Selective uptake and degradation of c-Fos and v-Fos by rat liver lysosomes

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Abstract The transcription factor c-Fos is a short-lived protein and calpains and ubiquitin-dependent systems have been proposed to be involved in its degradation. In this report, we consider a lysosomal degradation pathway for c-Fos. Using a cell-free assay, we have found that freshly isolated lysosomes can take up and degrade c-Fos with high efficiency. v-Fos, the oncogenic counterpart of c-Fos, can also be taken up by lysosomes, yet the amount of incorporated protein is much lower. c-Fos uptake is independent of its phosphorylation state but it appears to be regulated by dimerization with differentially phosphorylated forms of c-Jun, while v-Fos escapes this regulation. Moreover, we show that c-Fos is immunologically detected in lysosomes isolated from the liver of rats treated with the protease inhibitor leupeptin. Altogether, these results suggest that lysosomes can also participate in the selective degradation of c-Fos in rat liver.

Key words: Lysosome; Protein degradation; c-Fos; v-Fos; c-Jun

1. Introduction

A wide variety of extracellular signals which are implicated in cellular growth control and differentiation, rapidly and transiently induce the transcription of target genes, termed early response genes, via changes in the levels of second messengers [1]. It has been demonstrated that some of these immediate early genes belong to the group of so-called proto-oncogenes, mutated forms of which are involved in cellular transformation [2–5]. The protein products of some of these genes are transcription factors which link external stimuli to the expression of target late genes [4,6]. Although the number of early genes encoding transcription factors identified so far is limited, their regulatory effects on gene expression are pleiotropic and vary greatly between cell types and stimuli. Transcription factors encoded by immediate early genes have been found to share some common features such as: (1) a rapid and often transient induction elicited by physiological or pharmacological mitogens [6–8]; (2) short half-lives of messenger RNAs [9,10]; and (3) short half-lives (20–90 min) of their protein products [11,12].

Among the early response genes, c-*fos* and c-*jun* are members of two families of genes which encode proteins belonging to the AP-1 transcription complex. This complex is thought to mediate transcription through TREs (for TPA Responsive Elements) in response to activation of the protein kinase C intracellular signal transduction pathway [6,7]. Fos and Jun proteins associate to form homo- (Jun-Jun) or heterodimers (Jun-Fos) in all conceivable combinations, except Fos homodimers, which bind to the AP-1 sites of target genes and modulate their transcription [6,13,14].

The regulation of c-*fos* gene expression and its product is complex and involves transcriptional, post-transcriptional and post-translational mechanisms. At the level of transcription, both initiation and elongation are regulated by different signal transduction pathways, including protein kinases C and A routes and rises in intracellular Ca^{2+} concentration [13–17]. Post-transcriptional regulation of c-*fos* occurs via instability of the mRNA through the presence of AU-rich destabilizing sequences in the 3′-untranslated region [18]. At the post-translational level, the activity of the protein is modulated by changes in the pattern of phosphorylation, oxidation of specific amino acid residues or dimerization with Jun proteins or other transcription factors [6,19,20].

While the mechanisms regulating gene transcription and protein functions have been extensively studied, comparatively little is known about the intracellular degradation mechanisms of these proteins. These mechanisms may play an important role by controlling the intracellular levels of the protein and therefore its biological activity, thus representing another level of post-translational regulation [21,22]. In this respect, a series of previous observations suggest that at least two proteolytic pathways may be involved in c-Fos degradation: calpains [23–25] and ubiquitin-dependent systems [26–28]. However, these systems were not able to complete the degradation of c-Fos monomers, suggesting that additional pathways may also participate in c-Fos degradation. Furthermore, none of the reported systems was able to degrade v-Fos (the oncogenic counterpart of c-Fos). Therefore, we examined in this paper if lysosomes, a well-recognized pathway for protein degradation, could also participate in these degradative processes.

2. Materials and methods

2.1. General

c-Fos antibodies were obtained from Santa Cruz Biotechnology. Western blot analyses were carried out using alkaline phosphatase conjugated goat anti-rabbit IgG as secondary antibody.

The radioactivity associated with the c- and v-Fos proteins in the autoradiograms was quantified by phosphorimager analysis using a GS-250 Molecular Imager system (Bio-Rad). Statistical analyses were carried out with Student's *t*-test.

2.2. Fos and Jun proteins

As a source of c-Fos and v-Fos E300 proteins, a combined in vitro

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAP, potato acid phosphatase; TPA, phorbol 12-myristate 13-acetate; CQ, chloroquine; TREs, TPA responsive elements

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transcription-translation system was used. A plasmid that contained a rat cDNA encoding the wild-type c-Fos [29] or pTZE300 encoding v-Fos [30] was transcribed (after linearization with *Hin*dIII), essentially as suggested by the supplier, with either SP6 (c-Fos) or T7 (v-Fos) RNA polymerases (Promega). The resulting mRNAs (from 6 μg of template DNA) were used in a translation reaction (50 μl) that contained nuclease-treated rabbit reticulocyte lysate (40 μl; Promega) in the presence of 6 μl of L-[^{35}S]methionine (>1000 Ci/mmol; Amersham). The in vitro translation reaction was then treated with potato acid phosphatase (PAP) for 1 h at 25°C in the presence (mock-treated) or absence (PAP-treated) of a mixture of competitive and non-competitive PAP inhibitors (100 mM NaF, 15 mM Na_2MoO_4, 20 mM (Na_2)-*p*-nitrophenyl phosphate and 10 mM (Na_2)-bis-glycerophosphate), as described [31]. Before the cell-free assay, PAP-treated samples were quenched with PAP inhibitors.

cmv-H_6cJun protein was purified from untreated or TPA-treated transiently transfected HeLa TK$^-$ or 293 cells, as described [31]. Where indicated, aliquots of mock- or PAP-treated c-Jun (5 μl, ≈25 ng protein) were mixed with [^{35}S]c-Fos (5 μl that contained 1 μl of in vitro translated material), mock- or PAP-treated. Reaction mixtures were then incubated at 37°C for 30 min to enhance heterodimerization [31], brought to 30 μl (final volume) and used in the cell-free assay.

2.3. Preparation of subcellular fractions

Lysosomes were obtained from rat liver as described [32]. Briefly, a mitochondrial-lysosomal fraction from liver homogenates was prepared and resuspended in 57% metrizamide (pH 7.0), and 10 ml thereof was placed on the bottom of a discontinuous metrizamide gradient. The gradient was then centrifuged for 2 h in an SW28 rotor (Beckman) at 141 000×*g* and five different fractions were obtained. Lysosomes present in the top layer and in the 19.8–24.5 and 24.5–26.3% interfaces were collected, pooled together and used in the cell-free assay (see below). Mitochondria present in the 26.3–32.8% and 32.8–57% interfaces were also pooled together and used for Western blotting analysis.

Cytosol [32] and nuclei [33] were obtained from the homogenate as described.

In some experiments, rats were treated with leupeptin (2 mg/100 g body weight, intraperitoneally, 1 h before being killed) prior to the preparation of the subcellular fractions.

2.4. Cell-free assay: standard incubation conditions

Freshly isolated lysosomes were resuspended gently in medium S (10 mM Mes·NaOH, pH 7.0, 0.3 M sucrose). Where indicated, lysosomes (~5 mg/ml) were incubated for 10 min at 4°C with 10 mM chloroquine (CQ), before adding the substrate proteins. Lysosomes (10 μl, containing ~50 μg protein) and protein substrates (4 μl of reticulocyte lysate translation mixture) were incubated, with or without 10 mM CQ, in medium S (final volume 30 μl), in the presence of an ATP regenerating system (1 mM ATP, 8 mM creatine phosphate and 40 μg/ml creatine kinase) for 20 min at 37°C. Lysosomes were pelleted by centrifugation at 10 000×*g* for 5 min at 4°C in a Heraeus Biofuge 13, washed once with medium S and analyzed by SDS-PAGE and autoradiography. Where indicated, the incubation was followed by a treatment with trypsin (100 ng, 10 min on ice) and then with soybean trypsin inhibitor (200 ng, 10 min on ice) prior to the centrifugation steps.

3. Results

3.1. c-Fos is taken up in vitro by freshly isolated lysosomes

We have previously used an in vitro assay to monitor the uptake of proteins into rat liver lysosomes [32]. Here, [^{35}S]c-Fos was mixed with freshly prepared rat liver lysosomes, in medium S, in the absence or presence of chloroquine (CQ) (to inhibit intralysosomal proteolysis). After a 20 min incubation at 37°C, lysosomes were pelleted by centrifugation, washed twice in medium S, and analyzed by SDS-PAGE and autoradiography.

The amount of c-Fos found in the lysosomal pellets after the incubation was higher in the presence of CQ (Fig. 1A, compare lanes 2 and 3, 4 and 5), presumably due to the intralysosomal degradation in the absence of CQ. To analyze whether c-Fos was indeed within lysosomes, the incubation was followed by a trypsin treatment, to eliminate the protein which was simply bound to the external surface of the lysosomal membrane. Under these conditions, proteins which entered lysosomes are not sensitive to trypsin, since they are protected by the lysosomal membrane [32]. After trypsin treatment, >50% of c-Fos remained resistant to the enzyme when CQ was present (Fig. 1A, lane 4; see quantification in Table 1). In the absence of CQ, c-Fos was almost completely degraded (Fig. 1A, lane 5). In the presence of Triton X-100, c-Fos was completely degraded by trypsin, whether CQ was present or not (not shown). Thus, c-Fos uptake into lysosomes was very efficient, corresponding to ~55% of the protein added to the assay.

c-Fos expressed in a coupled transcription/translation system is phosphorylated [29,30]. Indeed, the inclusion of [γ-^{32}P]-ATP in the translation mixture yielded labeled protein (data not shown). We tested whether the phosphorylation state of c-Fos affects its incorporation and degradation by rat liver lysosomes. c-Fos was treated with PAP to remove all the accessible phosphate groups. No significant difference was observed in its uptake into lysosomes, as compared with c-Fos treated with inactivated (mock-treated) PAP (Fig. 1A, compare lanes 6–9 with lanes 2–5, and see Table 1). This result indicates that the phosphorylation state of c-Fos is not important for its uptake into lysosomes.

We then examined whether v-Fos was also taken up by lysosomes. As shown in Fig. 1B, v-Fos was incorporated into lysosomes, albeit 2–3 times less efficiently than c-Fos (Table 1). It should be noted that the two bands observed for v-Fos are always generated by in vitro transcription/translation of the v-Fos encoding construct pTZE300 [30,31], most likely the faster migrating species representing leakage in either of these processes.

As is the case for c-Fos, v-Fos synthesized in vitro is also phosphorylated, and its uptake into lysosomes was also independent of its phosphorylation state (Table 1).

3.2. Dimerization with c-Jun prevents c-Fos uptake, unless c-Jun is phosphorylated

c-Fos can heterodimerize with c-Jun through their leucine

Table 1
Binding and uptake of c-Fos and v-Fos by freshly isolated lysosomes

	Binding (%)	Uptake (%)
c-Fos	27.2 ± 5.4	56.7 ± 11.7
c-Fos-P	26.2 ± 4.2	54.7 ± 8.1
v-Fos	9.8 ± 0.8	25.2 ± 1.6
v-Fos-P	7.4 ± 1.4	21.3 ± 4.2

Quantification, using phosphorimager analysis, of the autoradiograms from three different experiments as in Fig. 1. The protein adsorbed to the external surface of the lysosomes (binding: lanes 3–5 or lanes 7–9) and the protein internalized (uptake: lanes 4–5 or lanes 8–9) are shown, expressed as a percentage of the total protein added to the assay (4 times the radioactivity present in lane 1, see legend to Fig. 1) [32,34]. For the quantification of v-Fos, the two observed bands (see Section 3) were taken into account [30,31]. The protein that was associated with lysosomes in the presence of chloroquine (lanes 2 and 6) roughly corresponds to the sum of the surface-bound and internalized proteins (data not shown, and [32,34]).

zippers to form the AP-1 transcription factor. The transcriptional activity and DNA binding properties of the heterodimer are regulated by signal-dependent phosphorylation and dephosphorylation events [19]. We therefore tested the influence of dimerization with c-Jun and phosphorylation in the uptake and degradation of c-Fos by freshly isolated rat liver lysosomes.

When c-Fos was dimerized with cmv-H_6cJun (an artificial derivative of c-Jun containing an hexahistidine sequence at its amino terminus to allow rapid purification from transfected cells while preserving its phosphorylation state [31]), no significant effect was seen on c-Fos lysosomal uptake (Fig. 2A, compare lanes 1–2 and 5–6; see quantification in Fig. 2B). However, when c-Fos was dimerized with cmv-H_6cJun treated with PAP to remove all accessible phosphate groups, uptake was significantly decreased (Fig. 2A, compare lanes 1–2 and 3–4). This suggests that the phosphorylation state of c-Jun may play a role in c-Fos uptake and degradation by liver lysosomes.

Moreover, c-Fos was dimerized with hypophosphorylated cmv-H_6cJun protein (purified from TPA-treated cells). Then, the uptake of c-Fos into lysosomes was significantly decreased (Fig. 2A, compare lanes 1–2 and 7–8). Altogether, these results imply that the phosphorylation state of c-Jun partially controls the incorporation and subsequent degradation of c-Fos by intact lysosomes.

3.3. Dimerization with c-Jun does not affect v-Fos uptake

We also investigated whether v-Fos uptake was modified upon dimerization with c-Jun, as was the case for c-Fos. As shown in Fig. 3, v-Fos uptake was not affected upon dimerization with cmv-H_6cJun, regardless of the phosphorylation state of the latter (see Fig. 3A and quantification in Fig. 3B). Therefore, v-Fos seems to escape the regulation mechanism observed for c-Fos.

3.4. Immunological detection of c-Fos in liver lysosomes

To test whether lysosomes could be involved in the degradation of c-Fos in vivo, we investigated if c-Fos was present in freshly isolated rat liver lysosomes. Rat liver fractions [32] were analyzed by SDS-PAGE followed by Western blotting with a c-Fos antibody (Fig. 4). c-Fos (62 kDa, lane 6) was detected in whole homogenates (Fig. 4, lane 1) and mainly distributed in the nuclei (Fig. 4, lane 2), where the protein exerts its function, and in the cytosol (Fig. 4, lane 3), where the protein is synthesized to be subsequently incorporated into the nucleus. c-Fos was not detected in mitochondria (Fig. 4, lane 4), as expected, but it was present in lysosomes of rats treated with leupeptin prior to being killed (Fig. 4A, lane 5). Under these conditions, lysosomal proteases are inhibited, providing a means to detect proteins within lysosomes which otherwise would be rapidly degraded [32,35]. No detectable signal was seen in lysosomes isolated from control rats, i.e. when the lysosomal proteases were not inhibited by leupeptin injection (Fig. 4B, lane 5), whereas other subcellular fractions contained similar amounts of c-Fos (i.e. nuclei or cytosol, compare lanes 2 and 3 in Fig. 4A,B). Furthermore, the amounts of c-Fos associated with lysosomal fractions were, at least partially, resistant to trypsin treatment. Addition of Triton X-100 to solubilize lysosomal membranes completely abolished this resistance, indicating that c-Fos was located inside the lysosomes (Fig. 4C). This suggests that in rat liver c-Fos may be degraded, at least in part, by a lysosomal mechanism.

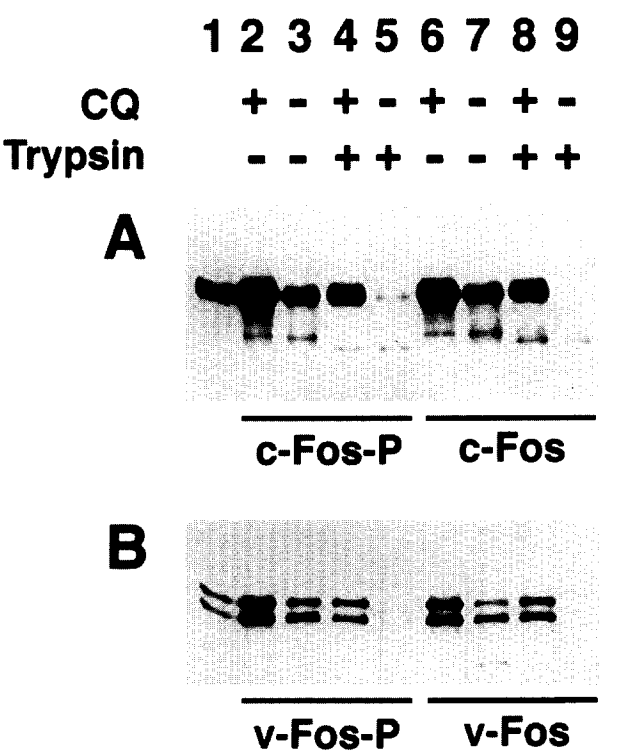

Fig. 1. c-Fos and v-Fos are taken up in vitro by freshly isolated lysosomes. A: A synthetic RNA encoding rat c-Fos protein was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The in vitro translation reaction was then treated with PAP in the presence (mock-treated) or in the absence (PAP-treated) of a cocktail of PAP inhibitors. Lysosomes, prepared as described in Section 2, were resuspended in freshly prepared medium S and incubated with (+) or without (−) 10 mM chloroquine (CQ) for 10 min on ice. Lysosomes (50 μg) were then incubated with c-Fos (4 μl of the reticulocyte lysate translation mixture), PAP-treated (c-Fos, lanes 6–9) or mock-treated (c-Fos-P, lanes 2–5), under the standard incubation conditions, in the presence (lanes 2, 4, 6 and 8) or absence (lanes 3, 5, 7 and 9) of CQ. Where indicated (lanes 4, 5, 8 and 9), the samples were treated with trypsin as described in Section 2. Finally, lysosomes were pelleted by centrifugation, washed in medium S, and analyzed by SDS-PAGE and autoradiography. B: The experiment was as in A, but using v-Fos (4 μl of reticulocyte lysate translation mixture), PAP-treated (v-Fos, lanes 6–9) or mock-treated (v-Fos-P, lanes 2–5), instead of c-Fos. Lane 1 contains 1 μl of the reticulocyte lysate translation mixture, containing c-Fos (A) or v-Fos (B).

4. Discussion

At least two different proteolytic pathways have been proposed to be involved in c-Fos degradation: ubiquitin/proteasomes [26–28] and calpains [23–25]. These studies have been focused on non-lysosomal pathways, because they have been mostly implicated in the degradation of short-lived proteins [36]. However, since it is possible that more than one pathway may be necessary to complete c-Fos degradation [24] and lysosomes are also a major site of intracellular protein degradation [37–39], we investigated if these organelles could also play a role in the degradation of c-Fos.

Indeed, uptake of c-Fos by freshly isolated lysosomes in a cell-free assay was very efficient (55% of the synthesized pro-

tein) and thus compatible with the short half-life exhibited by the protein [11,12]. Also, the detection of c-Fos in liver lysosomes isolated from leupeptin-treated rats is consistent with a lysosomal mechanism being involved in the degradation of c-Fos in rat liver.

We have studied if dimerization of c-Fos with c-Jun can affect the degradation of the protein in the lysosomal cell-free system. Our results clearly show that the lysosomal uptake of c-Fos for degradation depends on the c-Jun phosphorylation state: non-phosphorylated forms (PAP-treated) of c-Jun partially impair c-Fos degradation. This conclusion is further supported by the intermediate effect on c-Fos degradation of dimerization with hypophosphorylated forms (purified from TPA-treated cells) of c-Jun. In contrast, dimerization with phosphorylated or non-(hypo-)phosphorylated c-Jun did not change the rate of v-Fos lysosomal uptake.

Similar effects were observed for the degradation of c-Fos in a reticulocyte lysate [31] and the proteolytic pathway involved was recently identified as that comprising ubiquitin/proteasomes [27,28]. This system, which in contrast to lysosomes cannot degrade c-Fos monomers to any significant extent, can degrade c-Fos dimerized with phosphorylated c-Jun [27,28,31]. Whether dimerization also affects degradation of c-Fos by calpains remains to be investigated. However, calpains are unable to complete c-Fos degradation (i.e. degradation intermediates accumulate) and, as discussed by the authors [24], the calcium levels used to degrade c-Fos by calpains are high above the normal calcium levels found within cells

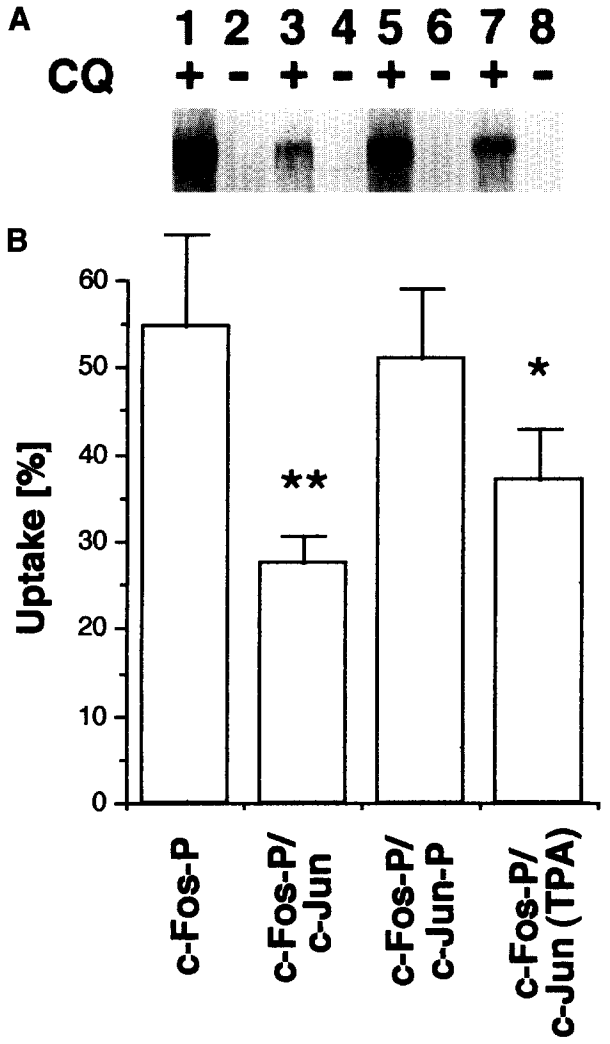

Fig. 2. Dimerization with c-Jun prevents c-Fos uptake by freshly isolated lysosomes, unless c-Jun is phosphorylated. A: The cmv-H_6cJun protein (lanes 3–6, ~50 ng) purified from transiently transfected HeLa TK$^-$ cells, PAP-treated (c-Jun, lanes 3–4) or mock-treated (c-Jun-P, lanes 5–6), or the cmv-H_6cJun protein purified from TPA-stimulated transiently transfected 293 cells [c-Jun (TPA), lanes 7–8] were mixed with [^{35}S]c-Fos (mock-treated, c-Fos-P). In lanes 1–2, c-Fos was not mixed with any Jun protein. Reaction mixtures were then incubated at 37°C for 30 min to enhance heterodimerization and then incubated with freshly prepared lysosomes, in the absence (lanes 2, 4, 6 and 8) or presence (lanes 1, 3, 5 and 7) of 10 mM CQ, as in Fig. 1. After the incubation, the samples were treated with trypsin, as described in Section 2. Finally, lysosomes were pelleted by centrifugation, washed in medium S and analyzed by SDS-PAGE and autoradiography. The figure depicts a typical gel. B: Quantification, using phosphorimager analysis, of the autoradiograms from three different experiments as in A. Each bar is the result of subtracting the radioactivity of the bands in the presence and absence of CQ; i.e. c-Fos-P, lanes 1 and 2, c-Fos-P/c-Jun, lanes 3 and 4, c-Fos-P/c-Jun-P, lanes 5 and 6, and c-Fos-P/c-Jun (TPA), lanes 7 and 8. Results are means ± S.D. Statistical significance with respect to c-Fos-P values: $*P < 0.025$ and $**P < 0.0025$.

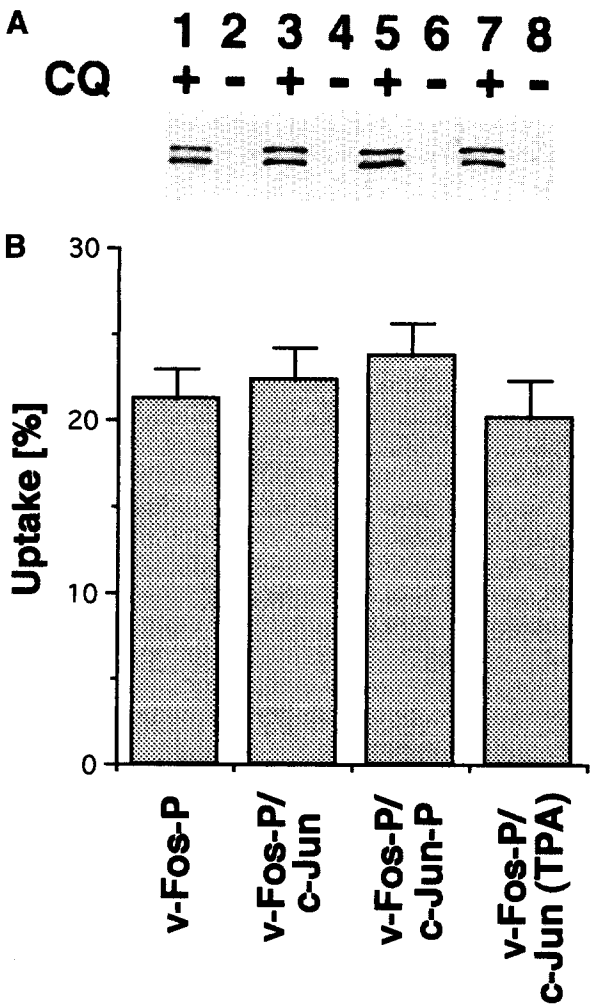

Fig. 3. Dimerization with c-Jun does not affect v-Fos uptake by freshly isolated lysosomes. The experiment was performed exactly as in Fig. 2, except that v-Fos protein was used instead of c-Fos. A: Typical gel from such an experiment. B: Quantification of the autoradiograms from three different experiments as in A.

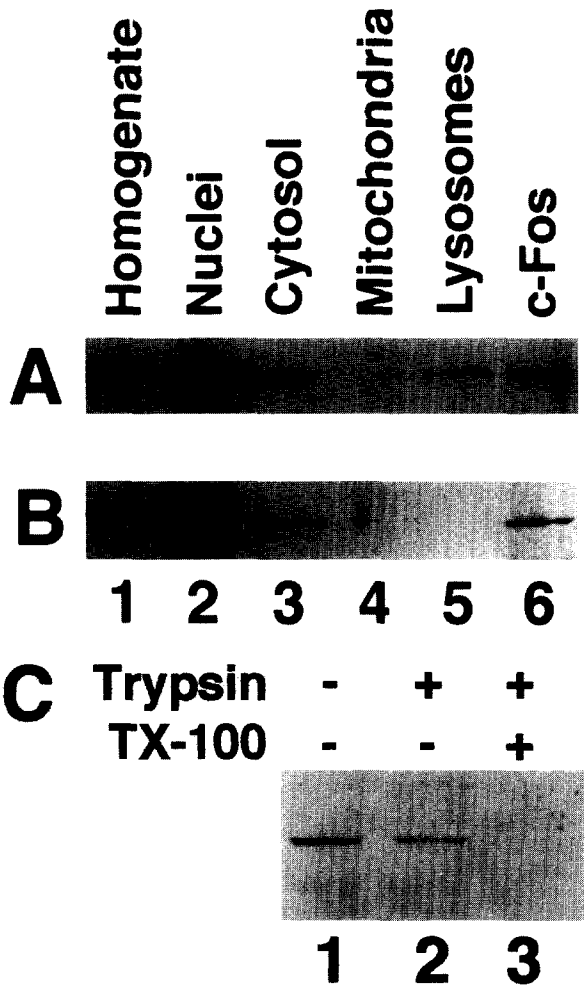

Fig. 4. Immunolocalization of c-Fos in rat liver fractions. Rats were treated (A) or not (B) with leupeptin (2 mg/100 g weight, intraperitoneally, 1 h before being killed) and liver fractions were prepared as described in Section 2. Proteins (15 μg) from the various fractions were separated by SDS-PAGE and immunoblotted with a polyclonal antibody against c-Fos and alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody. Lane 1, homogenate; lane 2, nuclei; lane 3, cytosol; lane 4, mitochondria; lane 5, lysosomes; lane 6, 1 μl of the reticulocyte lysate translation mixture containing in vitro synthesized c-Fos. C: Lysosomes (30 μg of protein) prepared as described in Section 2, from rats treated with leupeptin (as in A), were incubated with trypsin (0.5 μg), in the absence (lane 2) or presence (lane 3) of 1% Triton X-100 before SDS-PAGE and immunoblotting with the c-Fos antibody.

[40]. At any rate, it appears that the mechanism for destabilization of c-Fos is similarly recognized by both non-lysosomal (ubiquitin/proteasomes) [27,28,31] and lysosomal (this paper) degradative pathways. This probably increases the efficiency for selectively removing short-lived proteins and provides another example of the universality of signals for protein degradation, already exemplified by the ubiquitin targeting of proteins to non-lysosomal [41,42] and lysosomal degradative pathways [43,44].

In contrast to non-lysosomal pathways, lysosomes can also degrade v-Fos, albeit with lower efficiency than c-Fos (Table 1). This difference, together with the resistance of v-Fos to degradation by non-lysosomal proteolytic pathways [27,31], is likely to contribute to v-Fos oncogenicity. In this respect, the major change when comparing c-Fos to v-Fos sequences is the lack of 48 amino acid residues in the v-Fos C-terminus. Interestingly, the lysosomal efficiency in incorporating v-Fos is quite similar to that obtained when c-Fos heterodimerizes with non-phosphorylated forms (PAP-treated) of c-Jun. This suggests that two different lysosomal uptake mechanisms coexist in the cell-free assay of lysosomal degradation: non-selective (most probably, non-specific microautophagy [22]) and selective (either direct protein transfer across the lysosomal membrane [45] or specific microautophagy). The first mechanism would contribute to a basal degradation of all proteins, including v-Fos and the active form of AP-1, whereas the second mechanism would selectively recognize some still unidentified specific signals in the transcriptionally inactive c-Fos monomers and Fos-Jun heterodimers and degrade them. It should be noted that heterodimers of c-Fos and phosphorylated c-Jun are unable to bind TRE motifs, contrary to heterodimers formed between c-Fos and c-Jun isolated from TPA-treated cells (A.G. Papavassiliou, unpublished data). The signals which modulate c-Fos degradation, such as heterodimerization with different phosphorylated forms of c-Jun, the exact sequence(s) in the C-terminal region missing in v-Fos, or others need to be characterized more precisely.

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