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Commentary

The chlamydial protease CPAF: important or not, important for what?

Abstract

The protease CPAF is only found in *Chlamydiales* and in at least most bacteria that share with *Chlamydia* the biphasic life-style in a cytosolic inclusion. CPAF is intriguing: it appears to be secreted from the inclusion across the inclusion membrane into the cytosol. A bacterial protease ravaging in the cytosol of a human cell may cause a plethora of effects. Curiously, very few are known. The current discussion is bogged down by a focus on experimental artifact, while proposed functions of CPAF remain speculative. I here make the attempt to summarize what we know about CPAF.

Keywords: *Chlamydia*; CPAF; Proteolysis

1. Introduction

When first discovered [1], CPAF (chlamydial protease-like activity factor) attained quick fame as a chlamydial protein secreted into the cytosol of infected host cells, and since it was shown that it could cleave host transcription factors involved in antigen presentation, it was proposed to have important immunomodulatory functions. Subsequently, more host cell proteins have been discovered that were on one hand found degraded in lysates from *Chlamydia*-infected human or mouse cells, on the other hand cleaved by recombinant CPAF. These proteins may therefore be CPAF-substrates during infection.

At least in most cases they were chance discoveries: when investigating a cell biological question in infected cells it turned out that a certain protein was degraded. Naturally, each of these cleavage events may have biological relevance, and more or less plausible scenarios have been put forward how CPAF may affect cellular processes. While this path of discovery is consequent and straightforward for each cell biological question, from the viewpoint of understanding CPAF it is a random walk: a free protease in the cytosol is likely to cleave many proteins, and it is extremely unlikely that all cleavage events are important for the infection. From this angle the recent article by Chen et al. [2] was a welcome wake-up call to query the perhaps prevalent uncritical belief in the roles and importance of reported CPAF-dependent cleavage events. At the same time it is arguable that the report over-emphasizes the (very real) possibility of experimental artifact, does not do justice to at least part of the reported results and, by focusing on one part of the problem, detracts from at least suggestive evidence of the important role that CPAF may play for *Chlamydia*.

2. Cytosolic proteolysis: a riddle wrapped in a mystery inside a cell

There are not many proteases known in the human cytosol (other than the large machines regulating turnover, such as the proteasome), and the ones known are regulated by cellular systems. Calpains, for instance, are under the control of endogenous inhibitors and calcium-levels, and caspases are regulated by upstream signaling complexes. The presence of a free, active protease in the cytosol, as it probably occurs during chlamydial infection, is unusual. Experimental introduction of a protease into the cytosol can even kill a cell (death may be apoptotic or not [3,4]). Free CPAF may therefore be an important feature of chlamydial infection.

It is extremely difficult to assess proteolysis in the cytosol of an intact cell. Almost all experimental approaches rely on the analysis of cell extracts, most often by Western blotting for proteolytic fragments. It is principally possible to detect proteolytic events by other means, for instance by fixation without lysis and staining for neo-epitopes generated by proteolysis (an example is the detection of active caspase-3 by a specific antibody [5]). However, these methods have to be established in a lengthy process for each cleavage event and are therefore not readily available for the study of CPAF and chlamydial infection.

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The results of studies using protein extraction may be wrong for a number of reasons. Proteolysis may be underestimated because a fragment is unstable and quickly lost, because the antibody is less sensitive for the fragment, or the fragment is less easily mobilized. Proteolysis may be overestimated because of better mobilization of the fragment, because of higher sensitivity of the antibody for the fragment or because of artificial digestion of a protein during extract preparation. The latter is the problem that is suggested by the recent work on CPAF although the basis of this is not very clear.

Proteolytic extraction artifacts are a common problem since extraction with detergent typically removes membrane barriers to proteolysis. This problem is well illustrated by the case of activated T lymphocytes. When these cells are processed for Western blotting, cytotoxic granules are lysed by the detergent, and granzymes (serine proteases that, when delivered into the target cell, activate caspases and induce apoptosis) are released from their containment in these granules. Caspase-processing, previously interpreted as cytosolic caspase-activation during T cell activation, in fact only occurs during extraction [6].

A similar situation may also be encountered in chlamydial infection although it is not as clear. Western blotting of extracts from infected cells shows cleavage fragments. Either using harsh extraction conditions (chaotropic salt such as 8 M urea) or including the chemical CPAF-inhibitor lactacystin, which binds to and inhibits CPAF [1,7], reduced the CPAF-dependent cleavage below the detection threshold by Western blotting (this effect of lactacystin was shown for one CPAF-substrate, golgin-84, the urea effect for a number of them) [2]. In sum the data suggest that substantial CPAF-dependent proteolysis occurs during extraction of infected cells.

What then is happening during extraction to generate the artifact? At least some of the published proteins are unequivocally degraded either directly by CPAF or by a process activated by CPAF. This bold assumption derives from data of ectopic expression (i.e. without chlamydial infection) of active CPAF in human cells, which causes degradation of these proteins [8]. CPAF therefore can probably cleave these proteins but does so preferentially during extraction. Why is that so?

An obvious possibility is that a large share of CPAF is only liberated from the inclusion into the cytosol during extraction. Thereby proteolysis would be greatly enhanced when detergents dissolve the inclusion membrane. It is also possible that substrate epitopes are hidden in the intact cell, which become accessible only when cellular structures are dissolved. A further possibility is that secreted CPAF is complexed with an either bacterial or cellular inhibitor that is removed by detergent but again there is no evidence for this. In any case it appears likely that – if active CPAF is indeed secreted into the cytosol – there is at least a small amount of CPAF-dependent cleavage in infected cells that is amplified during detergent extraction.

All published experimental evidence agrees that CPAF is found in the cytosol plus more inside the inclusion. Although it has been speculated that CPAF may be contained in outer membrane vesicles that are somehow delivered into the host cell [9], there is no experimental evidence for that. Most images of CPAF-microscopy suggest that it leaks out at one pole of the inclusion and forms a gradient in the cell from there. It is also possible that there are certain cytosolic spots where CPAF accumulates to higher concentration (and mobilization from such spots may increase extraction associated cleavage) although at least some stains suggest homogenous localization (notably, the cytosolic portion of the recently reported FLAG-tagged CPAF appears to be distributed evenly [10]). It is also conceivable that mechanic stress increases the levels of CPAF in the cytosol, so for instance physical movement of patients may contribute.

Most of these scenarios predict that some active CPAF is in the cytosol where it has at least limited access to its substrates. It is therefore doubtful that there is absolutely no CPAF-dependent cleavage although it may be very little. And from there the step has to be taken to say that we have no handle whatever on the question how much of a substrate has to be cleaved to achieve a biological effect. Very moderate CPAF-dependent cleavage may activate a cellular enzyme; it may generate a dominant inhibitor of a cellular process; it may upset an intricate balance of processes. It would in my view therefore be rash, on the basis of the finding that cleavage is undetectable by Western blotting, to conclude that there is no relevant cleavage.

It should be added that the mystery of CPAF does not end with host cell substrates cleaved by CPAF. Although pro-apoptotic BH3-only proteins can be cleaved by CPAF directly [11], published evidence suggests that at least the BH3-only protein Bim is not cleaved directly by CPAF but rather diminishes in abundance through an indirect, unknown mechanism [8]. Bim-loss can be occasioned by ectopic CPAF-expression [8] and it occurs during chlamydial infection where its loss is not inhibited by 2% SDS [12] or 8 M urea (unpublished) in the extraction buffer.

3. Are then any of the CPAF-substrates that have been discovered important for *Chlamydia*?

If at all this question won't be answered soon. A CPAF-deficient strain will probably be described in the near future, and then we will have some idea, but even this is unlikely to give us a clear answer on the importance of a given substrate. As said above, the frequent random detection of CPAF-dependent, infection-associated cleavage events (artificial or not) suggests that a large number of host cell proteins can in principle be cleaved by CPAF (or a protease activated by CPAF). Indeed, when we expressed active CPAF in human cells we found over 3000 cleavage events (unpublished). At present it doesn't seem easy to identify the real ones, if they exist. The parallel to caspases is also not encouraging. Caspases are activated during apoptosis, and their activity is critically required for the cell to die with the morphological signs of apoptosis. About 250 caspase-substrates are currently listed in the casbase database, which collects reported caspase-substrates (and plenty more are likely to exist) [13,14]. There are some hot candidates for some apoptotic events, such as activation of the DNAse CAD by caspase-mediated cleavage of its inhibitor ICAD [15] but even in apoptosis it is mostly unclear how caspases cause cell death with the typical