A fluorescent residualizing label for studies on protein uptake and catabolism *in vivo* and *in vitro*

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Residualizing labels are tracers which remain in lysosomes after uptake and catabolism of the carrier protein and have been especially useful for studies on the sites of plasma protein degradation. Thus far these labels have contained radioactive reporters such as ^3H or ^{125}I . In the present paper we describe a fluorescent residualizing label, *NN*-dilactitol-*N'*-fluoresceinylethylenediamine (DLF). Modification of asialofetuin (ASF) or rat serum albumin (RSA) with DLF affected neither their normal kinetics of clearance from the rat circulation nor their normal tissue sites of uptake and degradation. After injection of DLF–ASF, fluorescent degradation products were recovered nearly quantitatively in liver and retained with a half-life of about 2 days. Fluorescent degradation products from DLF–RSA were recovered in skin and muscle, and were localized in fibroblasts by fluorescence microscopy. These results confirm previous studies with radioactive residualizing labels in which fibroblasts in peripheral tissues were identified as primary sites of albumin degradation. Fluorescent catabolites also accumulated in fibroblasts incubated with DLF–RSA *in vitro*, and residualized with a half-life of about 2 days. Overall, the data establish that DLF functions efficiently as a fluorescent residualizing label both *in vivo* and *in vitro*. The advantages of fluorescent, compared with radioactive, residualizing labels should make them valuable tools for studies on protein uptake and catabolism in biological systems.

INTRODUCTION

In order to identify the tissues and cells active in degradation of plasma proteins with long circulating half-lives, tracers have been developed which are retained in lysosomes at the site of protein catabolism. Using the residualizing label *NN*-dilactitol- ^{125}I -tyramine (DLT), we identified fibroblasts in skin and muscle as principal sites of albumin catabolism in rats, and from autoradiographic studies we concluded that only a subpopulation of fibroblasts were active in albumin degradation [1]. There was no means, using the radioactive tracer, to isolate this subpopulation of cells, but we reasoned that a comparable fluorescent residualizing label should allow their isolation by fluorescence-activated cell sorting (FACS). Since the dilactitol derivative of ^{125}I -tyramine has proven useful as a radioactive residualizing label [1–4], we prepared an analogous fluorescent compound that can be coupled to protein by the same techniques developed for ^{125}I -DLT. The synthesis and purification of this label, *NN*-dilactitol-*N'*-fluoresceinylethylenediamine (DLF), have been described in detail elsewhere [5]. In the present paper we describe experiments which establish that DLF serves as an efficient fluorescent residualizing label *in vivo* and *in vitro*.

A preliminary account of this work has been presented [6].

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate (FITC, isomer I), NaBH_3CN , insolubilized neuraminidase, Pronase, galactose oxidase and Sephadex G-50 were purchased from Sigma Chemical Co. Asialofetuin (ASF) was prepared from fetuin (Gibco) by using insolubilized neuraminidase [2]. Rat serum albumin (RSA) was purified from fresh rat serum and labelled with ^{125}I (Amersham

International) using Iodo-Gen (Pierce) as described previously [1]. DLF was synthesized from fluoresceinylethylenediamine and lactose by reductive amination with NaBH_3CN [5].

Preparation of labelled proteins

The means by which DLF is attached to protein is shown in Scheme 1. The experimental procedures are modelled on those

Scheme 1. Reaction scheme for the coupling of DLF to protein by reductive amination

Abbreviations: ASF, asialofetuin; DLF, *NN*-dilactitol-*N'*-fluoresceinylethylenediamine; DLT, *NN*-dilactitoltyramine; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; FL, fluorescein(yl); PBS, phosphate-buffered saline (137 mM- NaCl /2.7 mM- KCl /8.1 mM- Na_2HPO_4 /1.7 mM- KH_2PO_4 , pH 7.4); RSA, rat serum albumin.

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previously developed for the attachment of ^{125}I -DLF to protein [2]. Briefly, 200 nmol aliquots of DLF in 0.1 ml of 0.25 M-potassium phosphate, pH 7.7, were incubated with 7 units of galactose oxidase at 37 °C for 45 min to convert the galactose residues into the galactose C-6 aldehyde. The enzyme-treated glycoconjugate could be stored at -20 °C for at least 3 months with no loss in reactivity in the subsequent coupling reaction. After treatment with galactose oxidase, DLF was coupled to protein with NaBH_3CN . In preliminary experiments the extent of protein modification with DLF was found to be protein-dependent, so that the ratio of DLF to protein was different for each protein. In a typical preparation, 9 mg of RSA (132 nmol) or 2 mg of ASF (45 nmol) in 200–300 μl of phosphate-buffered-saline (PBS) was added to 200 nmol of enzyme-treated DLF; sufficient 1 M- NaBH_3CN in 0.5 M-potassium phosphate, pH 7.7, was then added so that the final concentration of reducing agent was 30–35 mM. The reaction mixture was incubated for 1 h at 37 °C, and then DLF-labelled protein was separated from unbound DLF by gel filtration on Sephacryl S-200 (Pharmacia) in Dulbecco's [7] phosphate-buffered saline (PBS). The amount of DLF coupled to protein was determined by measurement of A_{493} in 0.1 M-Tris (pH 8.5)/0.1% SDS to quantify fluorescein ($\epsilon_{493} = 77000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and by measurement of protein by using the method of Lowry *et al.* [8], with BSA as standard. Generally, 0.5–0.7 mol of DLF was incorporated per mol of protein. Proteolytic degradation products of DLF-labelled proteins were produced by digestion of 2 mg of protein with 2% Pronase in 0.5 M-potassium phosphate, pH 7.0, overnight at 25 °C. Protein fragments were separated from undigested protein by chromatography on a Sephadex G-50 column in PBS.

Proteins were labelled directly with fluorescein (FL) using minor modifications of a general literature procedure [8] employing FITC. Small volumes of FITC in acetone (20–30 μl ; 10–12 mg/ml) were added to 30–40 mg of protein in 10 ml of 0.1 M- Na_2CO_3 , pH 9.6. Reaction mixtures were incubated for 4 h at 4 °C, then labelled protein was separated from unbound FITC by chromatography on Sephacryl S-200. The ratio of FITC to protein was varied in order to obtain substitution comparable with that achieved with DLF; to prepare protein substituted to 0.5–0.7 mol of FL/mol of protein, the molar ratio of FITC to protein was approximately 1:1 for preparation of FL-RSA, and 2.5:1 for the preparation of FL-ASF.

Fluorescence measurements

To optimize detection of FL and eliminate protein quenching of fluorescence, plasma and tissue extracts were read in 0.1 M-Tris (pH 8.5)/1% SDS (Tris/SDS). Fluorescence was measured by using a Gilford Fluoro-IV spectrofluorimeter (excitation at 485 nm; emission at 520 nm). Relative fluorescence units were converted into nmol of DLF or FL using FITC as standard.

Experiments *in vivo*

Male and female Sprague-Dawley rats, (typically 140–190 g body weight each), were used for '*in vivo*' experiments. Rats were anaesthetized with diethyl ether and injected via the tail vein with fluorescent and/or radioactive proteins diluted into 200–300 μl of PBS. The kinetics of protein clearance from the circulation were monitored by removing blood samples from tail veins at desired time intervals and determining radioactivity and fluorescence in 25–50 μl aliquots of plasma dissolved in 1 ml of Tris/SDS. Fluorescence in plasma from uninjected rats, which was subtracted from experimental values, amounted to $\leq 2\%$ of initial experimental values.

Determination of fluorescence in liver homogenates

At various times after injection of DLF- or FL-ASF, animals were killed by over-anaesthetization with diethyl ether and perfused with iced PBS; the liver was removed and weighed, and then a weighed piece (~ 1 g) was homogenized in a Potter-Elvehjem Teflon-glass homogenizer in 3 vol. distilled water (liver/water, 1:3, w/v). Duplicate aliquots (350 μl) of the homogenate were diluted with 3 vol. of a Tris/SDS concentrate ($1.33 \times$) so that the final solution contained 0.1 M-Tris (pH 8.5)/1% SDS. The diluted homogenate was clarified by centrifugation for 5 min in an Eppendorf Microfuge, the supernatant filtered through a 0.45 μm -pore-size Acrodisc (Gelman) filter and the fluorescence then measured in the filtrate. Readings were corrected for endogenous fluorescence (10–30% of experimental values) measured in a liver extract prepared from an uninjected animal. An internal standard was then added to correct for endogenous quenching by the tissue extract, i.e., a known amount of fluorescent protein in a total volume of 20 μl was added to each sample to estimate recovery of added fluorescence. Recovery was typically 60–70%, i.e. 30–40% quench, and sample readings were corrected appropriately.

Determination of fluorescence in skin and muscle extracts

Rats injected with DLF- or FL-RSA were killed as described above, 4 days after injection, when approx. 75% of the administered protein had been catabolized. Sections of abdominal muscle and shaved abdominal skin were removed, then weighed pieces (~ 0.3 g) of each tissue were minced and extracted in 2 ml of Tris/SDS for 2 h at room temperature with shaking. The extracts were centrifuged, filtered, and the fluorescence in the filtrate determined as described above for liver. Background and quench corrections were applied; typical backgrounds were 10–20% of experimental values, and quench was 20–30%. When DLF-RSA or FL-RSA Pronase-digestion products were added to pieces of muscle and skin from uninjected animals, recoveries of fluorescence from intact and degraded protein were > 90 and 95% respectively.

Measurement of the extent of protein degradation in tissue extracts

Low-molecular-mass fluorescent degradation products from DLF- or FL-labelled proteins were co-precipitated with intact protein with 20% (w/v) trichloroacetic acid. An ultracentrifugation assay was used to distinguish radioactive and fluorescent degradation products from intact labelled protein. Aliquots (1 ml) of clarified tissue extracts were placed in Centricon-10 microconcentrators (Amicon; 10000- M_r cut-off) and centrifuged in a Beckman model J-21C centrifuge at 6000 rev./min for 90 min, when $\geq 95\%$ of the liquid had passed through the concentrator membrane. The microconcentrator membranes were then washed with 1 ml of Tris/SDS and the fluorescence was determined in the retained material and flow-through fractions. In control experiments, $\geq 89\%$ of DLF-RSA Pronase-digestion products and $< 4\%$ of intact DLF-RSA added to control tissue were recovered in the ultrafiltrate.

Localization of fluorescence in tissue sections

To obtain tissue sections for fluorescence microscopy, animals were injected with either DLF- or FL-RSA, and killed by over-anaesthetization at 4 days after injection. Tissues were removed rapidly, cut into 1 mm pieces in freshly prepared 2% (w/v) formaldehyde in PBS and fixed overnight in fresh aliquots of the same solution. The samples were then dehydrated through graded ethanol solutions and embedded in Sorvall JB-4 glycolmethacrylate (Polaron) according to the manufacturer's instructions

[1]. Tissue blocks were cut into 2–3 μm -thick sections, which were placed on glass slides; photographs were taken using a Zeiss IM35 microscope equipped for fluorescence and phase-contrast microscopy.

Isolation of skin cells containing fluorescent degradation products

At 4 days after injection of DLF–RSA, cells were isolated from skin by sequential collagenase digestions, using techniques described previously for rats injected with ^{125}I -DLT–RSA [1]. Cells were recovered from each harvest by centrifugation, washed once in Ham's F-10 medium (Hazelton Laboratories) containing 10% (v/v) fetal-calf serum (HyClone), and then resuspended in 1 ml of PBS. A 100 μl aliquot was removed for measurement of cell number with a haemocytometer. A 100 μl aliquot of 1 M-Tris (pH 8.5)/10% (w/v) SDS was added to the remaining sample, which was then centrifuged in a Centricon-10 instrument, and the fluorescence measured in the filtrate. It was not possible to measure fluorescence in the lysed cells without first clarifying the lysate by centrifugation through the microconcentrator.

In vitro experiments

Rat skin fibroblasts isolated as described previously [2] were cultured in Ham's F-10 medium containing 10% fetal-calf serum. Cells, generally at population doubling of 5–10, were seeded at 0.2×10^6 cells per 35 mm-diameter culture dish and used 5–6 days later at confluence. Cells were fed with 1 ml of medium containing either DLF- or FL–RSA and incubated at 37 °C; the medium was removed at desired time intervals, and the cells were washed three times with 2 ml of 2% (w/v) BSA in PBS, followed by three washes with 2 ml of PBS. Cells were harvested by adding 1 ml 0.1 M-Tris (pH 8.5)/0.2% SDS to the plate, the plate being left at room temperature for 5 min and the lysate then removed for measurement of fluorescence and cell protein. To measure the rate of loss of degradation products from the cells, fibroblasts were fed and washed as described above, but then re-fed with 1 ml of normal medium and reincubated at 37 °C for various periods. At the end of the wash-out period the medium was removed, and the cells were processed as described above. The albumin concentration in all media samples was determined by using a Bromocresol Green dye-binding assay [10]. At the concentrations used, DLF–RSA did not interfere with measurement of total albumin in media samples.

RESULTS

Coupling of DLF to protein

The sequence of reactions used for labelling proteins with DLF, shown in Scheme 1, is similar to that used previously for coupling radioactive glycoconjugates to proteins by reductive amination [2]. The only major difference in the labelling reaction described here is that stoichiometric amounts of the glycoconjugate are required for derivatization in order to make fluorescent protein and/or degradation products detectable in tissues and cells.

Behaviour *in vivo* of DLF-ASF

As a first step in the evaluation of the intracellular retention of degradation products from DLF-labelled protein, the label was attached to ASF. This protein was chosen because it is both rapidly cleared into hepatocytes by a receptor-mediated process and rapidly degraded to low-molecular-mass catabolites [11]. Thus, in effect, a bolus of degradation products is produced quickly in a single tissue, and by measurement of the rate of loss of the reporter molecule, an estimate of the efficiency of retention

Fig. 1. Recovery of fluorescence in plasma and after injection of DLF–ASF and FL–ASF

(a) Kinetics of plasma clearance of DLF–ASF ($\square$) and FL–ASF ($\circ$). For plasma studies $\sim 150 \mu\text{g}$ of each fluorescent protein substituted with 0.6–1 mol of label/mol of protein was injected into 160–185 g rats and aliquots of plasma were removed at the indicated times for the measurement of fluorescence. In competition experiments 2 mg of unlabelled ASF was co-injected with each fluorescent protein. Fluorescence in plasma at 30 and 100 s was taken as 100% in clearance and competition experiments respectively. Data points represent average values for two animals with each label for each experiment. (b) Recovery of fluorescence in liver after injection of DLF–ASF ($\square$) and FL–ASF ($\circ$). Animals were injected with fluorescent proteins (substituted with 0.4–0.6 mol of label/mol of protein), killed at the indicated times, and the livers were removed and processed for the measurement of fluorescence. For DLF–ASF experiments conducted at 1–3 days, 4–5 mg of protein was used in order to obtain accurate measurements over the full time period; for FL–ASF experiments and DLF–ASF at 1 h, 1 mg of protein was injected in order to obtain complete clearance of protein from the circulation within 1 h. The percentage of initial fluorescence was based on fluorescence in blood at the 60 s time point. Data are shown for each of two animals at each time point.

of the catabolites can be obtained. FL–ASF served as a control in these experiments, for comparison of a conventional label with the residualizing label. When small amounts ($< 200 \mu\text{g}$) of DLF- or FL–ASF were injected into rats, fluorescence was recovered in plasma with a half-life of ~ 200 s (Fig. 1a); by contrast, when 2 mg of unlabelled ASF was co-injected with either protein, the circulating half-life was extended to ~ 22 min. Thus attachment of either DLF or FL did not affect the normal receptor-mediated clearance of the protein.

The time course of recovery of fluorescence in liver after injection of DLF- and FL–ASF in liver is shown in Fig. 1(b). For DLF–ASF, 80–100% of the injected dose was recovered in liver 1 h after injection of 1 mg of protein, but for FL–ASF only 60% of the dose was recovered at this same time point, presumably because of poor retention of degradation products from the conventionally labelled protein. Thus, whereas $\sim 50\%$ of the injected dose of DLF–ASF was recovered in the liver at 2 days after injection, $< 5\%$ of injected FL–ASF remained in liver 4 h after injection. The approx. 2 days half-life for recovery of fluorescence from DLF–ASF was similar to that observed for recovery of degradation products from the radioactive residualizing label, ^{125}I -DLT [2]. To estimate the size of the fluorescent material in liver, clarified homogenates were analysed by

Fig. 2. Sephadex G-50 chromatography of liver extracts prepared at various times after injection of DLF-ASF

Extracts of liver from animals killed at 1 h (---) and 1 day (···) after injection of DLF-ASF were chromatographed on an 80 ml (1.5 cm × 47 cm) Sephadex G-50 column in Tris/SDS; 1 ml fractions were collected and the relative fluorescence was measured. Pronase-digested DLF-ASF (—) was used as a marker for elution of degradation products. The vertical arrow indicates the void volume.

Sephadex G-50 chromatography. As Fig. 2 shows, at both 1 h and 1 day after injection, most of the fluorescent products in liver were comparable in size with degradation products from a Pronase digest of DLF-ASF. At 1 h a portion of recovered fluorescence was eluted between fractions 50 and 70, slightly ahead of the major fraction of degradation products, but only a small amount of this material was still detectable at 1 day. At both 1 h and 1 day < 10% of recovered fluorescence was eluted at the void volume of the column with the intact protein.

Recovery *in vivo* of fluorescence from RSA

DLF and FL were each attached to RSA to determine whether the fluorescent labels would affect the kinetics of clearance of a long-lived plasma protein from the circulation. ^{125}I -RSA was co-injected with each fluorescent protein as an independent marker of protein clearance. The plasma clearance of RSA labelled with the various reporter molecules is shown in Fig. 3. Regardless of

Fig. 3. Kinetics of plasma clearance of RSA labelled with various probes

Animals were injected with mixtures of either DLF-RSA (□) and ^{125}I -RSA (△) or FL-RSA (○) and ^{125}I -RSA (△). Protein mixtures contained 6–10 mg of fluorescent protein (0.4–0.7 mol of label/mol of protein) and 50 µg of radioactive protein [(6–8) × 10⁶ c.p.m.]. Aliquots of plasma were removed at the indicated times for the measurement of radioactivity and fluorescence. The percentage of the dose remaining is based on fluorescence or radioactivity at the 100 s time point. Each data point represents the average value for three animals with each pair of labels; variance was < 10% of the value shown. The broken line represents the equilibration phase of the clearance curve.

Table 1. Recovery of various tracers after injection of RSA labelled with radioactive and fluorescent probes

Rats were injected with 7–12 mg of DLF- or FL-RSA (0.4–0.7 mol of label/mol of RSA); 16×10^6 c.p.m. ^{125}I -RSA was co-injected with each fluorescent protein. Animals were killed 4 days later, and tissues were removed and processed for the measurement of fluorescence, radioactivity and intact and degraded protein as described in the Materials and methods section.

Label	Dose recovered (%)*		Dose catabolized (%)†	
	Muscle	Skin	Muscle	Skin
^{125}I	5.0–5.5	5.6–6.0	0.5–0.6	0.5–1.1
FL	2.7–5.2	3.0–5.0	1.7–2.2	0–0.7
DLF	20–23	23–26	13–20	16–18

* Data represent range for a total of four animals in two separate experiments.

† Calculated by multiplying the percentage of injected dose by the percentage fluorescence or radioactivity recovered in the Centricon ultrafiltrate.

the label used, the plasma half-life of RSA was the same, namely about 2 days, indicating that neither the label nor the labelling procedures affected the rate, or presumably the mechanism, of protein degradation.

Since we have previously identified skin and muscle as the major tissue sites of albumin catabolism in the rat [1], the recovery of fluorescence and radioactivity in these two tissues was examined. As Table 1 shows, significant quantities of both total dose and degradation products from DLF-RSA were recovered in skin and muscle, compared with the amounts recovered from either FL- or ^{125}I -RSA. Not only was 4–5 times as much total fluorescence recovered in tissues from the residualizing label, but ~65% of this fluorescence was in the form of degradation products (on the basis of the Centricon assay). The values for the degraded dose of albumin in skin and muscle using DLF-RSA are similar to those previously obtained using the radioactive label ^{125}I -DLT [1]. There was a similar low recovery of total radioactivity and fluorescence from ^{125}I - and FL-RSA respectively in skin and muscle, and essentially all of this material was in the form of intact protein. The fluorescence photomicrographs in Figs. 4, parts A–D, show that fluorescence in skin and muscle is localized in fibroblasts, identified by their location in interstitial connective tissue and characteristic elongated shape. The detection of fluorescence in some, but not all, fibroblasts is consistent with the distribution of autoradiographic grains over selected cells observed after injection of ^{125}I -DLT-RSA [1]. Also, in agreement with the autoradiographic studies, no fluorescence was detected in vascular endothelium or myocytes. In control experiments no fluorescence was detected in fibroblasts or other cell types in skin and muscle from animals injected with FL-RSA (results not shown), consistent with the low amount of fluorescence recovered in these tissues (Table 1).

Recovery of fluorescence in skin cells

In studies using the radioactive probe ^{125}I -DLT we developed a procedure for harvesting intact skin cells containing radioactive degradation products [1]. This technique was applied to skin from animals injected with DLF-RSA in order to determine whether fluorescent degradation products could be recovered in an analogous fashion. The results in Table 2 show that fluorescent degradation products from DLF-RSA were recovered in cells through several sequential digests of skin. The 2-fold variation in fluorescence per cell was comparable with the variation in

Fig. 4. Fluorescence photomicrographs of skin and muscle from a rat injected with DLF-RSA

A 134 g animal injected with 10 mg of DLF-RSA (0.7 mol/mol of protein) was killed after 4 days and pieces of skin and muscle removed, fixed, embedded and sectioned as described in the Materials and methods section. The exposure time was 90 s; overall magnification $\times 625$. A, a section of dermis showing several fibroblasts containing punctate cytoplasmic fluorescence. B, connective tissue adjacent to muscle containing numerous fibroblasts with cytoplasmic fluorescence. C, section of skeletal muscle showing an elongated fibroblast lying between myocytes. The cell contains numerous punctate fluorescent vesicles, consistent with lysosomal localization of the label. Fluorescence is absent in the myocytes; an autofluorescent red blood cell can be seen at the bottom of the micrograph. D, section of skeletal muscle containing a small blood vessel (bv) with autofluorescent red blood cells. Several fibroblasts (arrows) containing label can be seen in the interstitial connective tissue between myocytes. No fluorescence was seen in vascular endothelium or myocytes.

Table 2. Recovery of fluorescence in cells following collagenase digestion of skin from an animal injected with DLF-RSA

A 110 g rat was injected with 7 mg of DLF-RSA (0.8 mol of DLF/mol of protein) and killed 4 days later. A section of skin was removed from the abdominal area, and six sequential collagenase digests of skin were carried out as described previously [1]. Cells were recovered from each digest, and fluorescence and cell number were determined as described in the Materials and methods section.

Digest	Fluorescence (units/cell pellet)*	No. of cells ($\times 10^{-6}$)	Fluorescence (units/cell)
1	100	0.71	148
2	126	1.0	132
3	140	2.0	74
4	78	1.1	75
5	61	1.0	64
6	81	1.1	77

* Fluorescence in the cell pellet from the first digest is arbitrarily defined as 100 units; the values shown have been corrected for background readings of 9 units/ 10^6 cells, measured in cell pellets isolated from a control, uninjected, animal.

recovered radioactivity per cell from animals injected with ^{125}I -DLT-RSA [1]. No effort was made to isolate skin cells from rats injected with FL-RSA because of the low fluorescence recovered in skin (Table 1).

Behaviour *in vitro* of DLF-RSA

To evaluate the usefulness of DLF for measuring protein uptake by cells in culture, rat skin fibroblasts were fed DLF- or FL-RSA, then harvested and the amount of fluorescence per mg of cell protein determined as function of time. The graph in Fig. 5(a) compares the kinetics of uptake of each protein. At 24 h the amount of total fluorescence accumulated in cells incubated with DLF-RSA was four times greater than in cells incubated with FL-RSA, i.e. 4.2 as against 1.2 μg of albumin/mg of cell protein (Fig. 5a). When degraded protein was measured by using the Centricon assay, the values for DLF- and FL-RSA were 80 and 33 % respectively after 24 h. Thus cells incubated with DLF-RSA contained 10 times as much albumin in the form of degradation products as did cells incubated with FL-RSA, i.e. 3.2 as against 0.3 μg /mg of cell protein. By contrast, by similar calculations, the amount of intact protein was comparable in cells fed either labelled protein, i.e. about 0.8 and 0.7 μg of albumin/mg of cell

Fig. 5. Uptake and loss of fluorescent proteins by rat skin fibroblasts *in vitro*

Data points show the mean $\pm$ s.d. Where error bars are not shown, the s.d. was less than the size of the symbol. (a) Cells were fed 1 ml medium containing 11–17 nmol DLF-RSA ($\square$) or FL-RSA ($\circ$) each substituted with 0.7–0.8 mol of label/mol of protein and harvested at indicated times for the measurement of fluorescence and protein. Data are from three individual experiments, with three dishes at each time point per experiment. Data for DLF-RSA are expressed as percentages of fluorescence measured at 24 h, which was equivalent to $4.2 \pm 0.2 \mu\text{g}$ of albumin/mg of cell protein. Data for FL-RSA are expressed as percentage of DLF-RSA uptake at 24 h; FL-RSA uptake was $1.25 \pm 0.3 \mu\text{g}/24 \text{ h}$ per mg. (b) Cells were fed with 14–19 nmol of DLF-RSA ($\square$) or FL-RSA ($\circ$), as described in (a) for 24 h, then washed and re-fed with normal medium; at indicated times the cells were harvested and the fluorescence and protein measured. Data are from two individual experiments, with three dishes at each time point per experiment.

protein for DLF-RSA and FL-RSA respectively. Residualization of fluorescence was measured in a series of wash-out experiments. After 24 h of protein uptake the cells were washed and re-incubated in normal medium, and the amount of fluorescence remaining was measured over time. Fig. 5(b) demonstrates that DLF was well retained in cells, with about 65% of label still in the cells after a 24 h wash-out period, whereas only about 20% of the FL label was recovered at the same time. At this point the cells incubated with DLF-RSA contained more than 10 times the fluorescence of cells incubated with FL-RSA. Fluorescence released into the medium was not measured in these experiments because it was too low to be detected reliably.

The photomicrographs in Fig. 6, parts A–D, are pictures of rat skin fibroblasts which have been incubated with medium containing DLF-RSA. After 24 h, one set of dishes was washed and then photographed (Fig. 6, parts A and B). There are numerous cells containing bright cytoplasmic fluorescence, although many

cells appear unlabelled. In this same experiment a replicate set of plates was also washed after 24 h, but these dishes were re-fed with normal culture medium and, after another 24 h, washed and photographed (Fig. 6, parts C and D). In agreement with the kinetic data shown in Fig. 5(b), these cells still have significant amounts of fluorescence. Fluorescence could not be seen under the same conditions by fluorescence microscopy during either uptake or wash-out experiments using FL-RSA (results not shown), although a small amount of fluorescence was detectable in cell lysates (Fig. 5) with a fluorimeter. Since most of the cell-associated fluorescence from FL-RSA was in intact protein, it seems likely that this protein may have been diffusely distributed either on the cell surface or in the cytoplasm and thus difficult to see by microscopy. By contrast the recovery of largely degraded protein from DLF-RSA suggests that most of this fluorescence was in lysosomes, consistent with the punctate, perinuclear fluorescence seen in many cells.

DISCUSSION

The availability of radioactive residualizing labels has made possible the identification of the tissue and/or cellular sites of catabolism of a number of circulating proteins, including albumin [1,12,13], IgA [4], IgG [14], low-density lipoprotein [15], β -very-low-density lipoprotein [4] and retinol-binding protein [16]. Here we have described a fluorescent residualizing label which can also be used for this purpose *in vivo* as well as for studies on the uptake and catabolism of labelled proteins by cells in culture. Fluorescein was chosen as a convenient, inexpensive fluorophore for the development of a fluorescent residualizing label. The coupling of DLF at 0.5–0.7 mol of label per mol of ASF or RSA was readily accomplished at reasonable molar ratios of label to protein (1–4:1). Several other proteins have also been labelled with similar efficiency, including β -very-low-density lipoprotein, IgG, and IgM (J. K. Wills, J. W. Baynes & S. R. Thorpe, unpublished work).

Preliminary experiments with DLF-ASF indicated that the label did not interfere with receptor-mediated uptake of the protein, and, in addition, fluorescent degradation products were readily detectable in liver and residualized with an efficiency comparable with that for retention of degradation products from ^{125}I -DLT-ASF. The attachment of DLF also had no effect on the kinetics of catabolism of RSA, and the recovery of fluorescent degradation products from DLF-RSA in skin and muscle (Table 1) indicated that the label was also retained well in peripheral tissues. The eventual localization of the label in fibroblasts by fluorescence microscopy (Fig. 4, parts A–D) confirmed the results of previous studies with ^{125}I -DLT-RSA in which autoradiography was used [1], and established the utility of residualizing fluorescent labels for studies on the cellular sites of protein uptake and catabolism.

Because RSA is catabolized slowly and diffusely throughout the body, studies on the sites of catabolism of DLF-RSA required the injection of rather large, 7–12 mg, amounts of protein, compared with the microgram amounts of tracer protein generally used with radioactive labels. However, this amount of RSA does not significantly alter the total body pool of albumin, since rat plasma contains $\sim 35 \text{ mg}$ albumin/ml, and the total albumin in the plasma and extracellular fluids of a 150 g rat is in excess of 300 mg [17]. In these initial experiments the extent of substitution of the proteins was deliberately limited to $< 1 \text{ mol}$ of DLF/mol of RSA in order to minimize possible structural alterations in the protein; however, higher degrees of substitution of proteins are readily achievable and are not necessarily deleterious. In preliminary experiments with IgM, for example, we have found that substitution with 6–8 mol of DLF/mol of

Fig. 6. Photomicrographs of rat skin fibroblasts fed with DLF-RSA

Cells were fed with 12 nmol of DLF-RSA, as described in the legend to Fig. 5, then washed and photographed using phase (A) and fluorescence (B) optics. A replicate set of plates was also washed after 24 h, but re-fed with normal medium, reincubated for 24 h, then washed again and photographed using phase (C) and fluorescence (D) microscopy. Fluorescence exposure time was 2 min; the overall magnification is $\times 170$.

protein had no effect on the circulating half-life of the protein (2.7 days in the rat; J. K. Weis, J. W. Baynes & S. R. Thorpe, unpublished work). Higher degrees of substitution or studies with shorter-lived, more rapidly endocytosed and catabolized proteins, or with proteins whose catabolism is localized to specific organs, would permit injection of smaller amounts of labelled protein.

Fluorescent labels offer several advantages over radioactive compounds in addition to their stability and ease of handling and disposal. As illustrated in the present study, DLF allowed the use of fluorescence microscopy for convenient identification of skin fibroblasts active in albumin catabolism. The same technique should be generally applicable for identification of the cell populations in tissues that are active in receptor-mediated uptake of specific ligands, for example, determining which subpopulation of non-parenchymal cells in liver are involved in the uptake of

specific protein ligands *in vivo*. For these applications, fluorescence microscopy should be more convenient than autoradiography or fractionation of the various cell populations by centrifugal techniques. In addition, the fluorescent label also offers sufficient sensitivity that it should be useful, with FACS, for the isolation and characterization of the cells most active in protein uptake and catabolism *in vivo* or *in vitro*.

It should be noted that, although conventional fluorescent ligands such as FITC have proven suitable for monitoring protein uptake by receptor-mediated endocytosis, specialized image-intensification systems are often required to obtain useful results [18]. By contrast, residualizing fluorescent labels are retained efficiently in fibroblasts *in vivo* and *in vitro* and yield substantially higher sensitivity (Figs. 5 and 6) compared with conventional labels. DLF has already been used for the characterization of cells by FACS based on fluid-phase endocytic processes,

e.g. albumin uptake by fibroblasts *in vitro* [19]. Overall, the results from studies both *in vivo* and *in vitro* described here demonstrate that fluorescent residualizing labels provide a powerful new tool for identification and isolation of cells active in protein uptake and catabolism.

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