Membrane Cofactor Protein (CD46) of Complement

PROCESSING DIFFERENCES RELATED TO ALTERNATIVELY SPliced CYTOplASMIC DOMAINS*

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Membrane cofactor protein (MCP, CD46), a widely distributed regulatory protein, inhibits complement activation on host cells and serves as a measles virus receptor. Most cells express four isoforms (with one of two cytoplasmic tails, CYT-1 or CYT-2). Previously, we noted that MCP precursors had variable intracellular processing. Therefore, we characterized the intracellular transport of individual MCP isoforms. Transfectants were used for pulse-chase analyses. MCP isoforms bearing CYT-1 localized into their mature, surface forms with a half-life (t0) of 10–13 min while those with CYT-2 required 35–40 min. The precursor of a tail-less mutant possessed a t0 of 160–165 min. Chimera were constructed that added both tails in opposite orientation into the isoform (i.e. CYT-1+2 or CYT-2+1). Chimera 1+2 precursor processed with a t0 of 35–37 min, similar to CYT-2. Chimera 2+1 had a t0 of 15–19 min, more closely resembling CYT-1. Thus, in both cases the carboxyl-terminal tail controlled the processing rate. Deletions were made in the beginning, middle, and carboxyl terminus of CYT-1. Deletion of the first or middle six amino acids had no effect on the processing rate. However, deletion of the terminal tetrapeptide (FTSL) slowed the rate to 30–32 min, suggesting that this sequence facilitates exit from the endoplasmic reticulum.

Membrane cofactor protein (MCP, CD46) is a widely distributed regulatory protein of the complement system that inhibits complement activation on host cells (reviewed in Ref. 1). MCP acts as a cofactor in concert with plasma serine protease and factor H. Of interest, complement receptor 2 (CD57) and 2 (CD21), and two plasma proteins, C4 binding protein and factor H. Other members of this multigene family are decay-accelerating factor (CD55), complement receptors 1 (CD36) and 2 (CD21), and two plasma proteins, C4 binding protein, and factor H. Of interest, complement receptor 2 serves as the Epstein-Barr virus receptor (7–9), and recently MCP has been shown to be a measles virus receptor (10–12).

MCP is expressed on nearly every cell examined (except erythrocytes) and displays an unusual pattern on SDS-PAGE in that it appears as a broad, heterogeneous doublet with a molecular mass of 58–68 and 48–56 kDa. The cDNA cloning (13) and genomic organization of MCP (14) have provided an explanation for this electrophoretic characteristic. On most human cells MCP consists of a family of at least four isoforms that arise by alternative splicing of a single gene (14) (Fig. 1). The amino terminus of the extracellular portion contains four of the C3b/C4b-binding modules known as short consensus repeats (1, 15–18). Following this area is a region enriched in serines, threonines, and prolines (STP), a site of extensive O-linked glycosylation. The STP region consists of 14 or 29 amino acids, depending on whether STP exon B (15 amino acids) is spliced out. The presence of STP-B produces the higher molecular weight protein isoforms whereas its absence generates the lower molecular weight forms of MCP (1, 14). The STP region is followed by a tract of 12 amino acids of unknown significance that is encoded by a separate exon. A transmembrane region, intracytoplasmic anchor, and one of two alternatively spliced cytoplasmic tails (CYT-1 of 16 amino acids and CYT-2 of 23 amino acids) form the carboxy terminus.

We have previously characterized two high mannose-containing pre-Golgi precursors in human cells whose processing times varied (19). We report that differences in precursor processing are directly related to the cytoplasmic tail of MCP.

EXPERIMENTAL PROCEDURES

Construction of cDNA Mutants and Subcloning into Expression Vectors—Four previously cloned MCP isoforms (14) were subcloned into the EcoRI sites of expression vectors pSFFV-neo (20) and pH3AP1-neo (21). For controls, an isoform was subcloned in reverse orientation in both vectors.

Six modified constructs of MCP are utilized in this investigation (Fig. 2). A "tail-less" mutant consisted of the isoform MCP-BC2 in which the intracytoplasmic tail was deleted making the intracytoplasmic anchor the carboxy terminus. The nomenclature for the MCP isoforms indicates which STP region (B or C) and which cytoplasmic tail (1 or 2) is expressed. The tail chimera designated MCP-BC2+1 added the amino acids of CYT-1 to CYT-2. The chimera designated MCP-BC1+2 added CYT-2 to CYT-1. Finally, three constructs were made with deletions in the tail of MCP-BC1 of the first six (TYLTDE), the middle six (THREVK), or the final four (FTSL) amino acids. Chimera and mutants were created using polymerase chain reaction methodology in which two "partners" were generated and then ligated together. Thirty-residue oligonucleotides were employed as amplification primers with the appropriate MCP cDNA isoform as template. The "outer" 5'-amplification primer incorporated an EcoRI site joined to a common sequence in the 5'-untranslated region of MCP cDNA. The outer 3'-amplification primer for the tail chimera consisted of a sequence complementary to MCP cDNA in the 3'-untranslated domain with an added EcoRI site at its terminus. "Internal" 3' - and 5'-oligonucleotide primers were phosphorylated prior to cDNA amplification. Following the generation of the polymerase chain reaction products, the products were blunt-end ligated. The ligated insert was restriced with EcoRI and subcloned into the expression vector. All constructs were sequenced in their entirety to verify the fidelity of created sequences.

Transfection: Plasmid DNA was isolated using the Midiprep method (22). Stably transfected cell lines were prepared in NIH-3T3 or

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Fig. 1. Diagram of the four commonly expressed isoforms of MCP. The NH2 terminus is composed of four contiguous short consensus repeats (SCR) in which there are three sites for N-linked glycosylation. Following these repeating modules is a domain composed of one or two exons (designated B and C) enriched in serines, threonines, and prolines (STP), which is a site for extensive O-linked glycosylation. The cytoplasmic domain follows a hydrophobic membrane-spanning segment and consists of a common intracellular anchor and a cytoplasmic tail of 16 (CYT-1) or 23 amino acids (CYT-2).

A. MCP Constructs

B. MCP-BC1 Tail Mutants

Chinese hamster ovary K1 (CHO) cells (American Type Culture Collection, Rockville, MD) using Lipofectin reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Transfected cells were selected for neomycin (Geneticin) resistance. NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 0.4 mg/ml (active concentration) Geneticin (Life Technologies, Inc.). CHO transfectants were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum and 0.4 mg/ml (active concentration) Geneticin (0.5 mg/ml). Some transfected cells were sorted for higher expression in an Epic 753 sorter (Coulter Corp., Hialeah, FL).

Pulse-Chase Analysis—Transfectants were starved 1 h in cysteine-free medium containing 10% dialyzed fetal calf serum. After this incubation, 100 μCi/ml L-[35S]cysteine (DuPont NEN) was added for a 15-min pulse. The label was removed and chased using fully supplemented medium for varying times. Cells were then washed with Dulbecco’s phosphate-buffered saline (Life Technologies, Inc.) and solubilized in lysis buffer (1% Nonidet P-40, 0.05% SDS, and 1 mM phenylmethylsulfonyl fluoride in Dulbecco’s phosphate-buffered saline). After incubating at 4 °C for 20 min, debris was pelleted at 10,000 x g for 10 min. Supernatant was stored at −70 °C. Immunoprecipitation, SDS-PAGE, and autoradiography were performed as described (14). Autoradiographs were scanned using a laser densitometer (Ultra scan, Pharmacia LKB Biotechnology Inc.) and software (GELscan) to analyze results.

RESULTS

Pulse-Chase Analysis of MCP Isoforms—Ballard et al. (19) previously noted a 4-fold difference in the processing times of MCP precursors in several human cell lines. Since these lines expressed multiple isoforms of MCP, it was not possible to correlate this finding with a particular species. This issue became approachable with the molecular definition and transfection of the four commonly expressed isoforms (14).

Pulse-chase analyses were performed on stably transfected CHO and NIH-3T3 cell lines, each expressing one of the four isoforms. MCP was immunoprecipitated, characterized on SDS-PAGE, and the half-life (t1/2) of the precursor determined by densitometric scanning of the resulting autoradiograms (Fig. 3 and Table I). Precursors of transfectants bearing CYT-1, the major tailless mutant, came approachable with the molecular definition and transfection of the four commonly expressed isoforms (14).
The goal of the present study was to examine if the cytoplasmic tails of MCP affected the processing of its precursors. We focused on differences in precursor processing rates because in a previous study Ballard et al. (19) had detected a 4-fold difference in the processing time of high mannose, pre-Golgi precursors of MCP in human cell lines. However, since multiple MCP species are expressed on human cells, it was not possible to determine which form(s) were being differentially processed. Consequently, we prepared stably transfected cell lines bearing the four common isoforms of MCP, employed pulse-chase methodology for analysis, and found that precursors of isoforms bearing CYT-1 chased several times faster than those possessing CYT-2 (summarized in Table I). Thus, these data clarify the earlier results and suggest that the cytoplasmic tail is responsible for this variability in MCP precursor processing time.

We next constructed a series of tail mutants and chimeras to further characterize the role of the cytoplasmic tail. If the cytoplasmic tail was deleted altogether (leaving the transmembrane domain and intracellular anchor), the processing rate slowed markedly (4–12-fold) compared with the native rate of both CYT-1 and CYT-2 isoforms, again demonstrating the importance of the cytoplasmic tail. The construction of chimeras in which both tails were present (in the order of 1+2 or 2+1) also slowed the processing rate further substantiated by the finding that deletion of the first or middle six amino acids of CYT-1 had little or no effect on precursor processing, while deletion of the last four amino acids retarded its processing rate.

These findings are consistent with recent evidence that cytoplasmic domains are important determinants for the intracellular transport of certain proteins (reviewed in Ref. 23). Several studies employing either cytoplasmic tails translocated from other proteins or tail mutants (23, 24) have generally resulted in impeded processing or retention in the ER. Few, if any, studies have demonstrated an accelerated processing rate when splicing a domain onto a protein. Thus, in considering several possibilities to account for these results, we favor a hypothesis that CYT-1 contains a positive sorting signal that is masked by the interaction of CYT-2. Thus, CYT-1 acts as a negative sorting signal that is eliminated upon interaction with CYT-2. This hypothesis is consistent with the observation that CYT-1 has little or no effect on precursor processing, whereas CYT-2 accelerates the processing rate.

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CYT-1 terminator or altered its conformation. Our results, then, suggest a specific process rather than a default pathway for transport of MCP proteins from the ER. However, alternative explanations for these results must be considered. If CYT-2 were to possess a signal for slower transport, the results would also be explained. However, it seems less likely that deletion of the last four amino acids of CYT-1 would slow its rate to nearly that of CYT-2.

We have considered several explanations to account for the cytoplasmic tail-directed differences in processing times. Following translation and entry into the ER, the carboxyl terminus of MCP is exposed on the cytoplasmic face of the ER membrane. As a result, it has ample opportunity to interact with host cell proteins (chaperones) that may direct its intracellular transport. Chaperone proteins are importantly involved in the control of protein structure, function, localization, and transport (reviewed in Refs. 25 and 26). Additionally, the cytoplasmic tails of MCP contain consensus signals for events such as phosphorylation and nuclear transport (Fig. 6). It is possible that a post-translational modification such as phosphorylation or the bidirectional transport of an MCP isoform into the nucleus (27) may have a bearing on its processing rate. These issues are being addressed utilizing MCP isoform transfectants, specific antibodies, and phosphorylation stimulators.

Another possibility is that clustering or oligomerization may affect precursor processing. Cytoplasmic domains may accelerate exit from the ER by inducing clustering of a protein at sites of transport vesicle formation (23). Additionally, it is possible that a tail signal may direct a process such as oligomerization in the ER. Many proteins undergo modifications in the ER including assembly into homo- or heterooligomers. The three-dimensional structure and oligomeric state of a protein may control not only its functional properties but also its intracellular transport, cellular localization, and lifespan (28). Cytoplasmic domains have been implicated in this process for some proteins (28, 29). The use of mild detergents and chemical cross-linking studies should allow us to determine whether MCP species form oligomers and if that is correlated with isoform processing rates.

Since MCP isoforms show differences in intracellular processing times, biologic advantages may be conferred as a result. For example, an isoform that can be more quickly recruited to the cell surface would be an advantage if MCP were up-regulated during complement activation at an inflammatory site. Additionally, a signaling ability mediated through a phosphorylation event could play a critical role in response to an inflammatory process. In these and other ways, a single cell that possesses multiple MCP isoforms may be protected more effectively from complement attack.

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