

## Transport of $\beta$ -lactoglobulin and $\alpha$ -lactalbumin in enterocyte-like Caco-2 cells

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**Summary** — The transport of [ $^{14}\text{C}$ ]-radiolabelled  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin through Caco-2 cell monolayers grown on permeable filters was studied in order to evaluate the different protein pathways through the intestinal epithelium.  $\beta$ -Lactoglobulin or  $\alpha$ -lactalbumin (0.25–3 mg/ml) was introduced on the apical side of the monolayer and both the transport and the release of labelled material from the cells were measured following different incubation times. The labelled material was analysed by either trichloroacetic acid precipitation or by high pressure liquid chromatography. Despite some differences between the 2 proteins, the overall mechanism followed approximately the same pattern. Part of the intact internalized protein was either recycled (10–15%) or transported via transcytosis (about 5%). Another pathway corresponded to the intracellular degradation of the protein. The calculation of the different routes followed by the proteins indicated that the main part of the degraded fraction (about 70%) was recycled whereas approximately 30% was transported to the other side. Moreover, 5–10% of the endocytosed material was retained intracellularly.

**milk protein / protein absorption / intestine / Caco-2 cells**

**Résumé** — Transport de la  $\beta$ -lactoglobuline et de l' $\alpha$ -lactalbumine dans les cellules de type entérocytaire Caco-2. Afin de quantifier les différentes voies de transport des protéines à travers l'épithélium intestinal, nous avons étudié le transport de la  $\beta$ -lactoglobuline et de l' $\alpha$ -lactalbumine, marquées au  $^{14}\text{C}$ , à travers des cellules Caco-2 cultivées en monocouches sur des filtres perméables. Après addition de la  $\beta$ -lactoglobuline ou de l' $\alpha$ -lactalbumine (0,25 à 3 mg/ml) dans le compartiment apical, le transport et le relargage des produits radiomarqués ont été mesurés à différents temps d'incubation. Le matériel marqué a été analysé par précipitation à l'acide trichloracétique et par chromatographie liquide haute performance. Malgré quelques différences entre les 2 protéines, leur mécanisme général de transport était comparable. Une partie des protéines intactes internalisées a été soit recyclée (10–15%) soit «transcytosée» (environ 5%). Les protéines peuvent également être dégradées à l'intérieur des cellules. La quantification des différentes voies indique que les produits de dégradation sont majoritairement recyclés (70%) alors que 30% environ sont transportés vers la face opposée. De plus, 5 à 10% du matériel internalisé est stocké dans les cellules.

**protéines du lait / absorption / intestin / Caco-2**

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## INTRODUCTION

The apical side of the intestinal cell is in contact with the proteins that are present in the lumen, *ie* endogenous secreted proteins and exogenous ingested proteins. Early investigations have shown the existence of a luminal protein transport through the intestinal epithelium (Danforth and Moore, 1959; Walker *et al*, 1972; Warshaw *et al*, 1974). Different functions are associated with this mechanism, including passive immunity (Walker, 1981; Gardner, 1988), ligand transport (Papiz *et al*, 1986; Brown *et al*, 1988) and various metabolic regulation mechanisms (Gardner, 1988; Lee *et al*, 1990; Sanderson and Walker, 1993). Results obtained with different proteins show that protein transport through the intestinal epithelium proceeds by a major transcellular degradative pathway together with a minor intact transport route (Cornell *et al*, 1971; Walker *et al*, 1972; Walker, 1981; Stern and Walker, 1984; Gardner, 1988; Marcon-Genty *et al*, 1989; Hughson and Hopkins, 1990; Le Bivic *et al*, 1990; Matter *et al*, 1990; Heymann *et al*, 1990; Caillard and Tomé, 1992, 1994; Heyman and Desjeux, 1992). Depending on their nature, proteins are generally taken up by the intestinal cells as in other epithelial cells and are subsequently internalized inside plasma-membrane-derived vesicles *via* either receptor-mediated endocytosis or non-specific fluid phase endocytosis (Walker, 1981; Gardner, 1988). The internalized macromolecules inside the vesicles are recycled back to the plasma membrane or transported to the opposite side by a process referred to as transcytosis or processed in the course of a multistep transport sequence through various intracellular organelles, such as endosomes, prelysosomes and lysosomes (Shen *et al*, 1992).

The aim of the present study was to evaluate the different pathways (*ie* uptake, internalization, recycling, degradation and tran-

scytosis) followed by 2 of the exogenous protein models,  $\beta$ -lactoglobulin ( $\beta$ -Lg) and  $\alpha$ -lactalbumin ( $\alpha$ -La) in a Caco-2 cell monolayer.  $\beta$ -Lg and  $\alpha$ -La are the major proteins in bovine milk whey and are generally considered to be major allergens, especially  $\beta$ -Lg, which is absent in human milk (Bahna, 1985).  $\beta$ -Lg is also believed to be resistant to peptic and chymotryptic digestion (Reddy *et al*, 1988) and to play a role as a carrier of small, lipid-soluble molecules (Papiz *et al*, 1986; Brown *et al*, 1988). Both proteins have already been recovered in human jejunal effluents in an intact antigenic form following milk ingestion (Mahé *et al*, 1991); it has been demonstrated that they are taken up and transported by the intestinal mucosa (Stern and Walker, 1984; Gardner, 1988; Marcon-Genty *et al*, 1989; Huneau and Tomé, 1990; Caillard and Tomé, 1992, 1994). Since the Caco-2 cell line grown on permeable filters forms a polarized monolayer, it has been proposed as an intestinal epithelium model (Pinto *et al*, 1983; Hidalgo *et al*, 1989). Several intestinal transport systems have already been studied in these cells (Smith *et al*, 1992), including ions (Pinto *et al*, 1983; Grasset *et al*, 1984), neutral amino acids, bile acids, cobalamin, vitamin D, dipeptides and proteins (Heyman *et al*, 1990; Hughson and Hopkins, 1990; Le Bivic *et al*, 1990; Matter *et al*, 1990). The differentiated Caco-2 cell monolayer forms an impermeable barrier to the passage of molecules, such as inulin, polyethylene glycol 4000, dextran and Lucifer yellow (Hidalgo *et al*, 1989) which are not freely transported across cell membranes. In order to characterize the pathways of  $\beta$ -Lg and  $\alpha$ -La in Caco-2 cells, their transepithelial transport was first measured across the cell monolayer after apical incubation. In another set of experiments, after incubation with labelled proteins, the filters were washed and reincubated in a marker-free medium to determine the release (recycling and transport) of endocytosed material. The results showed that the 2 proteins were either recycled,

degraded or transcytosed by the cells to nearly the same extent.

## MATERIALS AND METHODS

### Materials

Bovine milk  $\beta$ -Lg (MW 36 kD, A+B, crystallized 3 times) and  $\alpha$ -La (MW 14 kD, Type III) were purchased from Sigma and were [ $^{14}$ C]-radiolabelled (120 kBq/mg) by reductive methylation with [ $^{14}$ C]formaldehyde (Amersham international, plc) in the presence of NaCNBH<sub>3</sub> (Jentoft and Deaborn, 1979). Cell culture reagents were purchased from Gibco Laboratories (Life Technologies SARL, France). The human colon carcinoma derived cell-line Caco-2 was obtained from J Grosclaude (INRA, Jouy-en-Josas). The cells were cultured in a humidified incubator at 37°C with 5% CO<sub>2</sub> in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 1% non-essential aminoacids, 6 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml) (complete medium: DMEMc). They were seeded at 4 10<sup>4</sup> cells/cm<sup>2</sup> and grown in 75 cm<sup>2</sup> flasks (Corning). They reached confluence in 6–7 d and were detached with 0.5% trypsin 0.2% ethylene-diaminetetraacetic acid (EDTA). The medium was changed every other day. The cells were used between passages 30 and 65.

### Caco-2 cell cultures on filters

Caco-2 cells were cultured on polyethylene terephthalate filters 11  $\mu$ m thick and 4.91 cm<sup>2</sup> in diameter (inserts Falcon). The medium was added to both the apical and the basolateral sides of the cells (2.5 ml/each side). The cells remained in a monolayer under these conditions. The trans-epithelial electrical resistance (TEER), used as a control of the tightness of the monolayer, was measured with the Epithelial Voltohmmeter, EVOM (World Precision Instruments, New Haven, CT). In DMEMc the TEER reached 500  $\Omega$ .cm<sup>2</sup> after confluency and remained constant between days 7 and 17 (data not shown). When TEER was less than 400  $\Omega$ .cm<sup>2</sup>, the filters were discarded. The cells were used on day 14.

### Transport and release experiments

For transepithelial transport measurement, radio-labelled protein (74 kBq, 0.25 to 3 mg/ml), [ $^{14}$ C]- $\beta$ -Lg or [ $^{14}$ C]- $\alpha$ -La was added to the apical reservoir. Apical and basal media and filters were removed at different times.

For the release study, the filters were removed after 2 h with the labelled proteins, washed 3 times in phosphate-buffered saline (PBS) at room temperature, immersed 5 min in ice-cold PBS and then washed twice in PBS at room temperature. Fresh medium without labelled protein was then added to both sides and incubated for various periods of time. The media and the filters were then collected.

### Sample analysis

All of the apical and basal media collected were either directly used for radioactivity measurement and high-pressure liquid chromatography (HPLC) analysis or subjected to trichloroacetic acid (TCA) precipitation. In the latter case, the solution was adjusted to 10% TCA, incubated overnight at 4°C and centrifugated (1 300 g, 10 min, 4°C). The supernatant, which represented the non-proteinic nitrogen, was directly counted for radioactivity. The pellet (proteinic nitrogen) was rinsed twice with 10% TCA, resuspended in 0.3 M NaOH and used for radioactivity measurement and HPLC analysis. The filters were washed 3 times with 0.3 M mannitol, the cells on the filters were solubilized with 3 ml NaOH 1 N for 16 h at 37°C and then counted. For the HPLC analyses, the samples were injected into a Waters gradient system equipped with a C<sub>18</sub> Zorbax (5  $\mu$ m) column (250 x 4.6 mm) at 214 nm and a radiochromatography detector (Flo-one A-500, Packard, Radiomatic, Meriden, USA). The column was eluted at 40°C at a flow rate of 2 ml/min following a linear 4.5–85.5% gradient of acetonitrile in 0.1% trifluoroacetic acid in the 0–20 min period.

### Calculation

The results are expressed as means  $\pm$  SE. The statistical analysis was performed using unpaired Student's *t* test. The transport is expressed as [ $^{14}$ C]-protein equivalent ( $\mu$ g), *i.e.* the amount of

labelled-protein calculated from the measured radioactivity.

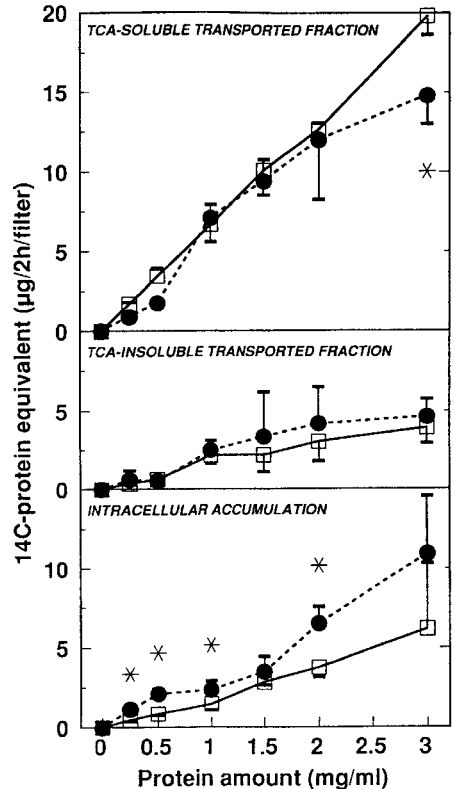
## RESULTS

In the first experiment, the TCA-soluble and insoluble fractions and the intracellular accumulation of labelled material were measured after 2 h of incubation with 0.25 to 3 mg/ml [ $^{14}\text{C}$ ]- $\beta$ -Lg or [ $^{14}\text{C}$ ]- $\alpha$ -La on the apical side of the Caco-2 cell monolayers (fig 1). The intracellular amounts of  $\beta$ -Lg and  $\alpha$ -La equivalent increased linearly with the initial protein amount. The accumulation was higher with  $\beta$ -Lg than with  $\alpha$ -La ( $P < 0.05$ ). The transported fractions, soluble or insoluble after TCA precipitation, both increased linearly with the initial protein amount. The radio-labelled material increased more rapidly for the transported TCA-soluble fraction. No significant difference was noticed between the proteins except at high protein concentrations. For an initial concentration of 3 mg/ml, it appeared that the transported TCA-soluble  $\alpha$ -La equivalent fraction was higher than that of  $\beta$ -Lg.

To study the nature of the different media, all of the incubation and transport media were analyzed by HPLC after 120 min of incubation with 1 mg/ml protein in the apical reservoir (fig 2). The overlapping of the radioactivity and standard absorbance curves indicated that neither protein was degraded and that the labelling occurred exclusively on the proteins. After 120 min in the incubation medium,  $\alpha$ -La was very slightly degraded compared to the standard. A small peak B appeared after a short retention time, but the intact protein peak A corresponded to more than 90% of the material. The same 2 fractions, A and B, were detected in the basal medium after 2 h incubation. For  $\beta$ -Lg, the 3 main fractions (A', B', C') were detected at 14.8, 10.8 and 1.5 min, respectively, in the incubation medium. The major fraction A corresponded to the pro-

tein, which was the only peak present in the standard profile. After 2 h protein incubation, these 3 fractions (A', B', C') could be detected in the basal medium. The intact protein fractions A and A' represented 25 and 30% of the transported material for  $\alpha$ -La and  $\beta$ -Lg, respectively, values near to those obtained by TCA-precipitation (around 25%).

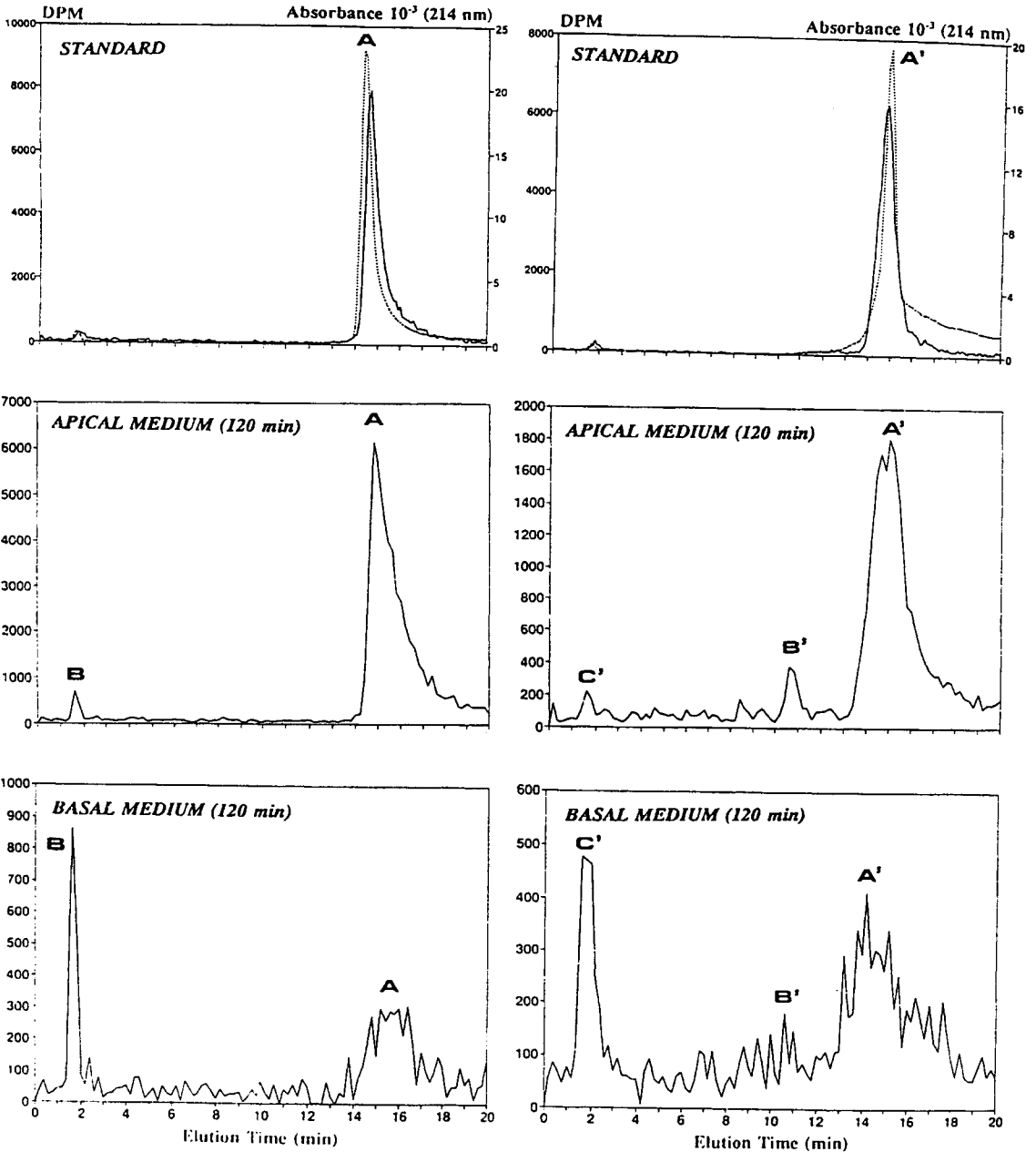
In another set of experiments, after apical incubation of Caco-2 cell monolayers



**Fig 1.** Dose-dependent transport of TCA-soluble and TCA-insoluble fractions and total intracellular accumulation of [ $^{14}\text{C}$ ]-protein equivalent. One milligram per millilitre (74 kBq) of [ $^{14}\text{C}$ ]- $\beta$ -Lg (dashed line) or [ $^{14}\text{C}$ ]- $\alpha$ -La (solid line) was incubated on the apical side of the Caco-2 cell monolayer for 2 h in DMEMc. Values are means  $\pm$  SE ( $n > 5$ ). \* The values of the given protein amounts are significant different between both proteins ( $P < 0.05$ ).

$\alpha$ -lactalbumin

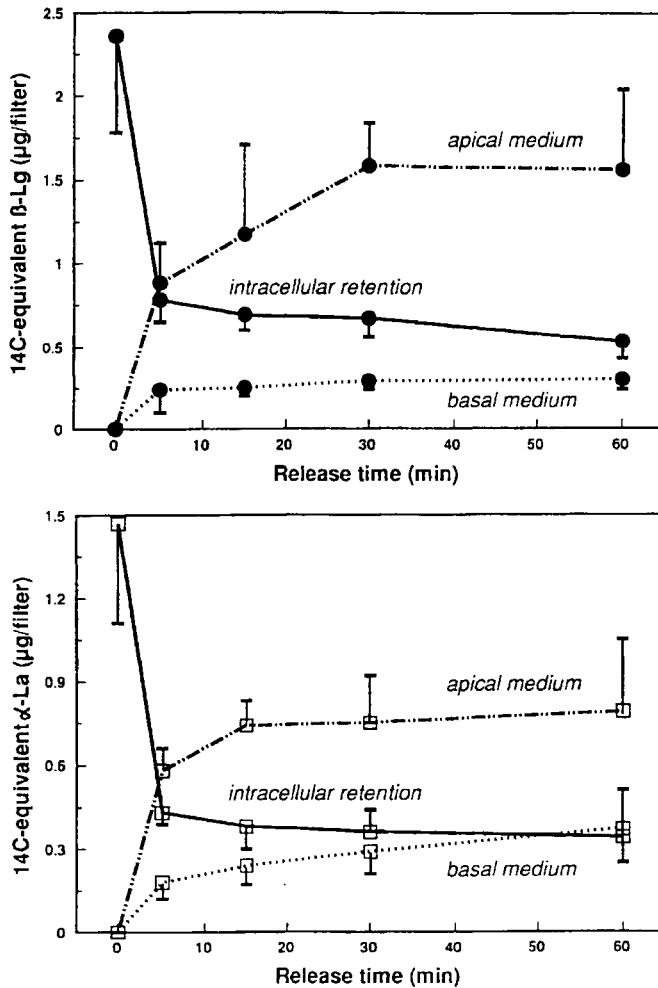
$\beta$ -lactoglobulin



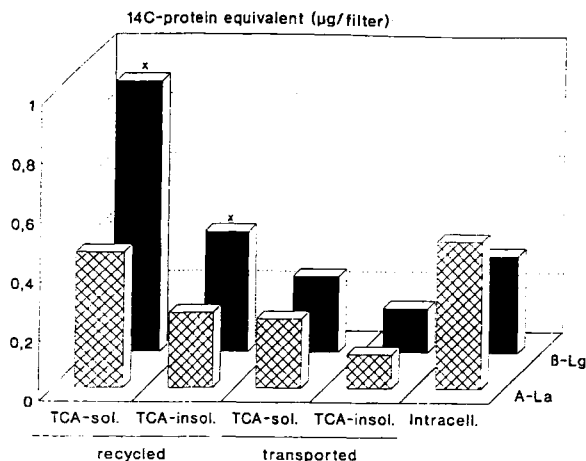
**Fig 2.** Typical reverse-phase high pressure liquid chromatography radioactivity (solid line) or UV spectrometry at 214 nm (dashed line) pattern of standards, apical and basal media after 120 min incubation of 3 mg/ml [ $^{14}$ C]- $\alpha$ -La (left) or  $\beta$ -Lg (right) on the apical side of a Caco-2 cell monolayer.

with 1 mg/ml labelled  $\beta$ -Lg or  $\alpha$ -La for 2 h, the cells were extensively washed and a marker-free medium was added to both sides of the monolayer. At different times (0–60 min), both the apical and basal media were measured for TCA-soluble and insoluble released labelled material fractions. The cells were then measured for the cellular retention of the labelled material (fig 3). The release of intracellular labelled material generally occurred mostly during the first 30 min and thereafter the intracellular reten-

tion of [ $^{14}$ C]-protein equivalent remained stable. Most of the labelled material released by the cells was recycled to the incubation side and a non-negligible fraction was transported to the opposite side. The intact (TCA-insoluble) and degraded (TCA-soluble) labelled fractions released in either the apical or the basal side and the cellular retention of labelled material were measured in the cells previously incubated for 2 h with 1 mg/ml [ $^{14}$ C]-labelled  $\beta$ -Lg or  $\alpha$ -La (fig 4). The TCA-soluble fractions were larger than



**Fig 3.** Time-dependent recycling (---●---), transport (····●····) and intracellular retention (—●—) of preinternalized [ $^{14}$ C]-protein equivalent. Caco-2 cells were preloaded for 2 h with 1 mg/ml (74 kBq)  $\beta$ -Lg (top) or  $\alpha$ -La (bottom) on the apical side of the monolayer. After washing, the cells layers were reincubated in a marker-free medium. At the indicated times, both apical and basal media were collected and the cells were washed, extracted and the remaining radioactivity in the cells was measured. Values are means  $\pm$  SE ( $n > 5$ ).



**Fig 4.** Dose-dependent recycling, transport and intracellular retention of preinternalized [ $^{14}\text{C}$ ]- $\beta$ -Lg or [ $^{14}\text{C}$ ]- $\alpha$ -La equivalent. Caco-2 cells were preloaded for 2 h with  $\beta$ -Lg or  $\alpha$ -La (1 mg/ml, 74 kBq) on the apical side of the monolayer. After washing, the cells layers were reincubated in a marker-free medium. At 60 min release time, both apical and basal medium were collected and submitted to TCA-precipitation, indicating the TCA-soluble and insoluble fractions. The cells were washed and extracted. The recovered radioactivity amounts increased linearly with the initial protein amount in the incubation medium. The results are expressed as  $\mu\text{g}$  protein recovered in each fraction per filter and per mg initial protein amount. The values marked with an x ( $n > 5$ ) are significantly different from the control values ( $P < 0.05$ ).

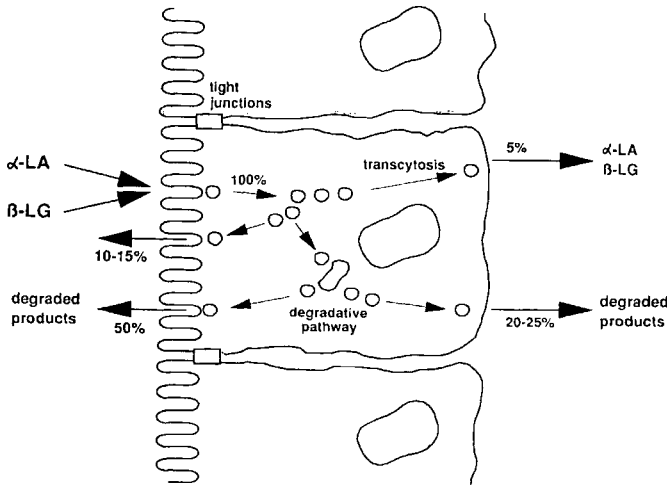
the TCA-insoluble fractions. No difference appeared between the 2 proteins concerning the transported fractions and the intracellular retention, whereas the  $\beta$ -Lg recycled fractions were more than twice those of  $\alpha$ -La.

## DISCUSSION

The present study was undertaken to evaluate the transport of the exogenous proteins  $\beta$ -Lg and  $\alpha$ -La across the intestinal epithelium model, Caco-2 cell monolayers, grown on permeable filters, from the apical to the basal side (in the physiological pathway). The present results show that both the food-type proteins  $\beta$ -Lg and  $\alpha$ -La are taken up and are subsequently recycled and transported in the Caco-2 cell monolayer. Despite some differences between the 2 proteins, the overall mechanism was approximately the same (fig 5). The traffic of the intact protein included uptake at the cell membrane and subsequent recycling, trans-

cytosis or degradation. The degraded products were excreted outside the cell (recycled or transported), with a small fraction being retained inside the cell. Binding or uptake was approximately 1.5 times higher for  $\beta$ -Lg than for  $\alpha$ -La. The intact and degraded recycled fractions always represented 10–20% and 45–50% respectively of the cell-associated material. In contrast, the transported fractions, both in TCA-soluble and insoluble forms, were quantitatively identical regardless of the protein.

The first step in the transport was the initial uptake of the protein at the cell membrane, probably by a non-specific endocytotic mechanism since the endocytosis appeared to be a non-saturable phenomenon (Walker *et al*, 1972; Warshaw *et al*, 1974; Leary and Lecce, 1976). A larger fraction of  $\beta$ -Lg than of  $\alpha$ -La (1.5:1) remained in the cell after incubation with the labelled proteins but no difference was detected for the fractions that remained in the cells after 2 h of release. This observation suggests that the difference is mainly



**Fig 5.** Schematic illustration of the transport of protein from the apical side of the Caco-2 cell monolayers. The values were estimated from the dose-dependent experiment (for 2 h incubation).

due to a higher fixation of  $\beta$ -Lg (compared to that of  $\alpha$ -La) to the cell membrane rather than to a higher intracellular accumulation. This is also confirmed by the higher recycling rate of  $\beta$ -Lg to the incubation medium and the equivalent or even higher (for the TCA-soluble fraction) transport rate of  $\alpha$ -La. The presence of a  $\beta$ -Lg receptor, or at least non-specific fixation sites in the cell membrane, has already been suggested in relation to a possible role as a lipid-soluble molecule transporter (Papiz *et al*, 1986; Caillard and Tomé, 1994).

Following endocytosis, part of the internalized protein in the plasma membrane-derived vesicles (endosomes) inside the cell was either recycled to the incubation side or moved *via* transcytosis to the opposite side. Bomsel *et al* (1989) previously mentioned an intact recycling pathway for HRP in MDCK (Madin-Darby canine kidney) cells and demonstrated the existence of distinct sets of early apical and basolateral endosomes where recycling and transcytosis primarily occur. Hughson and Hopkins (1990) have demonstrated that transferrin in Caco-2 cells is efficiently recycled back to the basolateral membrane. In the present study, the recycling pathway was quantitatively

higher for  $\beta$ -Lg than for  $\alpha$ -La, but was always in the 10–20% range when expressed as a percentage of the initial cell-associated material. For both  $\beta$ -Lg and  $\alpha$ -La, a quantitative apical-to-basal transcytosis of the proteins was quite evident. It has previously been suggested that microtubules are implicated in this transport (Marcon-Genty *et al*, 1989; Caillard and Tomé, 1992; Caillard and Tomé, 1994).

Another pathway for endocytosed protein in plasma membrane-derived vesicles is the intracellular degradation. The proportion of the protein using this pathway was larger for  $\beta$ -Lg than for  $\alpha$ -La. This pathway was not saturated and the degraded fraction increased with the concentration of the protein in the incubation medium. The main part of the degradation is believed to occur during the course of a multistep transport sequence through various intracellular organelles, such as endosomes, prelysosomes and lysosomes (Shen *et al*, 1992). The degraded products were either excreted outside the side or retained in the cell. The calculation of the different routes indicated that 65–70% of the degraded products were recycled and 30–35% were transported. The observation that most of the degraded



fraction was excreted in the incubation medium suggests that the degradation takes place near the cell membrane of the incubation side. In addition, membrane endoproteases are present in the brush-border membrane and have already been demonstrated to hydrolyse several proteins (Guan *et al*, 1988). Such a hydrolysis by membrane endoproteases cannot be excluded, especially for the apical recycling of degraded fractions.

Intestinal protein transport is believed to be an important physiological mechanism (Danforth and Moore, 1959; Lee *et al*, 1990; Smith *et al*, 1992; Sanderson and Walker, 1993). The present results give some data on the relative importance of the different pathways in the exogenous protein transport in an epithelium model system. It was observed that macromolecules such as  $\alpha$ -La and  $\beta$ -Lg can cross the epithelial barrier, but the role of the different pathways in the hypothetical functions, *ie* immune surveillance, metabolic regulation or gastrointestinal disease, still remains unknown. From a pharmacological aspect, transcytosis of active peptides or proteins or protein-like vectors could represent a useful pathway for their administration *via* the oral route (Lee *et al*, 1990; Smith *et al*, 1992). For instance, the structural analysis of  $\beta$ -Lg shows a partial sequence identity and a crystal structure which is similar to human retinol binding protein suggesting a carrier role of small, lipid-soluble molecules (Papiz *et al*, 1986; Brown *et al*, 1988).  $\alpha$ -La and  $\beta$ -Lg are generally considered as major allergens in milk (Bahna, 1985). There is evidence to suggest that adult animals, after closure, can continue to absorb large molecules in antigenic and biologically active quantities (Danforth and Moore, 1959; Warshaw *et al*, 1974). This mechanism is especially believed to play a role in the different physiological and immunological responses that contribute to the oral tolerance and its regulation. Numerous

results suggest a controlling role by intestinal cell in the control of the presentation of food antigens to immunocompetent cells. Partial processing of proteins and the generation of peptide fragments are necessary to present an antigenic peptide to a class II MHC (major histocompatibility complex). This idea may be supported by the observation that the gut epithelium expresses the class II glycoproteins (Bland and Kambrarage, 1991). In addition, although protein transport is of high physiological importance, it is probably not always necessary that luminal proteins penetrate the epithelium in order to act as immunological or metabolic effectors. For instance, the apical membrane of the epithelial cell expresses numerous receptors, some of which could recognize luminal proteins and initiate cellular events before or even without further penetration of the proteins (Sanderson and Walker, 1993). Research in these directions is also of great importance in order to further the understanding of the physiological and immunological role of luminal proteins.

## ACKNOWLEDGMENT

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