed in the presence of increasing amounts of rGST-RAP (black) or rGST (gray, negative control) ranging from 0.3 to 10 μg .

Figure 3. Scatchard analysis of binding. Radiolabeled ^{14}C - α -2-macroglobulin* (A) and ^{125}I - α -2-macroglobulin* (B) to Huh7 cells (cpm).

RESULTS AND DISCUSSION

Radiolabeling of α -2-Macroglobulin

α -2-Macroglobulin was radiolabeled using a ^{14}C -methylamine-glutaraldehyde conjugate. The association of ^{14}C with the protein was monitored during the labeling process by carrying out scintillation counting on the flow-through and material retained by the centrifugal filter during the washing procedure. A decline in ^{14}C was observed in the flow-through as washing proceeded; however, the protein retained by the filter remained associated with the radioisotope (Figure 1A). The association of ^{14}C with α -2-macroglobulin was found to be glutaraldehyde-dependent because, in the absence of dialdehyde, no radioactivity was detected in protein-containing fractions after washing (data not shown).

The α -2-macroglobulin was converted to its activated conformation, α -2-macroglobulin*, as described previously (4). The activated protein, glut- ^{14}C -MA- α -2-macroglobulin*, was analyzed using gel filtration chromatography, the BCA protein quantification assay, and scintillation counting. Figure 1B shows that both protein and ^{14}C were co-eluted as a single tight peak, indicating a physical association between the two and successful protein labeling.

Functional Integrity of the Labeled Protein

To confirm that the functional integrity of the protein had been maintained during the labeling process, the

specific binding of α -2-macroglobulin* to its cellular receptor, the α -2-macroglobulin receptor or low-density lipoprotein receptor related protein (LRP) (5,6), was confirmed using competitive binding assays. rRAP, which is

a specific ligand of the LRP and blocks the binding of α -2-macroglobulin* to LRP competitively, was produced as a GST fusion protein, GST-RAP (recombinant GST also was expressed for use as a negative control). Figure 2 shows binding of ^{14}C - α -2-macroglobulin* to cells and competitive inhibition of that binding in the presence of rGST-RAP (no competition was observed in the presence of increasing amounts of rGST). Therefore, the functional integrity of the ^{14}C - α -2-macroglobulin* was unaffected by the radiolabeling process. For comparison, competitive binding assays were also performed using ^{125}I - α -2-macroglobulin*, and binding of ^{125}I - α -2-macroglobulin* was found also to be inhibited by the presence of rRAP (data not shown).

Further analysis of the binding of ^{14}C - α -2-macroglobulin* to Huh7 cells was performed using Scatchard analy-

sis. For both ^{14}C - α -2-macroglobulin* and ^{125}I - α -2-macroglobulin*, analysis of the binding data yielded a nonlinear plot (Figure 3), suggesting the presence of two sets of receptors with different binding affinities and that do not show cooperativity (1). This corresponds with previous reports that α -2-macroglobulin* binds to both the LRP and to a G-protein coupled receptor, the α -2-macroglobulin signaling receptor (7,8), with Scatchard plots (9) corresponding well with the results presented here.

CONCLUSION

This procedure will not substitute completely for all applications of radioiodination, specifically those requiring proteins with unaltered pI values (e.g., 2-D gel electrophoresis of cell-surface proteins). Glutaraldehyde conjugation will undoubtedly result in changes in the isoelectric points of ^{14}C -labeled proteins because of the modification of lysine residues. Nevertheless, we believe that the technique described here offers an alternative to radioiodination for the sensitive labeling of proteins.

The entire procedure is performed easily within one day. Although resulting in a lower specific activity compared to iodination, the benefits of labeling proteins with ^{14}C include lower cost, increased safety, enormously prolonged half-life, and reduced equipment costs.

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