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CSF-1 and TPA stimulate independent pathways leading to lysosomal degradation or regulated intramembrane proteolysis of the CSF-1 receptor

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Abstract

The CSF-1 receptor is a protein-tyrosine kinase that has been shown to undergo regulated intramembrane proteolysis, or RIPPING. Here we have compared receptor downregulation and RIPPING in response to CSF-1 and TPA. Our studies show that CSF-1 is a relatively poor inducer of RIPPING and that CSF-1-induced receptor downregulation is largely independent of RIPPING. TPA is a strong inducer of RIPPING and TPA-induced receptor downregulation is mediated by RIPPING. We further found that RIPPING is dependent on TACE or a TACE-like protease, that CSF-1 and TPA use independent pathways to initiate RIPPING, and that the intracellular domain is targeted for degradation through ubiquitination.

Keywords

signal transduction; protein-tyrosine kinase; RIPPING; PKC; macrophage; ubiquitination

1. Introduction

Colony-stimulating factor 1 (CSF-1) is a critical regulator of monocyte proliferation, their differentiation into macrophages as well as macrophage activation [1-3]. The receptor for CSF-1 is a protein-tyrosine kinase [4]. It is well established that the CSF-1 receptor, like other receptor protein-tyrosine kinases, uses autophosphorylation sites to select and activate intracellular signaling proteins [5-11]. We have previously shown that the CSF-1 receptor also undergoes regulated intramembrane proteolysis resulting in the release of the intracellular domain into the cell [12].

Regulated intramembrane proteolysis, or RIPPING, is a highly conserved process that is used by a wide variety of organisms [13-15]. RIPPING involves two cleavage events. First, an integral membrane protein is cleaved within its extracellular region close to the plasma membrane. This results in ectodomain shedding and yields a truncated integral membrane protein with a very short extracellular domain [13-15]. The truncated product of the first cleavage is recognized by a second protease, often γ -secretase, that cleaves proteins within their transmembrane region [16,17]. Intramembrane cleavage results in the release of the intracellular domain (ICD) into

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the cytosol. The intracellular domain generally moves to the nucleus where it is likely to participate in the activation of gene transcription [16,17].

RIPping provides a mechanism for CSF-1 receptor downregulation, as well as a mechanism for signal transduction. In this study we have compared RIPping induced by CSF-1 with RIPping induced by TPA.

2. Materials and methods

2. 1. Reagents

Recombinant human CSF-1 was obtained from R&D Systems (Minneapolis, MN). A polyclonal antiserum was raised previously in our laboratory against the kinase insert domain of the murine CSF-1 receptor [10]. A monoclonal antibody against ubiquitin was purchased from Cell Signaling Technology (Danvers, MA), TAPI-0 was purchased from Calbiochem (San Diego, CA) and TPA was purchased from Sigma-Aldrich (St Louis, MO).

2. 2. Cell culture

The murine macrophage cell line, P388D1, was obtained from Dr. Ed Dennis (UCSD, San Diego, CA) and was grown in Iscove's modified Eagles media supplemented with 10% fetal bovine serum. Cells were grown at 10% CO₂. P388D1 cells were seeded at 2-3×10⁵ cells per ml in a 10 cm dish two days prior to stimulation.

2. 3. Immunoprecipitation

Cells were stimulated as described in the text, rinsed 2 times with cold TBS, and lysed in PLC lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 100 mM Na pyrophosphate, 100 mM Na orthovanadate, 1 mM AEBSF, 1 mM leupeptin, 1 mM benzamidine, and 1 mM E64). Lysates were cleared by centrifugation at 10,000 rpm in a microcentrifuge at 4°C, incubated with 0.5 μl polyclonal anti-CSF-1 receptor antiserum 1 hr on ice and subsequently for 1 hr with 100 μl 10% Protein A-Sepharose for 1 hr at 4°C on an agitator. Sepharose beads were collected by centrifugation and washed 4 times with PLC lysis buffer. Immunoprecipitates were boiled 3 min in 62.5 mM Tris/Cl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 5 mM DTT, 2.3% SDS, and 0.025% Bromophenol Blue (SDS-sample buffer) and resolved by SDS-PAGE on a 7% acrylamide gel.

2. 4. Immunoblotting

Proteins were transferred to PVDF membranes using a Biorad semi-dry blotting apparatus at 50 mA per gel for 45 min at room temperature. Membranes were blocked for 1 hr at room temperature in 10 mM Tris/Cl pH 7.4, 150 mM NaCl, 0.2% Tween 20 (TBST) containing 5% dried milk and incubated with 1:700 dilution of the polyclonal anti-CSF-1 receptor serum in TBST 5% milk for 1 hr at room temperature. Blots were washed 2 × 10 min with TBST and 2 × 5 min with 10 mM Tris/Cl pH 7.4 and 150 mM NaCl (TBS). Membranes were then incubated for 30 min with HRP-Protein A (Biorad, Hercules, CA) diluted 1:10,000 in TBST and washed as before. Reactive proteins were visualized by ECL (Amersham, Piscataway, NJ).

3. Results

3. 1. Concentration dependent processing of the CSF-1 receptor in response to CSF-1 or TPA

To determine at what concentration CSF-1 and TPA induce maximal levels of RIPping, P388D1 macrophage cells were incubated with various concentrations of these activators for 20 minutes. The cells were then lysed; receptors were isolated by immunoprecipitation and visualized by immunoblotting with an antiserum directed against the CSF-1 receptor kinase

insert domain (Figure 1). Protein bands of 150, 130 and 55 kDa were detected on these blots. The 150 kDa protein represents the mature receptor present on the cell surface; the 130 kDa protein corresponds to a precursor protein present in the endoplasmic reticulum; the 55 kDa protein is produced by RIPPING and is composed largely of the intracellular domain (ICD) of the CSF-1 receptor (Figure 1). It is only the 150 kDa mature receptor that is subject to RIPPING, the 130 kDa precursor is unaffected (Figure 1). Our results show a low level of constitutive CSF-1 receptor RIPPING. Stimulation with CSF-1 causes a small but reproducible increase in the level of the ICD. Maximal levels were observed with 100 ng/ml CSF-1 (Figure 1A). The results also show that the decrease in the levels of mature receptors correlates poorly with the increase in levels of the ICD. This is a consequence of the fact that CSF-1 causes most receptors to be internalized and degraded in the lysosomes, while a smaller fraction is subject to RIPPING.

Stimulation with TPA also results in a concentration dependent induction of RIPPING. We detected an increase in RIPPING with as little as 10 nM TPA, while maximal RIPPING was observed with 100 nM TPA (Figure 1B). In contrast to what was found with CSF-1, the decrease in levels of mature receptors correlates well with the increase in levels of the ICD.

3. 2. CSF-1 and TPA-induced RIPPING follow different time courses

To find out whether CSF-1 and TPA use the same pathway to induce CSF-1 receptor RIPPING we carried out a time course experiment. P388D1 macrophages were stimulated with either CSF-1 or TPA for various amounts of time and analyzed for CSF-1 receptor downregulation and the appearance of the ICD. The experiment shows that receptors start to disappear within minutes after addition of CSF-1. Maximal receptor downregulation is seen at 30 minutes (Figure 2A). The ICD first appears at 5 minutes after addition of CSF-1, maximal levels are seen at 20-30 minutes after which the ICD slowly disappears (Figure 2A). Downregulation of receptors may be slightly slower in response to TPA while the ICD levels peak a little earlier (Figure 2B). These observations show that both CSF-1 and TPA induce RIPPING, but that the kinetics of this process depends on the stimulus.

3. 3. CSF-1 is largely independent of PKC for the induction of RIPPING

To investigate whether CSF-1 depends on the presence of PKC for the induction of RIPPING, P388D1 cells were pretreated with TPA or DMSO for 24 hours to downregulate expression of PKC, prior to stimulation. CSF-1-induced disappearance of mature receptors and the appearance of the ICD were largely unaffected by the absence of PKC (Figure 3A). In contrast, the TPA-induced disappearance of mature receptors and the appearance of the ICD were both blocked completely following downregulation of PKC (Figure 3B). These observations suggest that CSF-1 and TPA are using different pathways to stimulate receptor downregulation as well as RIPPING.

3. 4. TACE inhibitors differentially affect CSF-1 and TPA-induced downregulation of the CSF-1 receptor

To investigate the involvement of TACE in CSF-1 and TPA-induced RIPPING and receptor downregulation, P388D1 macrophages were pretreated with the TACE inhibitor TAPI-0 for 1 hour before stimulation. Our results show that the inhibitor has very little effect on receptor disappearance in response to CSF-1, while it almost completely blocked receptor disappearance in response to TPA (Figure 4). These observations show that much of CSF-1-induced receptor downregulation is independent of TACE. In contrast, TPA-induced receptor downregulation appears to be completely dependent on TACE. This supports the notion that TPA-induced receptor downregulation is mediated by RIPPING, while CSF-1-induced receptor downregulation is not.

3. 5. Ubiquitination of the CSF-1 receptor intracellular domain

During the course of our experiments we observed that hydrogen peroxide cooperates with other stimuli in the induction of CSF-1 receptor RIPPING (results not shown). These experiments further showed the generation of a series of ICD-related proteins of increasing size. To investigate the possibility that the CSF-1 receptor ICD becomes ubiquitinated, CSF-1 receptor immunoprecipitates from hydrogen peroxide treated cells were analyzed by anti-ubiquitin immunoblotting. The results show indeed a series of proteins of increasing size that can be detected with an anti-CSF-1 receptor antibody (Figure 5, left panel). Most of these proteins can also be detected by anti-ubiquitin immunoblotting (Figure 5, right panel). These results show that the CSF-1 receptor ICD is modified by ubiquitination, likely marking the ICD for degradation in the proteasome.

4. Discussion

Macrophages play an important role in the defense against foreign intruders and in tissue development. In addition, they have been implicated in the etiology of various maladies including diabetes and heart disease [18,19]. The CSF-1 receptor regulates the renewal and survival of macrophages, recruitment of macrophages to specific sites within the body, and macrophage activation. [1-3,20]. We have shown that the CSF-1 receptor can undergo RIPPING [12]. CSF-1 receptor RIPPING and transient accumulation of the ICD are seen following activation of Toll-like receptors (Glenn and van der Geer, unpublished observations), suggesting that RIPPING may play a role during macrophage activation and initiation of the innate immune response. We believe that following its release from the plasma membrane the ICD travels to the nucleus, where it may contribute to regulation of pro-inflammatory gene transcription.

In this study, we have compared RIPPING in response to either CSF-1 or TPA. The results show that CSF-1 is a relatively poor inducer of RIPPING (Figure 1). This suggests that when cells are stimulated with CSF-1 alone, most receptors disappear through internalization and degradation in the lysosomes. Consequently, RIPPING makes only a small contribution CSF-1-induced signaling or receptor downregulation (Figure 6). On the other hand, RIPPING is more robustly induced by TPA, suggesting that CSF-1 receptor RIPPING is likely to contribute to signaling by receptors that strongly activate PKC, presumably through activation of phospholipase C [21]. In addition, the possibility exists that RIPPING is strongly induced when cells are stimulated with CSF-1 and one or more other cytokines. This is relevant because macrophages encounter various different cytokines during the onset of inflammation [22,23].

It has been shown previously that TACE is involved in CSF-1 receptor downregulation [24]. This prompted us to investigate whether TACE is essential for either CSF-1 or TPA-induced RIPPING. The results show CSF-1-induced receptor downregulation is largely unaffected by the presence of TACE inhibitors. This suggests that CSF-1-dependent receptor downregulation is not mediated by RIPPING (Figure 6). TPA dependent CSF-1 receptor downregulation, however, is completely blocked in the presence of 100 μ M TAPI-0. This suggests that TPA-induced receptor downregulation is mediated entirely by RIPPING (Figure 6). Interestingly, the appearance of the ICD is not completely blocked by TACE inhibitors (Figure 4B), suggesting that TPA may activate a TACE-related protease such as Kuz or one of the meltrins, all of which are known to act as ectodomain sheddases [25].

Time course experiments of CSF-1 and TPA induced CSF-1 receptor downregulation agree largely with previously published observations [26,27]. Small differences may be due to differences in the cell lines studied or in the methods used to assess the presence of the CSF-1 receptor. In response to CSF-1, the levels of the ICD keep rising for up to 30 minutes. In contrast, when cells are stimulated with TPA maximal levels of the intracellular domain can

be observed at 15 minutes after the onset of stimulation. It is unclear what causes this difference. However, it suggests that CSF-1 and TPA may activate different pathways that both lead to CSF-1 receptor RIPping. This model is further supported by results showing that TPA-induced RIPping depends on the presence of PKC, while CSF-1 receptor induced RIPping does not (Figure 2). Together these observations support the conclusion that CSF-1 and TPA activate independent pathways leading to receptor RIPping (Figure 6).

We have shown that CSF-1 receptor RIPping is initiated by cleavage within the receptor's extracellular domain that is mediated by TACE or a related protease. There are at least two independent pathways that lead to CSF-1 receptor RIPping (Figure 6). This suggests that RIPping is subject to regulation in response to various extracellular signals and that the release of the CSF-1 receptor intracellular domain into the interior of the cell plays a role in signaling by a variety of cytokines that regulate of macrophage activity. Following its release into the cytoplasm, the ICD is likely to move to the nucleus, becomes ubiquitinated and is finally directed to the proteasome for degradation (Figures 5 and 6).

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Fig. 1.

Dose-dependent RIPping of CSF-1 receptors in response to CSF-1 or TPA. P388D1 cells were stimulated with increasing amounts of CSF-1 (A) or TPA (B) for 20 minutes, the cells were lysed and CSF-1 receptor immunoprecipitates were analyzed by anti-CSF-1 receptor immunoblotting. Protein bands representing the mature receptor (CSF-1R), the CSF-1R precursor, and intracellular domain (ICD) are indicated.

Fig. 2. CSF-1 and TPA induce CSF-1 receptor RIPping with different kinetics. P388D1 cells were stimulated with CSF-1 (100 ng/ml) or TPA (100 nM) for varying lengths of time, the cells were lysed, and CSF-1 receptor immunoprecipitates were analyzed by anti-CSF-1 receptor immunoblotting.

Fig. 3.

CSF-1 and TPA-induced RIPping differ in their dependence on the presence of PKC. P388D1 cells were treated for 24 hours with 100 nM TPA or DMSO as a control. Cells were then stimulated with CSF-1 or TPA, the cells were lysed, and CSF-1 receptor immunoprecipitates were analyzed by anti-CSF-1 receptor immunoblotting.

Fig. 4.

The TACE inhibitor, TAPI-0, differentially inhibits CSF-1 and TPA-induced receptor downregulation. P388D1 cells were pretreated for 1 hr with 100 μ M TAPI-0, the cells were lysed, and CSF-1 receptor immunoprecipitates were analyzed by anti-CSF-1 receptor immunoblotting.

Fig. 5. P388D1 cells were treated with 3 mM hydrogen peroxide for varying lengths of time, the cells were lysed, and CSF-1 receptor immunoprecipitates were analyzed by anti-CSF-1 receptor and anti-ubiquitin immunoblotting.

Fig. 6.

CSF-1 and TPA induced receptor downregulation and RIPping. Stimulation with CSF-1 causes receptor activation. Most activated receptors are removed from the cell surface by internalization and degradation in the lysosomes (blue arrows). CSF-1 causes a small fraction of its receptors to undergo RIPping. Activation of PKC by TPA results in robust activation of TACE or a TACE-like protease followed by ectodomain shedding and intramembrane proteolysis. All TPA induced CSF-1 receptor downregulation is mediated by RIPping (pink arrows).