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Hydrophobic erythrocyte folate binding proteins are converted to hydrophilic forms by trypsin in vitro

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Human erythrocyte membranes contain high-affinity folate-binding proteins (FBPs) which on solubilization with detergents resolve into apparent 160000 M_r moieties on Sepharyl S-200 gel filtration in Triton X-100. These FBPs share antigenic and ligand binding characteristics with particulate FBPs from other human tissues. During studies to define the vectorial orientation of these FBPs on the erythrocytes, we trypsinized intact cells with 250 μg trypsin per ml packed cells and quantitatively analysed the remaining cell-associated FBPs as well as the products of proteolysed FBPs in the supernatant. Incubation of intact cells with trypsin resulted in a dose-dependent decrease in their capacity to bind ^{125}I-labelled pteroylglutamate (histamine derivative); at 250 μg/ml trypsin, folate binding was decreased by 77% compared to nontrypsin-treated control cells. While trypsinized cells contained proportionately lower quantities of apparent 160000 M_r FBPs than untreated control cells, the supernatant of trypsinized cells (soluble phase) contained a single species of M_r = 40000 which retained folate binding capacity. The sum of FBPs in trypsin supernatant and trypsin-treated cells was 87% of that found in untreated cells. Analysis of solubilized particulate erythrocyte FBPs and soluble (trypsin product) FBPs by sucrose density gradient ultracentrifugation in H_2O and ^2H_2O above the critical micellar concentration of Triton X-100 revealed that apparent 160000 M_r FBPs were detergent-binding (hydrophobic) species (which sedimented at M_r = 40000 in H_2O) while soluble FBPs (also sedimenting at M_r = 40000) were hydrophilic and did not bind Triton X-100. These are the first data which show that hydrophobic FBPs can be directly converted to hydrophilic FBPs by a trypsin-mediated proteolytic event. The trypsin-sensitive site is likely to be at the junction between the detergent-binding site and the major body of the protein (M_r = 40000) containing the folate binding site.

Introduction

We recently reported the identification of high-affinity folate binding proteins (FBPs) on erythrocyte membranes [1]. While these species are functional in the cellular uptake of folates in erythroid precursors during in vitro erythropoiesis [2], the majority of these proteins are converted to nonfunctional forms (while still attached to cells), shortly before release into the circulation [1,3]. Since circulating erythrocytes are the simplest of human cells in that they consist of a single membrane into which the FBPs were inserted during synthesis (before release of these cells from the bone marrow into the circulation) these cells appeared good models to investigate the vectorial orientation and degradative process of FBPs. Although we previously identified that > 99% erythrocyte membrane-associated FBPs were nonfunctional in folate binding [1,2], it has not been clear if these findings were reflective of that found on intact cells. This is due to the fact that the possibility that significant quantities of functional FBPs were released during lysis of cells preparatory to erythrocyte membrane preparation had not been ruled out. Therefore, before investigating the vectorial orientation of these FBPs in the lipid bilayer, it was necessary to confirm that the FBPs were quantitatively similar on intact cells as reported earlier with membranes.

Abbreviations: FBP, folate-binding protein; ^{125}I-PteGlu (HD), ^{125}I-labelled pteroylglutamate (histamine derivative); PBS, phosphate-buffered saline (0.01 M potassium phosphate, pH 7.5, containing 0.15 M NaCl); DTT, dithiothreitol; 5-CH_3H_4PteGlu, 5-methyltetrahydrofolate; PteGlu, pteroylglutamate (folic acid); 5-$CHOH_4$PteGlu, 5-formyltetrahydrofolate; MTX, methotrexate (amethopterin).

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Many human tissues contain both membrane-associated and soluble FBPs. Earlier, we biochemically compared these proteins after isolation, and reported that these two forms were biochemically very similar to one another in human milk [4], except that membrane-associated FBPs were extremely hydrophobic; following their solubilization out of membranes with the nonionic detergent Triton X-100, these FBPs bound up to three times their weight in Triton X-100. We subsequently identified through biosynthetic studies that similar hydrophobic FBPs in the membrane of cultured malignant human nasopharyngeal carcinoma (KB) cells, which function as folate transport proteins [5], are probably precursor to soluble hydrophilic FBPs which are released into the growth media [6]. No additional information is available regarding whether the relationship between hydrophobic or hydrophilic FBPs is the result of post-translational events involving the direct conversion of the hydrophobic to hydrophilic FBPs. Such an event could explain the origin of soluble FBPs in humans [7,9]. In this report, we show that FBPs on intact human erythrocytes have folate-binding sites within the main body of an externally-oriented hydrophilic portion which has an M_r of approx. 40000. This portion of the protein is distinct from (a) detergent-binding site(s) embedded in the erythrocyte membrane. These studies are the first to directly demonstrate that native intact hydrophobic erythrocyte FBPs have a trypsin-sensitive site at the junction between a minor detergent-binding fragment and the major body of the protein containing the ligand binding site.

Materials and Methods

^{125}I-labelled PteGlu (histamine derivative), (a histamine derivative of pteroylmonoglutamic acid, 1-carboxy-3-(*N*-(4-imidazolyl)carbamoyl)-1-pteroylamino propane, ^{125}I-PteGlu (HD)), with a specific activity of 2200 Ci/mmol and 3340 μCi/μg was obtained from Dupont/New England Nuclear (Boston, MA, U.S.A.), after HPLC purification to greater than 99% [1]. PteGlu (99% pure), Sephacryl S-200, DEAE cellulose, Triton X-100, sodium deoxycholate, α-cellulose, Sigmacell Type 50, bovine pancreatic trypsin treated with diphenylcarbamyl chloride to inactivate chymotrypsin, chicken egg-white purified ovomucoid trypsin inhibitor, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest analytical grade available from Sigma Chemical or Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Erythrocyte folate uptake studies. All studies were performed on peripheral venous blood from normal volunteers with reticulocyte counts < 1%, and the hematocrit, red cell and leukocyte counts were measured by a Coulter Counter (Model M430, Coulter Electronics, Hialeah, FL). A minor modification [1] of the method originally described by Beutler et al. [10] was used to purify leukocyte-free erythrocytes. Routinely, erythrocytes were sedimented by centrifugation at $1000 \times g$ for 10 min at 4°C in an IEC Centra 7R table-top centrifuge (International Equipment Company, Needham Heights, MA). The supernatant was aspirated and the pellet was resuspended in 15 volumes of ice-cold PBS. This centrifuge-wash cycle was repeated for a total of four times and the final sample of packed purified erythrocytes had a hematocrit of 60% (approx. $3 \cdot 10^9$ cells/ml)) and a purity of > 99.98%.

A final volume of 2 ml contained 150 mM NaCl and 10 mM potassium phosphate (pH 7.5), and 1000 μl erythrocyte suspension ($3 \cdot 10^9$ cells), to which ^{125}I-PteGlu (HD) was added in increasing concentrations. Routine incubations, unless otherwise specified, were performed for 90 min at 37°C in a shaking waterbath, followed by cooling to 4°C for 10 min. Unbound radioligand was removed by four centrifuge-wash cycles, as described above, and after transfer to 12×75 mm glass tubes, cells were counted for radioactivity in a Beckman gamma 5500 counter at 78% efficiency. Additional samples which, in addition, contained a 1000-fold excess of unlabelled PteGlu, were also routinely studied for each level of radiolabelled PteGlu to assess nonspecific folate uptake.

Competitive inhibition of ^{125}I-PteGlu (HD) uptake with various unlabelled folates was performed using increasing concentrations of unlabelled folates (determined from extinction coefficients given by Blakley [11]) incubated with 0.025 nM of ^{125}I-PteGlu (HD) and 1 ml of washed packed erythrocytes in a final volume of 2 ml in (PBS) containing 2 mM DTT.

Effect of trypsin on erythrocyte folate uptake. To define the external orientation of FBPs on intact erythrocytes, tubes containing 1 ml purified erythrocytes in PBS ($3 \cdot 10^9$ cells) were incubated with ^{125}I-PteGlu (HD), $7.5 \cdot 10^{-11}$ M (75 pM), for 1 h at 37°C, and after removal of unbound radioactivity, increasing concentrations of freshly prepared trypsin dissolved in 1 ml PBS were added to these cells. Control experiments were simultaneously performed on erythrocyte suspensions treated with similar concentrations of trypsin which were first inactivated by preincubation for 30 min at 37°C with a 2-fold excess of trypsin inhibitor. After mixing, the samples were incubated at 37°C for 1 h, and, after cooling to 4°C, cell-associated radioactivity was determined, as described above. A corollary experiment to control for the possibility that trypsin only displaced bound ^{125}I-PteGlu (HD) from intact erythrocytes was also performed. To tubes containing 1 ml of packed cells, increasing concentrations of trypsin dissolved in 2 ml PBS were first added and the mixture was incubated for 1 h at 37°C. The samples were then centrifuged at $1000 \times g$ for 10 min at 4°C, and the

uppermost 1 ml of each supernatant was aspirated, and boiled at 100°C for 30 min in 2 mM DTT. The remaining trypsin-treated erythrocyte pellets were subjected to three centrifuge-wash cycles, before radioligand uptake studies were repeated, as described above. The inhibitory potential of the supernatants of trypsin-treated cells was tested by incubating the boiled supernatant at each level of trypsin with 1 ml of fresh washed untreated erythrocytes and 75 pM ^{125}I-PteGlu (HD), following which cell-associated radioligand was determined.

Partial purification of erythrocyte FBPs. Separate 30-ml aliquots of packed cells treated with 250 μg trypsin per ml packed cells and control cells (treated with inactivated trypsin) in a final volume of 40 ml (in PBS) were incubated for 1 h at 37°C and cells were sedimented, as described above. These cells as well as their supernatants were separately processed as follows: cells were washed with 10 volumes of PBS for two cycles before lysis, as previously described [1]. The membranes were concentrated in a ultracentrifuge at 25000 rpm over 1 h at 4°C, the supernatant was discarded, and each pellet was subsequently solubilized with 2% Triton X-100, and 1% deoxycholate containing 0.5% sodium azide over 24 h at 4°C. The mixture was diluted 10-fold in 0.01 M sodium acetate-acetic acid (pH 4.5), and the pH adjusted to pH 4.5 with 1 M glacial acetic acid. The sample was subsequently applied to a 0.5×10 cm Econo-column containing 1 ml CM-cellulose that was equilibrated with 0.01 M sodium acetate-acetic acid (pH 4.5), containing 1% Triton X-100. The column was subsequently washed with 1 liter equilibration buffer following which it as batch-eluted with 4 ml equilibration buffer containing 1 M NaCl and the eluate was collected in its entirety. After adding potassium phosphate (pH 7.5) to achieve a final concentration of 100 mM potassium phosphate, the pH was adjusted to 7.5 by the addition of 1 M NaOH. The supernatants of trypsin-treated cells and control cells (10 ml each) were diluted 20-fold with CM-Cellulose equilibration buffer before partial purification of eluted FBPs over CM-cellulose, as described above.

Gel filtration. Gel filtration chromatography was performed at 4°C on a calibrated 2.5×30 cm Sephacryl S-200 column which was equilibrated and eluted with 10 mM potassium phosphate (pH 7.5) containing 1% Triton X-100, as previously described [1].

Results

Specific and nonspecific uptake of ^{125}I-PteGlu (HD) uptake by normal intact human erythrocytes shown in Fig. 1A. At PteGlu concentrations below 1 nM, the predominant uptake mechanism was a specific, saturable, high-affinity mechanism with a K_d of $3.3 \cdot 10^{-11}$ M (Fig. 1B). The inhibitory effect of various folates on uptake of ^{125}I-PteGlu (HD), 0.025 nM, by intact human erythrocytes is depicted in Fig. 1C. The concentrations of PteGlu and DL-5-CH_3H_4PteGlu which inhibited

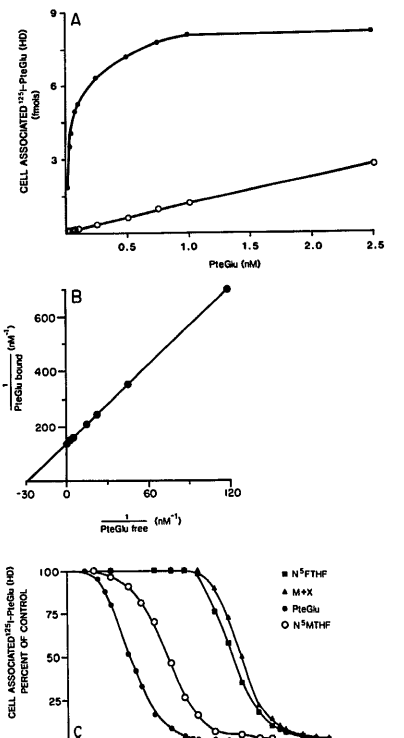

Fig. 1. Folate uptake by intact erythrocytes. (A) Erythrocytes (1 ml), were incubated with increasing concentrations of ^{125}I-PteGlu (HD) in the absence (●) and presence (○) of a 1000-fold excess of unlabelled PteGlu for 90 min at 37°C. They were subsequently washed four times with 14 volumes of PBS and counted for radioactivity. (B) K_d of intact erythrocyte interaction with ^{125}I-PteGlu (HD). (C) Competitive inhibition of ^{125}I-PteGlu (HD) binding to erythrocytes by unlabelled folate analogues. The maximal uptake (100%) was determined in the absence of unlabelled folate but in the same concentration of DTT as the test samples.

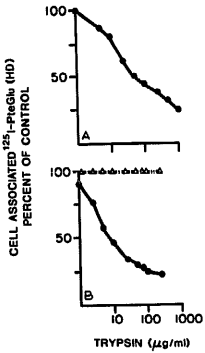

Fig. 2. The effect of trypsin on specific uptake of ^{125}I-PteGlu (HD), 75 pM, by erythrocytes. (A) 1 ml of cells containing 5.75 fmol of bound ^{125}I-PteGlu (HD) were incubated with increasing concentrations of trypsin for 1 h at 37°C. Following four centrifuge-wash cycles, cell associated radioactivity was determined. (B) after treatment with increasing concentration of trypsin for 1 h at 37°C, erythrocytes were washed to remove excess trypsin and digested externally-oriented membrane proteins, followed by incubation with radioligand. The data (●) are expressed as percent of maximum uptake obtained with control samples using inactivated-trypsin. Each supernatant following treatment of cells with different concentrations of trypsin was also tested for its inhibitory effect on subsequent radioligand uptake by another batch of untreated cells (△). The 100% control value (no inhibition) obtained, using the supernatant from cells incubated with inactivated-trypsin, was 5.75 fmol/ml of erythrocytes.

erythrocyte ^{125}I-PteGlu (HD) uptake by 50% (IC_{50}) were 0.025 nM and 0.2 nM, respectively. In contrast, the IC_{50} values for 5-$CHOH_4$PteGlu and MTX were much higher, being 6.5 nM and 10 nM, respectively. Thus, the observed binding by intact erythrocytes were both also quantitatively and qualitatively comparable and to earlier reported data [1] on folate binding to erythrocyte membranes.

Fig. 2A shows a dose-dependent effect of trypsin on the release of bound ^{125}I-PteGlu (HD) from cells which were preincubated with 75 pM ^{125}I-PteGlu (HD). The dose-dependent decrease in the capacity of trypsin-treated intact human erythrocytes to bind ^{125}I-PteGlu (HD) is shown in Fig. 2B. That the decrease in ^{125}I-PteGlu (HD) binding was not simply due to release of folate from intracellular stores (due to trypsin-induced hemolysis) is demonstrated by the inability of the boiled supernatant of trypsin-treated cells to inhibit ^{125}I-PteGlu (HD) binding to untreated erythrocytes (Fig. 2B). (Boiling the supernatant destroyed trypsin released FBPs and thus prevented them from competing with membrane-associated erythrocyte FBPs for ^{125}I-PteGlu (HD)). These data suggest that greater than 75% of the observed ^{125}I-PteGlu (HD) binding to intact erythrocytes was to externally oriented FBPs. Trypsin-treated cells and their supernatants as well as controls were subsequently quantitatively analysed by HPLC gel filtration. Fig. 3A shows a single ^{125}I-PteGlu (HD) binding peak from non-trypsin-treated detergent-solubilized cells, a coincident smaller peak from trypsin-treated cells, and an overlapping peak (with a longer retention time) from the supernatant of trypsin-treated cells. There was no folate-binding species identified in control cell supernatants. When the pooled fractions from trypsin-treated cell associated FBPs and supernatant peaks were each analyzed (Fig. 3B), ^{125}I-PteGlu (HD)-bound FBPs from the trypsin-treated cells eluted at $V_e/V_o = 1.25$ on Sephacryl S-200 gel filtration in the presence of Triton X-100. This corresponded to an apparent M_r of 160000. These characteristics are similar to solubilized particulate FBPs in human KB cells [5], milk [4], and erythrocyte membranes [1]. The supernatant from trypsin-treated cells contained a ^{125}I-PteGlu (HD)-binding moiety which eluted at $V_e/V_o = 1.56$, corresponding to an M_r of 40000, similar to that observed for soluble milk and KB cell FBPs [4,5]. The recovery of applied radioactivity in eluted fractions from HPLC was 98%. The sum of ^{125}I-PteGlu (HD)-binding to FBPs partially purified from trypsin-treated cells plus their supernatant was 87% of that from an equal volume of control cells, with less than 5% variation in three experiments. These results suggested a trypsin-mediated conversion of membrane-associated FBPs (having an apparent $M_r = 160000$) to a soluble extracellular FBP (having an M_r of 40000).

Since the apparent M_r of 160000 for solubilized particulate human KB cell and milk FBPs was in large part due to detergent binding [4,5], FBPs from non-trypsin exposed (control) erythrocytes were similarly analyzed by sucrose density gradient ultracentrifugation in H_2O and ^2H_2O above the critical micellar concentration of Triton X-100 (Fig. 4). A shift in sedimentation on ^2H_2O compared with H_2O noted for the solubilized erythrocyte membrane-associated FBPs (similar to that for solubilized particulate human KB cell FBPs) indicated a significant degree of Triton X-100 binding. No such shift was observed for the (trypsin-mediated) soluble FBP with $M_r = 40000$ (not shown) which had similar sedimentation profiles as soluble human KB cell and milk FBPs [4,6]. Thus, trypsin-mediated proteolysis of externally-oriented, membrane-associated FBPs on intact erythrocytes resulted in their conversion from hydrophobic forms to soluble hydrophilic forms that retained their ligand binding characteristics.

Discussion

Using a high specific activity folate analogue, ^{125}I-PteGlu (HD), which had a high degree of purity, the

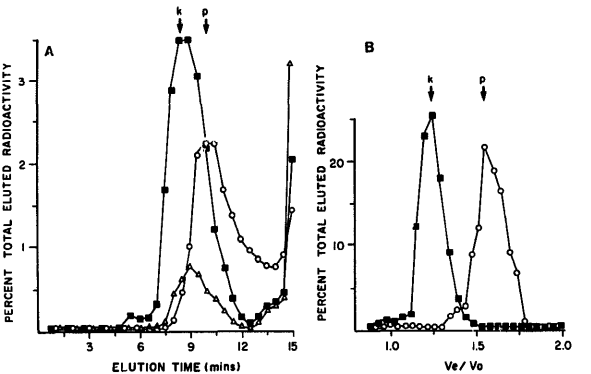

Fig. 3. Gel filtration of solubilized erythrocyte FBPs using (A) SW 300 Protein-Pak and (B) Sephacryl S-200 columns before (closed symbols) and after (open symbols) trypsin-treatment of 50 ml packed erythrocytes. FBPs from trypsin-treated erythrocyte membranes (△), trypsin-treated erythrocyte supernatants (○) and untreated control erythrocyte membrane (■) that were solubilized with 2% Triton X-100 and 1% sodium deoxycholate were partially purified over CM-cellulose as described in Materials and Methods and incubated with 500 fmol ^{125}I-PteGlu (HD) at 37 °C for 2 h. 2-ml samples were applied to a SW 300 Protein-Pak column that was equilibrated and eluted with 0.01 M potassium phosphate (pH 7.5) containing 1% Triton X-100 (flow rate 1 ml/min). The fractions (0.5 ml) eluting between 9 and 11.5 min (○) and those eluting between 7.5 and 10 min (△, ■) were pooled separately and applied to calibrated Sephacryl S-200 gel filtration columns, and eluted fractions were counted for radioactivity. Arrows indicate elution peaks of ^{125}I-PteGlu (HD) bound placental folate receptors (p, M_r 40000), and KB cell FBPs, (k, apparent M_r 160000). Free ^{125}I-PteGlu (HD) eluted at a single sharp peak at 15.5 min (not shown).

presence of specific, saturable, high-affinity folate binding sites on intact mature erythrocytes were identified. These externally-oriented binding sites on intact cells were proteins which, after solubilization from purified membranes and gel filtration chromatography, retained their folate binding characteristics and eluted at an apparent M_r of 160000 in the presence of detergent. Competitive inhibition studies on intact cells using unlabelled folate analogues demonstrated similar specificity of binding to intact erythrocytes, as previously reported with membranes [1]. Thus, both quantitatively and qualitatively, the data on intact erythrocytes was reflective of that reported earlier with erythrocyte membranes.

It has been demonstrated with membrane-associated FBPs purified from human milk [4] and KB cells [5,6], that although their gel filtration profile in the presence of Triton X-100 would suggest an M_r of 160000, this is due to the fact that these proteins bind significant amounts of detergent (75% of the apparent M_r of 160000 is due to Triton X-100 micelle binding). Their true molecular mass, as estimated on SDS-PAGE of detritonized purified preparations, was 44000 daltons and 50000 daltons, respectively. In contrast, soluble milk and KB cell FBPs (the latter are secreted into the growth medium of cultured KB cells) have M_r values of approximately 40000 on gel filtration in Triton X-100, and SDS-PAGE, and analysis by sucrose density gradients in H_2O and ^2H_2O indicated little to no detergent binding. The solubilized FBPs from untreated erythrocytes had many characteristics as hydrophobic milk and KB cell FBPs, since on Sephacryl S-200 gel filtration in Triton X-100 they eluted at an apparent M_r = 160000, while on sucrose density gradient ultracentrifugation in H_2O they eluted as approximately 40000 M_r proteins [4,5]. In contrast, studies in ^2H_2O revealed a dramatic shift in sedimentation to a similar extent as other hydrophobic FBPs [4,5] which indicated significant detergent binding. Due to the paucity of these FBPs on intact cells, it is not possible at this time to purify adequate quantities for detailed amino acid analysis to determine the true molecular weight of these species. However, based on similarities in (i) affinity, (ii) specificity for various folate analogues, (iii) M_r values by gel filtration and sucrose density gradients in H_2O and ^2H_2O above the critical micellar concentration of Triton X-100, (iv) shared antigenic determinants with other purified FBPs [1], our data support the conclusion that erythrocyte FBPs are also hydrophobic to the same extent as hydrophobic milk and KB cell FBPs.

Although our data indicated that a significant number of functional FBPs are externally oriented on KB

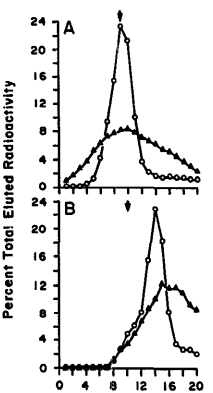

Fig. 4. Sucrose density gradient ultracentrifugation (3–15%) of partially purified erythrocyte FBPs (Δ) in (A) H_2O and (B) ^2H_2O. ^{125}I-PteGlu (HD) bound to placental folate receptors (arrow) and solubilized particulate KB cell FBPs (○) served as non-detergent-binding and detergent-binding marker proteins, respectively [4].

cells [5] and erythrocytes, some important differences were apparent among erythrocyte and KB cell FBPs after trypsin-mediated proteolysis of surface proteins on these cells. As with KB cells, trypsin was able to release a folate binding species from erythrocytes. This soluble FBP from erythrocytes, however, had uniquely different characteristics. In contrast to the release of a 160000 M_r species from KB cells [5], erythrocytes released a smaller M_r FBP. This species did not require the presence of detergent to maintain it in the soluble phase, and had an M_r of 40000 on Sephacryl S-200 gel filtration, in both the absence or presence of Triton X-100, corresponding to the elution position of other soluble hydrophilic FBPs [4,5]. Furthermore, it had an M_r of 40000 on sucrose density gradients in H_2O and ^2H_2O in Triton X-100, indicating that it did not bind detergent. The 40000 M_r species was not identified in the supernatant of untreated or inactivated trypsin-exposed intact erythrocytes. Since the method for analysis of these FBPs was based on binding to ^{125}I-PteGlu (HD), and the sum of the FBPs in soluble form and that which remained attached to trypsin-treated cells was 87% of that originally present in untreated cells, these findings support the conclusion that the soluble hydrophilic FBPs (released into the trypsin supernatant) were derived from externally oriented hydrophobic FBPs on intact erythrocytes. Since the molecular weight of the hydrophobic and hydrophilic FBPs on sucrose gradients in H_2O were between 45000 and 40000 M_r, it appears that the portion of the FBP (which confers detergent-binding characteristics to the hydrophobic species) occupies only a minor portion of the protein and is distinct from the main body of the protein containing the folate binding site. Moreover, based on the fact that trypsin (which does not enter cells) effected this conversion, these data also suggest that the junction between the detergent-binding site and the major portion of the protein (M_r 40000) was the locus of proteolytic cleavage. In contrast to the data with erythrocytes, trypsin treatment of intact KB cells resulted in the elution of apparent 160000 M_r hydrophobic species [5]. If the erythrocyte and KB cell FBPs are structurally identical, this would suggest that the orientation of the FBP within the erythrocyte (but not KB cell) membrane was such that its trypsin-sensitive site was accessible to the enzyme. Alternatively, minor differences in structure between these FBPs may explain this effect of trypsin on erythrocytes. Whether the hydrophilic erythrocyte FBP was derived through the direct action of trypsin on the hydrophobic FBPs, or indirectly (by activation of a proteinase adjacent to the native FBP species), remains to be determined with purified erythrocyte FBPs. The observations that there is a precursor-product relationship between membrane-associated apparent 160000 M_r and soluble 40000 M_r FBPs in KB cells in culture [6] nevertheless appears somewhat analogous to the sensitivity of erythrocyte FBPs to trypsin. The identification of trypsin-sensitive sites in erythrocyte FBPs, but not in membrane-associated KB cell FBPs, supports the hypothesis for a specific proteinase that effects this physiologic cleavage in KB cells (thereby releasing 40000 M_r soluble FBPs into the growth medium). Preliminary studies from our laboratory appear to substantiate the existence of such a proteinase in human placenta.

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