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Regulated Intramembrane Proteolysis of the Colony-Stimulating Factor 1 Receptor during Macrophage Activation

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Introduction

The colony-stimulating factor (CSF-1) receptor is a protein-tyrosine kinase expressed on monocytes and macrophages. Binding of CSF-1 to its receptor results in receptor dimerization, cross-phosphorylation, and recruitment of cellular signaling proteins. More recently, we discovered that the CSF-1 receptor is subject to regulated intramembrane proteolysis or RIPping. RIPping involves ectodomain shedding and release of the cytoplasmic region into the interior of the cell. It is carried out by tumor necrosis factor α-converting enzyme (TACE) and γ-secretase, following the encounter of macrophages with molecules that are derived from microbial pathogens. CSF-1 receptor RIPping is likely to play a role in macrophage activation in response to microbial infection.

The CSF-1 receptor uses tyrosine phosphorylation sites to recruit and activate cellular signaling proteins. CSF-1 is a cytokine that acts as a key regulator for growth, survival, differentiation, and activation of monocytes, macrophages, and other cell types [1,2]. It acts by binding to a receptor that is present on the surface of a variety of cell types, including primitive multipotent hematopoietic stem cells, B cells, neurons, placental trophoblasts, osteoclasts, monocytes and macrophages [3]. The CSF-1 receptor is a protein-tyrosine kinase, composed of an extracellular ligand-binding region, a transmembrane region, and a cytoplasmic region [4-6]. The cytoplasmic portion of the receptor is divided into a juxta membrane region, a kinase domain that is separated into two parts by a kinase insert, and a carboxy-terminal tail [2]. It is well established that ligand binding results in receptor dimerization and autophosphorylation on a number of tyrosine residues (Figure 1). Phosphorylation at tyrosine 807 in the activation loop increases kinase activity [7]. Additional phosphorylation sites have been characterized as binding sites for intracellular signaling proteins (Figure 1). These signaling proteins are activated directly or indirectly as a consequence of their interaction with the receptor and they relay information along intracellular signal transduction pathways [1,2]. Thus, like other receptor protein-tyrosine kinases, the CSF-1 receptor uses phosphotyrosine-containing protein-binding sites to recruit cytoplasmic signaling proteins. Upon binding to the receptor, these proteins activate signal transduction pathways that cause the biochemical changes that make it possible for the cell to respond to the presence of CSF-1.

Regulated intramembrane proteolysis

Regulated intramembrane proteolysis is a process that involves two cleavage events and that results in the release of the cytoplasmic region of an integral membrane protein into the interior of the cell [8]. Proteins that are present on the cell surface as well as proteins present in intracellular membranes have been shown to undergo RIPping [8]. Cell surface proteins are first cleaved in their extracellular region within 5-20 residues of the plasma membrane. This first cleavage event, which is usually regulated, results in release of the extracellular domain and the production of an integral membrane protein with a very short section extending from surface the cell and a longer cytoplasmic region that contains one or more functional domains. This cleavage product, in turn, is recognized by a second protease, resulting in cleavage within the transmembrane region, followed by release of a soluble protein product into the cytoplasm. Following its release from the membrane this protein can travel to other locations within the cell to carry out a particular function [8,9].

RIPping was first observed in the context of sterol-regulated gene transcription. During this process, the expression of proteins that participate in the uptake or biosynthesis of cholesterol is turned on in response to the absence of sterols [10]. Regulation of sterol sensitive gene transcription is mediated by sterol-regulatory element binding proteins or SREBPs. SREBPs are produced as integral membrane protein precursors that are localized in the endoplasmic reticulum in the presence of cholesterol. These precursor proteins are composed of an amino-terminal region that can function as a transcription factor and that project into the cytoplasm, a transmembrane region, a short loop projecting into the lumen of the endoplasmic reticulum, a second transmembrane region, and a regulatory region that is present in the cytoplasm [11]. In the absence of sterols, these proteins move from the endoplasmic reticulum to the Golgi apparatus where they are proteolytically cleaved, resulting in the release of their amino-terminal transcription factors [12]. Maturation of the SREBP precursor proteins involves two distinct cleavage events. The precursor protein is first cleaved within its luminal loop by the site 1 protease [13]. The amino-terminal half is subsequently recognized and cleaved within its transmembrane region by the site 2 protease [14]. This results in the release of the mature SREBP into the cytosol, followed by its translocation into the nucleus. Like many transcription factors, SREBPs are short lived; they are poly-ubiquitinated and degraded in the proteasome [15]. We recently discovered that the CSF-1 receptor is processed in a similar fashion during macrophage activation [16-20].

Regulated intramembrane proteolysis of the CSF-1 receptor

We followed up experiments showing that treatment of macrophages with 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, or lipopolysaccharides (LPS), a component of bacterial cell walls caused the disappearance of CSF-1 receptors from the cell surface [21,22]. To investigate the dynamics of this process, P388D1 macrophages were incubated with TPA for various amounts of time before the cells were lysed and analyzed

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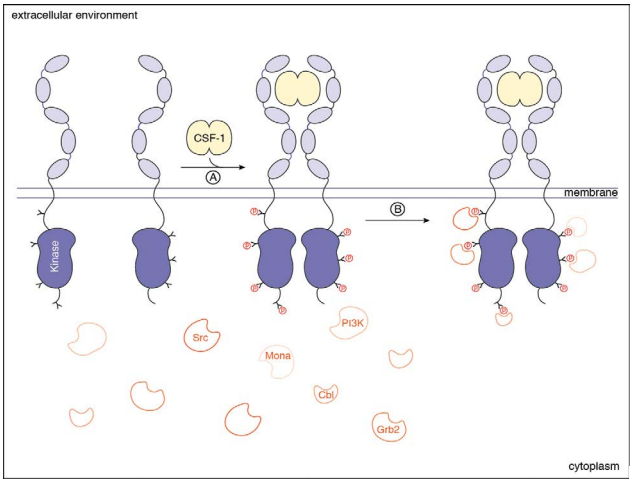

Figure 1: *The CSF-1 receptor uses tyrosine phosphorylation sites to recruit and activate cellular signaling proteins.* The CSF-1 receptor is a protein-tyrosine kinase expressed on the cell surface of a variety of cells. It contains an extracellular ligand binding domain (light purple) and a cytoplasmic kinase domain (dark purple). CSF-1 (yellow) is a dimer that contains two receptor-binding sites. Binding of CSF-1 to its receptor results in receptor dimerization (arrow A) making it possible for receptors to phosphorylate each other on tyrosine residues (tyrosine residues are indicated with the letter Y and phosphate groups are shown as an encircled p). A number of autophosphorylation sites have been identified in the CSF-1 receptor, including tyrosines 559, 697, 706, 721, 807 and 973 [42-46]. Phosphorylation on tyrosine 807 contributes to activation of the catalytic domain [7,47]. Other tyrosine phosphorylation sites act as binding sites for Src-homology domain-containing signaling proteins (red and orange globular structures in the cytoplasm). Upon autophosphorylation of the receptor, these proteins move from the cytoplasm to their docking sites on the receptor (arrow B). Cytoplasmic proteins known to bind to the activated CSF-1 receptor include: the protein-tyrosine kinase Src, growth factor receptor binding protein 2 (Grb2), signal transducer and activator of transcription 1 and 3 (STAT 1 and 3), phosphatidyl-inositol 3-kinase, the monocytic adaptor protein Mona, phospholipase Cγ, and the ubiquitin protein ligase c-Cbl [42,45,46,48-52]. Upon binding to the receptor, these proteins are activated resulting in the relay of information along cellular signal transduction cascades.

for the presence of CSF-1 receptors by immunoblotting. Following addition of TPA it takes 5 to 10 minutes before receptors start to disappear. Receptor downregulation reaches maximal levels after 1.5 hours [23]. As receptors disappear, a 55 kDa protein that represents the carboxy-terminal half of the receptor emerges [23]. Separation of the cells into particulate and soluble fractions showed that the formation of this CSF-1 receptor fragment involves two steps. First, the receptor is cleaved amino-terminally to the membrane, resulting in the formation of a 55 kDa membrane-associated fragment (Figure 2). The membrane-bound cleavage product is often referred to as the carboxy-terminal fragment or CTF. The CSF-1 receptor CTF is cleaved again, resulting in the release of a slightly smaller fragment into the cytoplasm (Figure 2). This soluble product is often referred to as the intracellular domain or ICD. The first cleavage can be blocked using inhibitors of TACE, a metalloprotease composed of an extracellular catalytic domain, a single transmembrane domain and a short cytoplasmic tail [21,24]. The second cleavage can be blocked using pharmacological inhibitors of γ-secretase or dominant interfering mutants [23].

γ-Secretase is a complex of several integral membrane proteins that cleaves proteins within their transmembrane region [25]. γ-Secretase has been implicated in the production of Aβ, which is a major constituent of amyloid plagues, and the onset of Alzheimer's disease [25]. Our results suggest that the CSF-1 receptor is cleaved in its extracellular region by TACE, followed by cleavage in the transmembrane region by γ-secretase.

Identification of the TACE and γ-secretase cleavage sites in the CSF-1 receptor

While we had good evidence showing that the CSF-1 receptor undergoes proteolytic processing, the exact location of the cleavage sites has remained unknown. Identification of the cleavage sites involves purification of the cleavage products followed by amino-terminal sequencing. To facilitate purification, we engineered CSF-1 receptors containing several affinity tags at their carboxy-terminus. Tagged receptors were stably expressed in 293A cells, cells were stimulated to initiate receptor processing, and cleavage products were purified using affinity chromatography [26]. The TPA-inducible TACE cleavage site was identified following pretreatment of cells with the γ-secretase inhibitor, L-685,458, for 1 hour, followed by stimulation with TPA for 20 minutes. The γ-secretase inhibitor was included to prevent the second cleavage. Edman degradation yielded the following amino-terminal sequence, SKQLPDES, indicating that the receptor is cleaved 12 amino acid residues before the start of the transmembrane domain (Figure 3). The γ-secretase cleavage site was identified from cells that were stimulated for 20 minutes with TPA in the presence of H_2O_2; H_2O_2 was included because in our experiments it appears to inhibit degradation of the soluble product in the proteasome [27]. Amino-terminal sequencing yielded a major and a minor sequence, LLLYKYKQKP and YKYKQKP respectively, thus identifying a major and minor cleavage site within the transmembrane region (Figure 3). Identification of the cleavage sites will provide insight into substrate recognition by both TACE and γ-secretase. Analysis of cleavage site mutants will help elucidate the role of CSF-1 receptor RIPping in macrophage activation.

Nuclear localization of the CSF-1 receptor ICD

In most cases studied, RIPping results in the transient appearance of cleavage products that move to the nucleus where they regulate gene expression, followed by their degradation in the proteasome. For example, the Notch ICD transiently translocates to the nucleus where it interacts with members of the CSL family of transcription factors before it is degraded [28,29]. Because the cytoplasmic cleavage products are usually instable, it has remained difficult to document their presence in the nucleus. Immunofluorescence data suggested that the CSF-1 receptor ICD, following its release from the plasma membrane, localizes in part to the nucleus [23]. The interpretation of these experiments is complicated by the fact that the CSF-1 receptor ICD becomes ubiquitinated followed by its degradation in the proteasome [23,27]. To further investigate the localization of the ICD following its release from the membrane, we have generated an amino-terminally tagged version of the cleavage product. Preliminary data suggest that this protein may be present in the nucleus. Together these observations suggest that the CSF-1 receptor ICD travels to the nucleus where it may be involved in regulation of gene transcription. It remains unclear, however, exactly how the CSF-1 receptor ICD is marked for translocation to the nucleus. Because a well-recognized nuclear localization signal is absent from the ICD, we propose that the ICD associates with an unidentified binding partner, which directs the ICD towards the nucleus. Alternatively, it is possible that the ICD contains an unusual nuclear localization signal. Expression of stable ICD mutants, which are currently being constructed, would make it possible to initiate a more thorough investigation of the localization of the ICD, following its release into the cytoplasm.

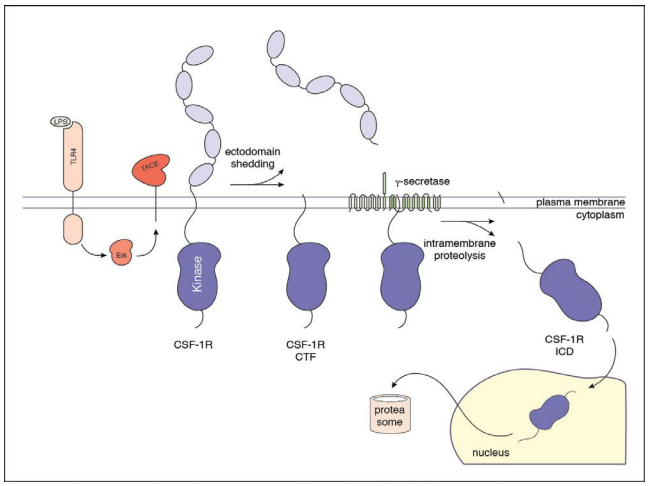

Figure 2: *Regulated intramembrane proteolysis of the CSF-1 receptor following the interaction of macrophages with pathogen-derived molecules.* Pathogen-derived molecules are recognized by dedicated families of receptors on the surface of macrophages. For example, LPS binds to Toll-like receptor 4 (TLR4). TLR4 activates various intracellular kinases, including Erk-1 and Erk-2 [40,41]. This leads to activation of TACE, a protease with a short cytoplasmic region and an extracellular catalytic domain [53]. TACE cleaves the CSF-1 receptor (CSF-1R) at a site 12 residues amino-terminal to the transmembrane region. This results in the release of most of the ligand-binding region into the extracellular environment (ectodomain shedding) leaving behind a protein with a very short extracellular region. This product is often referred to as the carboxy-terminal fragment or CTF. The CTF is cleaved by γ-secretase within its transmembrane region resulting in the release of the intracellular domain or ICD into the cytoplasm [53]. The ICD can be detected in the nucleus where it may be involved in the regulation of gene transcription. It is short lived and is degraded in the proteasome.

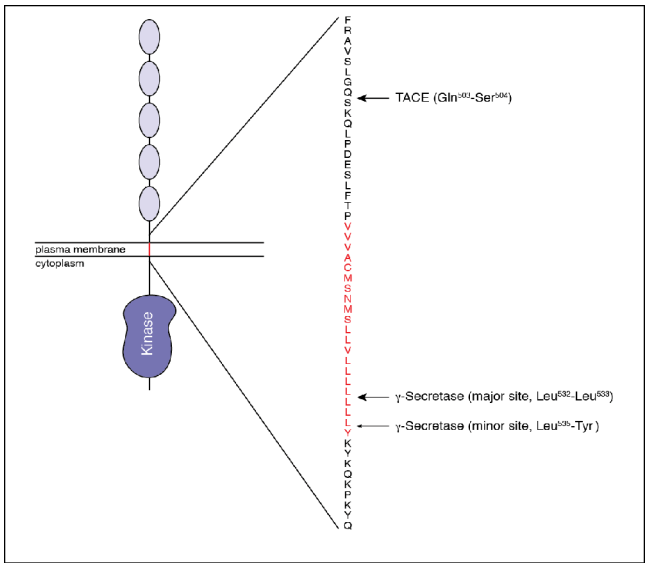

Figure 3: *Location of the TACE and γ-secretase cleavage sites in the CSF-1 receptor.* The sites were identified by automated Edman degradation following purification of the cleavage products [26].

CSF-1 receptor RIPping during the immune response

A successful response against microbial infections depends on the interplay between the innate and the adaptive immune systems [30,31]. The innate immune system recognizes the invader as foreign, based on the presence of pathogen-associated molecular patterns, such as LPS or double stranded RNA [32]. It initiates the anti-microbial response and helps to recruit and activate the adaptive immune system. The adaptive immune system generates responses against specific antigens associated with the intruder and solicits the help of the innate immune system to terminate the infection [33]. Macrophages, which are found in most tissues, form the first line of defense by recognizing invading microorganisms using pattern recognition receptors, initiating the anti-microbial response, and alerting the rest of the immune system by producing proinflammatory cytokines, including IL-1β, IL-6 and TNF [30]. Toll-like receptors (TLRs) are one of several families of pattern recognition receptors that are expressed on macrophages [34,35]. They recognize molecules that are associated specifically with microorganisms, including lipopolysaccharide, a major component of gram-negative bacterial cell walls (which binds to TLR4), bacterial lipoproteins (TLR2), double-stranded RNA (TLR3), and CpG islands in bacterial DNA (TLR9) [36-39]. Upon ligand binding, TLRs recruit death domain-containing adaptor proteins that relay information along signal transduction cascades, which leads to the activation of the stress activated protein kinases p38 and JNK, Tank-binding kinase 1, an IκB kinase, and the extracellular signal regulated kinases Erk-1 and Erk-2, ultimately resulting in macrophage activation [40,41].

Interestingly, we observed that LPS induces RIPping of the CSF-1 receptor in a dose and time dependent manner [21]. Other Toll-like receptor ligands such as Lipid A, Lipoteichoic acid, poly I: poly C, and bacterial DNA also stimulated proteolytic processing of the CSF-1 receptor [21]. These observations lend support to the idea that CSF-1 receptor RIPping and release of the ICD from the plasma membrane into the cytoplasm plays a role in macrophage activation during the response to a microbial infection. While this remains to be proven, it seems likely that the CSF-1 receptor ICD moves to the nucleus to contribute to the activation of pro-inflammatory gene transcription.

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