Fos family protein degradation by the proteasome

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Abstract

c-Fos proto-oncoprotein defines a family of closely related transcription factors (Fos proteins) also comprising Fra-1, Fra-2, FosB and ΔFosB, the latter two proteins being generated by alternative splicing. Through the regulation of many genes, most of them still unidentified, they regulate major functions from the cell level up to the whole organism. Thus they are involved in the control of proliferation, differentiation and apoptosis, as well as in the control of responses to stresses, and they play important roles in organogenesis, immune responses and control of cognitive functions, among others. Fos proteins are intrinsically unstable. We have studied how two of them, c-Fos and Fra-1, are degraded. Departing from the classical scenario where unstable key cell regulators are hydrolysed by the proteasome after polyubiquitination, we showed that the bulk of c-Fos and Fra-1 can be broken down independently of any prior ubiquitination. Certain conserved structural domains suggest that similar mechanisms may also apply to Fra-2 and FosB. Computer search indicates that certain motifs shared by the Fos proteins and putatively responsible for instability are found in no other protein, suggesting the existence of degradation mechanisms specific for this protein family. Under particular signalling conditions, others have shown that a part of cytoplasmic c-Fos requires ubiquitination for fast turnover. This poses the question of the multiplicity of degradation pathways that apply to proteins depending on their intracellular localization.

Introduction

The AP-1 (activator protein-1) complex is a family of dimeric transcription factors binding to DNA motifs {AP-1/TRE [PMA ('TPA')-responsive element]} found in many genes [1]. It is involved in the regulation of a flurry of processes both at the cellular and at the organism levels. The best-documented events are proliferation, differentiation, apoptosis and responses to stresses. AP-1 is also involved in organogenesis, the control of the immune response and that of memory formation and cognitive functions [2–7] as well as in various pathologies, notably cancer [4,8–11]. Indeed, certain of its components are oncogenes and/or tumour suppressors, depending on the context [4,8–11].

The best-known AP-1 components are the members of the Fos family, namely c-Fos, Fra-1, Fra-2, FosB and ΔFosB (the latter two proteins being generated by alternative splicing) and those of the Jun family (c-Jun, JunB and JunD) [1,5]. All AP-1 proteins share two adjacent, highly conserved domains: the basic DBD (DNA-binding domain) and the LZ (leucine zipper) mediating dimerization. Together, the DBD and the LZ constitute the bZip region. Fos proteins must heterodimerize with other AP-1 components to acquire transcriptional competence. In contrast, Jun proteins can also function as homodimers, even though heterodimerization with Fos partners is favoured. AP-1 recognizes the so-called AP-1/TRE or the related CRE (cAMP-response element) DNA motifs found in many genes. It can act either positively or negatively on transcription depending on its composition, the target gene, the cell context and the environmental cues [1]. The *fos* family genes are exquisitely regulated at multiple interwoven transcriptional and post-transcriptional levels. This limits, or prevents, the pathological manifestations that would otherwise result from dysregulation of just one of these controls.

c-Fos and Fra-1: physiopathological importance

c-Fos is the founder of the Fos family. It has been discovered in mutated and oncogenic forms in mouse osteosarcomatogenic retroviruses [12]. The other proteins have been identified by homology with c-Fos [12]. c-Fos (380 amino acids) and Fra-1 (271 amino acids) are expressed constitutively in a limited number of tissues where they exert diverse functions, some of them not or ill-identified [4]. Neither the c-Fos nor the Fra-1 transcriptome has been investigated extensively despite the fact that this would invaluably help in understand the multiple facets of their functions. c-*fos* and *fra-1* genes are best characterized as IEGs (immediate early genes) as they are rapidly induced, i.e. within 15 min and 2 h respectively in many cell types by numerous extracellular signals. This is necessary for transforming short-term stimuli, such as addition of growth factors, into long-term responses, such as cell proliferation. However, c-Fos and Fra-1 show different

Key words: activator protein-1 (AP-1), c-Fos, Fra-1, proteasome, protein degradation, ubiquitin.

Abbreviations used: AP, activator protein; DBD, DNA-binding domain; ERK, extracellular-signal-regulated kinase; LZ, leucine zipper; MAPK, mitogen-activated protein kinase; RSK, p90 ribosomal S6 kinase; TRE, PMA ('TPA')-responsive element.

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expression profiles. For example, on mitogen stimulation of quiescent cells, c-Fos accumulates transiently, disappearing within a few hours, whereas Fra-1 appears later and persists well beyond the G_1-phase. It is worth noting that (i) variations in abundances are not limited to c-Fos and Fra-1 in cells re-entering the cell cycle, but also concern other AP-1 family proteins, which are responsible for continuous and dynamic changes in AP-1 dimer composition [13–15] and (ii) c-Fos participates in transcriptional activation of *fra-1* [10,16].

Expression of c-Fos and Fra-1 is altered in many tumours [8,10]. c-Fos can participate in tumorigenesis either as an oncogene, as demonstrated in *in vitro* transformation assays and by bone tumour formation in transgenic mice, or as a necessary mediator of upstream oncogenic events as (i) shown using mouse skin tumour models [17] and (ii) suggested by its overexpression in a number of human tumours [4,8,11]. Importantly, c-Fos can also exert oncosuppressive actions depending on the cell context [18,19]. Fra-1 does not transform on its own [4,11]. However, it is associated with tumour progression, where it can contribute to cell survival [20], proliferation [21], invasiveness [21] and protection against apoptosis-inducing mitotic catastrophes [22]. Finally, c-Fos being an important regulator of bone formation, inflammation and immune responses, the mechanisms controlling its accumulation and activity may be exploited to develop new drugs for treating bone mass diseases, inflammatory bone and joint illnesses and modulating immunity.

ERK (extracellular-signal-regulated kinase) MAPKs (mitogen-activated protein kinases) control c-Fos and Fra-1 abundances and activities

c-*fos* and *fra-1* gene transcriptions are activated by various MAPK pathways. Their protein products are also targeted by ERK1/2 [10,23] (Figure 1) and ERK5 [24] cascades. Both ERK1/2 and their effector kinases RSK (p90 ribosomal S6 kinase) 1 and RSK2 phosphorylate c-Fos [25–32], which alters both its degradation rate [27,29,32,33] and its transcriptional activity [30–32]. Similarly, activation of the ERK5 cascade stabilizes and enhances the transcriptional activities of c-Fos and Fra-1 [24]. Finally, the pattern of AP-1 proteins, including Fra-1, is perturbed in Ras-, Raf- and Mek1-transformed cells where ERK1/2 play key roles in the expression and phosphorylation of several of them [34–36].

c-Fos protein degradation

In vivo c-Fos degradation has mostly been studied in two experimental systems where the protein is mostly nuclear. One is constitutive expression during asynchronous growth. The other is transient induction during the G_0/G_1 transition after mitogen stimulation of quiescent cells. In both situations, c-Fos is unstable, with a half-life in the hour range. We showed that the bulk of the protein is degraded by the proteasome [33,37–39] independently of any prior ubiquitination [38], despite the fact that a fraction of c-Fos can undergo ubiquitination *in vivo* in certain circumstances [38]. This is unusual, as most substrates require polyubiquitination for proteasomal degradation [40]. In line with this observation and the fact that c-Fos is not detectably ubiquitinated in serum-stimulated cells [38], Sasaki et al. [41] have reported lack of ubiquitination of unstable, nuclear c-Fos induced by PMA. However, in the same work, they also showed that c-Fos, when retained in the cytoplasm upon activation of the STAT3 (signal transducer and activator of transcription-3) pathway in the presence of pharmacologically inactivated ERK5, undergoes ubiquitination-dependent, proteasome-dependent degradation [41]. This suggests that alternative pathways may contribute to c-Fos degradation in different subcellular compartments. They may also explain why we originally found c-Fos more unstable in the cytoplasm than in the nucleus [42].

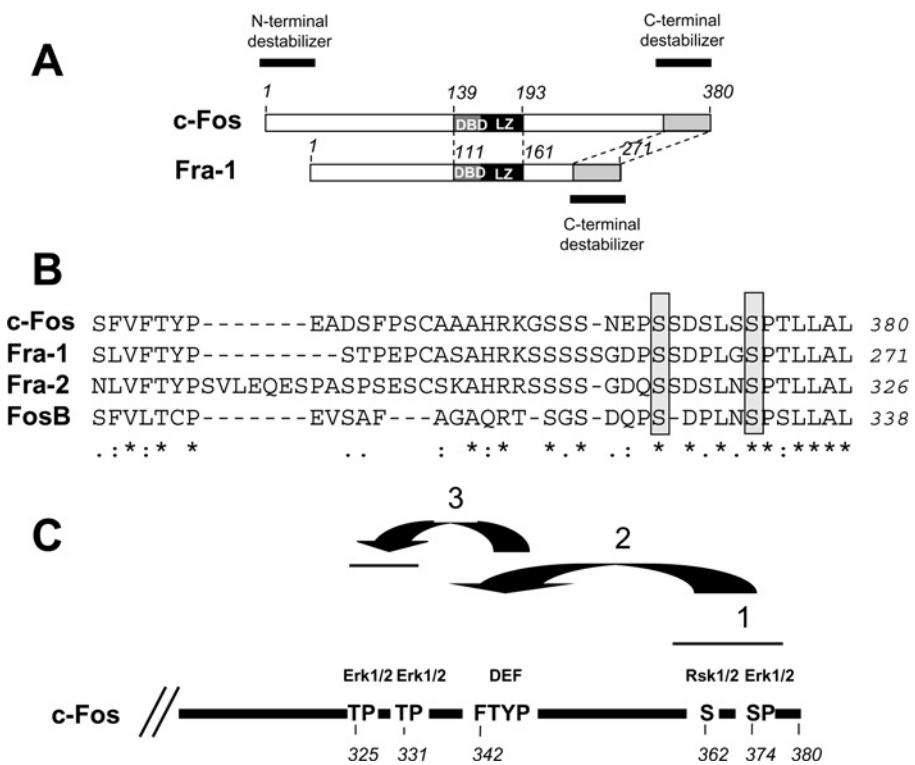
Interestingly, c-Fos breakdown is controlled by differentially regulated autonomous destabilizers, located at its two extremities [33,38]. A C-terminal element is functional in c-Fos during both asynchronous growth and the G_0/G_1 transition, whereas an N-terminal destabilizer is active only in G_0/G_1 [33,38]. Moreover, the cytoplasmic degradation of c-Fos described by Sasaki et al. [41] depends on a single destabilizer co-localizing with the N-terminal one active in G_0/G_1 cells. Further work will establish whether the c-Fos N-terminal region contains a single or two distinct destabilizing elements. Importantly, the activity of the C-terminal destabilizer is reduced on phosphorylation of two C-terminal serine residues (Ser-362 and Ser-374) by ERK1/2 and RSK1/2 [33,38]. As a consequence, this domain is less active in G_0/G_1 cells, where the ERK1/2 pathway is strongly activated, than in asynchronously growing cells, where ERK1/2 are weakly active [23]. c-Fos turnover is, however, maintained in G_0/G_1 by functional activation of the N-terminal destabilizer [33,38]. Finally, expression of Ras, Mos and Raf oncogenes, which activate the ERK1/2 pathway [23], also inhibit c-Fos C-terminal destabilizer activity via direct ERK1/2-dependent phosphorylation in proliferating transformed cells (Figure 1).

Degradation of Fra-1

We have combined genetic, pharmacological and signalling approaches to study Fra-1 turnover in non-transformed and cancerous cells [43]. Clearly, Fra-1 is intrinsically unstable under conditions of low ERK1/2 or low ERK5 activity. In these situations, degradation depends on a single destabilizer contained within the C-terminal 30–40 amino acids, i.e. a region highly conserved between all Fos proteins (Figure 1) and previously demonstrated to contain one of the two c-Fos destabilizers. This strongly suggests that the mechanisms whereby the various Fos proteins undergo proteasomal destruction are similar, if not identical. Supporting this possibility, we also showed that Fra-1, like c-Fos, belongs to a small group of proteins that may, at least under the conditions

Figure 1 | Degradation of c-Fos and Fra-1

(**A**) Structures of c-Fos and Fra-1 and positions of the identified destabilizers. The grey boxes at the C-termini of c-Fos and Fra-1 indicate the second region of high homology between the two proteins after the central DBD-LZ region (95 % homology). (**B**) Comparison of Fos protein C-terminal sequences. The most C-terminal 40 amino acids constitute the second region of high homology between the Fos proteins as indicated in the lower panel of (**A**). The symbols '*', ':' and '.' indicate identical, conserved and semi-conserved amino acids respectively. The numbers indicate amino acid positions. The grey boxes indicate the serine residues that are phosphorylated by the kinases of the ERK1/2 pathway in c-Fos and Fra-1 and that are conserved in the other Fos proteins. (**C**) Phosphorylation of c-Fos C-terminal domains. On activation of the MAPK pathway, Ser-374 is phosphorylated by ERK1/2 and Ser-362 is phosphorylated by RSK1/2, the latter kinases being activated by ERK1/2. If stimulation of the MAPK pathway is sufficiently sustained (**1**), ERK1/2 can dock on an upstream FTYP amino acid motif, called the DEF domain (docking site for ERKs, FXFP) (**2**), and phosphorylate Thr-331 and Thr-325 (**3**). Phosphorylation of Ser-362 and Ser-374 stabilizes c-Fos but has no demonstrated role in the control of transcriptional activity. On the contrary, phosphorylation of Thr-325 and Thr-331 enhances c-Fos transcriptional activity but has no demonstrated effect on protein turnover. The two serine residues (Ser-252 and Ser-265 respectively) and the two threonine residues, as well as the DEF domain, are conserved in Fra-1. The two indicated serine residues are phosphorylated by kinases of the ERK1/2 pathway and their phosphorylation stabilizes Fra-1. Phosphorylation of the two threonine residues does not stabilize Fra-1.



studied, undergo ubiquitin-independent degradation by the proteasome.

Work by several laboratories has pointed to Fra-1 stabilization upon ERK1/2 pathway activation. Thus, in the case of high ERK1/2 pathway activity resulting from either physiological stimulation by mitogens [25,44] or from oncogenic activation of upstream effectors in thyroid [22,45], colon [20] and breast [21] tumours, Fra-1 accumulates to high levels and shows a characteristic diffuse and retarded electrophoretic mobility due to phosphorylation at multiple, unmapped sites. By contrast, Fra-1 shows reduced phosphorylation and destabilization when ERK1/2 activity is reduced, as in control non-transformed thyroid cells [45], in cells treated with a pharmacological inhibitor of MEK1 (the kinase activating ERK1/2 by phosphorylation) [20] or upon mitogen withdrawal [25,44]. No causal link between ERK1/2 activity, phosphorylation of Fra-1 and protein stabilization had been demonstrated until we showed that two serine residues, Ser-252 and Ser-265 (the homologues of c-Fos Ser-362 and Ser-374), within the C-terminal destabilizer are phosphorylated by ERK1/2 pathway kinases (Figure 1). These phosphorylations compromise proteolysis both upon normal physiological induction during the G_0/G_0–S transition (conditions of high ERK1/2 activity) and in colon tumour cells showing high ERK1/2 activity triggered by Ras and B-Raf oncogenes. Finally, despite similarities, there are differences between Fra-1 and c-Fos degradations. In particular, the presence of a single destabilizer within Fra-1,

instead of two that are differentially regulated in c-Fos, explains the much faster turnover of the latter when cells traverse the G_0/G_0–S transition.

c-Fos and Fra-1 degradation: the next steps

Our work raises a number of questions.

Can several catabolic pathways target the same protein?

The answer to this question is yes for at least two reasons. First, depending on its localization, c-Fos protein degradation is dependent on or independent of prior ubiquitination. Thus our results show that proteolysis of the bulk of c-Fos, when this protein is predominantly nuclear, is independent of prior ubiquitination. This has been confirmed by Sasaki et al. [41]. However, these authors have also reported that a fraction of c-Fos is subjected to accelerated ubiquitin-dependent degradation involving the UBR1 E3 ligase, when the protein is cytoplasmic under particular signalling conditions [41]. The fact that ubiquitin-dependent degradation of c-Fos is possible raises the possibility that, under conditions where the protein is not ubiquitinated itself, polyubiquitin chains may be brought in *trans* by a ubiquitinated protein partner. Should this occur, such an interactor could not be an LZ dimerization partner since (i) LZ-deficient mutants of c-Fos and Fra-1 are as unstable as the wild-type proteins and (ii) EGFP (enhanced green fluorescent protein) is destabilized by the C-terminal 40 amino acids of c-Fos and Fra-1 not containing the LZ. Secondly, we have shown that c-Fos degradation in G_0/G_0 cells involves one destabilizer at each extremity functioning independently and additively [33]. As there is no structural homology between them and because they obey different control mechanisms, it is likely that how c-Fos is addressed to the proteasome is different when this involves its N- or C-terminus. Considering that the two destabilizers function independently, it is plausible that c-Fos is dragged to the proteasome via one extremity or the other but not by the two at the same time.

What about the other Fos proteins?

That the two destabilizers of c-Fos function differently does not exclude that there may be strong similarities in the mechanisms accounting for proteasomal degradation of the different Fos. As mentioned above, the 30–40 amino acids at the C-terminus, which contain one of the c-Fos destabilizers and the sole destabilizer of Fra-1, are highly conserved in Fra-2 and FosB. Work is therefore under way to investigate whether this region is destabilizing in all four Fos proteins with equal efficiency and whether Fra-2 and FosB destabilizers are also inhibited by ERK1/2 pathway-driven phosphorylation of C-terminal serine residues. It is also worth noting that the N-terminal regions of c-Fos, Fra-2 and FosB share several homology segments that are not found in Fra-1. It will consequently be important to address whether (i) Fra-2 and FosB carry another N-terminally located destabilizer as c-Fos and (ii) the dissimilarity between the N-terminal regions of c-Fos and Fra-1 explains why c-Fos, and not Fra-1, is degraded in a UBR1- and ubiquitin-dependent manner. Finally, it will be important to establish whether ubiquitination is dispensable, or not, for Fra-2 and FosB degradations and whether C-terminal phosphorylations may inhibit their degradations as for c-Fos and Fra-1.

How are proteins whose degradations are independent of prior ubiquitination recognized and degraded by the proteasome?

NMR studies have shown that the C-terminal half of c-Fos is essentially unstructured [46]. c-Fos and Fra-1 primary structures being closely related in the C-terminal region and no particular motif or structure being identifiable in bioinformatics analyses, the Fra-1 C-terminal domain is most probably also unstructured. This observation is important as ubiquitination-independent proteasomal degradation is believed to be primed by poorly structured polypeptide domains possibly exposing hydrophobic segments [47].

How can lack of structure favour Fos protein proteasomal degradation? Adaptor proteins have already been described for recruitment and delivery of ubiquitinated proteins to the proteasome [40]. A first possibility might be the involvement of comparable adaptors, recognizing loosely structured and/or hydrophobic peptide motifs. Asking whether chaperone-type structures may play this role may be rewarding. Another possibility is direct recognition by the proteasome as proposed for most proteins processed independently of prior ubiquitination. As there are several types of proteasomal complexes coexisting in any eukaryotic cells [48], this, in turn, poses the question of what is, or what are, the proteasomal complex(es) responsible for Fos protein degradation (see [43] for a detailed discussion)? Biochemical studies involving various proteasomal complexes are under way to identify which of them is (are) responsible for Fos protein hydrolysis, as well as whether recognition occurs directly via its C-terminus or indirectly through a peptidic adaptor.

Could ubiquitination of Fos proteins serve purposes that are not related to degradation?

It is worth noting that a fraction of c-Fos and Fra-1 can be found ubiquitinated in an transient transfection ubiquitination assay. Therefore it is possible that a small fraction of these proteins undergoes ubiquitination-dependent degradation in our experiments or that this ubiquitination corresponds to unspecific background of modification. Interestingly, Hoffmann et al. [49] have shown that ubiquitination of transfected Fra-1 is stimulated by activation of the ERK1/2 pathway, i.e. when the protein is stabilized. As ubiquitination is involved in the control of various protein functions independent of proteasomal proteolysis [50,51], it is possible that ubiquitination of Fra-1 may serve to modulate transcriptional activity. This is all the more interesting because activity has formerly been shown to be stimulatable by the ERK1/2 pathway [52]. Whatever the case, c-Fos and Fra-1 belong to the growing list of proteins that can be degraded predominantly by the proteasome independently of any ubiquitination (see [53] for a review).

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