Production of the Serum Form of the Transferrin Receptor by a Cell Membrane-Associated Serine Protease (43635)

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Abstract. **Recent investigations have demonstrated that the soluble form of the transferrin receptor in human serum is an 85-kDa fragment of intact receptor containing most of the extracellular domain. The recent demonstration of a remnant of the truncated receptor in cell membranes suggests that the soluble form arises from proteolytic cleavage of intact receptor. In the present investigation, domain-specific antibodies were used to further examine the subcellular location and nature of this proteolysis. HL60 cells were used in the investigation because the cells release an 85-kDa soluble form of the receptor to the culture supernatant that is identical to that found in serum. When intact, purified transferrin receptor from human placenta was incubated with the culture supernatant, no proteolytic activity could be demonstrated. However, when purified membrane fractions from HL60 cells were used in this incubation system, the 85-kDa fragment was produced. This activity was inhibited by serine protease inhibitors indicating that cell membrane fractions contain a serine protease capable of producing the serum form of the transferrin receptor.** [P.S.E.B.M. 1993, Vol 204]

The detection and quantitation of a soluble form of transferrin receptor in serum has proven to be an important new clinical tool for the assessment of erythropoiesis and identification of iron deficiency (1–3). Biochemical analysis has demonstrated that the circulating transferrin receptor is an 85-kDa fragment of intact receptor that lacks the first 100 amino acid residues (4). The truncation site is located between arginine 100 and leucine 101 in the extracellular domain adjacent to the cell membrane. The mechanism of production of the soluble transferrin receptor is not known. One possibility is that it is synthesized within the cell either as a discrete gene product (5) or as a consequence of alternate RNA splicing (6). However, because the truncation site is located adjacent to the cell surface and the amino terminus of the molecule is oriented to the cytoplasm, proteolytic cleavage of intact cellular receptor seems more likely. Evidence in support of a proteolytic mechanism was recently obtained in our laboratory using domain-specific antibodies that demonstrated a membrane-bound remnant of receptor (7). The present study was undertaken to further characterize the subcellular location and mechanism of production of soluble transferrin receptor.

Materials and Methods

Chemicals and Reagents. Hanks' buffered salt solution (HBSS), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), diisopropylfluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, EDTA, iodoacetamide, pepstatin A, keyhole limpet hemocyanin, Freund's adjuvant, ammonium sulphate, glycine, tris [hydroxymethyl]aminomethane (Tris), RPMI 1640, pyruvate, glutamine, fetal bovine serum, 5′ nucleotidase assay kit, prestained molecular mass standards, and 4-chloro-1-napthol were obtained from Sigma (St. Louis, MO). Sterile culture flasks, conical collection bottles, and disposable pipettes were obtained from Fisher Scientific (St. Louis, MO). Tissue culture antibiotic (penicillin and streptomycin) and antimycotic (amphotericin) solution was obtained from Gibco/BRL

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(Grand Island, NY). Peroxidase-conjugated swine immunoglobulin to rabbit IgG was obtained from Dako (Carpinteria, CA). Polyacrylamide gradient gels and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Richmond, CA). Activated metalloproteinases 1 (collagenase), 2 (72-kDa gelatinase), 3 (stermolysin), and 9 (92-kDa gelatinase), and L-trans-expoxysuccinyl-leucylamido-(3-methyl) butane (EP475) were provided by Dr. H. Nagase of the Department of Biochemistry at the University of Kansas.

Cell Culture. Culture studies with K562 and HL60 cells have demonstrated the production of a soluble form of transferrin receptor in the supernatant that is identical to serum receptor (8, 9). HL60 cells were used in the present study because they have been observed to produce soluble receptor at roughly two to three times the rate of K562 cells (unpublished observations). The cells were maintained in log phase growth in RPMI 1640 medium supplemented with glutamine, pyruvate, and an antibiotic/antimycotic solution. The cultures were enriched with 20% fetal calf serum. Cells were cultured in a 5% CO_2 environment at 37°C.

To examine the proteolytic activity of various cell culture fractions, 200 ml of propagated culture in log phase growth were centrifuged twice at 8,000×*g*. The supernatant containing culture supernatant and exosomes (8) was then concentrated 10-fold in an Amicon pressure filtration unit (Amicon, Lexington, MA) using a filtration membrane with a 10-kDa exclusion limit. The pelleted cell fraction was washed twice in HBSS at 4°C and washed a final time in 5 m*M* Tris-HCl, pH 7.6, containing 5 m*M* $CaCl_2$. After resuspension in 5 ml of the same buffer, the cells were sonicated twice on ice for 10 sec and centrifuged at 770×*g* for 15 min. The pellet obtained by centrifugation at 50,000×*g* for 20 min was resuspended in 1 ml of HBSS and layered on a discontinuous sucrose density gradient and centrifuged at 100,000×*g* for 3 hr. The fractions were mechanically aspirated, made to 13 ml in HBSS, and centrifuged at 100,000×*g* for 40 min. The pellets were then resuspended in 350 μl of HBSS, from which 40 μl were removed for assay of 5′ nucleotidase and 10 μl for protein determination. The remainder was used for studies of proteolysis.

Assay of Proteolytic Activity. Proteolytic activity of cell fractions and specific proteases were examined by determining their ability to produce the 85-kDa soluble fragment of transferrin receptor when incubated with intact transferrin receptor isolated from human placenta. The latter was purified by ligand affinity chromatography as described previously (10). The purified receptor was extensively dialyzed against HBSS to remove excess CHAPS used in the purification. When examining the proteolytic activity of the cell supernatant or membrane fraction, 10 μl of purified intact receptor in HBSS containing 0.2 μg protein/μl were incubated with 10 μl of culture supernatant or cell membrane fraction at 37°C for 5 hr. Generation of the soluble form of transferrin receptor was then examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and immunostaining with domain-specific antibody. In all assays of proteolytic activity, appropriate control incubations of purified transferrin receptor alone or cell fraction alone were processed simultaneously. The effect of various protease inhibitors was examined in this same system by adding these at the appropriate concentration.

The effect of activated metalloproteinases was similarly examined by incubation with intact transferrin receptor. Activated purified metalloproteinases 1, 2, 3, and 9 were incubated at a concentration of 0.2 μg/ml in phosphate-free buffer containing 50 m*M* Tris, 150 m*M* $NaCl_2$, 10 m*M* $CaCl_2$, and 0.02% sodium azide (TNC buffer). In these incubation studies, 36 μl of purified intact receptor in phosphate-free TNC buffer at a concentration of 0.2 μg/μl were incubated with 4 μl of activated enzyme containing 2 μg/ml at 37°C for 5 hr. The production of soluble transferrin receptor was examined with domain-specific antisera as described below.

A search for specific protease activity in the various cell fractions was made as follows. Serine protease activity was sought by means of inhibition by either DIFP or PMSF at a concentration of 1 m*M*. Metalloproteinase activity was screened for with 1,10-phenanthroline at a concentration of 1 m*M* or EDTA at a concentration of 5 m*M*. Thioprotease activity was evaluated by EP475 at a concentration of 50 μ*M* or iodoacetamide at a concentration of 10 m*M*. Aspartate protease activity was examined with inhibition by pepstatin A at a concentration of 1 μg/ml. The activity of 5′ nucleotidase was determined as described by the manufacturer. Protein was estimated by the method of Lowry (11).

Production of the soluble form of transferrin receptor was detected with a sequence-specific peptide antibody that identifies only the extracellular domain of transferrin receptor. To exclude the possibility that the appropriate-size proteolytic fragment was resulting from truncation from the opposite end of the receptor, a second sequence-specific peptide antibody was employed that identified the cytoplasmic domain. The amino acid sequences used for the preparation of antisera were chosen to maximize regional hydrophilicity while maintaining an optimal position of lysine residues for complexing the keyhole limpet hemocyanin carrier protein. The selected peptide in the extracellular domain was located six residues distal to the truncation site of intact receptor responsible for the serum receptor (4), but proximal to the putative trypsin cleavage site (10; Fig. 1). The peptide sequence in the cytoplasmic domain was chosen between residues 40 and 54 (Fig.

1). The peptides were synthesized commercially by the solid-phase method at Multiple Peptide Systems (San Diego, CA) and purified by high-performance liquid chromatography as described previously (12). Polyclonal antisera against the purified peptides were prepared in rabbits. The extracellular domain-specific antibody showed reactivity with intact and soluble receptor (12) but not a 70-kDa fragment produced by tryptic cleavage between arginine 121 and leucine 122 (unpublished observations). The cytoplasmic domain-specific antibody showed reactivity with intact receptor (12) and with the membrane-bound receptor remnant remaining after receptor truncation (7) but not with the serum receptor (12).

The intact and soluble forms of transferrin receptor were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions and transblotting to nitrocellulose membrane as previously described (4). After blocking with 1% nonfat, dry milk in phosphate-buffered saline for 1 hr, the membrane strips were incubated for 2 hr with 10 ml of peptide antibody which had been purified using protein A chromatography. After washing with phosphate-buffered saline, the nitrocellulose membrane strips were incubated with 1:1000 swine anti-rabbit IgG antibody conjugated to horseradish peroxidase. The immunoreactive proteins were detected after extensive washing with phosphate-buffered saline by adding 4-chloro-1-napthol.

Results

Incubation of intact transferrin receptor with the supernatant from HL60 cells failed to produce the soluble form of the receptor (Fig. 2). However, when the eight fractions obtained from density-separated cell membranes were incubated with intact transferrin receptor, fractions 4 to 8 produced peptides of molecular mass 84–88 kDa that reacted with the domain-specific antibody. The maximal activity occurred in fraction 7, which produced a single peptide of molecular mass 85 kDa (Fig. 3) as detected by extracellular domain peptide antibody. This was not detected with cytoplasmic domain antibody. The assays for 5′ nucleotidase activity and protein content also revealed a maximum enrichment for surface membrane in this fraction obtained with a sucrose density cut of 45%. To determine the class of protease responsible for this proteolysis, incubation studies were repeated in the presence of the previously mentioned inhibitors. PMSF and DIFP were able to completely block the production of the soluble form of transferrin receptor while the other inhibitors were not. This indicates that the proteolytic activity, capable of producing a truncated receptor ectodomain compatible with the serum form of the receptor and which is maximal in a cell membrane-enriched fraction, is mediated by a serine protease.

Because previous studies have shown that cancer cell membranes have associated metalloproteinase activity (13, 14), further studies were performed to determine the effect of various metalloproteinases on intact receptor. Soluble transferrin receptor could not be detected after incubation with activated metalloproteinases 1, 2, 3, and 9.

Discussion

Previous work from this laboratory has shown that the circulating transferrin receptor is a truncated fragment containing most of the extracellular domain, with cleavage between arginine 100 and leucine 101 (4). This 85-kDa fragment contrasts with the 70-kDa fragment that is produced by digestion of purified transferrin receptor with trypsin at neutral pH (10). This smaller fragment has been shown to result from a cleavage between arginine 121 and leucine 122 (10). The use of an antibody against a peptide sequence proximal to this cleavage site has permitted us to confine our observations to the production of the biologically relevant form present in human serum. That the 85-kDa form of the receptor produced was not the consequence of truncation nearer the carboxy-terminal end of the extracellular domain was established by nonreactivity of the amino-terminal portion which was detected by extracellular domain antibody with cytoplasmic domain-specific antibody. We cannot completely exclude the possibility that a smaller soluble fragment similar to that produced by trypsin is generated by HL60 cells, but monoclonal antibodies have failed to detect a smaller fragment in the culture supernatant or in hu-

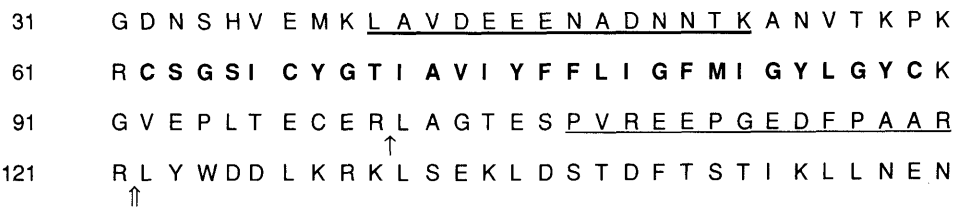

Figure 1. Partial amino acid sequence data indicating the extracellular domain peptide sequence (underlined) used to distinguish between the proteolytic product found in serum resulting from cleavage at ↑ (4) and that produced by tryptic digestion ↑↑ (10). The membrane spanning sequence is shown in bold lettering. The cytoplasmic domain peptide sequence used to exclude possible distal truncation as the mechanism of production is underlined in bold.

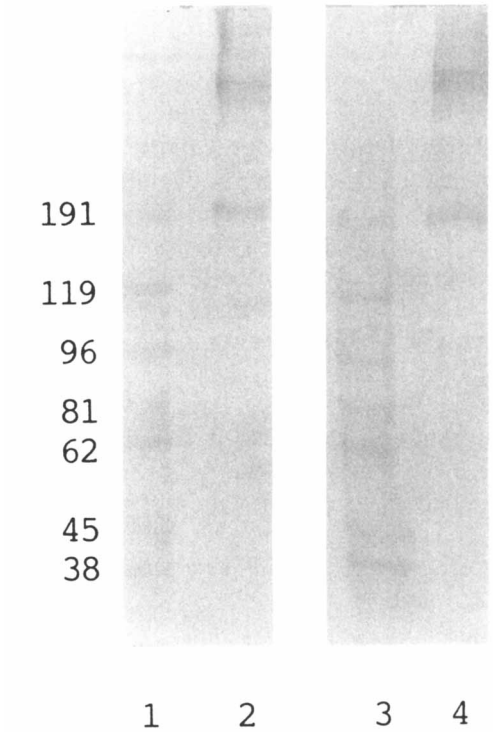

Figure 2. Western blot with extracellular domain antibody indicating the effect of incubating intact transferrin receptor (Lane 2) with concentrated culture supernatant (Lane 4). Molecular mass indicators, Lanes 1 and 3.

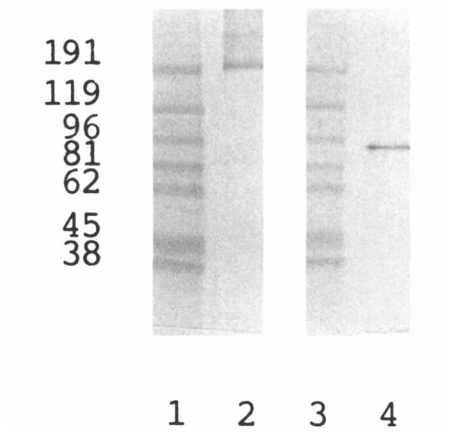

Figure 3. Western blot with extracellular domain antibody indicating the effect of incubating intact transferrin receptor (Lane 2) with a surface membrane fraction (Lane 4). Molecular mass indicators, Lanes 1 and 3.

man serum (4, 8). The mechanism of production of the 85-kDa fragment in human serum therefore appears to be independent of previously defined trypsin activity.

Although a variety of mechanisms could account for the presence of a truncated soluble form of transferrin receptor, recent studies from this laboratory using domain-specific antibodies have demonstrated a receptor fragment in cell membranes, thereby providing direct evidence for receptor proteolysis as the mechanism of soluble receptor production (7).

There are several possible subcellular locations at which the soluble fragment may be generated by proteolysis. Prior work had indicated that this is not mediated by a protease in serum because both K562 and HL60 cells produce the soluble receptor in serum-free conditions (8, 9). Our initial investigation focused on the activity of the culture supernatant, but incubation studies failed to demonstrate proteolytic activity in this fraction. Rather than being facilitated by extracellular proteases, the production of the soluble fragment occurs either at the cell surface or within an intracellular compartment.

Of relevance to the generation of soluble transferrin receptor is the demonstration that small 50-n*M* particles termed exosomes containing transferrin receptor are produced when sheep erythrocytes are cultured *in vitro* (15, 16). The exosomes are formed by internal bleb formation within the endocytic vesicle, resulting in a multivesicular endosome. The exosomes contained in this structure are released by exocytosis. Incubation of K562 and HL60 cells has demonstrated the production of exosomes (8, 9). It is possible that the proteolysis that produces the soluble receptor could occur after release of exosomes from cells, but we have observed that there is no shift of receptor content from exosomes into solution when culture supernatant is incubated (9). This suggests that if the soluble receptor is produced from the exosome, it occurs within the multivesicular endosome. This process could be facilitated by the interaction of the exosome membrane and the multivesicular endosome membrane, both of which, being derived from surface membrane, might be reasonably expected to be associated with the appropriate protease activity. Production of the soluble fragment from exosomes is consistent with our previous study showing that the remnant receptor endodomain is contained in a significantly higher concentration on exosomes than on cell membranes. It should be noted that the lysosome and the recycling endocytic vesicle are unlikely sites of proteolysis based on prior studies of receptor degradation (17, 18). The ability of the proteolytic activity of surface membrane-enriched cell membrane fractions in the present study to be fully inhibited by PMSF and DIFP indicates that the activity is mediated by a serine protease.

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