Decreased susceptibility to calpains of v-Fos^{FBR} but not of v-Fos^{FBJ} or v-Jun^{ASV17} retroviral proteins compared with their cellular counterparts

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The c-Fos and c-Jun transcription factors are rapidly turned over *in vivo*. One of the multiple pathways responsible for their breakdown is probably initiated by calpains, which are cytoplasmic calcium-dependent cysteine proteases. The *c-fos* gene has been transduced by two murine oncogenic retroviruses called Finkel-Biskis-Jenkins murine sarcoma virus (FBJ-MSV) and Finkel-Biskis-Reilly murine sarcoma virus (FBR-MSV); *c-jun* has been transduced by the chicken avian sarcoma virus 17 (ASV17) retrovirus. Using an *in vitro* degradation assay, we show that the mutated v-Fos^{FBR}, but not v-Fos^{FBJ} or v-Jun^{ASV17}, is resistant to calpains. This property raises the interesting

possibility that decreased sensitivity to calpains might contribute to the tumorigenic potential of FBR-MSV by allowing greater accumulation of the protein that it encodes in infected cells. It has also been demonstrated that resistance to cleavage by calpains does not result from mutations that have accumulated in the Fos moiety of the viral protein but rather from the addition of atypical peptide motifs at its both ends. This observation raises the interesting possibility that homologous regions in viral and cellular Fos either display slightly different conformations or are differentially accessible to interacting proteins.

INTRODUCTION

c-Fos and c-Jun proteins are transcription factors defining multigenic families, which include Fos-B, Fra-1 and Fra-2 in the case of Fos and Jun-B and Jun-D in the case of Jun (reviewed in [1,2]). Although both proteins are expressed constitutively in only a restricted number of tissues, their accumulation can be induced in numerous other cell types by stimuli of varied nature. For this reason, c-Fos has long been considered as a master switch for the transformation of short-term stimuli into long-term responses such as cell proliferation, differentiation and adaptive neuronal response. Gene ablation in mouse has recently called this view into question and supports the notion that redundancy in the Fos family is important because c-Fos-null mice are viable [3,4] and affected only in osteoclastic development [5]. Jun family members, in contrast, seem to substitute for one another to a much lesser extent. For example, *c-jun* gene knock-out leads to embryonic lethality in mouse [6,7], and microinjection of anti-c-Jun antibodies, but not of anti-c-Fos antibodies, in embryo fibroblasts blocks cell cycling [8]. Few target genes have been identified for either protein. It is nevertheless clear that both Fos and Jun exert their function through interaction with various partners. Thus (1) in the context of the AP-1 transcription factor complex, c-Fos family members can heterodimerize with those of the Jun family but they are unable to homodimerize as Jun proteins are; (2) c-Fos can dimerize with other transcription factors such as Nrl1, Maf1 and Fip, whereas Jun can dimerize with members of the CREB/ATF family; and (3) both Fos and Jun can associate with nuclear hormone receptors, the NF-AT transcription complex or myogenic transcription factors [1,2].

Although it is clear that c-Fos [9] and c-Jun [10,11] are very unstable proteins, little is known of the basic mechanisms responsible for their degradation. For example, although it has been clearly demonstrated that c-Fos undergoes rapid proteolysis in the cytoplasm [12], it has not yet been demonstrated whether this also holds true in the nucleus. In other words, one cannot formally exclude the possibility that the short nuclear turnover of

c-Fos that has been documented by numerous groups [2] merely reflects a return of the protein (perhaps in a modified form) into the cytoplasm for immediate breakdown. Another major issue is to determine whether both proteins are destroyed by a unique mechanism or whether several catabolic pathways, the relative contributions of which might vary according to the cell context and/or to the physiological conditions, can operate on them. In fact, several lines of evidence have accumulated supporting the notion that multiple mechanisms might participate in the destruction of c-Fos and c-Jun. On the one hand, it seems that ubiquitinylation might be determining for rapid breakdown *in vivo* because (1) Treir et al. [11] have shown that a fraction of c-Jun is ubiquitinated *in vivo* and that ubiquitinylation of c-Jun is correlated with short turnover in a transient transfection assay with human HeLa cells and (2) Stankovski et al. [13] have reported a 3–4-fold stabilization of c-Fos in a hamster cell line expressing a thermosensitive E1 ubiquitin-activating enzyme when grown at the non-permissive temperature. On the other hand, reports by different groups have shown that c-Fos and c-Jun can be degraded *in vitro* by the three major known intracellular proteolytic machineries. First, c-Jun can be degraded by the proteasome in both a ubiquitin-dependent [14] and a ubiquitin-independent [15] manner in different cell-free assays. Second, c-Fos is rapidly and selectively imported and degraded in purified lysosomes [16]. Thirdly, two lines of evidence support the idea that two abundant cytoplasmic calcium-dependent cysteine proteases called micro- and milli-calpains, according to the calcium concentration necessary for their activation *in vitro* (2–75 and 200–800 μ M for half-maximal activity, respectively) (reviewed in [17]), can initiate degradation of c-Fos and c-Jun: (1) the two proteins have been shown to constitute actual substrates for these proteases *in vitro*, both in purified form and in cytoplasmic extracts [18–22], and (2) specific modulation of calpain activity *in vivo* modifies c-Fos- and c-Jun-dependent AP-1 transcription complex activity in a transient co-transfection assay ([21], and M. Pariat, unpublished work). It must be emphasized that in this situation (1) another proteolytic system

Abbreviations used: FBJ-MSV, Finkel-Biskis-Jenkins murine sarcoma virus; FBR-MSV, Finkel-Biskis-Reilly murine sarcoma virus; ASV17, avian sarcoma virus 17.

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must take over the action of calpains because the latter cleave their substrates only to a limited extent and (2) this pathway concerns only the cytoplasmic degradation of c-Jun and c-Fos because of the restricted intracellular distribution of calpains.

The *c-fos* gene is carried by two murine, Finkel-Biskis-Jenkins murine sarcoma (FBR-MSV) and Finkel-Biskis-Reilly murine sarcoma (FBJ-MSV), and one avian (NK24) retroviruses [2,23], whereas *c-jun* is carried by one avian sarcoma retrovirus (ASV17) [24]. In all situations, mutations that have accumulated during and after the transduction process participate in the increased tumorigenic potential of transduced genes (see [2,25] and the Discussion section). Notably, the nuclear turnover of viral Fos and Jun proteins are significantly decreased [11], thus allowing higher accumulation in infected cells. Interestingly, stabilization of v-Jun^{ASV17} correlates with decreased ubiquitinylation of the protein, which is likely to be due to the loss of the δ domain [11].

Along the same lines, we report here that v-Fos^{FBR}, but not v-Fos^{FBJ} or v-Jun^{ASV17}, displays a markedly decreased sensitivity to calpains. This raises the interesting possibility that resistance to calpains contributes to the tumorigenic potential of FBR-MSV by allowing a greater accumulation in the cytoplasm of intact viral protein available for transport into the nucleus of infected cells. v-FOS^{FBR} is a protein that harbours deletions and point mutations in its proto-oncoprotein-derived moiety and is fused both to part of the retroviral Gag protein at its N-terminus and to a C-terminal aberrant peptide motif. We show here that resistance to calpains is not due to mutations that have accumulated in the Fos part of the viral protein but to the addition of new peptide motifs. This indicates that either accessibility and/or conformation of the viral Fos region is different from that of its homologous domain in the cellular proto-oncoprotein. Whatever the case, this raises the possibility of modified interactions with natural protein partners of c-Fos.

MATERIALS AND METHODS

Chemicals

Bovine milli-calpain (calcium-activated neutral protease), aprotinin and soybean trypsin inhibitor were from Sigma; leupeptin, bestatin and α_2 -macroglobulin were from Boehringer Mannheim. Solid-phase synthesis of the calpastatin peptide inhibitor [26] was performed on a Miligen 9050 peptide synthesizer with the fluorenyl-9-ylmethoxycarbonyl group as temporary amino protection.

Cells and cytoplasmic cell extracts

Jurkat and Daudi cells were obtained from the American Type Cell Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. After harvesting, they were washed twice in PBS [150 mM NaCl/10 mM sodium phosphate (pH 7)] and then incubated for 5 min on ice in a hypotonic lysis buffer [20 mM Hepes (pH 7.5)/10 mM potassium acetate/1.5 mM magnesium acetate; 2 ml for 5×10^8 cells] without any detergent. Lysis was completed with a Dounce homogenizer and verified by microscopic examination. Nuclei were removed by low-speed centrifugation (2000 *g* for 15 min) and supernatants were centrifuged at 100 000 *g* for 1 h in a Beckman SW60 rotor. Aliquots of cytoplasmic extracts (S100; 5–12 mg/ml of protein) were then frozen and stored at -80°C until use [19,20].

Plasmids and cloning

The PM83.31 and pG5-5-1 plasmids [24] carry the genomic v-

Fos^{FBR} and v-Jun^{ASV17} proteins respectively. They have been used as starting materials for subsequent PCR amplifications and clonings. PM83.31 was generated by cloning the 2.5 kb *Bam*HI–*Xho*I fragment of the FBR-MSV genome [27,28] into the *Bam*HI and *Xho*I sites of pKSII(M13+) (Clontech). PCR amplifications were performed with the *Tfi* DNA polymerase (Epicentre Technologies) in accordance with the specification of the supplier. Primers used were the following (see Figure 1 for details): 943, 5'-ACCATGTTCTCGGGTTTCAAC-3'; 944, 5'-TCCCTAGGTCGACGGGAACCTTTG-3'; 1019, 5'-TCGAC-CATGGACAGCCTTCTACTACCATTCC-3'; 1020, 5'-TCATCAGCCACTGTGTCCAGATGATGCCGG-3' 1022, 5'-GGAATTCAATTTCTAAGTCCTATTGCCACCCT-3' 1029, 5'-CCCATAUGTTCTCGGGCTTCAACGCAGAC-3' 1030, 5'-GGGGGATGCGCCTTCTCTGACTGCTCACAG-3'; 3.1, 5'-CCCGAATTCTCAAAACGTTTGCAACTGTTG-3'; 5.1, 5'-CCCAAGCTTATGGAAGCCGTCATAAAGGTG-3'; 5.2, 5'-CCCAAGCTTATGAGTGTTTGTATCCTTCCCTG-3'; MP2012, 5'-CTACTATTGCCACCCTGCCATGCTAATAA-AGCCACTGGGCCTAGATGATGCCGG-3'.

c-Fos, v-Fos^{FBR} and v-Jun^{ASV17}-derived PCR-amplified DNA fragments were cloned into the *Sma*I site of pKS II(M13+). PM74.3 bears the mouse c-Fos coding sequence that was amplified by using primers 1029 and 1030 from a full-length cDNA clone (gift from Dr. S. Vincent). AMS2.2 corresponds to Gag-FOS^v, AMS1.9 to Fos^v-Fox, AMS3.5 to c-Fos^v, PM75.33 to Fos^v and AMS5.6 to p10-Jun^v. AMS4.2 encodes Jun^v and was generated by inserting the *Sac*II–*Eco*RI fragment from pG5-5-1 into the *Sac*II and *Eco*RI sites of pKSII(M13+). PM55.41 bears the v-Fos^{FBJ} coding sequence that was PCR-amplified from the FBJ-2 genomic clone [29] and cloned into the *Sma*I site of the pBS vector (Clontech). PM151.15 carries the chicken c-Jun sequence and was generated by cloning the 1.85 kb *Cl*aI fragment from pACJ-1 [30] into the *Cl*aI site of pKSII(M13+). AMS11.83 carries Gag-c-FOS^v-Fox; it was constructed in two steps. First, a c-Fos^v-Fox-carrying plasmid (AMS10.11) was constructed by cloning into the *Sma*I site of pKSII(M13+) a c-Fos^v-Fox fragment generated by PCR with oligonucleotides 1019 and MP2012. Then the 0.8 kb *Bgl*II–*Bgl*II fragment from PM83.31, which carries the Gag sequence from v-Fos^{FBR}, was cloned into the *Bam*HI and *Bgl*II sites of AMS10.11. Sequencing of the various constructs was performed with the T7 sequencing kit from Pharmacia.

Transcription and translation *in vitro*

For transcription, plasmids were linearized with appropriate restriction enzymes and subsequently extracted with phenol. Transcription with T3 and T7 RNA polymerases was performed in accordance with the supplier's specifications (Promega), as was translation in rabbit reticulocyte lysates in the presence of [³⁵S]methionine (Amersham). Alternatively, the TNT transcription/translation kit from Promega was used with circular plasmids.

Degradation assay and protein analysis

Degradation experiments were performed at 37°C in a final volume of 40 μl . For degradation in cytoplasmic extracts, 1 μl of the translation mixture and 39 μl of pure or diluted S100 extract were mixed and prewarmed at 37°C . As a reference (time 0), 4 μl of the mixture was sampled, mixed with 10 μl of electrophoresis loading buffer containing 1% (w/v) SDS and kept at room temperature until electrophoresis. CaCl_2 (1 mM final concentration) and protease inhibitors when necessary (see below) were then added in a volume of 4 μl at a time taken as t_0 of the

degradation kinetics. When one component was omitted, the volume was adjusted to 40 μ l with PBS. When degradation kinetics were performed in the presence of pure milli-calpain, the latter was added at a final concentration of 50 μ g/ml. For degradation kinetics, 4.4 μ l aliquots of the reaction mix were sampled at various time points and the reaction was stopped by the addition of electrophoresis loading buffer containing 1 % SDS. Samples were then subjected to electrophoresis through 15 % (w/v) gels by the method of Laemmli [31] and electrotransferred to nitrocellulose for autoradiography. When needed, protease inhibitors were used as follows: calpastatin peptide at 0.5 mg/ml, leupeptin at 5 μ g/ml, aprotinin at 200 μ g/ml, PMSF at 1 mM, bestatin at 40 mg/ml, α_2 -macroglobulin at 12.5 mg/ml and soybean trypsin inhibitor at 0.85 μ g/ml.

Protein sequence analysis

Protein sequences or nucleotide sequences were recovered from Genbank. PEST motifs were identified with the PESTFIND algorithm obtained from Dr. M. Rechsteiner [32]. All details on the latter can be found in Rechsteiner et al. [33].

RESULTS

v-Fos^{FBR}, but not v-Fos^{FBJ}, is resistant to calpains

v-Fos^{FBJ} and v-Fos^{FBR} are mutant forms of c-Fos (see Figure

1A). v-Fos^{FBJ} is a 381-residue protein that has accumulated five scattered point mutations and one frameshift, changing the last 48 residues into 49 new ones. v-Fos^{FBR} is a Gag-Fos-Fox hybrid protein of 554 residues, of which 310 are encoded by parental retrovirus *gag*-derived sequences, 236 by Fos sequences and eight are derived from a genomic locus called *fox* [27,28] or *fau* [34]. Moreover, the first 24 and last 98 residues of c-Fos are deleted and five point mutations plus two in-frame deletions of thirteen and nine residues are scattered throughout the molecule.

The basic degradation assay used here has been described elsewhere [19,20]. First, proteins are produced by translation of T7 or T3 RNA polymerase-derived transcripts in the presence of [³⁵S]methionine in the rabbit reticulocyte lysate, which has been shown elsewhere [19,20] to be devoid of any detectable level of calpain activity. Depending on both the protein and the reticulocyte lysate used, complex patterns were sometimes obtained with higher-molecular-mass products due to post-translational modification and/or lower-molecular-mass products due to abortive translation or internal initiation (see [19,20] and Figure 2 for examples). Aliquots of translation reactions were then mixed with calpain-containing human Jurkat or Daudi cell S100 cytoplasmic extracts [19,20] and analysed by electrophoresis as described in the Materials and methods section. Whatever the origin of the cell extract, no significant difference was observed in the final outcome of each experiment, except that calpain activity was higher in Jurkat cell extracts. Under these conditions,

Figure 1 Normal and mutant viral and cellular Fos and Jun

Names of proteins are indicated in italic capitals (see text for details on mutations); names of corresponding expression plasmids are indicated underneath in parentheses. In cases of cloning of PCR-generated DNA fragments, names of oligonucleotides are indicated above the arrows (for details, see the Materials and methods section). In the specific case of AMS 11.83, the 1019 oligonucleotide is in parentheses to indicate that the construction of the vector required two cloning steps. (A) Fos proteins; (B) Jun proteins.

Figure 2 Differential sensitivity of cellular and viral Fos to calpains

c-Fos, v-Fos^{FBJ} and v-Fos^{FBR} translated *in vitro* were incubated for various periods either in S100 Daudi cell extracts [(A), (B) and (C) respectively] or in the presence of purified bovine milli-calpain [(D), (E) and (F) respectively] as described in the Materials and methods section. Reactions were performed in the presence of either 1 mM CaCl₂ or 1 mM CaCl₂/10 mM EGTA. The positions of molecular mass markers are shown at the right. Abbreviation: ', minutes.

calpains were shown to be the only proteases able to cleave most members of the AP-1 family [19]. It is of note in this assay that discrete proteolytic fragments (see Figures 2 and 3 for examples) that may be stable for several hours are observed, indicating that cleavage by calpains is limited [19,20]. Alternatively, the addition of pure bovine milli-calpain to the newly synthesized protein-containing reticulocyte lysate was used for degradation kinetics analysis with similar outcomes. Because calpain activity can vary from one cell extract to another, the comparison of different proteins was always performed in kinetics experiments conducted in parallel with the same extract batch. Non-specific degradation was ruled out by showing that proteins such as glyceraldehyde-3-phosphate dehydrogenase or dihydrofolate reductase were stable in the assay ([19,20], and results not shown).

Degradation kinetics of c-Fos, v-Fos^{FBJ} and v-Fos^{FBR} proteins by calpains were compared in parallel experiments as described above. In the various experimental conditions we tested (i.e. involving variations in concentrations of cytoplasmic extracts or of purified milli-calpain in the assay), c-Fos and v-Fos^{FBJ} always decayed with similar rates (results not shown), whereas v-Fos^{FBR} always remained unattacked. Precise quantification of the difference in sensitivity to calpain between c-Fos and v-Fos^{FBJ} on the one hand, and v-Fos^{FBR} on the other, was, however, not

possible owing to the extent of this difference. It can nevertheless be estimated to be at least a 50-fold factor: as can be seen in Figure 2, c-Fos and v-Fos^{FBJ} were quantitatively degraded in less than 5 min, whereas no detectable degradation of v-Fos^{FBR} was observed after 1 h of reaction in a typical experiment involving a Daudi cell extract (Figures 2A–2C). Such a difference in sensitivity was also observed when pure milli-calpain was added directly to the reticulocyte lysate (Figures 2D–2F). This indicates that the structural alteration of v-Fos^{FBR}, but not those of v-Fos^{FBJ}, confers resistance to cleavage by calpains.

Comparable sensitivities of the chicken c-Jun and v-Jun^{ASV17} to calpains

The chicken c-Jun is 310 residues in length whereas v-Jun^{ASV17} (Figure 1B) is a fusion protein in which 283 Jun-encoded residues are fused to 11 residues derived from the 5' non-coding region of c-jun mRNA plus 220 residues from the gag region of the parental retrovirus (which include p19^{gag} and part of p10^{gag}). Moreover, 27 residues are deleted from the N-terminal region (δ region) and three point mutations are scattered into the Jun moiety of the molecule [35].

Studies of the degradation kinetics of c-Jun and v-Jun^{ASV17} were conducted in parallel assays with either a Jurkat cell extract (for examples see Figures 3A and 3B) or by adding purified milli-calpain to the reticulocyte lysate (for examples see Figures 3C and 3D). In both situations, no detectable difference in sensitivity to calpains was observed in the different experiments conducted. Because of the importance of the structural alterations of the v-Jun^{ASV17} protein and more particularly the addition of the Gag motif, it was important to demonstrate formally that degradation was actually due to calpains and not to one or more other proteases activated under the same conditions. To this end, two types of experiment were conducted. In a first set of experiments (Figures 3E and 3F), the involvement of calpains was confirmed by the use of a series of protease inhibitors [18] because these enzymes are (1) insensitive to aprotinin, bestatin and soybean trypsin inhibitor, (2) partly sensitive to PMSF and α_2 -macroglobulin and (3) fully inhibited by EGTA, leupeptin and above all by the highly specific physiological inhibitor, calpastatin, which was replaced in our experiments by a 27-mer peptide (calpastatin peptide) as described by Maki et al. [26]. In a second set of experiments, evidence that Fos and Jun mutants are actual substrates of calpains and not substrates of other proteases activated by calpains was provided by a two-step degradation assay. First, newly synthesized proteins were incubated in the cytoplasmic extract for 30 min in the presence of Ca²⁺ ions to achieve complete degradation. Secondly, fresh protein was added and the reaction was pursued for another 30 min, either under the same conditions, to show that the proteolytic activity was not exhausted, or in the presence of calpastatin or EGTA to test the involvement of calpains, which are inhibited under these conditions. Experiments shown in Figures 3(G) and 3(H) clearly demonstrate that degradation of v-Jun^{ASV17} in the assay is due to calpains.

Finally, because of the chimaeric structure of v-Jun^{ASV17}, it was important to determine whether susceptibility to calpains was due to the presence of the Jun moiety of the molecule or to the presence of Gag motifs that might compensate for a possible stabilizing effect of mutations within the Jun part. For this purpose, degradation rates of c-Jun, p10-Jun^v (which corresponds to v-JUN^{ASV17} deleted from p19^{gag}) and Jun^v (which corresponds to the mutated Jun moiety of v-Jun^{ASV17}) were compared in parallel experiments. Studies of degradation kinetics

Figure 3 Differential sensitivity of cellular and viral Jun to calpains

c-Jun and v-Jun^{ASV17} translated *in vitro* were incubated for various periods of time in S100 Daudi cell extracts [(A) and (B) respectively] or in the presence of bovine milli-calpain [(C) and (D) respectively]. For testing the effect of the different protease inhibitors, degradation experiments (E, F) were performed in Daudi cell extracts for 1 h in the presence of 1 mM CaCl₂ and calpastatin peptide (Calpast.), leupeptin (Leu.), aprotinin (Apro.), PMSF, bestatin (Best.) or soybean trypsin inhibitor (STI) as indicated in the Materials and methods section. To demonstrate the involvement of calpains, the two-step degradation assay (G, H) used was the following: newly synthesized protein was incubated for 30 min in Daudi cell extract in the presence of 1 mM CaCl₂ (T0) or 1 mM CaCl₂/10 mM EGTA (EGTA.1) as described in the Materials and methods section. At this time (T1) a comparable amount of fresh radioactive protein was added to the reaction mixture, one-third of which was incubated for a further 30 min under the same conditions (T2) and each other one-thirds was incubated separately in the presence of either calpastatin or 10 mM EGTA (EGTA.2). The positions of molecular mass markers are shown at the right of (A) and (B). Abbreviation: ', minutes.

Figure 4 Degradation of v-Jun^{ASV17} mutants by calpains

Experiments were conducted as described in the legend to Figure 3. (A, B) Degradation kinetics; (C, D) test of protease inhibitors; (E, F) two-step degradation assay. (A), (C) and (E) correspond to p10-Jun and (B), (D) and (F) to Jun^V. Degradation kinetics were conducted with a Daudi cell extract and must be compared to those presented in Figure 3. The positions of molecular mass markers are shown at the right. Abbreviations: ', minutes; CalpaSt, calpastatin; Apro, aprotinin; Leu, leupeptin; Best, bestatin; α2-macro, α₂-macroglobulin.

presented in Figures 4(A) and 4(B) (compare with Figure 3A) show that both mutants are as sensitive as c-Jun to Daudi cell extract Ca²⁺-dependent proteases. Similar results were obtained

in degradation experiments performed with pure milli-calpain (results not shown). Moreover, the use of protease inhibitors (Figures 4C and 4D) along with that of the two-step degradation

Figure 5 Degradation of c-Fos and v-Fos^{FBR} mutants by calpains

Degradation experiments were conducted as described in the legend to Figure 3. Left panels: degradation kinetics of mutants translated *in vitro* in Daudi cell extracts in the presence of 1 mM CaCl₂ or 1 mM CaCl₂/10 mM EGTA. They should be compared with those shown in Figures 2(A)–2(C). Middle panels: tests of the protease inhibitors. Right panels: two-step degradation assay. (A), (E) and (I) correspond to Gag-Fos, (B), (F) and (J) to Fos-Fox, (C), (G) and (K) to Fos^v and (D), (H) and (L) to c-Fos^v (see text and Figure 1 for details). The positions of molecular mass markers are shown at the right. Abbreviations: ', minutes; CalpaSt, calpastatin; Apro, aprotinin; Leu, leupeptin; Best, bestatin; STI, soybean trypsin inhibitor.

assay (Figures 4E and 4F) demonstrated the involvement of calpains in the breakdown of these mutants.

Addition of peptide motifs, rather than mutations in the proto-oncoprotein moiety, is responsible for resistance of v-Fos^{FBR} to calpains

To determine whether the resistance to calpains of v-Fos^{FBR} was due to mutations having accumulated in the Fos moiety of the protein and/or to fusion with atypical peptide motifs, three deletion mutants were constructed and tested for their sensitivity to calpains. They were Gag-Fos^v, Fos^v-Fox and Fos^v, which are v-Fos^{FBR} proteins with Fox, Gag and Fox+Gag sequences respectively deleted (see Figure 1A). All three mutants proved to be very sensitive to Ca²⁺-dependent breakdown (Figure 2) in degradation experiments performed with cytoplasmic extracts (Figures 5A–5C), indicating that the addition of both Gag and Fox motifs is necessary to stabilize the viral protein. Comparable results were obtained when purified milli-calpain was added to newly synthesized proteins instead of the cytoplasmic extract (results not shown). The use of protease inhibitors (Figures 5E–5G; results not shown for α₂-macroglobulin) and the two-step degradation assay described above (Figures 5I–5K) demonstrate the involvement of calpains in the cleavage of mutant proteins.

Analysis of the Fos^v construct strongly suggests that accumulation of mutations in the Fos moiety of v-Fos^{FBR} does not contribute detectably to the resistance to calpains. This point was confirmed by the fact that sensitivity of the c-Fos^v construct, which corresponds to the region of the cellular Fos that has been transduced by FBR-MSV (i.e. it is devoid of any mutation except the N- and C-terminal deletions), is comparable with that of c-Fos (Figures 5D, 5H and 5L). Finally, the analysis of a Gag-c-Fos^v-Fox mutant (Figure 6), in which the mutated Fos^v sequence has been replaced by its cellular homologue (Figure 1A), rules out the possibility that stabilization of v-Fos^{FBR} was due to a co-operation between the addition of Gag and Fox motifs and the internal mutations in the Fos part of the molecule because this mutant protein also revealed resistance to calpains. In conclusion, the addition of both Gag and Fox motifs is necessary to stabilize v-Fos^{FBR}.

DISCUSSION

A catabolic pathway operating differentially on Fos and Jun family members [19,21] is most probably initiated by calpains, which are abundant cytoplasmic proteases [17]. This pathway very probably participates in the regulation of the activity of AP-1 transcription factor through the control of the abundance of the different protein components of this transcription complex

Figure 6 Analysis of the Gag-c-Fos^v-Fox mutant

Left panels: degradation experiments were conducted with a Jurkat cell extract as described in the Materials and methods section. Right panels: purified milli-calpain was added to the reticulocyte lysate. (A), (B) and (C) correspond to c-Fos, v-Fos^{FBR} and Gag-c-Fos^v-Fox respectively. The positions of molecular mass markers are shown at the right. Abbreviation: ', minutes.

available for nuclear transport. However, it is indirect and compartmentalized within the cell because AP-1 family proteins acquire protection from calpains once they enter the nucleus. We report here that the mutated form of c-Fos carried by an oncogenic retrovirus, FBR-MSV, is resistant to calpains. Interestingly, rather than mutations having accumulated during and after the transduction process in the Fos moiety of the viral protein, resistance to breakdown is due to the addition of exogenous peptide motifs, either altering the conformation or masking the cleavage sites.

Several lines of evidence indicate that both deregulated expression and alteration of biochemical properties of protein products are necessary for revealing the tumorigenic potential of viral *fos* and *jun* genes (reviewed in [25,36]). To allow sufficient accumulation of the transforming proteins, most of the numerous transcriptional and post-transcriptional levels of regulation operating on the cellular genes (reviewed in [1]) no longer exert their action on viral genes: (1) constitutive long terminal repeats replace complex and exquisitely regulated promoter regions; (2) in *c-fos*, blocking the elongation of nascent RNA transcripts in intron 1 and regulating pre-mRNA splicing are no longer possible owing to the elimination of introns; (3) mRNA species are stabilized owing to the loss of a potent destabilizer located in 3' untranslated regions; (4) in *fos* the transport of viral proteins into the nucleus is no longer regulated but constitutive; and (5) nuclear turnover of proteins is significantly decreased. Along this line, the resistance of v-Fos^{FBR} to calpains raises the possibility of a new contribution, indirectly allowing a greater accumulation of this protein in the nucleus of FBR-MSV-infected cells. Two

mechanisms by which resistance to calpains might contribute to the tumorigenic potential of this retrovirus can be proposed. The most obvious one is certainly a decreased cytoplasmic breakdown that allows the nuclear importation of increased amounts of viral proteins during interphase. Testing this possibility will, however, have to await the means of making precise measurements of degradation rates of transcription factors within the cytoplasm. Although this is possible for c-Fos, whose retention in the cytoplasm and cytoplasmic half-life determination are achievable in serum-starved fibroblasts, it is not possible for v-Fos^{FBR}, which evades the nuclear transport control by extracellular signals and is thus constitutively transported into the nucleus [12]. The other mechanism, which does not exclude the first one, might be the stabilization of viral proteins during mitosis, because calpains have access to new substrates during this phase of the cell cycle owing to disruption of the nuclear envelope.

Despite the large number of peptides and proteins that have been studied, the recognition and hydrolysis of substrates by calpains still remain a puzzle. Several points are, however, worth noting (for more details see [17]). First, small synthetic peptides are generally poor substrates compared with proteins. Secondly, protein substrates are generally hydrolysed to a limited extent. Thirdly, even if the amino acid residues of identified scissile bonds are not random, no consensus peptide motif for cleavage by calpains can be proposed. Taken together, these observations suggest that (1) conformational determinants of substrates, rather than primary sequence motifs, are responsible for recognition by calpains (however, whether cleavage sites themselves or binding domains distant to them are recognized is not yet clear) and (2) because of this property, calpains might be used in some instances as topological probes for studying protein conformation. The comparable rates of degradation of c-Fos, Fos^v and c-Fos^v (see the Results section) suggest that most, if not all, cleavage sites for calpains have been neither deleted nor mutated in the Fos moiety of v-Fos^{FBR}. Indeed, we have recently characterized one of the major cleavage sites in c-Fos between residues 90 and 91 in Fos, i.e. within a region that is not modified in v-Fos^{FBR} (M. Pariaat and M. Piechaczyk, unpublished work). In consequence there are two possible non-exclusive mechanisms by which Gag and Fox motifs might confer resistance to calpains: the first consists of simple steric hindrance's masking cleavage and/or recognition sites; the second implies structural constraints linked to the addition of exogenous motifs that alter the conformation of cleavage and/or recognition sites for calpains. Whatever the explanation, the resistance to calpains that we observed raises the interesting possibility that the addition of Gag and Fox motifs might alter interactions with putative partners of c-Fos and consequently influence the activity of v-Fos^{FBR}. Consistent with this idea, the deletion of Gag motifs significantly decreases the transformation efficiency of v-Fos^{FBR} in a transformation assay involving mouse embryo fibroblasts [37].

One final point concerning calpain substrate-recognition motifs is worthy of note. PEST motifs (hydrophilic regions of varying length, rich in Pro, Glu, Asp, Ser and Thr and, found in most unstable proteins) have been proposed to constitute built-in signals for recognition by calpains [32]. Site-directed mutagenesis has recently allowed us to demonstrate that none of the three PEST sequences (spanning residues 128–139, 205–250 and 360–380 and showing scores of +10.1, 5.7 and 4.4 respectively; for more details on PEST motifs, see [33]) harboured by c-Fos is necessary for hydrolysis by calpains [20]. Moreover, a survey of a series of calpain substrates has indicated that PEST motifs are neither necessary nor sufficient for conferring susceptibility to calpains [20]. Strengthening this conclusion, it is interesting to consider that the Gag moiety of v-Fos^{FBR} contains several high-

score PEST motifs (spanning residues 104–125, 142–178, 178–196, 200–212 and 301–317 and showing scores of +7.9, +10.3, +8.9, +9.6 and +6.2 respectively) but still confers resistance to calpains in the presence of the Fox sequence.

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