Vitamin A Metabolism in the Human Intestinal Caco-2 Cell Line†

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ABSTRACT: The human intestinal Caco-2 cell line, described as enterocyte-like in a number of studies, was examined for its ability to carry out the metabolism of vitamin A normally required in the absorptive process. Caco-2 cells contained cellular retinol-binding protein II, a protein which is abundant in human villus-associated enterocytes and may play an important role in the absorption of vitamin A. Microsomal preparations from Caco-2 cells contained retinal reductase, acyl-CoA–retinol acyltransferase (ARAT), and lecithin–retinol acyltransferase (LRAT) activities, which have previously been proposed to be involved in the metabolism of dietary vitamin A in the enterocyte. When intact Caco-2 cells were provided with β-carotene, retinyl acetate, or retinol, synthesis of retinyl palmitoleate, oleate, palmitate, and small amounts of stearate resulted. However, exogenous retinyl palmitate or stearate was not used by Caco-2 cells as a source of retinol for ester synthesis. While there was a disproportionate synthesis of monoenoic fatty acid esters of retinol in Caco-2 cells compared to the retinyl esters typically found in human chylomicrons or the esters normally synthesized in rat intestine, the pattern was consistent with the substantial amount of unsaturated fatty acids, particularly 18:1 and 16:1, found in the *sn*-1 position of Caco-2 microsomal phosphatidylcholine, the fatty acyl donor for LRAT. Both ARAT and LRAT have been proposed to be responsible for retinyl ester synthesis in the enterocyte. When Caco-2 cells were pretreated with phenylmethanesulfonyl fluoride (PMSF), previously shown to inhibit LRAT but not ARAT, cellular accumulation of exogenous retinol was unaffected whereas retinyl ester synthesis was reduced by >90%, even at supraphysiological levels (30 μM) of retinol exposure. Examination of microsomes from PMSF-treated cells confirmed the inhibition of LRAT activity and the retention ARAT activity as well as the ability to generate acyl-CoA. These data suggest that LRAT may be the physiologically important enzyme for the esterification of retinol in Caco-2 cells.

The Caco-2 cell line, derived from a human colon adenocarcinoma, has proven useful in the study and characterization of a number of the biochemical processes of the small intestinal enterocyte. Postconfluent cultures of these cells spontaneously differentiate, forming monolayers of polarized, columnar epithelium with junctional complexes and apical, microvillar brush-border membrane. When grown on an impermeable support, the formation of domes of cells in the monolayer is apparent, indicative of transepithelial ionic transport (Pinto et al., 1983). On permeable membranes, Caco-2 cells synthesize and release from the basolateral surface the major classes of intestinal lipoproteins (Traber et al., 1987; Hughes et al., 1987) and cholesterol ester transfer protein (Faust & Albers, 1988). Caco-2 cells also contain certain biochemical elements characteristic of enterocytes including brush-border disaccharidases and peptidases (Zweibaum et al., 1983; Hauri et al., 1985), the capability of folic acid uptake (Vincent et al., 1985), Na-dependent P_i uptake (Mohrmann et al., 1986), and receptors for epidermal growth factor (Hidalgo et al., 1989) and vasoactive intestinal peptide (Laburthe et al., 1987). However, to our knowledge, vitamin A metabolism has not previously been examined in this cell line.

The absorption of vitamin A by the small intestine is a complex process that requires several metabolic conversions [reviewed in Goodman and Blaner (1984)]. Dietary retinyl esters are hydrolyzed within the lumen of the intestine, and retinol is taken up by the enterocyte by a specific transport mechanism. Dietary β-carotene is absorbed intact within mixed micelles of lipid and bile salts and then may be cleaved within the enterocyte to form *all-trans*-retinaldehyde, which is subsequently reduced to *all-trans*-retinol. Retinol is esterified with long-chain fatty acids prior to incorporation into chylomicrons. Both retinaldehyde and retinol have been shown to bind to cellular retinol-binding protein, type II [CRBP(II)[1] (MacDonald & Ong, 1987)], and these protein–ligand complexes serve as substrates for microsomal retinal reductase and lecithin–retinol acyltransferase (LRAT), respectively (Kakkad & Ong, 1988; MacDonald & Ong, 1988). In addition to LRAT, a second retinol esterification activity has been described, acyl-CoA–retinol acyltransferase or ARAT (Helgerud et al., 1983), which acylates free retinol, but apparently not retinol bound to CRBP(II) (Ong et al., 1987).

We report here that Caco-2 cells were found to contain a number of the components of intestinal vitamin A metabolism, particularly the ability to internalize and esterify retinol. The synthesis of these retinyl esters appeared to be attributable to LRAT, but not ARAT, even at high concentrations of retinol in the culture medium.

Experimental Procedures

Materials. Caco-2 cells were obtained from the American Type Culture Collection (ATCC HTB 37, Freeze 7547/passage 19). Dulbecco's modified Eagle's medium, fetal bovine serum, MEM nonessential amino acids, L-glutamine, genta-

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[1] Abbreviations: ARAT, acyl-CoA–retinol acyltransferase; bSA, bovine serum albumin; CRBP(II), cellular retinol-binding protein, type II; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earl's balanced saline solution; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LRAT, lecithin–retinol acyltransferase; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline.

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mycin reagent, and trypsin–EDTA were from Gibco. Earle's balanced saline solution, dithiothreitol, sodium taurocholate, phenylmethanesulfonyl fluoride, diisopropyl fluorophosphate, β-carotene, *all-trans*-retinol, *all-trans*-retinaldehyde, *all-trans*-retinyl acetate, *all-trans*-retinyl palmitate, palmitoyl-CoA, palmitoleoyl chloride, oleoyl chloride, stearoyl chloride, fatty acid free bovine serum albumin, and protein A were from Sigma. NADH was obtained from Pharmacia. Dimethyl sulfoxide and aluminum oxide (activated, neutral, Brockmann 1) were from Aldrich. All organic solvents were HPLC grade from Burdick and Jackson Laboratories.

Cell Culture Conditions. Caco-2 cells were cultured in DMEM with 4.5 g/L glucose, 1% nonessential amino acids, 2 mM L-glutamine, 50 μg/mL gentamycin sulfate, and 20% FBS. Cells were seeded at a density of (~2–3) × 10^4 cells/cm^2 in 100-mm tissue culture dishes and maintained in a 37 °C humidified incubator in an atmosphere of 5% CO_2 in air. Culture were refed every other day and typically reached confluency 3–4 days postseeding. All experiments were conducted between days 11 and 21 postseeding using cultures between passages 25 and 45. Cells were routinely passaged at a split ratio of 1:4 when 70–80% confluent by rinsing with PBS, pH 7.4, containing 1 mM Na_2EDTA followed by incubation with 2 mL of trypsin–EDTA for 15–20 min. Cells were counted after trypsinization with a Coulter counter.

Preparation of Cytosol and Microsomes. Caco-2 cells cultured on 100-mm plastic dishes 11–21 days postseeding were washed 1× with EBSS and scraped into 10 mL of ice-cold EBSS. The cells were centrifuged at 700*g* for 10 min at 4 °C. The supernatant liquid was discarded, and the cells were sonicated in 0.5 mL of PBS with 1 mM dithiothreitol (DTT) using a Branson sonifier fitted with a microtip for 20 × 1 s pulses at a power output of 2. The broken cell suspension was centrifuged at 20000*g* for 20 min at 4 °C, followed by centrifugation of the supernatant fraction at 113000*g* for 60 min at 4 °C. The supernatant fraction (cytosol) was removed, the microsomal pellet was rinsed gently with 0.2 M KH_2PO_4, pH 7.2, containing 1 mM DTT, and the pellet was resuspended in the same buffer to a final protein concentration of ~2–10 mg/mL. Aliquots of cytosol and microsomes were frozen in a dry ice/methanol bath and stored at −80 °C. Protein concentrations were determined by the BCA assay (Pierce) using bSA as a standard.

Radioimmunoassay and Western Blot Analysis. Pure rat CRBP(II) was purified as described (Schaefer et al., 1989). Radioiodination and radioimmunoassay of rat CRBP(II) were conducted essentially as described (Quick & Ong, 1989). Cytosolic proteins were submitted to Laemmli SDS–polyacrylamide gel electrophoresis (4% stacking/12% separating gel) and transferred to nitrocellulose membrane (Towbin et al., 1979). Immunodetection of CRBP(II) on the Western blot membrane using an affinity-purified rabbit anti-rat CRBP(II) antibody, and ^{125}I-labeled protein A was conducted as previously described (Ong & Page, 1987). Protein A was radioiodinated by the same method as for rat CRBP(II).

Microsomal Esterification and Reductase Assays. Analyses of microsomal lecithin–retinol acyltransferase (LRAT) activity and acyl-CoA–retinol acyltransferase (ARAT) were conducted essentially as previously described (Ong et al., 1987). The reactions were stopped by addition of ethanol containing 100 μg/mL butylated hydroxytoluene (BHT) and extracted with hexane with 100 μm/mL BHT. The hexane extracts were dried under N_2, dissolved in 100 μL of methanol, and analyzed by reverse-phase HPLC using two Vydac peptide/protein guard columns (Rainin 218-GCC54) in tandem with a Nova Pak C_{18} 3.9 × 150 mm column (Waters). An isocratic methanol mobile phase was used at a flow rate of 2 mL/min. Retinyl esters were quantified by comparison of their integrated peak areas to calibrated areas from pure retinyl ester standards (E_{325} ~52 000 in ethanol). Retinyl palmitoleate, retinyl oleate, and retinyl stearate standards were prepared as described (Huang & Goodman, 1965). For studies on the dependence of microsomal LRAT activity on the concentration of [^3H]retinol–CRBP(II), 100 μL of a 120-μL reaction was extracted into hexane and applied to a 1.2-g 10% H_2O-deactivated alumina column (Ong et al., 1987). ^3H-Labeled retinyl esters were batch-eluted with 2% diethyl ether in hexane, counted by liquid scintillation spectrometry, and quantitated on the basis of the specific activity of [^3H]retinol (5 Ci/mmol).

Determination of the microsomal reduction of retinal bound to CRBP(II) was conducted as previously described (Kaddad & Ong, 1989) using straight-phase HPLC. Quantitative estimation of *all-trans*- and 13-*cis*-retinol production was made by comparison of their integrated peak areas to the calibrated area from a pure retinol standard (E_{325} ~52 000 in ethanol).

All esterification and reductase assays were conducted under conditions of linearity for time and Caco-2 microsomal protein concentration and at the respective pH optima previously established for these activities in rat small intestine (unpublished observations; Kakkad & Ong, 1988).

Retinyl Ester Synthesis in Intact Cells. Caco-2 cells cultured on 100-mm dishes were washed 1× with EBSS with 10 mM sodium taurocholate (EBSS-T) and incubated in 10 mL or EBSS-T with additions as indicated in the figure legends. Stock retinoid solutions in ethanol with 100 μg of BHT were added directly to EBSS-T. Stock β-carotene in hexane was dried under N_2 and dispersed in 100 μL of Me_2SO and 100 μL of 3% Tween 40 by vortexing, prior to addition of 10 mL of EBSS-T and sonication for 60 × 1 s pulses at a power output of 2. PMSF in Me_2SO was not completely soluble when added to EBSS-T, and, consequently, the solution was sonicated for 20 × 1 s pulses at a power output of 2 (thus, the effective concentration of PMSF must be considered to be somewhat less than 5 mM). All additions of retinoids in EtOH with 100 μg/mL BHT or of Me_2SO (with or without PMSF) to the medium constituted less than 1% of the total volume and had no effect on cell viability (>90% viable by trypan blue exclusion) over the period of the experiments.

After incubation, cells were washed with EBSS-T, scraped into 10 mL of ice-cold EBSS with 5 mM PMSF (sonicated as above; note: PMSF was added as an antiesterase to prevent the hydrolysis of retinyl esters in the cell extracts), and centrifuged at 700*g* for 10 min at 4 °C. The supernatant liquid was discarded, and the cells were sonicated in 0.5 mL of ice-cold EBSS with 5 mM PMSF for 20 × 1 s pulses at a power output of 2. The cell suspension was then extracted in 10 mL of 2:1 $CHCl_3$/MeOH (Folch et al., 1957). The Folch extract was dried under N_2, dissolved in hexane, and applied to a 1.2-g 10% H_2O-deactivated alumina column as described above. The retinyl esters were batch-eluted from the column with 2% diethyl ether in hexane, dried under N_2, redissolved in 100 μL of methanol, and analyzed by reverse-phase HPLC as described above for microsomal esterification assays.

Fatty Acid Composition of Microsomal Phosphatidylcholine. Phosphatidylcholine (PC) was isolated from total lipid extracts of microsomes by thin-layer chromatography. The Folch extract of microsomes was dried under N_2, dissolved in a small amount (~50 μL) of $CHCl_3$, applied in a band to a HPTLC plate (Adsorbosil, Alltech Assoc., Inc.), and developed

in a solvent system of ethyl acetate/*n*-propanol/$CHCl_3$/CH_3OH/0.25% aqueous KCl (25:25:25:13:9 v/v) (Mori et al., 1987). The HPTLC plate was covered with foil so that only the vertical edges were exposed, and the phospholipid bands were revealed by spraying with Phospray (Supelco). The PC band was identified by a pure standard (Sigma P-6638), the adjacent unsprayed portion of the band was scraped from the plate, and the silica scrapings were eluted as described by Skipski and Barklay (1969). The PC extract was dried under N_2 and low heat, dissolved in 0.25 mL of $CHCl_3$/CH_3OH (1:1 v/v), and passed over a neutral alumina column (Wells & Hanahan, 1969).

The fatty acid at the *sn*-2 position of the isolated PC was hydrolyzed by phospholipase A_2 essentially by the method of Wells and Hanahan as described (Brockeroff, 1975). Aliquots of PC (~150–250 μg) in $CHCl_3$ were transferred to 2-mL reacti-vials, dried under N_2, and dissolved in 400 μL of 95% diethyl ether and 5% methanol. Twenty microliters of a 10 mg/mL solution of lyophilized *Crotalus adamanteus* snake venom (Sigma V-6875) in 0.22 M NaCl, 0.02 M $CaCl_2$, 0.001 M Na_2EDTA, and 0.05 M MOPS, pH 7.2, was added and the reacti-vial shaken vigorously for 30 s and rocked for 2 h at room temperature. The reaction mixture was then dried under N_2 and extracted with 2.5 mL of $CHCl_3$, 2.5 mL of MeOH, and 2.25 mL of H_2O.

The lysophosphatidylcholine (LPC) and free fatty acids (FFA's) fom PC hydrolysis were separated on HPTLC plates as described above. After the plates were developed, the top portion was sprayed with 0.1% fluorescein in 95% ethanol to identify the FFA band, while the lower portion of the plate was sprayed with Phospray to detect LPC and PC (note: typically no PC was evident on the HPTLC plate, indicating that the hydrolysis had gone to completion). The FFA band was scraped from the plate, and the FFA's were eluted with three washes of diethyl ether (Skipski & Barclay, 1969). The extract was dried, and the FFA apos were methylated with BF_3/CH_3OH (Morrison & Smith, 1964). Fatty acid methyl esters of LPC were prepared by adding BF_3/CH_3OH directly to the silica scrapings and heating at 100 °C for 30 min. The fatty acid methyl esters were extracted with hexane and analyzed by using a Hewlett Packard 5890A gas chromatograph equipped with a 6 × 6 1/8 in. glass column packed with 10% SP-2330 on 100/120 Chromasorb W (Supelco), flame ionization detectors, and an HP 3365 ChemStation.

Results and Discussion

Presence of CRBP(II) in Caco-2 Cells. It has previously been established that the villus-associated enterocytes of the human small intestine contain cellular retinol-binding protein type II, a protein that appears to play an important role in vitamin A processing in the gut (Ong & Page, 1987). As the initial step in characterizing vitamin A metabolism in the Caco-2 cell, soluble protein extracts from Caco-2 cells cultured 11–21 days were examined for the presence of CRBP(II), utilizing an antiserum against rat CRBP(II) that previously has been shown to cross-react with human CRBP(II) but not human CRBP (Ong & Page, 1987). Adding increasing amounts of the Caco-2 extract resulted in an incremental displacement of rat ^{125}I-CRBP(II) in a radioimmunoassay (Figure 1A). The slope of the displacement curve was essentially the same as observed previously with extracts of human jejunum (Ong & Page, 1987).

The soluble protein extract from Caco-2 cells was next examined by SDS–polyacrylamide electrophoresis and Western blot analysis (Figure 1B). A single protein band (lane 2) was observed at a migration position corresponding to a molecular

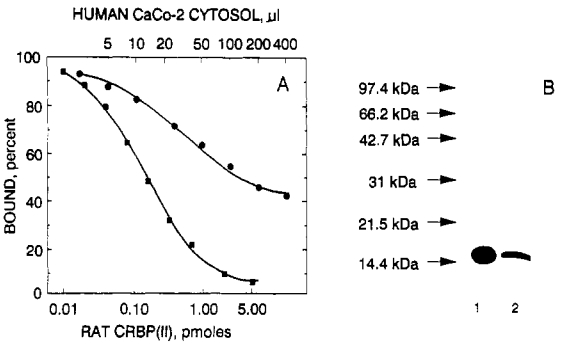

FIGURE 1: Evidence of CRBP(II) in the cytosolic fraction from Caco-2 cells. A sigmoidal displacement curve (panel A) was evident when increasing amounts of Caco-2 cytosol (●) were added to the standard incubation mixture containing ^{125}-labeled rat CRBP(II) and rabbit anti-rat CRBP(II) serum. Antibody-bound ^{125}I-CRBP(II) was precipitated by addition of immobilized protein A and expressed as a percentage of radioactivity precipitated when no rat CRBP(II) was added. The displacement curve for pure rat CRBP(II) is also shown (■). Western blot analysis (panel B) using anti-rat CRBP(II) antibody following SDS–PAGE of 0.21 μg of pure rat CRBP(II) (lane 1) and 110 μL (110.8 μg of total protein) of the cytosolic fraction from Caco-2 cells (lane 2) revealed a cytosolic protein in Caco-2 cells at a similar migration position to that of pure rat CRBP(II). Details of the radioimmunoassay and immunodetection of the Western blot are presented under Experimental Procedures.

weight of ~15.6K, and identical with the migration of pure rat CRBP(II) (lane 1). This was also similar to the position of a band previously observed in an extract of human jejunum (Ong & Page, 1987), providing strong evidence for the presence of CRBP(II) in Caco-2 cells.

On the basis of the previously determined quantitative displacement of rat CRBP(II) by human CRBP(II) in the radioimmunoassay mentioned above (Ong & Page, 1987), CRBP(II) was estimated to be ~0.03% of the total soluble protein in Caco-2 cells compared to 0.4% previously determined for extracts of human jejunum. Although the content of CRBP(II) was considerably lower in the Caco-2 cells than expected in small intestine (based on rat and limited human data), this still was equivalent to ~3–4 million molecules of CRBP(II) per cell.

Presence of Vitamin A Metabolizing Enzymes in Caco-2 Cells. Two different enzymes have been proposed to carry out the esterification of retinol in the small intestine. Acyl-CoA–retinol acyltransferase (ARAT) esterifies free retinol and has been shown to be present in human intestine (Helgerud et al., 1983). ARAT has little ability, however, to esterify retinol bound to CRBP(II) (Ong et al., 1987). In contrast, lecithin–retinol acyltransferase (LRAT) esterifies retinol bound to CRBP(II) as well as free retinol. LRAT activity is present in the rat small intestine, but human intestine has not yet been examined (Ong et al., 1987; McDonald & Ong, 1988).

LRAT activity in Caco-2 cells was demonstrated by incubating microsomal preparations with free retinol or retinol–CRBP(II) and observing the production of retinyl palmitoleate (16:1), retinyl oleate (18:1), retinyl palmitate (16:0), and trace amounts of retinyl stearate (18:0) (Figure 2a, upper tracing). This activity was sensitive to pretreatment of the microsomes with the anti-esterase PMSF (lower tracing), decreasing the esterification of either free retinol or retinol bound to CRBP(II) to less than 10% of control values. Sensitivity to PMSF has been shown to be characteristic of LRAT but not of ARAT (MacDonald & Ong, 1988). Retinyl ester synthesis was similarly abolished by heating the microsomes prior to the addition of retinol or retinol–CRBP(II) (data not shown).

The microsomal esterification reaction was saturable with increasing concentrations of retinol–CRBP(II) (Figure 2B).

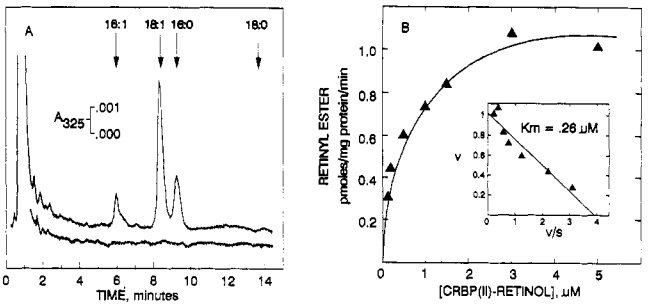

FIGURE 2: Demonstration of Caco-2 microsomal retinyl ester synthesis from retinol–CRBP(II). (Panel A) Following a 10-min preincubation of microsomes (125 μg of protein) with 2.5 μmol of PMSF in Me_2SO or with Me_2SO alone, retinol (1.5 nmol) bound to CRBP(II) was added to the reaction mixture (500-μL total volume) and incubated for 30 min at 37 °C. The lipid extracts of the incubation mixtures without PMSF (upper tracing) and with PMSF (lower tracing) were analyzed by reverse-phase HPLC as described under Experimental Procedures. 16:1, retinyl palmitoleate; 18:1, retinyl oleate; 16:0, retinyl palmitate; 18:0, retinyl stearate. The tracing is representative of duplicate determinations from three experiments. (Panel B) Increasing amounts of [^{3}H]retinol–CRBP(II) were added to 30 μg of microsomal protein in a total reaction volume of 120 μL and incubated for 10 min at 37 °C. Total retinyl esters were isolated by alumina column chromatography and quantitated by liquid scintillation spectrometry as detailed under Experimental Procedures. Each point is the average of duplicate determinations. The inset displays a linear transformation of the [^{3}H]retinol–CRBP(II) saturation curve.

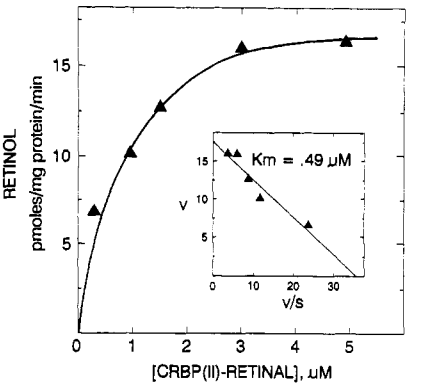

FIGURE 3: Retinal–CRBP(II) concentration dependence of retinal reductase activity in microsomal preparations from Caco-2 cells. Increasing amounts of retinal–CRBP(II) were added to 60 μg of microsomal protein and 30 nmol of NADH in a total volume of 500 μL. The mixture was incubated for 10 min at 37 °C and extracted into hexane, and the retinol product was quantitated by straight-phase HPLC as described under Experimental Procedures. Each point is the average of duplicate determinations. The inset displays a linear transformation of the retinal–CRBP(II) saturation curve.

The kinetic constants were derived from a linear transformation of the data (inset). The observed K_m was 0.26 μM, and the V_{max} was 1.02 pmol of retinyl ester synthesized per minute per milligram of microsomal protein. This K_m is quite similar to the value previously observed for LRAT in rat small intestine (~0.24 μM), but the V_{max} is considerably lower [44 pmol min^{-1} (mg of microsomal protein)$^{-1}$] (Ong et al., 1987). While the amount of LRAT expression and/or activity in Caco-2 cells may be considerably less than what is present in normal enterocytes, LRAT in human intestine, as mentioned above, has not yet been examined, and, consequently, a direct comparison cannot yet be made.

ARAT activity was also present in Caco-2 microsomes as indicated by the synthesis of the appropriate retinyl ester when the microsomes were provided with free retinol and an acyl-CoA (data not shown). When microsomes were incubated with 3 μM retinol and 40 μM oleoyl-CoA, ARAT activity (i.e., synthesis of retinyl oleate) was 10 times greater than LRAT activity (i.e., total retinyl esters synthesized in the absence of oleoyl-CoA) with 3 μM retinol–CRBP(II). Preincubation of the microsomes with 5 mM PMSF prior to addition of retinol and oleoyl-CoA resulted in a 13% greater recovery of retinyl oleate, confirming the previously observed insensitivity of microsomal ARAT activity to PMSF. The greater yield of retinyl esters in the presence of PMSF was probably due to an inhibition of microsomal esterase activity which otherwise would reduce recovery by the hydrolysis of newly formed retinyl ester.

A second metabolic activity of interest in vitamin A absorption is the reduction of retinaldehyde to retinol. Retinaldehyde is a product of the cleavage of dietary β-carotene within the absorptive cell and can be bound by CRBP(II). The bound retinaldehyde has been shown to be reduced to retinol by a microsomal reductase present in rat small intestine (Kakkad & Ong, 1988). This retinal reductase activity was also demonstrable in microsomes from Caco-2 cells. The production of retinol was absolutely dependent on the inclusion of NADH in the reaction and was abolished by preheating the microsomes (data not shown). The reaction exhibited saturation kinetics with increasing amounts of retinal–CRBP(II) (Figure 3), with an apparent K_m of 0.49 μM and a V_{max} of 17.4 pmol of retinol product min^{-1} (mg of microsomal protein)$^{-1}$ (inset). As was observed for the kinetic constants for LRAT in Caco-2 microsomes, the K_m of the reductase activity was very similar to that previously found in rat small intestine (0.46 μM), while the V_{max} was considerably lower than observed for rat [~325 pmol min^{-1} (mg of microsomal protein)$^{-1}$. The approximate 20-fold lower V_{max} for reductase activity in Caco-2 compared to the rat may reflect the presence of much less enzyme in these cells (under the conditions in which they were cultured) than normally found in enterocytes. However, like LRAT, retinal reductase has not yet been examined in human intestine.

We have been unable to develop a reliable in vitro assay for the enzymatic cleavage of β-carotene. Consequently, whole cell experiments, presented in the next section, were required to assess this activity.

β-Carotene Metabolism by Intact Caco-2 Cells. When Caco-2 cells cultured on 100-mm plastic dishes were incubated for 2 h with 3 μM β-carotene, the synthesis of retinyl palmitate, retinyl oleate, and retinyl palmitoleate and a trace amount of retinyl stearate was observed, as shown in Figure 4 (lower panel). The synthesis of retinyl esters from β-carotene demonstrated that these Caco-2 cells were capable of β-carotene cleavage and, subsequently, retinal reduction and retinol esterification (as was expected from the earlier demonstration of the requisite enzyme activities in vitro). On the basis of several experiments, the rate of β-carotene cleavage in Caco-2 cells, assuming an approximate 1:1 molar ratio between β-carotene and retinyl ester product (Ganguly & Sastry, 1985), was ~12–20 pmol h^{-1} (100-mm culture dish)$^{-1}$. This extrapolates to a rate of ~250–400 μg of β-carotene cleaved per day in the small intestine of an adult human. This activity would be insufficient to process the current National Research Council recommended daily allowance of 6 mg of β-carotene (1 mg of retinol equivalent) for an adult male (NRC, 1989) and may indicate a lower expression of the enzyme(s) and/or other factors necessary for β-carotene cleavage in these cells compared to normal enterocytes.

Later passages of these cells (after passage 35), grown continuously under the same conditions, apparently lost the ability to utilize β-carotene as a precursor for retinyl ester synthesis. Caco-2 cells which had been frozen at an earlier

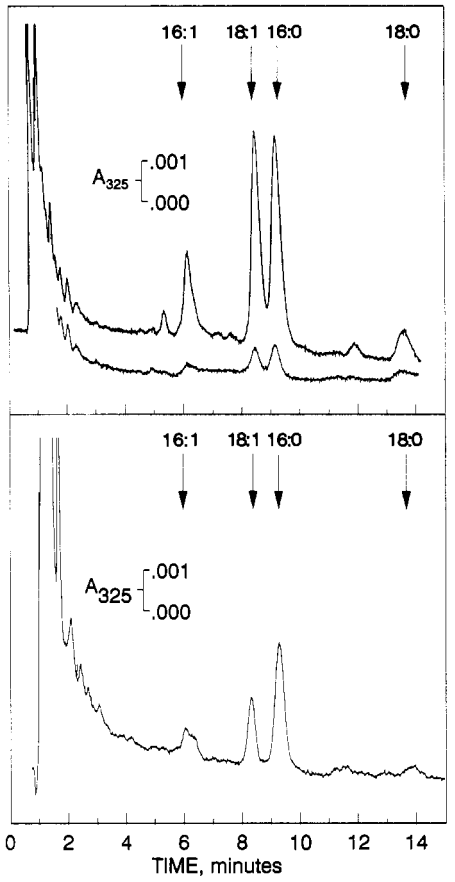

FIGURE 4: Demonstration of retinyl ester synthesis from retinol and β-carotene in intact Caco-2 cells. (Upper panel) Caco-2 cels cultured on 100-mm dishes were incubated in EBSS-T with 1 μM retinol for 30 min at 37 °C following a 30-min preincubation in EBSS-T with 5 mM PMSF in Me_2SO (lower tracing) or Me_2SO alone (upper tracing). The cells were then harvested and extracted, and retinyl esters were analyzed by reverse-phase HPLC as described under Experimental Procedures. (Lower panel) Caco-2 cells cultured on 100-mm dishes were incubated with 3 μM β-carotene in EBSS-T for 2 h at 37 °C. Retinyl esters were extracted from harvested cells and analyzed by reverse-phase HPLC as described under Experimental Procedures. The data are representative of duplicate determinations from at least two experiments.

passage (30) and subsequently returned to culture were able to synthesize retinyl esters from exogenous β-carotene initially, but then also lost this ability after several additional passages. Acquisitions of later passages of Caco-2 cells from the ATCC have also been cultured and tested but failed to demonstate retinyl ester synthesis from β-carotene. Interestingly, retinal reductase and retinol esterification activities in these cells or in microsomes from these cells were unaltered. It is possible that culture conditions may affect the ability of these cells to cleave β-carotene. Jiao et al. (1990) recently reported that Caco-2 cells grown on plastic synthesize apolipoprotein B-100, the form expressed primarily in fetal enterocytes, but, when cultured on Millicell HA inserts (Millipore), these cells switch to the synthesis of apolipoprotein B-48, the truncated form of B-100 associated with the adult phenotype of the enterocyte. Indeed, a single preliminary experiment with Caco-2 cells grown on HA inserts demonstrated a recovered ability to utilize β-carotene for the synthesis of retinyl esters (data not shown).

Metabolism of Exogenous Retinyl Esters by Caco-2 Cells. While β-carotene can enter enterocytes intact, dietary retinyl esters appear to be resistant to absorption and must be hydrolyzed in the lumen prior to internalization (Mahadevan et al., 1963a,b). A rather nonspecific pancreatic lipase activity has been described in the intestine of both rat (Calame et al., 1975) and human (Lombardo et al., 1979) which hydrolyzes retinyl esters. Retinyl esterase activity has also been demonstrated in preparations of brush-border membranes from rat small intestine (David & Ganguly, 1967), but it has not been established whether this activity is derived from the pancreas or is intrinsic to brush-border membrane.

The ability of Caco-2 cells to utilize exogenous retinyl esters for the synthesis of other retinyl esters would provide a priori evidence that retinyl esterase activity is an intrinsic property of this cell line and, possibly, of small intestinal absorptive cells. Long-chain fatty acid esters of retinol were indeed synthesized by Caco-2 cells incubated with 1 μM retinyl acetate for 30 min at 37 °C, producing a pattern of esters similar to that by cells incubated with β-carotene, and thus demonstrating an ability to hydrolyze retinyl acetate and utilize the liberated retinol for subsequent ester synthesis (data not shown). However, no similar ability was evident when cells were incubated with exogenous retinyl palmitate or retinyl stearate, the major dietery esters of vitamin A. It is not clear whether this inability to utilize long-chain fatty acid esters of retinol was due to a problem with solubility and the conditions in which these esters were presented to the cells in culture, a specific defect in the Caco-2 cell (as observed with the loss of β-carotene cleavage in later passages of Caco-2 cells maintained in continuous culture), or whether these findings may actually be extended to the normal human enterocyte. The possible lack of brush-border esterase activity for long-chain fatty acid esters of vitamin A in humans could have important physiological implications, particularly under conditions of pancreatic failure.

Utiization of Retinol by Caco-2 Cells. Exogenous retinol was used more efficiently than either β-carotene or retinyl acetate by Caco-2 cells for the production of retinyl esters. The composition of retinyl esters synthesized by intact cells incubated with retinol (Figure 5, upper panel, upper tracing) was similar to that observed for LRAT in vitro (Figure 2A, upper tracing), although the relative proportions of individual esters were somewhat different. There was also a disproportionate synthesis of monoenoic esters (18:1 and 16:1) by both Caco-2 cells or microsomes compared to the normal pattern of retinyl esters synthesized by the human intestine. Chylomicrons in human intestinal lymph contain retinyl palmitate, stearate, oleate, and linoleate in an approximate ratio of 57:25:12:6 (Goodman et al., 1966). Because LRAT utilizes predominantly, if not exclusively, the fatty acid in the *sn*-1 position of phosphatidylcholine (PC) for the transesterification of retinol (MacDonald & Ong, 1988), this unusual pattern of retinyl esters synthesized by the Caco-2 cell suggested that the fatty acid composition of PC in these cells might be different than that in normal human intestine. Indeed, a recent study reported relatively elevated levels of 16:1 and 18:1, depending on culture conditions, in the total fatty acids of microsomes isolated from Caco-2 cells (Murthy et al., 1988). Consequently, we compared the fatty acid composition at the *sn*-1 position of microsomal PC from both Caco-2 cells and normal human small intestine, as shown in Table I. Whereas saturated fatty acids, particularly 16:0 and 18:0, predominated at the *sn*-1 position of PC in microsomes from human intestine, the monoenoic acids, 18:1 and 16:1, were relatively elevated in PC from Caco-2 cells. There was also appreciably less 18:0 and 18:2 at *sn*-1 of Caco-2 PC. While the relative proportions of fatty acids at *sn*-1 were not identical with the pattern of retinyl ester synthesized by Caco-2 cells (or microsomes), they did reflect the relatively greater synthesis of retinyl oleate and palmitoleate and the decrease in retinyl

Table I: Fatty Acid Composition of Phosphatidylcholine Isolated from Caco-2 Microsomes and the Associated Microsomal and Cellular Synthesis of Retinyl Esters[a]

fatty acid	phosphatidylcholine fatty acid composition		retinyl ester synthesis	
	Caco-2 *sn*-1	human[a] *sn*-1	cells	microsomes
14:0	4.0[b]	0.8	ND[c]	ND
16:0	46.0	48.0	30.1	12.4
16:1	10.0	1.7	18.7	21.9
18:0	9.0	23.1	5.2	TR[d]
18:1	31.0	21.6	45.9	65.7
18:2	ND	4.8	ND	ND
20:4	ND	ND	ND	ND

[a] Microsomes were isolated from scraped mucosa from a 1-m segment of jejunum obtained at autopsy from a 70-year-old male. [b] All values are expressed as a percent of the total. [c] Not detectable. [d] Trace.

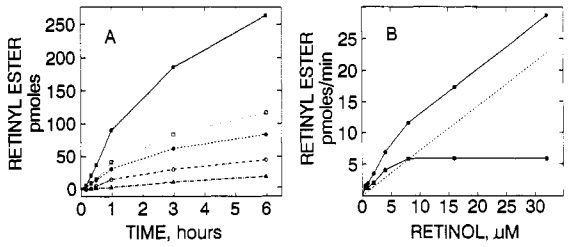

FIGURE 5: Effects of time and retinol concentration on retinyl ester synthesis by Caco-2 cells in culture. Caco-2 cells cultured on 100-mm dishes were incubated in EBSS-T with 1 μM retinol at 37 °C for increasing periods of time (panel A). At the end of the incubation, the cells were scraped into 10 mL of EBSS-T containing 5 mM PMSF in Me_2SO. Lipid extracts of the cells were analyzed for retinyl oleate (□), retinyl palmitate (●), retinyl palmitoleate (○), and retinyl stearate (▲) by reverse-phase HPLC as described under Experimental Procedures. Total retinyl ester (■) represents the sum of the four retinyl esters. In a similar experiment (panel B), Caco-2 cells on 100-mm dishes were incubated with increasing concentrations of retinol at 37 °C for 30 min. The cells were then harvested, extracted, and analyzed for retinyl esters as described above. The saturable component of retinyl ester synthesis (■) was estimated by the graphical method of Neame and Richards (1972) by subtracting the nonsaturable component (---) from the total retinyl ester synthesis (●). All points in the two experiments represent the average of duplicate determinations.

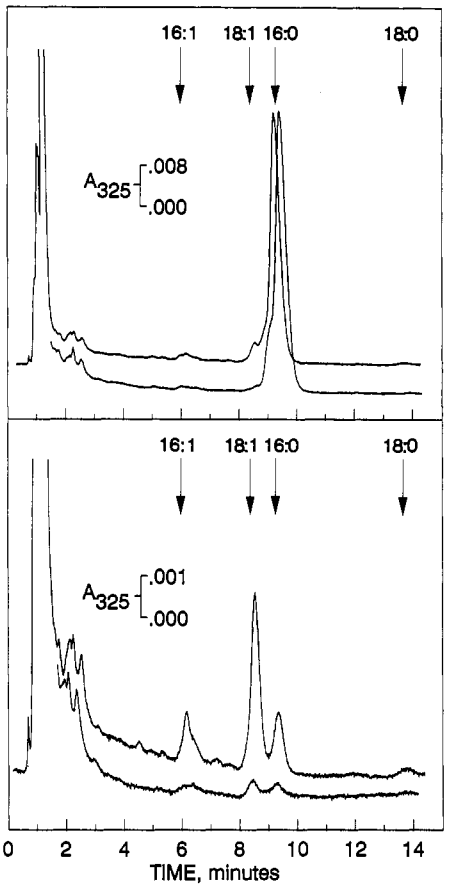

FIGURE 6: ARAT and LRAT activity in microsomes from Caco-2 cells treated or untreated with PMSF. Caco-2 cells cultured on 100-mm dishes were incubated for 30 min at 37 °C in EBSS-T containing 5 mM PMSF in Me_2SO or Me_2SO alone. Microsomes (125 μg of protein) prepared from PMSF-untreated cells (upper tracings in both panels) and PMSF-treated cells (lower tracings in both panels) were incubated for 30 min at 37 °C with 1.5 nmol of retinol in the presence (upper panel) or absence (lower panel) of 40 μM palmitoyl-CoA and μM bSA. Retinyl esters were extracted and analyzed by reverse-phase HPLC as described under Experimental Procedures. The data are representative of duplicate determinations in two experiments.

stearate and linoleate in these cells compared to normal intestine. We have not yet examined whether LRAT displays some preference for monoenoic acids at *sn*-1 of PC, which might account for the relatively greater proportions of retinyl oleate and palmitoleate in the Caco-2. It is also possible that the local pool of PC available to LRAT may have a different fatty acid composition than that determined from total microsomal PC in Caco-2 cells. Further, some component of this process may be altered or lacking in the Caco-2 cell, such as a phospholipid carrier protein, e.g., phosphatidylcholine transfer protein (Khan & Helmkamp, 1990), shown to modify enzyme reactions involving PC.

Esterification activity in the whole cell was further examined as a function of time and concentration of retinol in the culture medium. As shown in Figure 5A, the rate of individual and total retinyl ester synthesis in Caco-2 cells incubated with 1 μM retinol was linear between 10 and 60 min but then moderated between 1 and 6 h. Consequently, retinyl ester synthesis in intact cells was usually examined after a 30-min incubation. The rate of retinyl ester synthesis as a function of concentration of added retinol was biphasic, as shown in Figure 5B. It seemed possible that this might be evidence for the production of ester by LRAT and by ARAT, whose K_m is ~40-fold higher than LRAT [based on an estimated K_m of ~10 μM for human intestinal ARAT from Helgerud et al. (1983)] and, thus, would be the predominant activity at higher concentrations of retinol. Indeed, the existence of both LRAT and ARAT activities in the Caco-2 cell provided an opportunity to assess the relative importance of these enzymes in cells exposed to varying concentrations of exogenous retinol by exploiting their differential sensitivities to PMSF. Preincubation of Caco-2 cells with 5 mM PMSF for 30 min prior to incubation with 1 μM retinol—a concentration considered to be physiological (Hollander & Muraldhara, 1977)—decreased the recovery of retinyl esters by >90% compared to controls (Figure 4, upper panel, lower tracing), implicating LRAT as the major esterifying activity at this level of substrate addition. This decrease was apparently not due to an effect of PMSF on the internalization of added retinol, as PMSF-treated cells remained viable over the course of the experiment (>90% by trypan blue exclusion) and these cells accumulated retinol at a rate distinguishable from untreated cells when examined for up to 30 min of incubation (data not shown). To confirm that LRAT activity in Caco-2 cells had indeed been inhibited by preincubation with PMSF, microsomes were isolated from PMSF-treated cells and subsequently incubated with retinol–CRBP(II). The LRAT activity of these microsomes was decreased by ~90% compared to the activity of microsomes from untreated cells (Figure 6, lower panel), consistent with the decrease in retinyl ester synthesis observed in whole cells treated with PMSF. As expected, the ARAT activity of these

microsomes was essentially the same as the activity of microsomes from untreated cells (Figure 6, upper panel). The slightly increased recovery of ester observed here (~17%) may have been due to inhibition of esterases, as suggested above. Similar microsomal production of retinyl ester was observed when palmitoyl-CoA was replaced with an acyl-CoA-generating system (ATP, CoASH, $MgSO_4$, and palmitic acid) (Ross, 1982), providing evidence that PMSF had not blocked the ability of microsomal enzymes to produce acyl-CoA (data not shown).

If retinyl ester synthesis in Caco-2 cells incubated with high levels of retinol were largely due to ARAT activity, then the specific inhibition of LRAT accomplished with PMSF treatment would be expected to only modestly decrease the total amount of ester produced. However, PMSF treatment reduced cellular retinyl ester synthesis by ≥90%, even at the highest level of exogenous retinol examined (30 μM), a concentration of retinol greatly in excess of physiological levels (<1 μM) normally encountered within the lumen of the intestine (Hollander & Muralidhara, 1977). Thus, we have failed to find any evidence for ARAT esterification of retinol in the whole cell, even under conditions where LRAT activity was largely blocked, supraphysiological concentrations of retinol were provided, and the level of CRBP(II), which can sequester its retinol ligand from the action of ARAT, was considerably lower than expected in normal enterocytes. Consequently, we conclude that the true physiological function and substrate of ARAT in the Caco-2 cell and, by extension, in the normal enterocyte are yet to be established.

The biphasic production of retinyl esters in Caco-2 cells may instead be due to the uptake of retinol by the combined processes of carrier-mediated transport and passive diffusion, as first proposed by Hollander and Muralidhara (1977). According to the graphical method of Neame and Richard (1972), the nonsaturable or diffusion component of uptake can be estimated from a line whose slope is determined by the production of esters at high concentrations of exogenous substrate but with the y intercept moved to the origin (Figure 5B). Subtracting this component from total ester synthesis generates a substrate concentration curve which appeared to be saturable with an approximate K_m of 3 μM, similar to the apparent K_m for retinol uptake (8 μM) previously reported for everted gut sacs from adult rat jejunum (Said et al., 1988). Thus, it appears that retinol may enter these cells predominantly by a saturable, carrier-mediated process at lower concentrations of retinol (0.5–8 μM) and by a nonsaturable, diffusion-dependent process at higher concentrations (8–30 μM). Further work will be required to test this hypothesis.

In summary, although the ability of Caco-2 cells to metabolize both β-carotene and retinol, at least under the conditions in which they were examined here, is probably not as great as expected for the human enterocyte, this cell line still appears to be a useful tool for the study of intestinal vitamin A metabolism in humans. These cells can be manipulated in ways that in this study, for example, have allowed the assessment of the relative importance of different enzyme activities, i.e., LRAT vs ARAT. Further, the control available for substrate addition provides suggestive evidence for a specific transporter for the uptake of retinol by these cells. Future work with the Caco-2 cell line should bring new insights into the complex process of vitamin A absorption in the human.

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Registry No. ARAT, 81295-48-9; LRAT, 117444-03-8; 14:0 fatty acid, 544-63-8; 16:0 fatty acid, 57-10-3; 16:1 fatty acid, 373-49-9; 18:0 fatty acid, 57-11-4; 18:1 fatty acid, 112-80-1; 18:2 fatty acid, 60-33-3; 20:4 fatty acid, 506-32-1; retinol reductase, 130095-93-1; vitamin A, 68-26-8; β-carotene, 7235-40-7; retinyl acetate, 127-47-9; retinyl palmitoleate, 79464-27-0; retinyl oleate, 631-88-9; retinyl palmitate, 79-81-2; retinyl stearate, 631-87-8; retinyl ester (C14:0), 1181-93-7; retinyl ester (C18:2), 631-89-0; retinyl ester (C20:4), 79279-27-9.

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The Human *N*-Formylpeptide Receptor. Characterization of Two cDNA Isolates and Evidence for a New Subfamily of G-Protein-Coupled Receptors[†,‡]

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ABSTRACT: Two variants of the human *N*-formylpeptide chemoattractant receptor have been isolated from a CDM8 expression library prepared from mRNA of human myeloid HL-60 cells differentiated to the granulocyte phenotype with Bt2cAMP. Both recombinant receptors, fMLP-R26 and fMLP-R98, are 350 amino acids long (M_r 38 420); they differ from each other by two residue changes at positions 101 and 346 and by significant differences in the 5′ and 3′ untranslated regions. Both clones were able to transfer to COS-7 cells the capacity to specifically bind a new and highly efficient hydrophilic derivative of *N*-formyl-Met-Leu-Phe-Lys, referred to as fMLPK-Pep12. Photolabeling experiments revealed that the glycosylated form of the fMLP receptor in COS cells has a molecular weight (M_r 50 000-70 000) similar to that observed for the native receptor in differentiated HL-60 cells. Northern blot analysis revealed a major transcript of 1.6-1.7 kb and two minor hybridization signals of 2.3 and 3.1 kb, suggesting a related family of receptors. The complex hybridization pattern obtained with restricted genomic DNA was consistent with either two genes encoding fMLP receptor isoforms or a single gene with at least one intron in the coding sequence. Sequence comparison established that the fMLP receptor belongs to the G-protein-coupled receptor superfamily. The structural similarities observed with RDC1, a receptor isolated from a dog thyroid cDNA library, which shares weak homologies with other members of the family, suggests that the fMLP receptor is representative of a new subfamily.

Polymorphonuclear neutrophils are phagocytic cells specialized in the destruction of microorganisms. To accomplish their essential role in host defense against bacterial and fungal infections, they emigrate from blood vessels to the sites of infection by active amoeboid movements. The directed locomotion of the neutrophils is triggered by specific substances, termed chemotactic factors, which include the platelet activating factor (PAF) (O'Flaherty et al., 1986), arachidonate metabolite leukotriene B4 (LTB4) (Goldman & Goetzl, 1982), anaphylatoxin complement fragment C5a (Shin et al., 1968; Chenoweth & Hugli, 1978), and interleukin 8 (IL-8/NAP-1) (Baggiolini et al., 1989), and a number of *N*-formylmethionyl peptides. The *N*-formylated peptides are believed to derive from bacterial protein degradation (Schiffman et al., 1975; Marasco et al., 1984) or to arise from mitochondrial proteins upon tissue damage (Carp, 1982).

In addition to the directed migration of the cell, PAF, C5a, LTB_4, IL-8, and fMLP[1] stimulate a variety of coordinated biochemical and cellular responses in neutrophil and macrophages, including aggregation, phagocytosis of particles,

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[‡] The nucleotide sequences in this paper have been submitted to GenBank under Accession Numbers M33537 (for fMLP-R98) and M33538 (for fMLP-R26).

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[1] Abbreviations: BSA, bovine serum albumin; fMLP, *N*-formylmethionine-leucine-phenylalanine (fMLP is the commonly used, but nonstandard, abbreviation, which incorrectly assigned the letter P for phenylalanine); fMLPK, *N*-formylmethionine-leucine-phenylalanine-lysine; fMLPK-Pep12, *N*-formyl-Met-Leu-Phe-N^{ϵ}[*m*-benzoyl-(3-*S*-Cys-Tyr-Asp-Lys-Leu-Phe-Ser-Leu-Ala-Gln-Asp-Ser-*N*-acetyl)dihydromaleimide]Lys; HBS, Hanks' balanced salt solution; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Rt, retention time.

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