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Polymerization of MIP-1 chemokine (CCL3 and CCL4) and clearance of MIP-1 by insulin degrading enzyme

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 June 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees appreciate the structural analysis on the MIP-1 chemokine. However, referee #2 also raise several important concerns and finds that the present analysis is not well suited for publication here at this stage. The referee recognizes that we gain new insight into the regulation of MIP-1 activity, but also finds that there is too limited functional data provided to support that the observed MIP-1 polymerization as well as the IDE-mediated MIP-1 degradation is physiological relevant and this contributes to the chemotactic gradient and affects inflammation or cell migration. In other words, we would need some further functional data to support the biological significance of the structural and biochemical work. I recognize that such further analysis might not be straight-forward, but this is needed for further consideration here. Should you be able to add such further data then we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

I am available to discuss the manuscript and revisions further if you would find this helpful.

Thank you for the opportunity to consider your work for publication.

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Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

These are long awaited structures and the results explain quite nicely, a lot of mutational data on these proteins and open up many new questions. Overall, the results with the polymerization, effects of heparin and effects of IDE address the complex regulation that chemokines are subject to. The technical quality of the manuscript is very high. The writing requires minor editing and the manuscript should be made a bit more concise. Nevertheless, it should be published in EMBO.

Referee #2 (Remarks to the Author):

Summary of the manuscript major points.

The authors' goals were to elucidate the specific structural basis for MIP-1 aggregation and subsequent degradation of the MIP-1 aggregates, corresponding to activation and inactivation of the MIP-1 signal, respectively. The authors determined the polymer structures of three MIP-1 proteins: MIP-1α, MIP-1β, and a MIP-1α de-aggregation mutant that alters polymerization. Polymerization was shown by using SAXS analysis and size exclusion chromatography, as well as through heparin treatment. It was found that IDE can bind and proteolytically inactivate MIP-1 monomers, along with the observation of an inverse relationship between IDE levels and MIP-1 levels in a mouse microglia cell-line. The interaction between IDE and MIP-1 was investigated by proteomic and structural analyses. MIP-1α forms two different polymer structures: one similar to wild type, and an octamer form that is potentially an unstable intermediate in the polymerization of MIP-1α. MIP-1 polymer formation is reversible. The specific sites of proteolysis in MIP-1a targeted by IDE were found

General comments on the value of the experiments and how they contribute to advancing the field

While this study is quite interesting and may yield new insight into the regulation of MIP-1 activity in areas of inflammation, the authors need to make a stronger case for the importance of this regulation under physiological conditions. In addition, there are some aspects of this work that appear to be at odds with previous studies, including those that implicate GAG binding in chemokine oligomer formation and suggest that GAG binding actually improves downstream signaling, at least in other chemokines. The authors need to provide a better context for understanding the importance and relevance of this information, and need to make a better case for their suggestion that the polymerization and degradation of MIP-1 might cause its effects to be exerted primarily at the periphery of areas of inflammation. Overall, while this study is interesting, and a great deal of data is shown, the authors need to make a better, more logical case for their conclusions, and must address the more controversial aspects of their findings. In addition, there are several issues (detailed below) that must be resolved and the demonstration of the importance of the work within the chemokine field is unclear, at least in the mind of this reviewer.

Major general criticisms, questions, and comments

• Several years ago, when researchers were crystallizing the chemokine IL-8, they found evidence suggesting that IL-8 formed oligomers due to the high concentrations needed for crystallography. Furthermore, they found that mutations which disrupted this oligomer formation did not appreciably affect chemokine function. In this paper, the authors mention again that the polymer length is dependent upon the concentration of MIP-1 present. Due to the fact that under most circumstances only small amounts of chemokines are present in vivo, how likely is it that these dimmers/polymers actually form under physiological conditions? And under what kinds of conditions? It would be best

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if the authors were to show that these oligomers actually form in vivo.
• The authors mention the fact that MIPs bind to GAGs and that this is critical for their function, and also that the GAGs may promote oligomer formation. Is GAG binding necessary in order to promote aggregation, or is this merely one effect of GAG binding, in addition to receptor presentation?
• The authors mention that an area of MIP-1 that is necessary for receptor binding is sequestered within the MIP-1 polymer. This suggests that the dimer or multimer would not be able to bind and activate the receptor. The authors also explain that this binding site would be hidden. How then can their findings that the MIP-1 oligomers can induce migration be explained? Do they need to depolymerize in order to function? Or is the effect independent of the classical MIP receptors and/or the classical receptor-binding domain of MIP-1?
• The authors propose that monomers are rapidly degraded by the IDE protease, while polymers are not. Does this happen in vivo?
• The ability of heparin to decrease polymerization of MIP-1 seems unusual. Binding of the chemokines to heparin generally increases their local concentrations, which should increase their ability to polymerize, not decrease it. This should be discussed.
• The study focuses quite a bit on the ability of IDE to cleave and inactivate the MIP-1α protein. Why did the authors focus on this protease? There are other proteases that are present in inflamed or injured areas which could presumably also cleave MIP-1α-is IDE a more physiological or more effective MIP-1α-degrading enzyme?
• In figure 5C, the authors incubate the MIP-1α with an excessive amount of IDE (more than was used in 5B to block chemotaxis), which only partially blocks MIP-1α-induced Ca2+ mobilization. Is it possible that the cleavage of MIP-1α by IDE primarily affects its chemoattractant activity, with a lesser effect on Ca 2+ influx? If so, what are some potential mechanisms for this disparity? Differential receptor usage? Alterations in polymerization when a high concentration of MIP-1α was used in the Ca2+ assay? Or perhaps not enough IDE was present? It would be a good idea to perform the experiment again with multiple doses of MIP-1α and multiple doses of IDE to determine whether the conditions are optimal for this assay.
• The authors suggest that GAGs may mediate MIP-1 depolymerization because their binding site to MIP-1 is present at the dimer interface. However, if these dimers are very stable, GAGs should not be able to bind this region and thus disrupt polymerization. The authors need to explain their reasoning in more detail. If anything, the data suggests that GAGs may bind to the monomers and prevent them from polymerizing, which could reduce the concentration of free monomers, thereby reducing polymerization, but it seems unlikely that GAGs would induce depolymerization, but rather block polymerization. The authors introduce an analogy to actin polymerization that is quite interesting and should be expanded upon-in actin polymerization, reduction of the pool of free monomers would ultimately reduce the rate of subunit addition, potentially below the rate of subunit release and leading to depolymerization. This may provide a mechanism for GAG-induced depolymerizatin if explained in more detail.
• The authors propose that a higher concentration of chemokines in areas of inflamamtion will increase dimerization/polymerization, decreasing the ability to be degraded by proteases (like IDE), and increasing their duration and effective range. However, what is not account for is the fact that the receptor-binding portion of the chemokine is not exposed when polymerized-how can polymerization increase the effective and functional range if the chemokine cannot bind to its receptor?

Specific comments

• In the last sentence of the first introduction paragraph, the authors mention that the chemokine receptors have a relationship with HIV infection. This sentence appears out of nowhere, with no previous mention. Either the authors should go into more detail about this relationship or delete the sentence.
• There are grammatical issues; for example, the first sentence of the second intro paragraph states "MIP-1α and MIP-1β form aggregates (described as oligomer or polymer) and this process is a key regulatory step for MIP-1 function." This is a run-on sentence, and there is a plural problem. It should read: "MIP-1α and MIP-1β form aggregates (described as oligomers or polymers), and this process is a key regulatory step for MIP-1 function." The remainder of the paper should be read carefully and edited accordingly.
• In the first paragraph of the introduction, page 2, lines 10-12, the use of "responses" twice in the same sentence is distracting.
• In the second paragraph of the introduction, page 3, line 7, "b strands" should have a hyphen

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between "b" and "strands."
• In the third paragraph of the introduction, page 3, lines 22-23, the phrase "MIP-1 aggregation is influenced by GAG but the molecular basis remains elusive" seems presumptive, for the literature references cited up to this point described MIP-1 activation, not aggregation, as being influenced by GAG. The authors should consider revising the statement for clarity, or simply omitting the sentence all together, as it disrupts the flow of ideas with a rather hollow statement.
• In the third paragraph of the introduction, page 3, line 23, "are" should be "is", or "activity" should be "activities".
• In the fourth paragraph of the introduction, page 4, line 2, "an" should be "a".
• On page 7, line 4, the authors' claim that the F29 residue is "conserved" and "contribute[s] to MIP-1 polymerization via hydrophobic interactions." It is in fact not conserved, as observed in the sequence alignment of MIP-1a and MIP-1ḅ(Figure 1A). Also, claiming that it contributes to the polymerization is presumptuous as residue 29 in MIP-1ḅis a tyrosine, a polar residue that would not contribute to hydrophobic interactions.
• In Figure 1A, the numbers don't line up with the corresponding amino acid beginning with 31 and on to 61.
• In Figure 1A, residue 20 was not highlighted in blue even though it was included in the calculation for % sequence identity.
• The numbering scheme for the supplemental figures does not fit with the paper, for Figure S8 is cited before Figure S7; the numbering for these figures should be swapped.
• On page 14, lines 20-23, and page 15, lines 1-4, the authors' describe numerous interacting residues between IDE and MIP-1a, as was done with the MIP-1 dimer. They included a table for the MIP-1 dimer showing all of the specific bonding distances determined from the structure (Figure S5), but not for the IDE-MIP-1a structure. A similar table should be included for this structure as well.
• On page 15, line 10, "b strand" should have a hyphen between "b" and "strand."
• On page 15, line 11, "comparing" should be "compared."
• On page 17, line 22, the period (.) after "4.8" should be a comma (,).

Referee #3 (Remarks to the Author):

Ren et al. describe a mechanism how two closely-related chmokines, CCL3 and CCL4, are assembled into high-order polymers, and that insulin-degrading enzyme (IDE), an ubiquitous protease, is involved in the selective degradation of CCL3 and CCL4. The findings are conclusive, and is consistent with previous literature and the broad landscape of the field. These results are of interest to a broad audience, and are clearly a comprehensive study as benefited from the combination of a wide array of methods. The manuscript is well suitable for EMBO J, and should be published as soon as possible.

I only have a few minor points.

1. page 7. In describing the D27A mutant structure, the author should present a more detailed analysis of the structural consequence of the D27A mutation, e.g., the reduction of interactions, the atomic differences around the position of this mutation. From Fig. 1F, it seems that D27 is rather central in organizing the polymer, and that the D27A mutant can form the same type of polymer is somewhat surprising, although apparently factual.
It would be beneficial for the readers if Fig. 1F marks the N-terminii and C-terminii. Also for the closeup views, the lower panel should have the carbon atoms of the side chains colored differently, as in the upper panel. Maybe a close but different color from the ribbons would be best.
It is also not quite clear how F24, F29 and Y28 form hydrophobic interaction from Fig. 1F. Some details describing the specific interactions in the text are suggested.

2. Two segments of MIP-1alpha were found in the IDE complex. While the definition of the larger piece from electron density (Fig. 6B) is convincing, the authors should be more cautious in attributing the the smaller fragment to the N-terminus, given the limited resolution and quality of the electron density. It is therefore also better in the text to describe the identity of this fragment as a possibility rather than a certainty.

3. Table 1. Space group "P6222", the first "2" should be subscript.

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4. Fig. S4. A short discussion should be included to address whether the structural difference between crystallography and NMR represents an authentic difference resulted from in solution, or in harsh condition measurement of the NMR, or simply is the inaccuracy of NMR measurement due to restraint ambiguity. The NOE restrains of the NMR structures could be used to test whether they also fits the crystal structure perfectly.

1st Revision - authors' response 09 September 2010

Response to referees:

We first would like to thank the recommendation of acceptance by referee #1 and #3 and the constructive criticism from all three referees, particularly referee #2 and #3. We have performed additional experiments to address the comments raised by the referees and made the following major modifications:

1. Our structural and biophysical analyses reveal that MIP-1α and MIP-1β reversibly form the rod-shaped double helical polymers with the variable lengths, instead of fixed length oligomer. We are asked to provide some evidence for the role of MIP-1 polymerization in the biological functions. To address this, based on our MIP-1 structures and previous work on other CC chemokines, we have created and characterized an additional depolymerization mutant, MIP-1α P8A. Polymerization buries receptor-binding sites of MIP-1α, thus we predict that depolymerization mutants would have enhanced potency in the MIP-1 mediated response under certain condition. Indeed, we show that two independent depolymerization mutations, D27A and P8A have similar effects in enhancing MIP-1α to arrest monocytes onto activated human endothelium. However, our mathematical modeling reveals that, for a long-range chemotaxis of MIP-1, polymerization could serve to protect MIP-1α from proteases selectively degrading monomeric MIP-1α. Consistent with this modeling, we also show that same depolymerization mutations render MIP-1α ineffective in mouse peritoneal cell recruitment. These data support a vital role of MIP-1α polymerization for the biological functions.

2. Referee #3 requires a better analysis in the comparison between our MIP-1 polymer structures and the NMR structures of MIP-1 dimer. We follow this suggestion and find that the crystal structures of MIP-1 monomer fit nicely with the available NMR structures of wild type MIP-1β and MIP-1α D27A mutant. This is significant since low pH and other harsh treatments were used to reduce the oligomerization of MIP-1 in order to determine the NMR structures of MIP-1 dimer. We find no evidence that such treatments alter the MIP-1 monomer structure such as to make it different from MIP-1 polymer structures. However, the comparison of MIP-1 dimer between NMR and crystal structures reveals a noticeable rigid body movement between MIP-1 monomers. The center of mass of MIP-1β monomer shifts 7Å and undergoes 27o rotation (relative to the Cα atom of threonine 9) while MIP-1α has a smaller translation (5Å) and rotation (7o). This movement renders the dimer of MIP-1α and MIP-1β inside the MIP-1 polymers to be more compact for the favorable dimer-dimer interactions. This could form the molecular basis in the structural transition from the MIP-1 dimer to MIP-1 polymer.

3. Referee #2 noted that the degradation of MIP-1α by IDE seems to have more profound effects on the chemotaxis than the ability of MIP-1α to raise intracellular calcium concentration. Thus, we are asked to confirm this finding. We have performed a careful titration experiments to ensure that our experiments were done in the MIP-1 concentration that is optimal to detect the effect of IDE. We have also done a series of IDE dosedependent experiments to confirm this differential effect.

4. We have rewritten the introduction and discussion sections to address the comments raised by referee #2 and #3. The specific changes are listed below as the responses to the referees.

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Specific responses: The comments from referees are underlined and our responses start with "response". To make this coherent, we also group some comments so that we can address them together.

Referee #1 (Remarks to the Author):

These are long awaited structures and the results explain quite nicely, a lot of mutational data on these proteins and open up many new questions. Overall, the results with the polymerization, effects of heparin and effects of IDE address the complex regulation that chemokines are subject to. The technical quality of the manuscript is very high. The writing requires minor editing and the manuscript should be made a bit more concise. Nevertheless, it should be published in EMBO.

Response: We have gone over a careful editing of the document to improve its clarity.

Referee #2 (Remarks to the Author):

Summary of the manuscript major points.

The authors' goals were to elucidate the specific structural basis for MIP-1 aggregation and subsequent degradation of the MIP-1 aggregates, corresponding to activation and inactivation of the MIP-1 signal, respectively. The authors determined the polymer structures of three MIP-1 proteins: MIP-1α, MIP-1β, and a MIP-1α de-aggregation mutant that alters polymerization. Polymerization was shown by using SAXS analysis and size exclusion chromatography, as well as through heparin treatment. It was found that IDE can bind and proteolytically inactivate MIP-1 monomers, along with the observation of an inverse relationship between IDE levels and MIP-1 levels in a mouse microglia cell-line. The interaction between IDE and MIP-1 was investigated by proteomic and structural analyses. MIP-1α forms two different polymer structures: one similar to wild type, and an octamer form that is potentially an unstable intermediate in the polymerization of MIP-1α. MIP-1 polymer formation is reversible. The specific sites of proteolysis in MIP-1α targeted by IDE were found.

General comments on the value of the experiments and how they contribute to advancing the field

While this study is quite interesting and may yield new insight into the regulation of MIP-1 activity in areas of inflammation, the authors need to make a stronger case for the importance of this regulation under physiological conditions. In addition, there are some aspects of this work that appear to be at odds with previous studies, including those that implicate GAG binding in chemokine oligomer formation and suggest that GAG binding actually improves downstream signaling, at least in other chemokines. The authors need to provide a better context for understanding the importance and relevance of this information, and need to make a better case for their suggestion that the polymerization and degradation of MIP-1 might cause its effects to be exerted primarily at the periphery of areas of inflammation. Overall, while this study is interesting, and a great deal of data is shown, the authors need to make a better, more logical case for their conclusions, and must address the more controversial aspects of their findings. In addition, there are several issues (detailed below) that must be resolved and the demonstration of the importance of the work within the chemokine field is unclear, at least in the mind of this reviewer.

Major general criticisms, questions, and comments

1. Several years ago, when researchers were crystallizing the chemokine IL-8, they found evidence suggesting that IL-8 formed oligomers due to the high concentrations needed for crystallography. Furthermore, they found that mutations which disrupted this oligomer

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formation did not appreciably affect chemokine function. In this paper, the authors mention again that the polymer length is dependent upon the concentration of MIP-1 present. Due to the fact that under most circumstances only small amounts of chemokines are present in vivo, how likely is it that these dimmers/polymers actually form under physiological conditions? And under what kinds of conditions? It would be best if the authors were to show that these oligomers actually form in vivo.

Response: Our studies together with the previous biophysical characterization show that MIP-1α and MIP-1β reversibly form ordered rod-shaped polymers. The dissociation constant for the dimer-dimer interaction is estimated to be in the 100-300 nM range. While the detectable serum MIP-1 concentration is low, the local concentration upon secretion could reach a level that is approximately three orders of magnitude higher than the concentration required for MIP-1 dimer to polymerize. For example, it is shown that immune cells can secrete high levels of MIP-1 proteins upon LPS stimulation; up to 1 pg MIP-1β/hr/dendritic cell (Sallusto et al, 1999). It is also reported that MIP-1 and RANTES are secreted as high MW aggregates from HIV-1-specific cytotoxic T lymphocytes (Wagner et al, 1998). As described above, we have created two MIP-1α depolymerization mutants and show that both mutations, D27A and P8A, render MIP-1α less effective in cell recruitment into mouse peritoneum. We also show that same mutations render MIP-1α more potent to arrest peripheral blood monocytic cells onto the TNF-α activated human umbilical vein endothelial cells. Together, our data suggest that high molecular weight MIP-1 polymers could exist in physiologically relevant settings and polymerization plays a key role in the biological functions of MIP-1. We have incorporated these findings into our revised manuscript.

Questions #2, #5, and #8 are related to the interaction of MIP-1 with GAGs:

2. The authors mention the fact that MIPs bind to GAGs and that this is critical for their function, and also that the GAGs may promote oligomer formation. Is GAG binding necessary in order to promote aggregation, or is this merely one effect of GAG binding, in addition to receptor presentation?

5. The ability of heparin to decrease polymerization of MIP-1 seems unusual. Binding of the chemokines to heparin generally increases their local concentrations, which should increase their ability to polymerize, not decrease it. This should ne discussed.

8. The authors suggest that GAGs may mediate MIP-1 depolymerization because their binding site to MIP-1 is present at the dimer interface. However, if these dimers are very stable, GAGs should not be able to bind this region and thus disrupt polymerization. The authors need to explain their reasoning in more detail. If anything, the data suggests that GAGs may bind to the monomers and prevent them from polymerizing, which could reduce the concentration of free monomers, thereby reducing polymerization, but it seems unlikely that GAGs would induce depolymerization, but rather block polymerization. The authors introduce an analogy to actin polymerization that is quite interesting and should be expanded upon-in actin polymerization, reduction of the pool of free monomers would ultimately reduce the rate of subunit addition, potentially below the rate of subunit release and leading to depolymerization. This may provide a mechanism for GAG-induced depolymerizatin if explained in more detail.

Response: The effects of GAGs on chemokine functions are very complex. Many studies have addressed this important issue. A number of papers using the GAG binding mutants have shown that GAG binding is key for chemokine functions *in vivo*. Our paper takes advantage of heparin as an experimental model GAG to probe how GAG could affect MIP-1 polymerization in the test tube and how the presence of GAG could facilitate the degradation of MIP-1 by IDE. This study was not designed to specifically address the role of GAGs in the *in vivo* functions of MIP-1.

Specifically, we have shown that MIP-1α and MIP-1β form rod-shaped polymers of variable length. This process is dynamic and reversible. Thus, biological molecules that bind MIP-1 proteins such as GAGs can influence MIP-1 polymerization. In our test tube

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assay, we found that the presence of soluble heparin, a form of GAG, can reduce the degree of polymerization of both MIP-1α and MIP-1β. The positively charged residues (R18, K45, and R46) residing at the dimer-dimer interface of MIP-1 polymers are critical for GAG binding (Handel et al, 2005; Koopmann et al, 1999; Proudfoot et al, 2003). Thus, it is reasonable to observe that the binding of heparin could reduce the degree of MIP-1 polymerization by capping the end of MIP-1 polymer or affecting the equilibrium of MIP-1 polymerization.

However, we envision that the effect of GAG binding in MIP-1 polymerization *in vivo* is likely far more complicated. In different tissues, the heparan sulfate GAG chains are highly diverse in structure and their linked protein partners also vary. In addition to depolymerization, GAG could facilitate MIP-1 polymer formation by anchoring and clustering MIP-1 polymers together on the cell surface or extracellular matrix as well. While substantial knowledge has been gained by using GAG binding mutants, future investigations will be need to specifically address the effect of GAGs on the reversible MIP-1 polymerization *in vivo* and MIP-1-mediated biological functions.

In response to these questions, we have expanded in the discussion section and included our view on how GAGs could affect MIP-1 polymerization. We have used an analogy of MIP-1 polymerization with actin polymerization to explain the effect GAG might have on MIP-1 compared to the effect of actin binding proteins on actin polymerization.

Questions #3 and #9 deal with the issue whether MIP-1 polymers need to be dissociated into MIP-1 monomer for the receptor binding:

3. The authors mention that an area of MIP-1 that is necessary for receptor binding is sequestered within the MIP-1 polymer. This suggests that the dimer or multimer would not be able to bind and activate the receptor. The authors also explain that this binding site would be hidden. How then can their findings that the MIP-1 oligomers can induce migration be explained? Do they need to depolymerize in order to function? Or is the effect independent of the classical MIP receptors and/or the classical receptor-binding domain of MIP-1?

Response: Our working model is that MIP-1 monomer is the sole species that bind CCR effectively. However, MIP-1 monomer is also more sensitive to proteolytic inactivation than MIP-1 dimer or polymers. The dimerization/polymerization are used to store these ligands during the transportation either by diffusion or binding with other extracellular molecules such as GAGs. The dimerization/polymerization of MIP-1 prevents the proteolytic degradation by the extracellular proteases such as IDE. However, MIP-1 dimer or polymers need to be dissociated into the monomer in order to bind its cognate CCR. The dissociation should occur since this process is reversible and highly dependent upon the MIP-1 concentration. Our model does not evoke a novel receptor for MIP-1 dimer or polymers since the existence of such a receptor has not been demonstrated.

9. The authors propose that a higher concentration of chemokines in areas of inflamamtion will increase dimerization/polymerization, decreasing the ability to be degraded by proteases (like IDE), and increasing their duration and effective range. However, what is not account for is the fact that the receptor-binding portion of the chemokine is not exposed when polymerized-how can polymerization increase the effective and functional range if the chemokine cannot bind to its receptor?

Response: Again, we propose that MIP-1 monomer is the active form for the receptor binding and activation and MIP-1 polymers can reversibly dissociate into MIP-1 monomers. In fact, based on our mathematical modeling for the reversible MIP-1 polymerization, the concentration of MIP-1 monomer is always highest at any given MIP-1 concentration. When considering the effective biological function such as chemotaxis (Figure 4A), we also only consider the concentration of MIP-1 monomer. One can easily envision that, with less proteolytic inactivation, MIP-1 protein in its polymer/dimer form will have more time to diffuse than the MIP-1 monomer. Thus, MIP-1 dimer/polymers would reach farther in distance since it would not be attacked by extracellular protease(s). Once

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MIP-1 becomes a monomer, it will be a race for it to either bind CCR or be degraded (of course, it can reform MIP-1 dimer or polymer to avoid the degradation but it would not be able to bind the receptor as well). Thus, we envision that it is a race for MIP-1 to reach its targeted cells before it is inactivated by the clearance mechanism. The reversible polymer formation of MIP-1 would allow MIP-1 to be delivered farther away from the source by its ability to protect MIP-1 from the proteolytic inactivation.

Questions #4 and #6 deal with the degradation of MIP-1 by IDE:

4. The authors propose that monomers are rapidly degraded by the IDE protease, while polymers are not. Does this happen in vivo?

6. The study focuses quite a bit on the ability of IDE to cleave and inactivate the MIP-1α protein. Why did the authors focus on this protease? There are other proteases that are present in inflamed or injured areas which could presumably also cleave MIP-1α. Is IDE a more physiological or more effective MIP-1α-degrading enzyme?

Response: We use IDE as an exemplary protease that can selectively degrade the monomeric form of MIP-1 and leave MIP-1 dimer/polymers intact. We show that the IDE knockdown could increase the accumulation of MIP-1α and MIP-1β level in the cultured microglia cell-line, BV-2. We also provide the molecular basis for such selectivity. Thus, it is reasonable to consider that IDE could play a role in modulating the MIP-1 level *in vivo* in certain physiological setting(s). We do envision that other proteases also serve to selectively degrade MIP-1 monomer and thus contribute to regulation of MIP-1 mediated biological functions. As reported before, chemokines are subjected to the proteolytic degradation by a wide variety of proteases (review in Wolf et al 2008). Based on MIP-1 dimer structure, the N-terminus of these chemokines is buried upon dimerization. Thus, one can easily envision that the extracellular protease that targets the N-terminus of MIP-1 would be ineffective in cutting the N-terminal region when MIP-1 is dimerized or polymerized. Future work will be required to address how various proteases work together in modulating MIP-1 mediated functions in the physiological settings.

7. In figure 5C, the authors incubate the MIP-1α with an excessive amount of IDE (more than was used in 5B to block chemotaxis), which only partially blocks MIP-1α-induced Ca2+ mobilization. Is it possible that the cleavage of MIP-1α by IDE primarily affects its chemoattractant activity, with a lesser effect on Ca2+ influx? If so, what are some potential mechanisms for this disparity? Differential receptor usage? Alterations in polymerization when a high concentration of MIP-1α was used in the Ca2+ assay? Or perhaps not enough IDE was present? It would be a good idea to perform the experiment again with multiple doses of MIP-α and multiple doses of IDE to determine whether the conditions are optimal for this assay.

Response: As described above, we have done the experiments with multiple doses of IDE and MIP-1 to quantify the ability of MIP-1 to raise intracellular calcium concentrations. We confirm the subtle difference on the effect of the degradation of MIP-1 by IDE on intracellular calcium level and chemotaxis. In this case, IDE seems to have the more profound effect on the chemotaxis than calcium level. It is conceivable that the IDEcleaved MIP-1α products that are held together by the disulfide bonds are still able to partially activate CCR receptor for the transient increase of [Ca2+]i but lose their potency in chemotaxis, which occurs on a much longer time scale. We have included these data in our manuscript and have a brief discussion on this issue.

Specific comments

1. In the last sentence of the first introduction paragraph, the authors mention that the chemokine receptors have a relationship with HIV infection. This sentence appears out of nowhere, with no previous mention. Either the authors should go into more detail about this relationship or delete the sentence.

Response: The specific sentence is modified as the following: Based on the

protective activity of hematopoietic stem cells and the inhibition of HIV-1 infections by their binding with the HIV-1 coreceptor, CCR5, MIP-1 proteins were also explored as possible therapeutics for cancer and AIDS (Cocchi et al, 1995; Dunlop et al, 1992; Graham et al, 1990; Horuk, 2009).

2. There are grammatical issues; for example, the first sentence of the second intro paragraph states "MIP-1α and MIP-1β form aggregates (described as oligomer or polymer) and this process is a key regulatory step for MIP-1 function." This is a run-on sentence, and there is a plural problem. It should read: "MIP-1α and MIP-1β form aggregates (described as oligomers or polymers), and this process is a key regulatory step for MIP-1 function." The remainder of the paper should be read carefully and edited accordingly.

Response: This paragraph is completely revised to highlight the functional role of chemokine oligomerization.

3. In the first paragraph of the introduction, page 2, lines 10-12, the use of "responses" twice in the same sentence is distracting.

Response: The sentence is revised as the following: MIP-1 can activate several chemokine receptors (CCR1 and CCR5), which initiate diverse cellular responses that regulate both acute and chronic inflammation.

4. In the second paragraph of the introduction, page 3, line 7, "b strands" should have a hyphen between "b" and "strands."

Response: It is changed as advised.

5. In the third paragraph of the introduction, page 3, lines 22-23, the phrase "MIP-1 aggregation is influenced by GAG but the molecular basis remains elusive" seems presumptive, for the literature references cited up to this point described MIP-1 activation, not aggregation, as being influenced by GAG. The authors should consider revising the statement for clarity, or simply omitting the sentence all together, as it disrupts the flow of ideas with a rather hollow statement.

Response: The specific sentence is deleted.

6. In the third paragraph of the introduction, page 3, line 23, "are" should be "is", or "activity" should be "activities".

Response: It is modified as advised.

7. In the fourth paragraph of the introduction, page 4, line 2, "an" should be "a".

Response: It is modified accordingly.

8. On page 7, line 4, the authors' claim that the F29 residue is "conserved" and "contribute[s] to MIP-1 polymerization via hydrophobic interactions." It is in fact not conserved, as observed in the sequence alignment of MIP-1α and MIP-1β (Figure 1A). Also, claiming that it contributes to the polymerization is presumptuous as residue 29 in MIP-1β is a tyrosine, a polar residue that would not contribute to hydrophobic interactions.

Response: Conservation can refer to sequence identity or homology. In this case, we highlight the conserved hydrophobic nature between tyrosine and phenylanaline, which contributes to the hydrophobic interaction based on our MIP-1 structures. We also specify that we consider conservation only among MIP-1α, MIP-1β, and RANTES, three CC chemokines that have high propensity to form high molecular weight aggregates.

9. In Figure 1A, the numbers don't line up with the corresponding amino acid beginning with 31 and on to 61.

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Response: It is fixed.

10. In Figure 1A, residue 20 was not highlighted in blue even though it was included in the calculation for % sequence identity.

Response: It is fixed.

11. The numbering scheme for the supplemental figures does not fit with the paper, for Figure S8 is cited before Figure S7; the numbering for these figures should be swapped.

Response: It is fixed.

12. On page 14, lines 20-23, and page 15, lines 1-4, the authors' describe numerous interacting residues between IDE and MIP-1a, as was done with the MIP-1 dimer. They included a table for the MIP-1 dimer showing all of the specific bonding distances determined from the structure (Figure S5), but not for the IDE-MIP-1a structure. A similar table should be included for this structure as well.

Response: We have included a new table as requested.

13. On page 15, line 10, "b strand" should have a hyphen between "b" and "strand."

Response: It is modified accordingly.

14. On page 15, line 11, "comparing" should be "compared."

Response: It is modified accordingly.

15. On page 17, line 22, the period (.) after "4.8" should be a comma (,).

Response: It is modified accordingly.

Referee #3 (Remarks to the Author):

Ren et al. describe a mechanism how two closely-related chmokines, CCL3 and CCL4, are assembled into high-order polymers, and that insulin-degrading enzyme (IDE), an ubiquitous protease, is involved in the selective degradation of CCL3 and CCL4. The findings are conclusive, and is consistent with previous literature and the broad landscape of the field. These results are of interest to a broad audience, and are clearly a comprehensive study as benefited from the combination of a wide array of methods. The manuscript is well suitable for EMBO J, and should be published as soon as possible.

I only have a few minor points.

1. page 7. In describing the D27A mutant structure, the author should present a more detailed analysis of the structural consequence of the D27A mutation, e.g., the reduction of interactions, the atomic differences around the position of this mutation. From Fig. 1F, it seems that D27 is rather central in organizing the polymer, and that the D27A mutant can form the same type of polymer is somewhat surprising, although apparently factual.

Response: Based on our reversible polymer model, D27A mutation does cause the decrease of binding affinity by about 10 fold. Thus, it is conceivable that MIP-1 D27A can still form polymers although it requires higher concentrations to reach the same degree of polymerization as wild type MIP-1.

It would be beneficial for the readers if Fig. 1F marks the N-terminii and C-terminii. Also for the closeup views, the lower panel should have the carbon atoms of the side chains colored differently, as in the upper panel. Maybe a close but different color from the

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ribbons would be best.
It is also not quite clear how F24, F29 and Y28 form hydrophobic interaction from Fig. 1F. Some details describing the specific interactions in the text are suggested.

Response: We have included a new panel in Figure 1G to depict the role of F24, F28, and Y28 in dimer-dimer contact.

2. Two segments of MIP-1alpha were found in the IDE complex. While the definition of the larger piece from electron density (Fig. 6B) is convincing, the authors should be more cautious in attributing the smaller fragment to the N-terminus, given the limited resolution and quality of the electron density. It is therefore also better in the text to describe the identity of this fragment as a possibility rather than a certainty.

Response: We agree that it is challenging to place N-terminus of MIP-1α to the exosite with limited electron density and low resolution (3 angstrom). However, such model is reasonable based on the charge complementary of MIP-1 with IDE catalytic chamber and available electron density. We have modified our document to reflect this.

3. Table 1. Space group "P6222", the first "2" should be subscript.

Response: It is modified as requested.

4. Fig. S4. A short discussion should be included to address whether the structural difference between crystallography and NMR represents an authentic difference resulted from in solution, or in harsh condition measurement of the NMR, or simply is the inaccuracy of NMR measurement due to restraint ambiguity. The NOE restrains of the NMR structures could be used to test whether they also fits the crystal structure perfectly.

Response: As described above, we have done a careful structural comparison and found the major structural difference between the NMR MIP-1 dimer structures and X-ray crystallographic MIP-1 polymer structures reside in the rigid body movement of two MIP-1 monomers. The center of mass of MIP-1β monomer shifts 7Å and undergoes 27o rotation (relative to the Cα atom of threonine 9) while MIP-1α has a smaller translation (5Å) and rotation (7o). This movement renders the dimer of MIP-1α and MIP-1β inside the MIP-1 polymers to be more compact for the favorable dimer-dimer interactions.

Additional Correspondence 22 September 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #2 to review the revised version and I have now heard back from this referee. Referee #2 finds that the introduced changes has strengthened the manuscript and supports publication in the EMBO Journal. This referee has no further comments to the authors. Given the comments provided, I am pleased to proceed with the publication of the study in The EMBO Journal. You will receive the formal acceptance letter shortly.

Best wishes

Editor
The EMBO Journal

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