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A new method for detecting endocytosed proteins

Mark S.Bretscher and René Lutter

Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

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A new reagent, DPSgt, is described which has been designed to label cell surface proteins at 0°C. The reagent is easily made; it is water soluble and contains a reactive impermeant ester at one end, a tyrosine which can be radioiodinated at the other, and a disulphide in-between. The label can be removed from cells by cleaving the disulphide linkage in it with glutathione at 0°C. When cells are warmed to 37°C between labelling and reduction, labelled proteins which are endocytosed acquire resistance to reduction. This provides a simple way of measuring the endocytosis of surface proteins. The intracellular pools of transferrin and LDL receptors in K562 cells and fibroblasts have been estimated. The results indicate that intracellular receptors are in non-reducing compartments, and that uptake of average cell surface (by non-coated pit processes) in K562 cells is small.

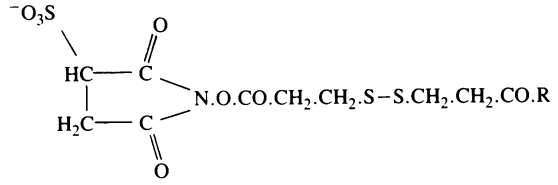
Key words: endocytosis/circulating receptors/cell surface labelling

Introduction

Most eukaryotic cells internalize segments of the plasma membrane, a process initiated by coated pits. Some membrane proteins, like the LDL receptor (Anderson *et al.*, 1976) and transferrin receptor (TFR) (Bleil and Bretscher, 1982), are concentrated into these coated pits, whereas others are excluded (Bretscher *et al.*, 1980). The receptors internalized are usually transported through a variety of intracellular compartments and later returned to the cell surface to undergo another cycle of endocytosis (see e.g. Pearse and Bretscher, 1981).

Much of the evidence for receptor recycling is morphological—biochemical measurements are more difficult. The best defined case at a biochemical level is that of the TFR where its internalization can be measured directly. If cells are labelled by surface iodination at 0°C, where endocytosis is arrested, only receptors in the cell surface are labelled. These are all sensitive to proteases added to the cells at 0°C. However, if the cells are incubated at 37°C between labelling and proteolysis, labelled receptors acquire resistance as they become part of the intracellular pool (Bleil and Bretscher, 1982; Watts, 1985).

A more general method for studying this process is desirable, especially as some receptors are extremely resistant to proteolysis of intact cells at 0°C. Here we describe a new class of reagent which can be used for this purpose. These molecules are based on an impermeant active ester containing a tyrosine residue which can be radioiodinated. Between the active ester and the label there is a disulphide bond which can be cleaved, at will and at 0°C, by glutathione (see Figure 1). The structures of the reagents used here are:



where R is glycyl-tyrosine (gt) or glycyl-tyrosyl-cholamine^{+} Cl^{-} (gtc). These reagents are referred to as DPSgt or DPSgtc (where DPS peptide stands for 3,3′ dithiopropionyl, 1 sulphosuccinimidyl, 1′peptide). When labelled in the tyrosine residue with radioiodide, these reagents are abbreviated to DPSgt* and DPSgtc*.

Results

We first tested the abilities of each reagent, DPSgt* and DPSgtc*, to label the TFR on K562 suspension cells. Washed cells were labelled at 0°C and then dissolved in a nonionic detergent. The TFRs were isolated with an anti-TFR antiserum, and this labelled material fractionated on an SDS gel. In each case, the TFR was effectively labelled (Figure 2, lanes a and d): in each of these lanes there are other major contaminants.

The band labelled TFR was identified as the *bona fide* receptor because, when isolated from cells labelled with lactoperoxidase and radioiodide, it has a mol. wt of 180 kd on non-reducing SDS gels, and 90 kd on reducing gels (Sutherland *et al.*, 1981) (not shown). When labelled with DPSgt* or DPSgtc*, all the label present in the TFR is attached via a reducible linkage, because if the isolated receptors are reduced with dithiothreitol in SDS sample buffer prior to SDS gel fractionation, all the label is removed (Figure 2, lanes g and h). This shows that no direct iodination of the protein occurs.

Figure 2 also shows that endocytosis of the TFR can readily be detected with either reagent. Labelled cells (equivalent to those used in lanes a and d) were either incubated at 37°C for 15 min (lanes c and f) or held at 0°C (lanes b and e); they were then washed in a glutathione buffer to remove surface label before isolation of the TFRs. The label associated with the TFR in cells held at 0°C in lanes b and e has been reduced by ~98% and 90% (compared to the receptor in lanes a and d respectively). However, incubation of the cells at 37°C prior to surface reduction at 0°C (lanes c and f) shows that ~60% of the TFRs are now protected from this reduction, i.e. they have been endocytosed. This conclusion is similar to that reported previously using proteases to detect intracellular TFRs in suspension HeLa cells (Bleil and Bretscher, 1982). The

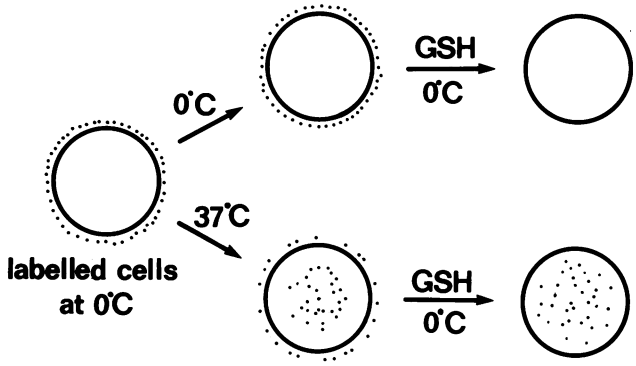


Fig. 1. Outline of standard experiment. Cells are labelled at 0°C, washed and split into three equal portions. One is held at 0°C; another is held at 0°C and (in parallel with the third portion) washed in glutathione buffer; the third portion is held at 37°C to permit endocytosis, then chilled to 0°C and washed in glutathione buffer at 0°C.

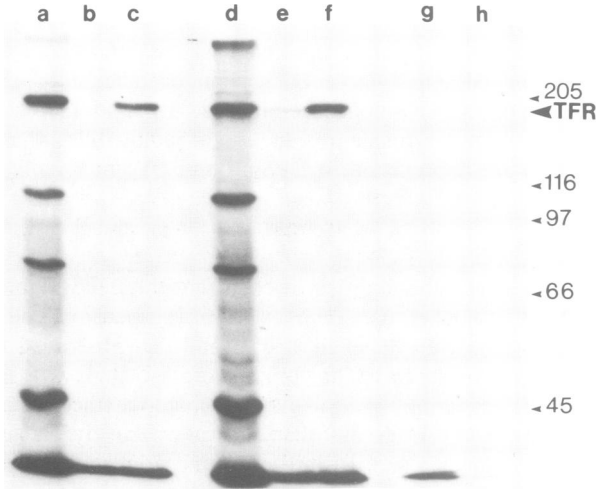


Fig. 2. SDS gel of the TFR from K562 cells. TFR was isolated from cells labelled at 0°C with either DPSgt* (lanes a–c and g) or DPSgtc* (lanes d–f and h). **Lanes a** and **d**: receptors from labelled cells. **Lanes b** and **e**: receptors from cells labelled and reduced at 0°C. **Lanes c** and **f**: receptors from cells labelled at 0°C, incubated 15 min at 37°C, reduced at 0°C. **Lanes g** and **h**: receptors from labelled cells reduced in SDS sample buffer. Note that the only band in lanes a or d which is protected when cells are reduced with glutathione is TFR (compare lane b with c; lane e with f).

other, contaminating, bands present in Figure 1, lanes a and d, are not so protected: evidently they do not enter the intracellular pool to an appreciable extent.

We also looked at the behaviour of the TFR in MRC-5 lung fibroblasts. Figure 3 shows a parallel experiment to that of Figure 2 lanes a–c. Less of the TFR is present intracellularly; the intensity of the protected receptor after a 37°C incubation (lane c) is ~45% of that in the total (lane a).

We wished to compare the intracellular pool of TFRs with the equivalent pool for a different receptor in these two cell types. We chose the LDL receptor, because little is known about the proportion of these which are intracellular, although in fibroblasts the level is very low (Basu *et al.*, 1981). We used a monoclonal antibody to the receptor, IgG-C7 (Beisiegel *et al.*, 1982) to isolate the receptor. Figure 4 shows that the antibody fishes out the correct molecule

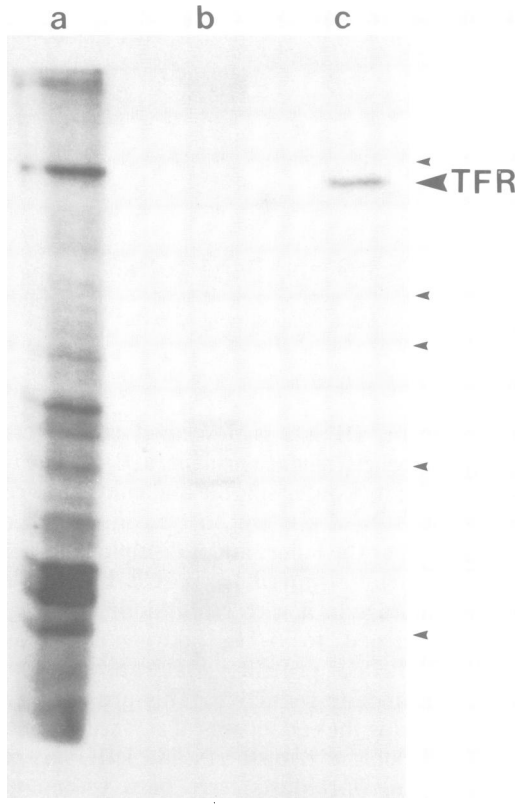


Fig. 3. SDS gel of the TFR from MCR-5 fibroblasts. **Lane a**, receptor from cells labelled with DPSgt*. **Lane b**, receptor from cells labelled and reduced at 0°C. **Lane c**, receptor from cells labelled at 0°C, incubated at 37°C, then reduced at 0°C.

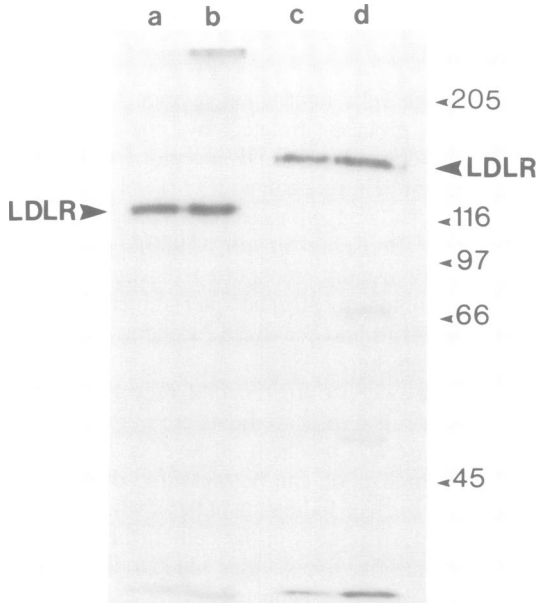


Fig. 4. Detection of LDL receptor. Cells were labelled with lactoperoxidase and radioiodide. LDL receptors were isolated using 1 μg (**lanes a** and **c**) or 2 μg (**lanes b** and **d**) of anti-LDL receptor monoclonal IgG-C7 antibody. The eluted receptors were fractionated on an SDS gel without (lanes a and b) or with 0.1 M dithiothreitol (lanes c and d) in the sample buffer.

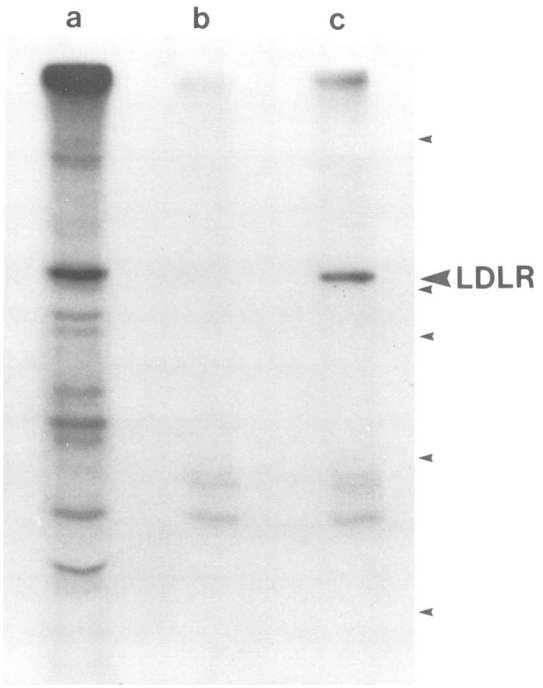


Fig. 5. SDS gel of the LDL receptor from K562 cells. Lanes as in Figure 3.

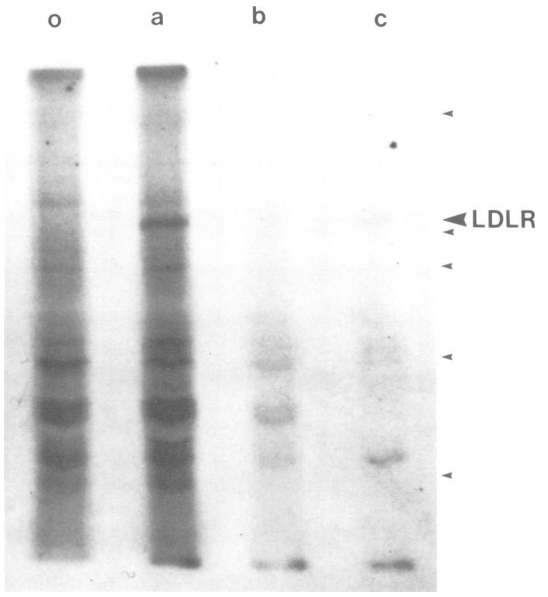


Fig. 6. SDS gel of the LDL receptor from MRC-5 fibroblasts. **Lanes a–c** as in Figure 3; in **lane o**, the LDL receptor isolation was carried out in the absence of added calcium ions, and in the presence of 1 mM EDTA. The LDL receptor, which is not recognized by monoclonal IgG-C7 under these conditions, is absent.

from lactoperoxidase catalysed iodide-labelled K562 cells which had been induced for the LDL receptor. The receptor has an apparent mol. wt of 120 kd (non-reducing conditions in lanes a and b), and after reduction with dithiothreitol in sample buffer, this changes to 160 kd (lanes c and d).

K562 cells and MRC-5 fibroblasts were induced for LDL receptors and then labelled at 0°C with DPSgt*. In the now familiar design of the experiment, the results for K562 cells are shown in Figure 5 and those for MRC-5 fibroblasts in Figure 6. This shows that some 50% of the LDL receptor is found intracellularly in K562 cells, and less, ~15%, in MRC-5 fibroblasts. A time course of endocytosis of the LDL receptor in K562 cells shows that the intracellular pool is saturated within ~2 min at 37°C (not shown).

We next tried to look at the total pool of internalized receptors in K562 cells. For this purpose we chose DPSgtc* which would be expected to be less permeant. Figure 7a shows a 2-dimensional fractionation of the proteins labelled at 0°C on the surface of K562 cells. Figure 7b shows the same from cells which had been kept at 0°C and the surface label depleted by glutathione reduction. Figure 7c shows the pattern of labelled proteins found when a 15 min incubation at 37°C is included between labelling and reduction at 0°C. Comparison of Figure 7b and c shows that several proteins have become resistant to reduction and thus entered into these cells at 37°C. These are indicated by arrows, and amongst them the TFR can be tentatively identified (X).

Discussion

Reagent

The two reagents described here are efficient cell-surface labelling agents. That containing glycyl-tyrosyl-cholamine (DPSgtc, which carries a fixed positive charge) would be expected to be extremely impermeant; its preparation does require synthesis of this tripeptide. The other reagent (DPSgt), containing glycyl-tyrosine, is easy to make and use: the two negative charges on it make it very water soluble. The value of both reagents depends on the ability to cleave the label from cell surface proteins at 0°C. This can be achieved using impermeant thiols: glutathione has been used here, but cysteine can also be used. However, the efficiency of this cleavage varies with the surface protein and the reagent. Thus, ~98% of the label is removed from the TFR when labelled with DPSgt*, but only ~90% when DPSgtc* is used. This resistance to removal of the final few percent of the label must be due to inaccessibility of the −S−S− linkage because all the label is removed by dithiothreitol in sodium dodecyl sulphate solution. This residue of uncleavable label is important, because it provides the natural background to, and thus limits the sensitivity of, the method. Thus, in studying the TFR on K562 cells, where ~60% of the total receptor pool is located intracellularly, the background using either reagent presents no problem. However, when the intracellular pool is small, the background may make its determination difficult. The reagents could clearly be improved: a longer spacer between the active ester and the disulphide bond might make the latter more accessible to glutathione. A different way of solving the same problem would be to incorporate two separated and reducible −S−S− linkages in a reagent, so that reduction of either −S−S− bond would remove the label.

Specific receptors

We show here that, after a 15 min incubation at 37°C, ~60% of the labelled TFRs from the surface of K562 cells have become non-reducible by externally added glutathione. We interpret this to mean that these receptors are now inaccessible to reduction and are therefore in an intracellular compartment. This result is similar to that concluded earlier

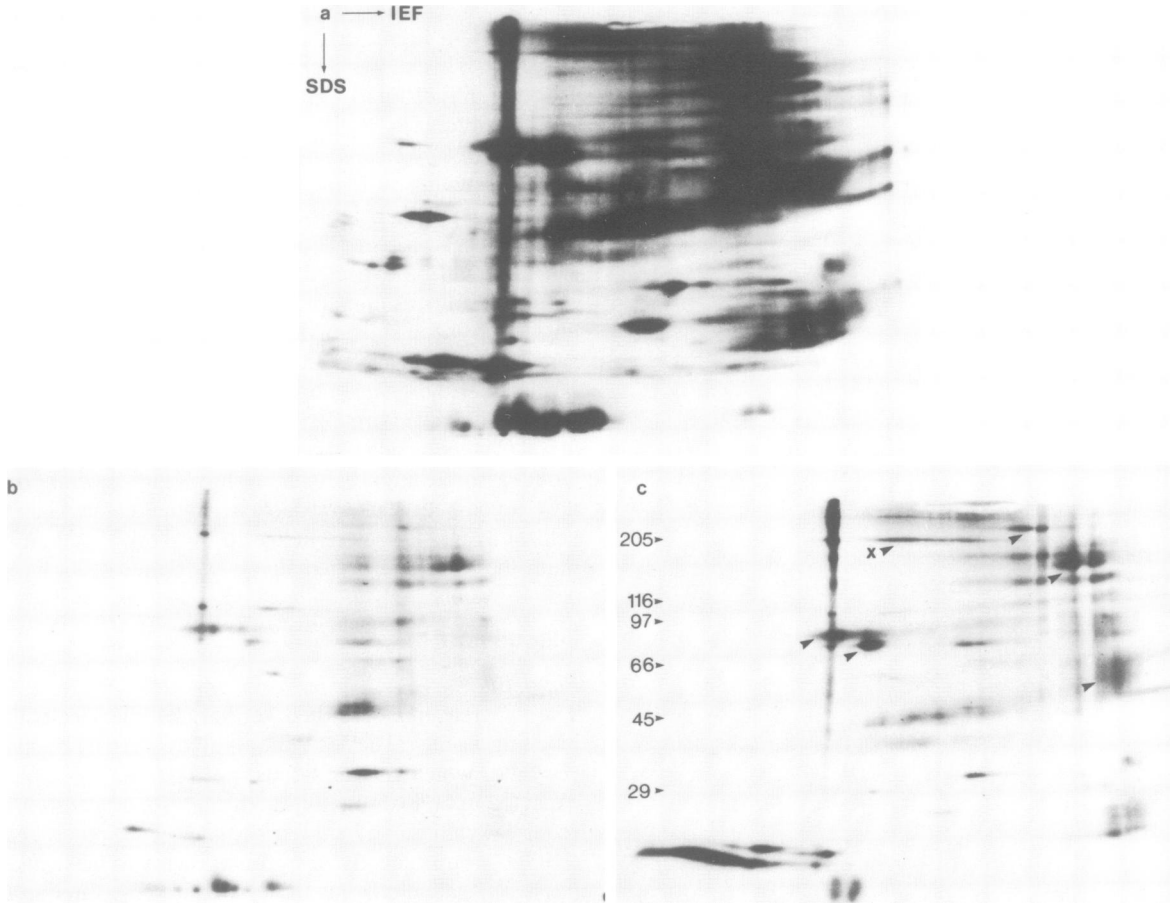


Fig. 7. Two-dimensional separation of labelled proteins from K562 cells. Cells were labelled with DPSgtc*. (**a**) Total soluble extract of labelled cells dissolved in nonionic detergent. (**b**) Extract from cells labelled and reduced at 0°C. (**c**) Extract from labelled cells incubated at 37°C and then reduced at 0°C. The band at 180 kd which streaks from the origin in gel c (labelled X with a pI of ~5–6) is probably the TFR. Some of the bands protected from reduction by incubation at 37°C (compare gel b and gel c) are indicated with arrowheads. The nature of the large labelled band at the front of gel c is unknown.

for HeLa and K562 cells and in which the half-life for these receptors to enter either cell type was found to be ~5 min (Bleil and Bretscher, 1982; see also Klausner *et al.*, 1983). Thus, although 15 min may not be quite sufficient to equilibrate the receptors along their intracellular routes, it is long enough to estimate the intracellular proportion of the receptor (see, e.g. Basu *et al.*, 1981; Bleil and Bretscher, 1982). For all other measurements we chose 15 min incubations at 37°C, it being assumed that this is long enough to provide an estimate of the intracellular pool of a particular receptor.

In MRC-5 fibroblasts, the TFRs are more weakly labelled, presumably because they are scarcer than on K562 cells. The intracellular proportion of them is ~45%. The LDL receptor on K562 cells is about equally partitioned between the intracellular and plasma membranes. In MCR-5 fibroblasts the internal proportion is significantly lower, being ~15%.

We have also tried to look at the total pool of circulating proteins by 2-dimensional electrophoresis. Whilst the quality of the gels leaves something to be desired, they do show that there are several unidentified molecules which are rapidly endocytosed, and which may therefore be circulating receptors (see also Watts, 1984, in which the proteins in the endosomal compartment were selectively labelled). In principle, a reagent could be devised in which a hapten is included distal to the reactive acylating end. It might be possible to use such an agent to isolate those proteins which are rapidly endocytosed (and which may therefore contain new circulating receptors).

A conclusion to be drawn from our observations is that a substantial fraction of each recycling receptor is intracellular. In addition, we may conclude that the intracellular compartments in which these labelled molecules reside are not strongly reducing (as otherwise the label would be removed from them). And, finally, the major contaminating bands seen in Figure 2 (lanes a and d) do not appear in an intracellular pool after the K562 cells are incubated at 37°C. This presumably means that they are excluded from coated pits. It also shows that generalized surface uptake (by a process not involving coated pits) does not contribute to the intracellular pool and therefore may not exist in these cells.

Materials and methods

Reagent synthesis

In a typical synthesis of the glycyl-tyrosine reagent, DPSgt, 4 μmol 3,3′-dithiobis(sulphosuccinimidylpropionate) (2.43 mg; DTSSP; Pierce Chemical Company) was dissolved in 40 μl water and immediately added to a mixture of 20 μl 0.1 M glycyl-tyrosine (2 μmol) and 40 μl 1 M sodium

phosphate pH 7.0 and held at 0°C for 15 min. It was then diluted with 2.3 ml 0.05 M acetic acid at 0°C, parcelled out in small (10 μl) aliquots and held at −70°C.

A similar protocol was used for the glycyl-tyrosyl-cholamine reagent, DPSgtc: to 4 μmol DTSSP in 60 μl water was added a mixture of 20 μl 0.1 M glycyl-tyrosyl-cholamine chloride hydrochloride plus 160 μl 0.025 M disodium hydrogen phosphate at 0°C, the mixture vortexed and held at 0°C for 15 min. A cloudiness appears which redissolves when the reaction is diluted in 2.3 ml 0.05 M acetic acid. The reagents appear stable for months at −70°C.

Reagent labelling

The standard reaction of 20 μl water contained 1−2 mCi ^{125}I. To this was added 1 μl 3 M NaCl, 2 μl 1 M sodium phosphate pH 7.0, 1 μl stock reagent (i.e. ~1 μg of DPSgt or DPSgtc) and 1 μl 5 mg/ml chloramine T and this mix held at 0°C for 15 min. To this was then added 2 μl of a solution of 1M sodium *p*-hydroxybenzoate containing 0.1 M NaI; the reaction goes light brown and then colourless. After 2−3 min at 0°C, the reagent is used. The purpose of adding this latter mix is to chase all the potentially reactive iodine species (apart from the reagent) into inert derivatives of hydroxybenzoate, and destroy excess chloramine T.

The reagent is clearly a mixture of molecules. There will be unlabelled and intact DTSSP which, in principle, could cross-link cell surface proteins. This is not a problem, as can be seen in the figures, because of the very low concentration of reagent used. There will also be labelled dithiobis (propionyl peptide) in which both ends of the DTSSP have reacted with the peptide, but this is unreactive and will not cause any labelling. There will be the desired reagent, the labelled peptide joined via the −S−S− linkage to the active ester end of the molecule. And finally, some of the active ester ends will have been hydrolysed in reagent preparation, but these do not matter as they are either unlabelled or inert, or both.

Glycyl-tyrosyl-cholamine chloride hydrochloride

Boc-glycyl-tyrosine. 1 mmol glycyl-tyrosine (242 mg) was converted to the t-BOC peptide, by vortexing in 4 ml 0.25 M NaOH in 50% dioxane with 0.24 ml (1.0 mmol) *t*-butyl pyrocarbonate at room temperature for 30 min. The mixture was concentrated by rotary evaporation to 2 ml and 2.2 ml 1 M $NaHSO_4$ and 2 ml ethyl acetate added and vigorously mixed. A second ethyl acetate extraction was done, the two organic phases pooled and back-extracted twice with 2 ml water. The organic phase was dried over anhydrous sodium sulphate, and then dried down to yield 335 mg product.

Boc-glycyl-tyrosyl-N-hydroxysuccinimide. To the protected peptide were added 120 mg N-hydroxysuccinimide in 2 ml dioxane plus 240 mg dicyclohexylcarbodiimide in 2 ml dioxane, and the mixture held at 0°C for 1 h, then at room temperature for 1 h. The precipitated dicyclohexylurea was filtered off and washed with a little dioxane; the pooled dioxane fractions were concentrated to ~2 ml. The active ester was not isolated but used *in situ.*

Boc-glycyl-tyrosyl-cholamine. To the crude active ester in dioxane was added a solution containing 2 mmol cholamine chloride hydrochloride plus 2 mmol triethylamine in 1 ml water at 0°C; the reaction was immediately vortexed and the cloudy reaction soon became clearer; it was held at room temperature overnight. Analysis of an aliquot of the reaction by pH 3.5 paper electrophoresis, followed by a spray to detect phenols (Ames and Mitchell, 1952), showed that ~70% of the original blocked dipeptide had been converted to the cholamine derivative.

An attempt was made to purify the product on a Dowex-1 column; the reaction was dried down to an oil, taken up in 5 ml water, filtered (to remove remaining dicyclohexylurea) and applied to a triethylammonium Dowex-1 column at pH 5.5. An applied gradient of triethylammonium acetate at pH 5.5 eluted the product (detected as above by pH 3.5 electrophoresis) in a large smear. The main fractions were pooled, dried down, taken up in a small volume of water and applied to a large C-18 HPLC column. This was eluted with a gradient of 2% acetic acid in water to 2% acetic acid in methanol. One large major peak emerged which was dried down to a glass [170 mg; when dissolved in water there were 454 OD_{274} U, or 0.34 mmol product (based on a molar extinction coefficient for tyrosine of 1340); 0.34 mmol would weigh 164 mg for the anhydrous acetate salt of t-BOC-glycyl-tyrosyl-cholamine (mol. wt 482)].

Glycyl-tyrosyl-cholamine chloride hydrochloride. The peptide was deacylated in 5 ml 95% trifluoroacetic acid at room temperature for 1 h, concentrated to dryness, dissolved in 2 ml 0.6 M HCl and extracted thrice with 2 ml ether. The deacylated peptide was concentrated to a glass, and finally held overnight over P_2O_5 and NaOH. There was 125 mg material (mol. wt of expected product is 394 as chloride hydrochloride salt; 0.32 mmol), giving a single spot of the expected mobility on pH 3.5 electrophoresis. A stock solution of 0.1 M was held at −20°C.

Cells

K562, a human leukaemic suspension line and MRC-5, a human lung fibroblast line, were maintained in DMEM plus 10% fetal calf serum (FCS). In some cases (for looking at LDL receptors or at total receptors) the cells were grown for 24 h in DMEM plus 10% human serum depleted of LDL prior to use in order to induce these receptors (Goldstein and Brown, 1974). Fibroblasts were removed from flasks with EDTA. All cells were washed twice in DPBS at 0°C. Cells were labelled either with lactoperoxidase and radioiodide (Bleil and Bretscher, 1982) or with one of the reagents described here.

To label cells with DPSgt* or DPSgtc*, a pellet of ~0.05 ml packed cells was suspended and held in 0.1 M Na_2HPO_4 at 0°C (for ~20 min until the reagent was almost ready). They were then pelleted and up to 50 μl 0.1 M Na_2HPO_4 added to produce a fairly dense cell slurry. The labelling agent was added directly to the cells, immediately vortexed and held at 0°C for 20 min. The cells were then washed twice in DPBS containing 10% FCS. This labelling procedure has no deleterious effects on cell viability, monitored on CHO cells with DPSgt.

To measure endocytosis, labelled cells were put into a small volume of DPBS/FCS and split into three equal portions. (i) One was held at 0°C. (ii) One was held at 0°C and, in parallel with the third sample, treated with glutathione. (iii) The third sample was diluted into 10 volumes of DPBS/FCS at 37°C, held there for 15 min with occasional shaking, chilled to 0°C, spun out and resuspended in a small volume of DPBS/FCS at 0°C.

Glutathione stripping

To cell samples (ii) and (iii) were added 5 ml of a glutathione solution at 0°C made as follows: 155 mg glutathione (0.5 mmol, free acid; Sigma), 0.15 ml 5 M NaCl, 0.1 ml 0.1 M EDTA in 9.0 ml water, to which were added just prior to use 0.075 ml 10 M NaOH (i.e. 0.75 mmol) and then 1 ml FCS or 1 ml 10% BSA. The NaOH added is that required to neutralize the carboxyl group and deprotonate half the cysteine residues in glutathione. It is thus strongly buffered at the pK of this cysteine, which is ~pH 8.6.

The cells were mixed in the glutathione buffer at 0°C and held there for 20 min. They were gently spun out, resuspended in 0.5 ml DPBS/FCS and then reincubated in fresh glutathione buffer for 30 min at 0°C. The cells were then washed in DPBS/1% BSA.

Cell lysates

All cells were washed in DPBS/1% BSA. Cell pellets were then resuspended at 0°C in 100 μl buffer containing 0.10 M NaCl, 0.05 M Tris pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 5 mg/ml iodoacetamide, 40 μg/ml pancreatic trypsin inhibitor, 1% BSA (except if the samples were to be subjected to isoelectric focusing) and held at 0°C for ~10 min. During this time extracellular (and probably also intracellular) free -SH groups will be derivatized by the iodoacetamide. 20 μl 10% NP-40 was then added, the lysates warmed to room temperature for 5 min and nuclei and other large debris removed by centrifugation for 10 min in a microfuge, followed by a spin for 20 min at 4°C at 70 000 r.p.m. in a Beckman TLA 100.1 rotor to remove ribosomes and other large macromolecules.

Receptor isolation

Receptors were isolated in one of two ways. For the TFR the cell extract was added to 20 μl of a 50% suspension of anti-receptor antibody coupled to Affigel-10 plus 10 μl 0.1 M EDTA pH 8 and vigorously shaken overnight at 4°C. For the LDL receptor, the extract was mixed with 1 μg anti-LDL receptor monoclonal antibody for 10 min at 0°C (with or without the addition of 10 μl 0.1 M EDTA), followed by vigorous shaking for 90 min with 20 μl 50% suspension of protein A−Sepharose (Boehringer). The Affigel or Sepharose beads were washed on a small column with 3 ml buffer containing 0.15 M NaCl, 0.05 M Tris pH 7.4, 1 mM PMSF, 1 mg/ml iodoacetamide, 1% BSA, 1% NP-40 and (for the transferrin, but not LDL, receptors) 1 mM EDTA at 4°C, and then with 0.2 ml of this buffer diluted 10-fold. The columns were eluted with SDS sample buffer (0.1 M CHES pH 9.6, 2% SDS, 10% glycerol, no reducing agent) and the eluates warmed briefly to 50°C before running on a 7.5% SDS acrylamide gel. Dithiothreitol was added, where indicated, to 10 mM. Gels were stained with Page blue G90 (BDH), dried and autoradiographed at −70°C on flashed film for 2−10 days. Comparison of the label present in gel bands was either done directly by counting excised bands in a γ-counter, or by densitometry of the autoradiograms.

2-dimensional analysis of labelled proteins was done as described by O'Farrell (1975) focusing in Pharmalyte pH 3.5−10 in 8 M urea and 1%

NP-40 using a T5C3 polyacrylamide gel; the second dimension was done on a 7.5–15% gradient acrylamide SDS gel.

Antibodies

A rat anti-human TFR serum from rats (Bleil and Bretscher, 1982) was used. A crude IgG fraction (50% ammonium sulphate precipitate) was prepared, dialysed extensively against PBS and then coupled to Affigel-10 (Biorad) using ~2 mg IgG to 1 ml packed beads. The mixture was rolled overnight at 4°C, the gel washed in PBS and stored in PBS/azide until required. The monoclonal anti-LDL receptor antibody was mouse IgG-C7 (Beisiegel *et al.*, 1982).

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