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Characterization of novel nuclear targeting and apoptosis-inducing domains in FAS associated factor 1

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SUMMARY

FAS associated factor 1 (FAF1) has been described as an unusual protein that binds to the intracellular portion of the apoptosis signal transducing receptor FAS/Apo-1 and potentiates apoptosis in L-cells. By means of mRNA differential display we have identified the avian homologue (qFAF) as a fibroblast growth factor-inducible gene in pluripotent cells from E0 quail embryos during mesoderm induction in vitro. Later during embryonic development, qFAF expression is ubiquitous. We confirm that qFAF is associated with FAS, and show that it is phosphorylated on serine residues and localized to the nucleus. By in vitro mutagenesis we have delimited a novel nuclear targeting domain to a short 35 amino acid alpha-helical region in the amino-terminal half of the protein. The nuclear function of qFAF remains unclear. However, a probably dominant negative deletion mutant of qFAF causes apoptosis of transfected cells. This function resides in the carboxy-terminal domain of qFAF which shares remarkable sequence homologies with a putative ubiquitin conjugating enzyme from *Caenorhabditis elegans*. Our data indicate a complex function for FAF, which may be executed during FAS signalling and/or in the ubiquitination pathway, and may be essential for cell differentiation and survival.

Key words: FAF1, FAS, Apoptosis, Ubiquitin conjugating enzyme, FGF, Differential display, Avian embryo

INTRODUCTION

The development of a vertebrate organism during embryogenesis is the result of proliferation, differentiation and, in many organs, selective cell death. For example, regression of the interdigital tissue contributes to the formation of the digits from the zeugopodium. The cavities of the heart enlarge, as one mechanism among others, by regression of the inner cells of the chamber wall. The vasculature of the lens capsule disappears to facilitate vision, and during maturation of the nervous system the initial number of neurones is largely reduced. All these very different examples of tissue remodelling during ontogeny are executed by programmed cell death. This type of cell death is called apoptosis and is morphologically and biochemically distinct from necrosis, which results from a sudden injury to a cell. Morphologically, apoptotic cell death is defined by condensation of nuclear chromatin and subsequent fragmentation of the nucleus and division of the whole cell into apoptotic bodies which are subsequently phagocytosed. Each cell of the body possesses the potency to undergo apoptosis, e.g. upon withdrawal of 'survival' factors. Therefore, the molecular mechanism that enables cells to execute programmed cell death is believed to be an ubiquitous property of any cell (for review see Cohen, 1993; Nagata, 1996; Peter et al., 1997; Wyllie et al., 1980, 1981).

During the last decade several details of the molecular mechanisms underlying apoptosis have been elucidated. Signals, both from inside and outside the cell, have been identified which are capable of irreversibly inducing cell death via the apoptotic pathway. The severe damage of chromosomal DNA (e.g. after irradiation) as well as receptor mediated signals from other cells can mediate apoptotic cell death. One of the major suicidal molecular responses of the target cell to death-inducing signals is the fragmentation of the chromosomal DNA down to the nucleosomal level and the proteolytic cleavage of specific substrates executed by a distinct family of proteases known as caspases (Salvesen and Dixit, 1997; Villa et al., 1997; Walker et al., 1993). In the immune system, the TNF-receptor related transmembrane protein FAS/Apo-1 has been demonstrated to transduce death inducing signals inside the cell upon binding of the FAS-ligand and subsequent trimerization of the receptor (Oehm et al., 1992; Nagata, 1996; Green and Ware, 1997). In addition, several molecules have been identified to interact with the intracellular portion of the FAS receptor, via the death domains, and are involved in the intracellular transduction of the death signal by further recruitment and activation of caspases (Chinnaiyan et al., 1995; Stanger et al., 1995). The search for intracellular FAS ligands using a two hybrid screen led to the discovery of a novel FAS associated factor, FAF1, which lacks a characteristic death domain and can enhance but not initiate FAS-mediated

apoptosis when overexpressed in L-cells (Chu et al., 1995). However, the mechanism by which FAF1 may support FAS mediated signalling is still unknown. On the basis of sequence homology a relationship of FAF1 to proteins of the ubiquitination pathway was postulated (Becker et al., 1997). Members of the family of ubiquitin conjugating enzymes transfer ubiquitin onto target proteins and these ubiquitinated proteins are destined for degradation by the proteasomal machinery (Hochstrasser, 1995). Ubiquitin conjugating enzymes have been shown to be associated with FAS (Becker et al., 1997; Wright et al., 1996). Therefore it was suggested that the half-life time of the FAS/FAS ligand complex and intracellular signalling generated by this complex is limited by ubiquitination and that FAF1 is involved in this process.

Using a completely different approach we identified an avian FAF1 homologue. A systematic search for genes that are expressed, when blood islands are induced in the early embryonic mesoderm, was performed using differential display of mRNA (Liang et al., 1992). For this purpose we used an established in vitro model, in which undifferentiated cells of E0 quail embryos can be induced to differentiate into blood and endothelial cells after treatment with basic fibroblast growth factor (bFGF) (Flamme and Risau, 1992). A variety of inducible gene products including a quail FAF1 homologue (referred to as qFAF) were found. Further characterization of qFAF suggests that this molecule is a ubiquitously expressed factor in the quail embryo. qFAF coprecipitates with FAS but it also localizes to the nucleus. The deletion of a distinct domain at the carboxy terminus of qFAF that shares significant homology with a putative ubiquitin conjugating enzyme from *C. elegans* (Wilson et al., 1994), results in apoptosis of the cells overexpressing this mutant. These data support the hypothesis that qFAF is a nuclear factor with functional domains that are involved in the FAS-mediated apoptotic signalling pathway.

MATERIALS AND METHODS

Cell culture

E0 quail embryos were isolated and cultured in suspension in the presence of bFGF (100 ng/ml, Progen, Heidelberg, FRG) as described previously (Krah et al., 1994). Control cultures were grown without the addition of bFGF. After defined incubation intervals from 30 minutes to 20 hours the cells were harvested and RNA was prepared.

RNA extraction and northern analysis

Total RNA was isolated using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Aliquots of 10 μg were separated on 1.2% agarose gels containing 0.66 M formaldehyde in 1× MOPS-buffer (40 mM 3-(N-morpholino)propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA, pH 7.2) and transferred to a nylon membrane (Hybond-N, Amersham) in 20× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate) following a standard protocol. The probes for the qFAF cDNA (631 bp *Hin*dIII-*Not*I fragment) and for β -actin were labelled with [^{32}P]dCTP using a random primer labelling kit (Boehringer). Hybridizations were carried out as described previously (Flamme et al., 1995).

Differential display of mRNA

Differential display conditions were modified from those originally described (Liang et al., 1992, 1993). Purified total RNA was reverse transcribed using T_{11}XY primers (MWG Biotech) where XY represents every possible combination of nucleotides with the exception of T(AGCT). An aliquot of 0.2 μg of total RNA was used for each reaction. Subsequent PCR amplification of 1/10 of the resulting cDNA, with the appropriate T_{11}XY primer in combination with an arbitrary 10mer primer (MWG Biotech), was performed in a TC 9600 thermocycler (Applied) using Taq DNA polymerase under the following cycling conditions: 94°C for 30 seconds, 42°C for 1 minute and 72°C for 1 minute for 40 cycles, followed by 72°C for 5 minutes. The PCR fragments were labelled by amplification in the presence of 0.25 μM = 5 μCi [α-^{35}S]dATP (1,000 Ci/mmol), separated in a 6% denaturing polyacrylamide gel and then exposed under a Kodak XOMAT AR Xray film. Bands which were differentially expressed were excised from the dried gel, boiled in 100 μl of 1× TE for 20 minutes, precipitated with ethanol and redissolved in 10 μl of H_2O. A sample of 5 μl of recovered cDNA was used for reamplification. The parameters for the reamplification steps were the same as in the initial amplification except that a higher concentration of dNTPs (200 μM) was used. The reamplified cDNAs were cloned into the *Sma*I site of pBluescript KS+ vector (Stratagene) by standard procedures and sequenced on an Applied Biosystems DNA sequencer. cDNAs were used as probes for library screening and Southern blotting. In the current study, we used 8 arbitrary primers with all possible combinations of 12 T_{11}XY primers for a total of 96 differential display comparisons per selected incubation time. Amplification of the qFAF cDNA fragment was obtained using the primers dT_{11}GG and d(GATTCAGTGG).

Semiquantitative PCR and southern blotting

The cDNAs derived from E0 quail embryo cell cultures incubated for 30 minutes to 20 hours were amplified with sequence specific primers using: d(TCTGCAAAGTGAGTACGGTGGAGAG) as an upstream and d(CATCTACATCTCCAGTCTTCCACCC) as a downstream primer. The cycling parameters were as follows: 94°C for 30 seconds, 61°C for 30 seconds and 72°C for 30 seconds for 20 cycles, followed by 72°C for 5 minutes. The amplified products were separated on a 2% agarose gel and analysed by Southern blotting.

Screening an E1 quail cDNA library

To identify full length qFAF clones, 5×10^5 recombinat plaques from a E1 quail embryonic λgt10 cDNA library (Flamme et al., 1995) were screened with the [^{32}P]dCTP labelled qFAF cDNA clone from differential display. The prehybridization and hybridization steps were performed as described (Maniatis et al., 1982). Hybridized nylon filters were washed in 0.1× SSC/0.1% SDS at 42°C and exposed to Kodak autoradiographic XOMAT AR films. Positive clones were isolated, and inserted cDNAs were recloned into the *Not*I site of pBluescript KS+ vector (Stratagene) and sequenced.

In situ hybridization

In situ hybridizations on paraffin sections of E6 quail embryos were performed as described previously (Flamme et al., 1995). Whole mount in situ hybridizations were carried out according to the protocol of Hemmati-Brivanlou et al. (1990). qFAF antisense and sense mRNA probes were generated from the qFAF differential display clone (bp 304-688) in pBluescript KS+ by transcription with T3 and T7 RNA polymerase (New England Biolabs) according to the manufacturer's instructions in the presence of 100 μCi [α-^{35}S]UTP or digoxigenin-11-UTP (Boehringer) for use in in situ hybridization on paraffin sections or for whole mount in situ hybridization, respectively. Digoxygenin-labelled hybrids were visualized using the Dig-nucleic acid detection kit (Boehringer).

Construction of qFAF fusion proteins

In order to create a plasmid encoding the full length amino-terminal FLAG-tagged qFAF the open reading frame was amplified by PCR from the qFAF cDNA using KlenTaq (Promega) and modified PCR primers which introduced a *Not*I restriction site just upstream of the initiating AUG codon and a *Kpn*I restriction site just downstream of

the termination codon. The primers used were: qFAF Not u1, d(GGCCGCCGGCGGCCGCCATGGCGT), qFAF Kpn d1, d(GATGGTACCTACAATCTTGTCTGATCTATT). After the open reading frame with the introduced restriction sites was digested with *Not*I and *Kpn*I, the resulting fragment was inserted into the pFLAG-CMV2 eucaryotic expression vector (Kodak) predigested with *Not*I/*Kpn*I. For qFAF constructs containing deletions of the carboxy terminus, three further *Kpn*I sites were introduced downstream using further oligonucleotideprimers in combination with the qFAF Not u1 oligonucleotide in order to create the deletion constructs $FqFAF^{M1-G467}$, $FqFAF^{M1-V309}$, and $FqFAF^{M1-V113}$, respectively. In qFAF constructs containing deletions of the amino terminus, the downstream oligonucleotideprimer qFAF Kpn d1 was used in combination with three further upstream oligonucleotides, which led to the introduction of a *Not*I site, in order to obtain the deletion constructs $FqFAF^{M482-E648}$, $FqFAF^{V309-E648}$, and $FqFAF^{D111-E648}$, respectively.

The plasmids which encoded FLAG-tagged qFAF fused with bacterial alkaline phosphatase (BAP) were generated by inframe ligation of PCR amplified fragments from qFAF into the *Hin*dIII site of pFLAG-CMV2-BAP. This restriction site is located between the FLAG-tag encoding sequence and the BAP open reading frame. Pairs of PCR primers which introduce *Hin*dIII sites were used and fragments were ligated into pFLAG-CMV2-BAP after digestion with *Hin*dIII restriction endonuclease.

Transfection of cells and immunfluorescence microscopy

3T3 fibroblasts and COS7 cells were cultured in DMEM supplemented with 10% fetal calf serum (Boehringer) and antibiotics. Cells were seeded onto 3.5 cm Petri dishes (Falcon) and transfected with plasmid DNA by calcium phosphate co-precipitation on the following day (Cepko, 1992). Briefly, 3.5 μg of plasmid DNA was precipitated in 250 μl 1× HBS (140 mM NaCl, 25 mM Hepes, 1.4 mM Na_2HPO_4) in the presence of 120 mM $CaCl_2$ and was added to the cells which were then reincubated for a period of between 3-8 hours. The cells were washed with serum free medium and then subjected to glycerol shock for 90 seconds by the addition of 250 μl of glycerol shock solution (15% glycerol in 1× HBS). The cells were washed again and cultured in medium supplemented with serum and antibiotics. At 8 to 72 hours after transfection the cells were fixed in 4% paraformaldehyde in PBS for 15 minutes, permeabilized in methanol for 30 seconds and immunostained with a monoclonal mouse anti-FLAG-tag antibody (M2, 10 μg/ml, Kodak). The bound antibody was visualized using a rhodamine-conjugated anti-mouse IgG secondary antibody (Dako). The cell nuclei were counterstained with Hoechst 33324 (Sigma), and specimens were examined with a Zeiss Axiophot fluorescence microscope. Apoptotic cells were detected using the ApopTag™ in situ apoptosis detection Kit (Oncor) according to the manufacturer's instructions.

Western blotting

The cells to be used for western blotting were grown on 6 cm dishes, incubated for 24 hours after transfection, washed twice with cold PBS and then lysed by the addition of 200 μl ice-cold RIPA buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% desoxycholic acid, 1% Triton X-100). The cell lysates were collected into tubes and centrifuged for 15 minutes at 4°C. The samples were then diluted 1:1 with 2× SDS-PAGE sample buffer and boiled prior to loading. Proteins were separated by 8% SDS-PAGE and electrotransfered to nitrocellulose membranes (Schleicher and Schüell). Non-specific protein binding was prevented by treating the membranes with 3% BSA for 1 hour. The membranes were subsequently incubated with M2 antibody (10 μg/ml, Kodak) for 1 hour at 25°C. After five washes in PBS (0.1% Tween-20), the blots were incubated with peroxidase-conjugated sheep anti-mouse Ig (Amersham, 1:5,000) and bands were visualized using the enhanced chemoluminescence (ECL) method (Amersham).

Immunoprecipitation

In co-immunoprecipitation experiments, COS cells were transiently transfected using the calcium phosphate method with either the FLAG-qFAF-pCMV2 full length construct alone or together with mouse FAS-pCDNA3 construct (kindly provided by Dr Nagata). At 48 hours after transfection transfectants were lysed in NP40-lysis buffer (150 mM NaCl, 1% NP-40 (Boehringer), 50 mM Tris-HCl, pH 8.0) including protease inhibitors (1 mM PMSF, aprotinin 10 mg/ml, leupeptin 1 mg/ml; Sigma). Cell lysates were used for immunoprecipitation using the anti-FLAG-tag M2 antibody (Kodak). Immune complexes were anaylzed on 8% SDS-PAGE followed by western blotting with monoclonal anti-FAS antibody (Transduction Laboratories, 1:2,500) and the blots were visualized following addition of a peroxidase-conjugated sheep anti-mouse IgG as secondary antibody and the ECL detection system (Amersham).

The phosphorylation of qFAF on serine residues was demonstrated by transiently transfecting COS cells, then lysing the cells in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (1 mM PMSF, aprotinin 10 μg/ml, leupeptin 1 μg/ml, 0.1 mM Na-vanadate, 1 mM ocadaic acid, 1 mM NaF; Sigma): PAGE and western blotting were carried out as previously described using a monoclonal anti-phosphoserine antibody (Sigma).

RESULTS

Cloning of quail FAF1

The differentiation of blood islands, consisting of blood cells and endothelial cells, is one of the earliest events of cell lineage diversification in the vertebrate embryo mesoderm (Risau and Flamme, 1995). This event can be mimicked in vitro by inducing pluripotent embryonic cells from quail blastodiscs to differentiate into blood islands after the addition of basic FGF (Flamme and Risau, 1992). In order to identify genes which are induced by FGF, we applied the technique of mRNA differential display (Liang et al., 1992) to induced versus uninduced in vitro cultures of quail blastodisc cells. Amongst the gene products which were identified, a 387 bp PCR fragment was isolated from a quail blastodisc cell culture induced for 20 hours with bFGF. This fragment was further used as a probe for the screening of a λgt10 cDNA library made from E1 quail embryos (Flamme et al., 1995). Five independent cDNA clones, one of which (2,642 bp) contained a complete open reading frame of 1,944 bp with a consensus start site, were isolated. A sequence similarity search was performed using the BLAST algorithm (Altschul et al., 1990), and by searching both the GenBank and the SwissProt databases revealed an 80.5% overall homology of the nucleic acid sequence of the open reading frame with a recently published murine mRNA sequence of FAS associated factor 1 (FAF1). The predicted protein (Fig. 1A) with a length of 648 amino acids shared an amino acid sequence similarity of 88% with FAF1, and contained 550 (84%) identical amino acids (Fig. 1B). FAF1 was described as an FAS/Apo-1 associated protein factor (Chu et al., 1995) and the identified gene described in this study is most likely the quail homologue of murine FAF1, and was therefore named qFAF.

An analysis of the predicted amino acid sequence of qFAF showed that it is a highly hydrophilic protein containing 28.4% charged residues and 26.4% residues with polar side chains. The major characteristics of this protein are two highly charged regions which have a predicted alpha-helical secondary structure (α1: Q278-G308; α2: Q488-L589) (Fig. 1A). The

A

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                GCGGCCGCCTGAGCTGAGGCAGCGCTGCTCGGCTCCGGCTCCCGGCCCTCCTCCTCT   57
          TTCTCCTCTTCTTCCTCCTCCTCTTCCGACCCTCCTTCTCCTCTCGGTCCGGGCCCGCCGTC  120
          TCCATGAGCCCGTAGTTCCCTCCGGCACAGCTCGCTCTACGCGGGGCAAAGCGGAGCCGGGGG  183
    1                M  A  S  N  M  D  R  E  M  I  L  A  D  F  Q  A
          CCGCCGCGGGCCGCCATGGCGTCCAACATGGACAGAGAGATGATCCTGGCCGACTTCCAGGCT  246
   17     C  T  G  I  E  N  I  D  E  A  I  T  L  L  E  Q  N  N  W  D  L
          TGTACTGGTATAGAAAATATCGATGAAGCTATAACTTTGCTTGAACAAAATAACTGGGATTTA  309
   38     W  A  A  I  N  G  V  I  P  Q  E  N  G  I  L  Q  S  E  Y  G  G
          TGGGCAGCTATAAATGGTGTAATACCACAAGAAAATGGCATTCTGCAAAGTGAGTATGGTGGA  372
   59     E  T  L  Q  G  P  A  Y  G  P  T  S  H  S  T  T  A  S  S  S  S
          GAGACTTTACAAGGACCAGCATATGGCCCCACAAGTCATTCTACAACTGCTTCTTCATCTTCT  435
   80     S  A  F  R  H  V  M  P  S  T  Q  I  V  E  R  Q  P  R  M  L  D
          TCAGCATTTCGCCATGTAATGCCATCTACACAAATAGTGGAAAGACAACCTCGAATGCTTGAC  498
  101     F  R  V  E  Y  R  D  R  N  V  D  V  V  L  E  D  S  S  T  V  G
          TTCAGGGTTGAGTACAGAGACAGAAATGTGGATGTAGTACTTGAAGACAGCTCTACAGTTGGA  561
  122     D  I  T  H  I  L  E  N  E  L  Q  I  P  A  S  K  M  L  L  K  G
          GATATCACACATATTCTAGAAAATGAACTTCAGATTCCTGCGTCTAAAATGCTGTTAAAGGGG  624
  143     W  K  T  G  D  V  D  D  S  T  V  L  K  T  L  H  L  P  K  N  N
          TGGAAGACTGGAGATGTAGATGATAGTACTGTTTTAAAAACTCTACACTTGCCAAAAAATAAT  687
  164     S  L  Y  Y  V  L  T  P  D  L  P  P  P  S  S  S  H  S  G  A  L  Q
          AGTCTTTATGTTCTTACACCTGACCTGCCTCCTCCTTCTTCAAGTCATTCTGGAGCCCTGCAG  750
  185     E  S  L  N  Q  N  F  M  L  I  I  T  H  R  E  V  Q  G  E  Y  N
          GAGTCATTAAATCAAAACTTCATGCTGATCATCACCCATCGAGAAGTCCAGGGGGAGTACAAC  813
  206     L  N  F  S  G  S  S  T  I  Q  E  V  K  R  N  V  Y  D  L  T  S
          CTCAACTTCTCAGGAAGCAGTACCATTCAGGAGGTGAAACGGAATGTGTATGACCTAACCAGT  876
  227     I  P  V  R  H  Q  Q  W  E  G  W  P  P  S  A  T  D  D  S  M  T
          ATCCCTGTCCGTCACCAGCAGTGGGAGGGCTGGCCTCCTTCTGCCACAGACGACTCTATGACT  939
  248     L  A  V  S  G  V  N  F  P  C  H  R  L  T  V  G  R  R  S  S  P
          CTAGCTGTATCAGGAGTGAACTTTCCTTGCCATCGACTTACAGTTGGAAGAAGATCTTCACCT 1002
  269     V  Q  T  R  E  Q  S  E  E  Q  C  T  D  V  H  M  V  S  D  S  D
          GTACAAACAAGGGAACAGTCAGAGGAGCAATGCACTGATGTCCATATGGTTAGTGACAGTGAT 1065
  290     G  D  D  F  E  D  A  T  E  F  G  V  D  D  G  E  M  F  G  V  A
          GGAGATGACTTTGAAGATGCTACGGAGTTTGGAGTTGATGATGGAGAAATGTTTGGGGTAGCA 1128
  311     S  S  A  L  R  K  S  P  M  M  P  E  N  A  E  N  E  G  D  A  L
          TCATCTGCCTTGAGGAAATCGCCAATGATGCCAGAAAATGCTGAAAATGAAGGAGATGCCTTA 1191
  332     L  Q  F  T  A  E  F  S  S  R  R  Y  G  D  C  H  P  V  Y  F  I
          TTGCAATTTACAGCAGAGTTTTCTTCGAGAAGATACGGTGATTGCCATCCTGTTTATTTTATT 1254
  353     G  S  L  E  A  A  F  Q  E  A  F  Y  G  K  A  R  D  R  K  L  L
          GGATCATTAGAGGCTGCTTTTCAAGAAGCCTTCTATGGAAAAGCTAGAGATAGAAAGCTTCTT 1317
  374     A  I  Y  L  H  H  D  E  S  V  L  T  N  V  F  C  S  Q  M  L  F
          GCTATCTACCTCCACCATGATGAAAGTGTGCTAACCAACGTCTTCTGCTCACAAATGCTGTTT 1380
  395     A  E  S  I  V  S  Y  L  T  Q  E  F  I  T  W  G  W  D  L  T  K
          GCGGAATCTATTGTCTCCTATCTGACTCAAGAATTCATAACCTGGGGTTGGGACCTGACAAAA 1443
  416     E  A  N  R  A  S  F  L  T  M  C  T  R  H  F  G  D  V  V  A  Q
          GAAGCAAACAGAGCGAGTTTTCTGACGATGTGCACAAGACACTTCGGGGATGTTGTAGCCCAA 1506
  437     T  I  R  T  Q  K  T  D  Q  F  P  L  F  L  I  I  M  G  K  R  S
          ACAATTCGAACTCAGAAGACAGATCAGTTCCCACTTTTCCTTATTATCATGGGAAAACGATCA 1569
  458     S  N  E  V  L  N  V  I  Q  G  N  T  T  V  D  E  L  M  M  R  L
          TCTAACGAAGTATTGAATGTAATACAAGGTAACACAACAGTGGATGAGCTAATGATGAGGCTG 1632
  479     M  A  A  M  E  I  F  S  A  Q  Q  Q  E  D  I  K  D  E  D  E  R
          ATGGCTGCAATGGAGATCTTCAGTGCCCAGCAGCAGGAAGATATAAAGGATGAGGATGAACGT 1695
  500     E  A  R  E  N  V  K  R  E  Q  D  E  A  Y  R  I  S  L  E  A  D
          GAAGCCAGAGAGAATGTGAAAAGGGAGCAGGATGAAGCGTACCGCATTTCACTCGAAGCTGAC 1758
  521     R  A  K  R  E  A  Q  E  R  E  M  A  E  Q  F  R  L  E  Q  I  R
          AGAGCAAAGAGGGAAGCACAAGAGAGAGAAATGGCAGAGCAGTTTCGTTTGGAACAGATTCGG 1821
  542     K  E  Q  E  E  E  R  E  A  I  R  L  S  L  E  Q  S  L  P  P  E
          AAAGAACAGGAGGAGGAACGTGAGGCTATCAGGCTCTCTCTTGAGCAGTCTTTGCCTCCAGAA 1884
  563     P  K  E  E  S  T  E  S  V  S  K  L  R  I  R  T  P  S  G  E  F
          CCAAAAGAGGAAAGCACCGAGTCAGTCAGCAAACTGCGTATCCGGACACCCAGCGGAGAATTC 1947
  584     F  E  R  R  F  L  A  S  S  K  L  Q  V  V  F  D  F  V  A  Y  K
          TTTGAGCGACGTTTCCTGGCCAGCAGCAAGCTGCAGGTTGTCTTTGATTTTGTGGCTTACAAG 2010
  605     G  F  P  W  E  E  F  K  L  L  G  T  F  P  R  R  D  V  T  Q  L
          GGATTTCCCTGGGAGGAATTCAAGCTTCTGGGCACCTTTCCGAGGAGAGATGTCACCCAGCTG 2073
  626     D  P  N  K  S  L  Q  E  V  K  L  Y  P  Q  E  T  L  F  L  E  A
          GATCCAAATAAATCATTACAGGAGGTAAAACTGTATCCTCAAGAAACCCTTTTCCTAGAGGCG 2136
  647     K  E
          AAAGAATAGATCAGACAAGATTGTAGGACTAATCACCTTTTGGCAAGCCAGAGGCACAGCATC 2199
          GAGAGGAACACTTCATCAACACCATCTTGTACTATCAGCCAACTCAACTCAATGTCACAACTT 2262
          TGCCTCTTGCAAGAATCCTGAAAGTCTGAAATAACATAGCTAAAGTTCCACATACATGAGTAT 2325
          ATGTTTCAACCTCGTTTAAATGAGCCGAGAATATAGCTCTGCTTTGAACACTGAACATTATGA 2388
          CTATGTTGCTCACTTATCATCCAGCTTTTGTGATATTTAATGCACCGTTAAAATGTGAGTGAT 2451
          TGTTAAATAGAATTTTTACTCCTCAAATGCCCCTTTTTCACAAAGAAATCTTGGACATTTCTG 2514
          CCTGTACTTTTGACACTAGAATGCGGTATATTTGATATCTCGCTGAGCCAGTGCTCTCATATA 2577
          TATGCATTTTATCTCTGTGTTTGAATTTCTACTTTGTCACCTAATTTAAAAAGGCAAAAAAAA 2640
          AA
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B

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   1 MASNMDREMILADFQACTGIENIDEAITLLEQNNWDLWAAINGVIPQENGILQSEYGGET
   1 ------LP-----------------------------V----------------DF----

  61 LQGPAYGPTSHSTTAS SSSSAFRHVMPSTQIVERQPRMLDFRVEYRDRNVDVVLEDSST
  61 MP--TFD-A--PAP--TP------P----R-----------------------------C-

 120 VGDITHILENELQIPASKMLLKGWKTGDVDDSTVLKTLHLPKNNSLYVLTPDLPPPSSS
 121 --E-KQ---------VP------------E------S----------------------S

 179 HFGALQESLNQNFMLIITHREVQGEYNLNFSGSSTIQEVKRNVYDLTSIPVRHQQWEGWP
 181 -A--------------------R-----------V------------------L-----

 239 PSATDDSMTLAVSGVNFPCHRLTVGRRSSPVQTREQSEEQCTDVHMVSDSDGDDFEDATE
 241 A-------C--E--LSY----------T------------S-----------------S-

 299 FGVDDGEMFGVASSALRKSPMMPENAENEGDALLQFTAEFSSRRYGDCHPVYFIGSLEAA
 301 -------V--M---T--------------------------- -S-----FY-------

 359 FQEAFYGKARDRKLLAIYLHHDESVLTNVFCSQMLFAESIVSYLTQEFITWGWDLTKEAN
 360 ------V--------------------------C--------S-N----A-----DT-

 419 RASFLTMCTRHFGDVVAQTIRTQKTDQFPLFLIIMGKRSSNEVLNVIQGNTTVDELMMRL
 420 --R-----N----S-I-------------------------------------------

 479 MAAMEIFSAQQQEDIKDEDEREARENVKREQDEAYRISLEADRAKREAQEREMAEQFRLE
 480 ------------------------------------L-----------H-----------

 539 QIRKKEQEEEREAIRLSLEQSLPPEPKEESTESVSKLRIRTPSGEFFERRFLASSKLQVV
 540 ---- ---------------A--------NA-P-------------L-------N---I-

 599 FDFVAYKGFPWEEFKLLGTFPRRDVTQLDPNKSLQEVKLYPQETLFLEAKE
 599 -----S-----D-----S----------------L--N-F-------Q---
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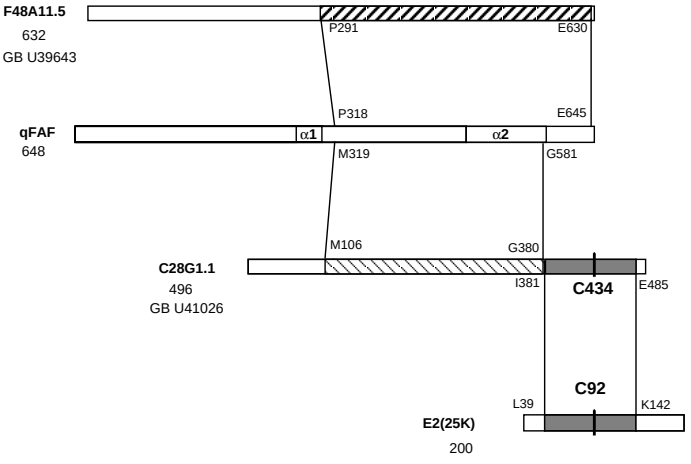
C



sequence motif SSXY (X = T; H; P; A; K, and Y = V; F; I; L) occurs six times (Fig. 1A). The database searches for highly homologous molecules yielded no other molecules than FAF1 but the similarity to several sequence motifs present in proteins of the ubiquitination pathway were detected. At the amino terminus a repeated sequence motif which is homologous to ubiquitin is present (Becker et al., 1997), and embraces the region between R-108 and T-261 that is directly followed by the alpha-helix-1. The carboxy-terminal region of the qFAF molecule from M-319 to G-581 exhibits significant homology with *C. elegans* ORF C28G1 (Wilson et al., 1994) which encodes a protein that contains sequence homology to the core domain of ubiquitin conjugating enzymes in its carboxy terminus (Fig. 1C,D). Another putative *C. elegans* cosmid translation product, F48A11.5 (Wilson et al., 1994), aligns with the entire carboxy-terminal half of qFAF and shares slightly higher similarity with qFAF than the C28G1 translation product (Fig. 1C,D). In addition, the alpha helix-2 of qFAF displays amino acid sequence homology to alpha helices of several nuclear proteins which have clusters of amino acids

D

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qFAF    318 PMMPENAENEGDALLQFTAEFSSRRYGDCH-----PVYFIGSLEAAFQEAF-YGKARDRK
            PM+P+   +  DAL  F A FS R           P +Y   L AA +EAF +  +  R+
F48A11  291 PMIPDGFSSVSDALRNFVAIFSDRFCSTPQTQAFMPPFYTEPLPAAVKEAFDHPNSEHRR

            LLAIYLHHDESVLTNVFCSQMLFAESIVSYLTQEFITWGWDLTKEANRASFLTMCT-RHF
             L  Y++HD S+  N+F SQ+L +E++ + +   ++ + WD+T D+N   FL      +
            PLLFYINHDRSIAANIFASQVLCSETVSTLIRHQYVLFPWDITSDSNLMLFLEYLQAANM

            GDV--VAQTIRTQKTDQFPLFLIIMGKRSSNEVLNVIQGNTTVDELMMRLMAAMEIFSAQ
            GDV  + Q +   K + FPL  I++ +R+S  +++  +G  T D++M +L++ +  +S
            GDVRTIIQRLAMSKIESFPLMAIVVKERNSYRLVDYCRGTDTSDQVMEKLLSGVSEYSDI

            QQEDIKDEDEREARENVKREQDEAYRISLEADRAKREAQEREMAEQFRLEQIRKEQEEER
            +  +  +  ERE RE ++ +Q+  Y+ SL AD+A+ EA ++E+ EQ RLE+ RK +EEE
            RMNEQSERREREEREAIRNQQEAEYKASLAADKARMEAKQQEIEEQ-RLEEERKLREEEE

            EAIRLSLEQSLPPE--PKEESTESVSKLRIRTPSGEFFERRFLASSKLQVVFDFVAYKGF
            E +R     S  PE  P       +  ++ R P G    RRF     +Q + ++++SKG+
            ECVRRQTVASTVPEEPPASAPLAEIINVKFRLPEGGQDMRRFRRLESIQTLINYLSSKGY

            PWEEFKLL-GTFPRRDVTQ-LDPNKSLQEVKLYPQETLFLE 646
              D+FK++ S FP++++T+ +D + ++ + K   +E +F+E
            SPDKFKYFNSDFPKKEITRHFDLSHNFADTKWPAREQIFVE 630


qFAF    319 MMPENAENEGDALLQFTAEFSSRRY--GDCHPVYFIGSLEAAFQEAFYGKARDR-KLLAI
            MMP +++   DAL  +T  F++RY   S   P FY  +L  A +EAF  +     + LA
C28G1   106 MMPHHSQPIPDALKCYTDNFNTRYQIGSTVMPKFYTDTLRNAVKEAFDQENPILCRPLAF

            YLHHDESVLTNVFCSQMLFAESIVSYLTQEFITWGWDLTKEANRASFL------TMCTRH
            +++++ S  T  F + +L  E + SYL   +I + WD+T+  N  RFL       M + H
            FINNENSSNTRNFVTNVLCNELVTSYLATKYILYPWDVTEPRNLDRFLRILTDSNMSSMH

            FGDVVAQTIRTQKTDQFPLFLIIMGKRSSNEVLNVIQGNTTVDELMMRLMAAME------
            +     ++    ++ +FP   I++ K +  EV+  + G + ++E++  L    E
            YN---LKSFNESESHRFPYIAIVLRKGNWFEVIREVNGTSDLNEVVNALYEGFEELQEEK

            --IFSAQQQEDIKDEDEREARENVKREQDEAYRISLEADRAKREAQEREMAEQFRLEQIR
              + S Q +   K     E R+ +  +Q+E Y  +L+ D       ++ +A +  +E  R
            LRVLSKQVERKTKQAKVEEERKLID-QQNEVYYKNLKID--TDNLNKKRIAAKSEIETTR

            KEQEEEREAIRL--SLEQSLPPEPKEESTESVSKLRIRTPSG 581
             E + +   +++     Q LP EP    T  V+ ++ R P G
            PETKPKITPMKVISVTPQNLPEEPNVSETNIVT-VKFRLPKG 380


qFAF    477 RLMAAMEIFSAQQQEDIKDEDEREARENVKREQDEAYRISLEADRAKREAQEREMAEQ 534
            RL   +++ + +++++   E+ + A+E  K++++E   +  +  R KRE  E+E AE+
CAF-I   325 RLGKQLKLRAEREEKEKLKEEAKRAKEEAKKKKEEEKELKEKERREKREKDEKEKAEK 382
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Fig. 1. The sequence, structure, and homologies of qFAF mRNA and the putative translation product. (A) The nucleotide sequence and the predicted amino acid sequence of a qFAF 2642 bp cDNA clone from an E1 quail embryo λgt10 cDNA library. The nucleotide sequences which were targeted by the differential display oligonucleotide primers are underlined. In the amino acid sequence, SSXY motives are underlined (see text for detail). Sequences in the open boxes represent the putative α-helical domains. (B) A comparison of the amino acid sequences of qFAF (upper sequence) and murine FAF1 (lower sequence). The overall amino acid identity is 84%. (C) A schematic representation and structural alignment of qFAF (648 amino acids) with the putative translation products of the open reading frames encoded by F48A11.5 (632 amino acids, GenBank accession no. U39643) and C28G1.1 (496 amino acids, GenBank accession no. U41026) from the *C. elegans* 2.2 Mb cosmid sequence published by Wilson et al. (1994). The C28G1.1 translation product is highly homologous to the core region of ubiquitin-conjugating enzymes (Ubc). As an example, alignment with the 200 amino acid bovine E2(25K) Ubc is depicted which reveals highest homology with C28G1.1 in the BLAST search. In C28G1.1 the cysteine at position 434 corresponds to the cysteine 92 of E2(25K), which serves as an acceptor/donor site for ubiquitin and is highly conserved among the Ubc family members. (D) Alignments of qFAF and homologues proteins: F48A11.5 and C28G1.1 translation products from *C. elegans* and human chromatin assembly factor p150 subunit.

with highly charged side chains, e.g. the p150 subunit of chromatin assembly factor 1 (Kaufman et al., 1995) (Fig. 1D).

Expression of qFAF mRNA

Since qFAF1 mRNA was initially cloned using the differential display technique from pluripotent embryonic cells 20 hours after they were induced with basic FGF to differentiate into blood islands, the time course of this induction was examined using sequence-specific primers and reverse transcription PCR. qFAF mRNA was detected as early as two hours after induction but was barely detectable in non-induced cultures. The level of qFAF mRNA gradually increased up to 20 hours after induction, at which time the message for qFAF was also detectable in the uninduced cultures (Fig. 2A). The inducibility of qFAF was restricted to the pluripotent embryonic cells that did not express the mRNA at detectable levels, while differentiated cells constitutively expressed qFAF (not shown).

Northern blot hybridization of quail embryonic RNAs revealed a transcript size of approximately 3,000 nt when a fragment from the 3′-untranslated region of the 2,642 bp qFAF clone (nt 1,993-2,624) was used. This transcript size corresponded to the approximate size of the isolated cDNAs. The difference of about 400 nucleotides may be due to the lack of parts of the 3′ and/or 5′ untranslated regions in the cDNA clones. A high level of the qFAF transcript were detected in brain, heart, liver, lung, intestine, and kidney, with lower levels seen in skin and spleen (Fig. 2B).

To investigate the potential roles of qFAF during early development, we determined the expression pattern of mRNA in different stages of quail development in situ. Whole mount in situ hybridization of E1 embryos (at the definitive primitive streak stage) revealed an intense and ubiquitous staining throughout the three embryonic germ layers (Fig. 3A,B, and data not shown). In situ hybridization analysis of sections through E6 quail embryos confirmed the ubiquitous expression of qFAF mRNA during early stages of quail embryonic development. However, there was a clear accentuation of the signal in the CNS. As an example of hybridization signal for qFAF sections through the dorsal region of an E6 embryo including the spinal cord are shown in Fig. 3C,D.

qFAF is associated with FAS

The murine FAF1 was described as a FAS associated protein that was implicated in signal transduction during apoptosis

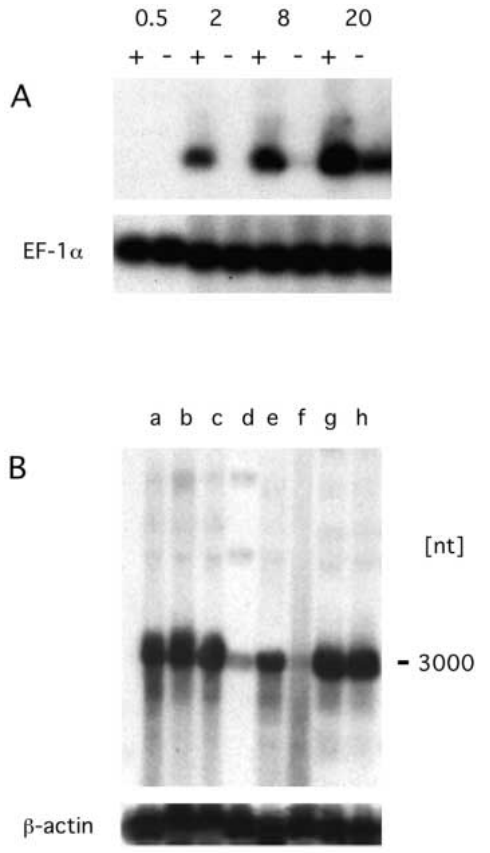

Fig. 2. Expression of qFAF mRNA. (A) Induction of qFAF mRNA transcripts following addition of basic FGF in cultures of pluripotent cells from E0 quail blastodiscs. The cells were cultured for 0.5, 2, 8, and 20 hours with or without 100 ng/ml bFGF as a mesoderm inducer and the RNA was extracted. Equal amounts of RNA were subjected to RT-PCR using qFAF specific primers. As a loading control the same mRNAs were amplified using EF-1α specific primers. The identity of the PCR products was determined by Southern hybridization with qFAF and EF-1α specific probes, respectively. (B) A northern blot hybridization of RNAs from various tissues of E12 quail embryos using a qFAF specific ^{32}P-labelled cDNA probe. A total of 10 μg of RNA were loaded per lane: a, brain; b, heart; c, liver; d, spleen; e, lung; f, skin; g, intestine; h, kidney. The same blot was hybridized with a chicken β-actin probe as a loading control.

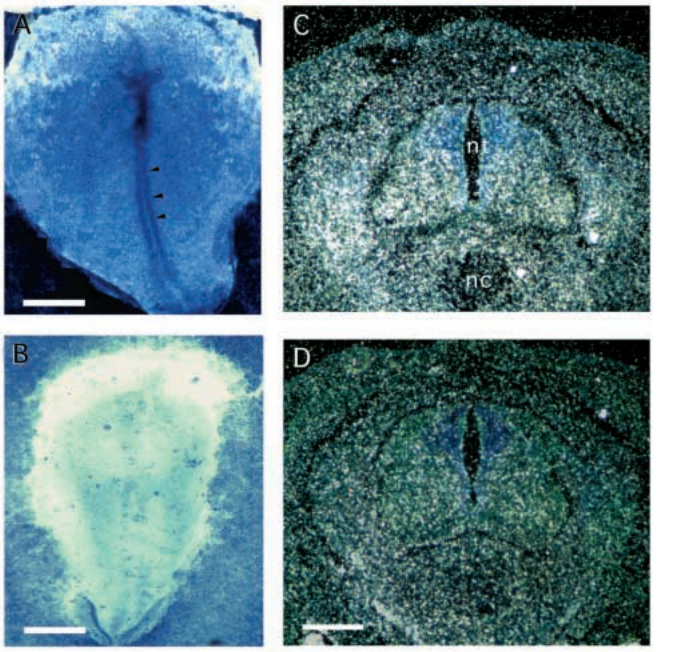

Fig. 3. Localization of qFAF mRNA in quail embryos as detected by in situ hybridization. (A) Whole mount in situ hybridization of a quail embryo at the stage of definitive primitive streak (about 24 hours of incubation) using a digoxygenin-labelled qFAF antisense RNA probe. The signal is ubiquitous but accentuated along the primitive ridges of the primitive streak (arrowheads). (B) Sense control using digoxygenin-labelled qFAF sense RNA probe. (C) Transverse section through an E6 quail embryo hybridized with a ^{35}S-labelled qFAF antisense probe after autoradiography under dark field illumination. nt, neural tube; nc, notochord. (D) The corresponding sense control to C using a ^{35}S-labelled qFAF sense RNA. Bars, 200 μm.

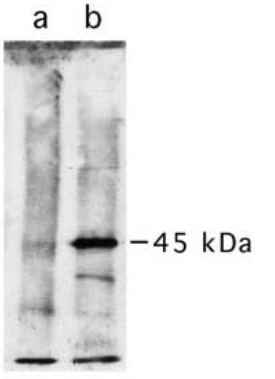

Fig. 4. Coimmunoprecipitation of FAS with FqFAF from COS cells overexpressing both FqFAF and FAS. Cell lysates were precipitated with anti-FLAG-tag M2 monoclonal antibody and the western blot was analyzed with anti-FAS antibody. Lane a: COS cells transfected with FqFAF, lane b: COS cells transfected with both, FqFAF and FAS.

(Chu et al., 1995). We tested the hypothesis that the avian homologue, qFAF, was also FAS associated and performed immunoprecipitation of qFAF from COS cells which overexpressed both, the FLAG-tagged qFAF and the human FAS/Apo-1 receptor. The blots were probed with anti-FAS antibody and a signal was detected at a molecular mass of 45 kDa which corresponds to the size of hFAS known from the literature (Suda and Nagata, 1994) (Fig. 4). We thus concluded that qFAF is the functional homologue of FAF1.

Expression of qFAF in vitro

In order to analyze the cellular function of qFAF, COS cells were transfected with a plasmid containing the coding sequence for an amino-terminal FLAG-tagged qFAF. The in vitro expression of the qFAF-FLAG construct and subsequent analysis by western blotting with an anti-FLAG antibody revealed a protein of 85 kDa (Fig. 5A). The 10 kDa difference in molecular mass to the predicted value of 75 kDa may be due to glycosylation, as 4 potential N-glycosylation sites are present in qFAF, and/or phosphorylation e.g. of serine residues which are very abundant in the qFAF protein. This latter possibility was substantiated by the result of anti-phosphoserine immunoblotting of qFAF-FLAG protein immunoprecipitated from COS cells (Fig. 5B). Consistent with the lack of a signal peptide consensus sequence at the amino terminus, the protein was exclusively detected in the cell lysates but not in the supernatants (not shown).

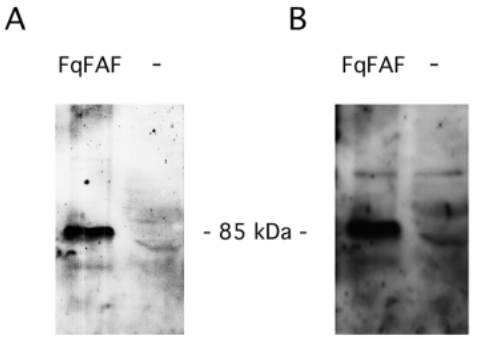

Fig. 5. Western blot analysis of FLAG-tagged qFAF (FqFAF) which is overexpressed in COS cells. (A) Using the anti-FLAG-tag M2 monoclonal antibody, FqFAF is detected as a 85 kDa protein. (B) After immunoprecipitation from the lysates using the M2 antibody FqFAF is detected as a serine phosphorylated protein using an anti-phosphoserine antibody.

Immunofluorescence analysis revealed that the qFAF protein accumulated in the interphase nuclei of transfected COS-cells, NIH 3T3 fibroblasts, and chicken embryonic fibroblasts (Fig. 6A,B). The protein was distributed homogeneously over the nuclei but was excluded from the nucleoli. The nuclear localization was not dependent on the FLAG epitope, because a FLAG-tagged form of bacterial alkaline phosphatase (BAP) was localized to the cytoplasm and was completely excluded from the nuclei (Fig. 6P).

The determinant for nuclear localization of qFAF is represented by the α-helix 1

Unlike many other nuclear proteins, qFAF/FAF does not contain a highly basic nuclear localization sequence (for review see Hicks and Raikhel, 1995). Recently a non-classical nuclear targeting sequence, termed M9, has been described for hnRNP molecules (Pollard et al., 1996; Siomi and Dreyfuss, 1995). There is only a weak homology between parts of qFAF and M9 (not shown), so it is unlikely that the nuclear targeting of qFAF is similar to M9. To identify the regions of qFAF which are important for nuclear localization, we constructed a series of amino- and carboxy-terminal deletion constructs (Figs 6, 7). To identify the sequence in qFAF that is responsible for nuclear localization parts of qFAF were overexpressed as FLAG-tagged fusion proteins in COS cells and their cellular localization was analyzed by immunofluorescence microscopy. The primary structure of wild-type qFAF is shown schematically in Fig. 7A. In a first series of experiments the

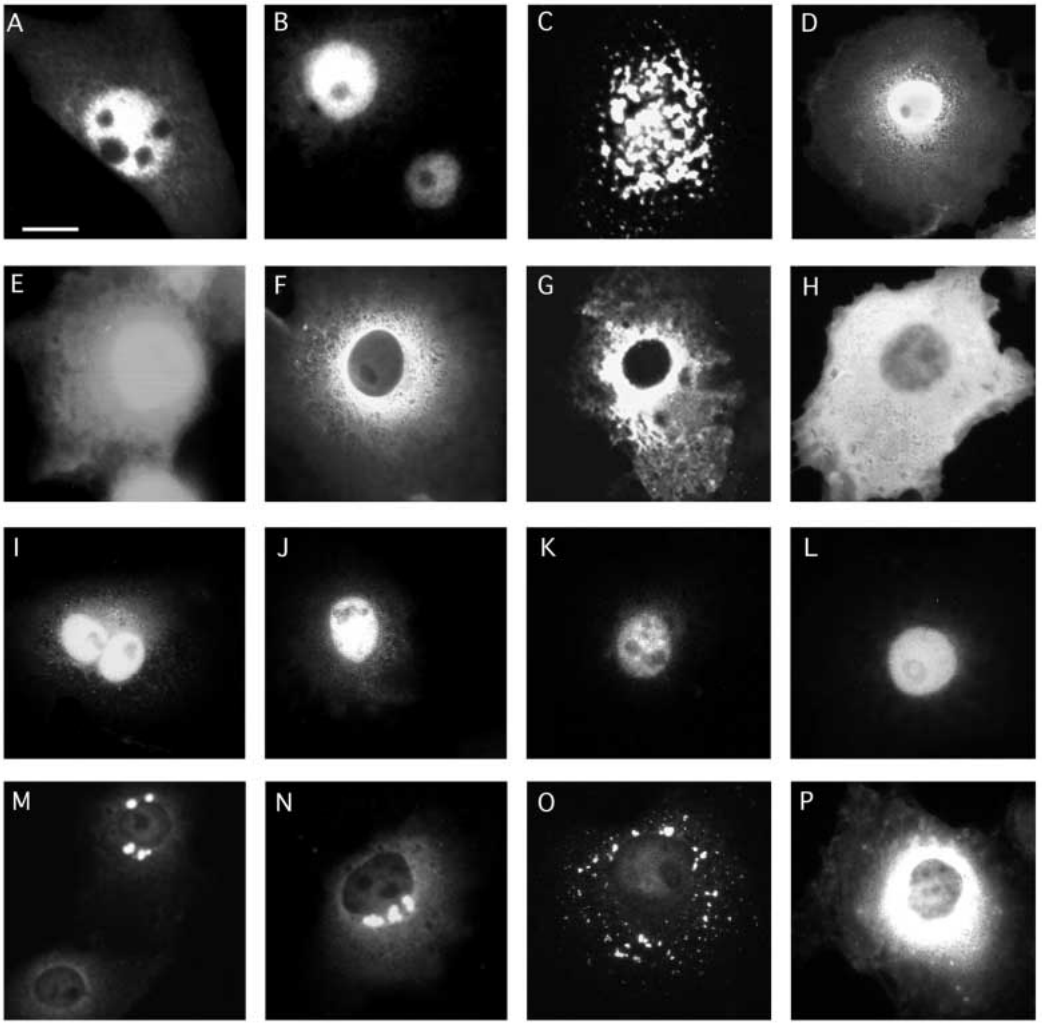

Fig. 6. The detection of FqFAF and FqFAF deletion mutants expressed in tissue culture cells 48 hours after transfection by immunofluorescence microscopy using the anti-FLAG-tag M2 antibody and rhodamine-conjugated secondary antibody. (A) NIH 3T3 fibroblasts, (B-P) COS cells. Transfected constructs are depicted in Fig. 7 and are as follows: FqFAF (A and B); FqFAF$^{M1\text{-}G467}$ (C); FqFAF$^{M1\text{-}V309}$ (D); FqFAF$^{M1\text{-}V111}$ (E); FqFAF$^{M482\text{-}E648}$ (F); FqFAF$^{V309\text{-}E648}$ (G); FqFAF$^{D111\text{-}E648}$ (H); FqFAF$^{D6\text{-}E322}$-BAP (I); FqFAF$^{D111\text{-}E322}$-BAP (J); FqFAF$^{N220\text{-}E322}$-BAP (K); FqFAF$^{S275\text{-}E322}$-BAP (L); FqFAF$^{D6\text{-}W217}$-BAP (M); FqFAF$^{D111\text{-}W217}$-BAP (N); FqFAF$^{P174\text{-}W217}$-BAP (O); FBAP (P). Bar, 20 μm.

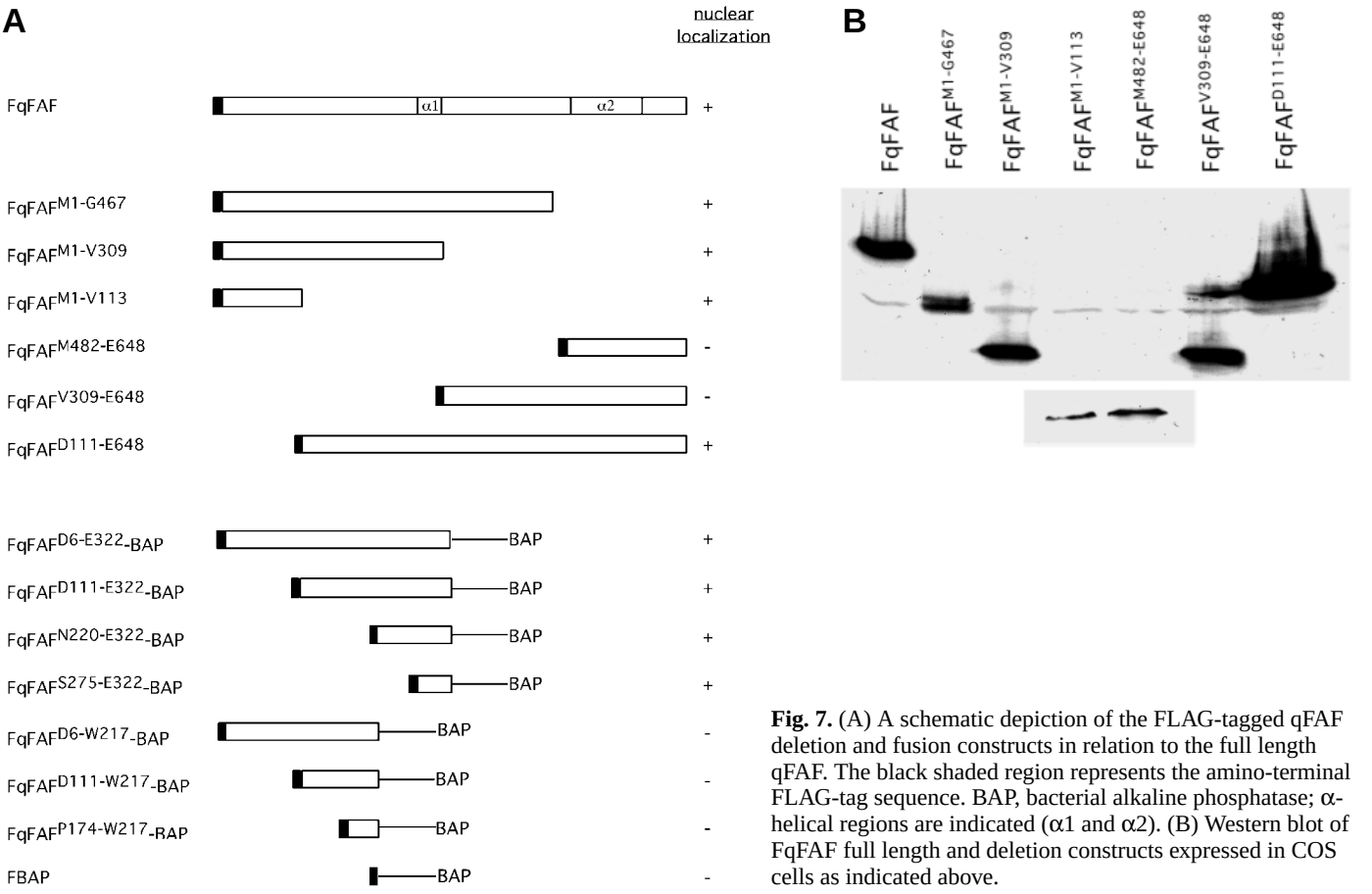

Fig. 7. (A) A schematic depiction of the FLAG-tagged qFAF deletion and fusion constructs in relation to the full length qFAF. The black shaded region represents the amino-terminal FLAG-tag sequence. BAP, bacterial alkaline phosphatase; α-helical regions are indicated (α1 and α2). (B) Western blot of FqFAF full length and deletion constructs expressed in COS cells as indicated above.

qFAF protein was subdivided into three pairs of corresponding amino and carboxy-terminal deletion mutants (Fig. 7). The FLAG-qFAF fusion proteins which contained parts of the amino terminus (Fig. 6C-E, FqFAF$^{M1-G467}$, FqFAF$^{M1-V309}$, FqFAF$^{M1-V113}$) localized to the nucleus, whereas the longest carboxy-terminal molecule (Fig. 6H, FqFAF$^{D111-E648}$) localized to both, the nuclear and the cytoplasmic compartments. Transfection of qFAF$^{M1-G467}$ (the molecule with the shortest carboxy-terminal deletion) resulted in unexpected cellular changes as described below in detail (Fig. 6C). The smallest deletion construct FqFAF$^{M1-V113}$ was localized to both, the cytoplasm and the nucleoplasm, which is consistent with its low molecular mass which allows free diffusion through the nuclear pore complex (Fig. 6E). qFAF-FLAG constructs which contain approximately 25% (FqFAF$^{M482-E648}$) or 50% (FqFAFV$^{309-E648}$) of the amino acid sequence of qFAF from the carboxy terminus, respectively, were retained in the cytoplasm (Fig. 6F,G). We therefore, conclude that the nuclear localization sequence of qFAF resides in the amino-terminal half of the protein between amino acids D111 and V309.

To further delineate the sequences responsible for the nuclear localization of qFAF, parts of the FLAG-tagged amino-terminal domain of qFAF were fused to cytoplasmatic bacterial alkaline phosphatase (BAP, 48 kDa) and, after transfection into COS cells, the expressed fusion proteins were detected with the anti-FLAG antibody (Figs 6, 7). The largest protein (FqFAF$^{D6-E322}$-BAP) containing the majority of the amino-terminal 50% of qFAF fused to BAP was completely localized to the cell nucleus, whereas BAP by itself was cytoplasmic (Figs 6I,P and 7). These data suggest that import of qFAF into the nucleus is mediated by an active process as the size of the fusion proteins prevent import by diffusion through nuclear pores (Gerace, 1992). All the amino-terminal fusion proteins which contained the complete amino-terminal domain of qFAF up to amino acid 322 acted as targeting signals which were capable of directing BAP into the nucleus (Figs 6I-L, 7). The smallest portion of qFAF which conferred nuclear targeting when fused to BAP was the region from S275 to E322 (Fig. 6L). Fusion proteins which lacked this portion failed to localize to the nucleus and accumulated around the nucleus in a spotted pattern (Fig. 6M-O).

We conclude from these data that the nuclear targeting signal of qFAF resides in the amino acid sequence from S275 to V309, which includes the α-helix 1 from Q278 to G308. This small amino-terminal fragment of qFAF is necessary and sufficient to target expression of qFAF to the nucleus and also to a large heterologous protein that is normally excluded from the nucleus. Sequence analysis of the amino acid sequence of this nuclear targeting domain revealed that there were no significant sequence homologies to domains of any other known proteins.

Deletion of the α-helix 2 of qFAF causes apoptosis in transfected cells

The cells which overexpressed a mutant form of qFAF

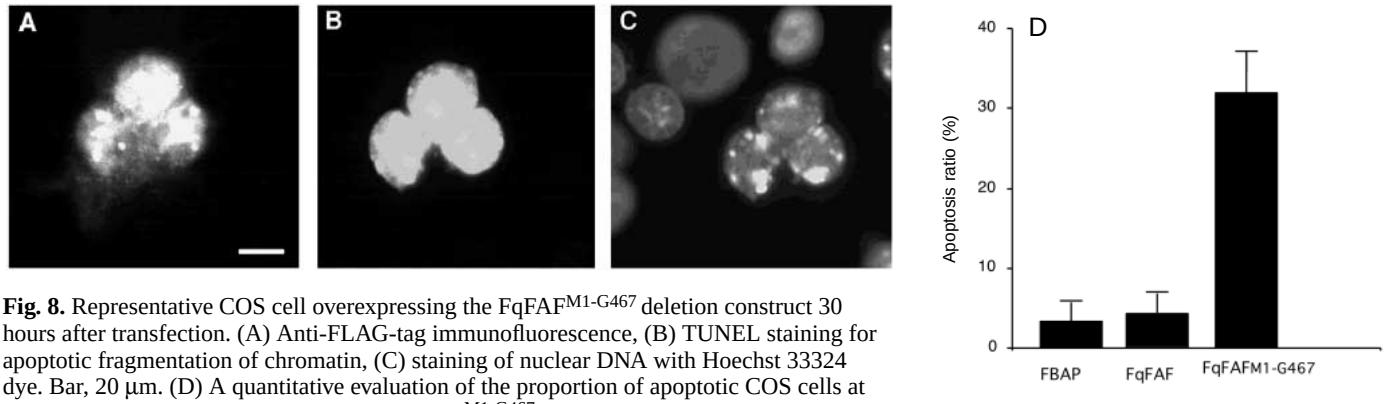

Fig. 8. Representative COS cell overexpressing the FqFAF$^{M1\text{-}G467}$ deletion construct 30 hours after transfection. (A) Anti-FLAG-tag immunofluorescence, (B) TUNEL staining for apoptotic fragmentation of chromatin, (C) staining of nuclear DNA with Hoechst 33324 dye. Bar, 20 μm. (D) A quantitative evaluation of the proportion of apoptotic COS cells at 30 hours after transfection with pCMV2-FqFAF$^{M1\text{-}G467}$, or control constructs pCMV2-BAP or pCMV2-FqFAF. Data from three independent experiments are presented.

(FqFAF$^{M1\text{-}G467}$) that lacks the carboxy-terminal amino acids Q468 to E648 (including the α-helix 2; Fig. 7) underwent apoptotic cell death, independent of the cell line used (COS, 3T3). At 16 hours after transfection cells demonstrated a normal nuclear morphology with the mutant qFAF localizing to the cell nucleus, however, from 24 hours to 30 hours membrane blebbing was apparent and by 48 hours a high proportion of cells with misshaped, pyknotic nuclei and condensed chromatin were visible. qFAF protein could be found at this time point in spot like aggregates distributed over the whole cell which was possibly due to a disruption of the nuclear envelope (Fig. 6C). By 72 hours after transfection no viable cells expressing FqFAF$^{M1\text{-}G467}$ could be found in the cultures. When we employed the TUNEL technique and double immunofluorescence with anti-FLAG antibody, the cellular changes were characteristic of apoptotic cell death (Fig. 8A-C). The electrophoretic separation of cellular DNA revealed the typical laddering pattern of DNA fragments seen during apoptosis, which is consistent with the results obtained by the TUNEL technique (not shown). At 48 hours after transfection of the FqFAF$^{M1\text{-}G467}$ deletion construct about 30% of transfected COS cells were apoptotic, whereas the amount of apoptotic cells in control experiments, in which either full length FqFAF or FBAP were used, remained at a constant low level (Fig. 8D). We therefore conclude that the amino-terminal fragment of qFAF, FqFAF$^{M1\text{-}G467}$, is capable of initiating the programme of apoptotic cell death.

DISCUSSION

We report here the cloning of the avian homologue of the FAS associated factor FAF1 (qFAF) from quail embryonic cells during induction of mesoderm in vitro, and the characterization of two novel domains within FAF which mediate nuclear localization and apoptosis. The amino acid sequence of FAF1 is highly conserved between mammals and birds, which may indicate an essential function for this protein. Like FAF1, qFAF can be co-precipitated with the apoptosis signal transducing receptor FAS/Apo-1 from cells, which overexpress both FAF and FAS. When qFAF is overexpressed in different cell lines, it is found to be localized to the nuclei. This finding raises the questions of what function this factor may exert in the nucleus, and whether this function is related to the enhancement of FAS-mediated apoptosis observed in certain cell lines (Chu et al., 1995). The fact that qFAF is induced in pluripotent embryonic cells by FGF during the early steps of mesoderm induction, but not in differentiated cells which express qFAF continuously, may provide an option for differentiated embryonic cells to enter the apoptotic pathway. In fact, at E1, when the qFAF message was maximally induced, apoptotic cells can be readily detected in avian embryos (T. Fröhlich and I. Flamme, unpublished data). However, it is not clear whether FAS or other members of the TNF-receptor family are involved in this early embryonic apoptosis. Moreover, the mechanism by which FAF interacts with FAS is still not clear. Coexpression of both FAS and wild-type qFAF did not result in translocation of qFAF to the plasma membrane, and by coimmunoprecipitation, a domain of qFAF which is responsible for the association with FAS could not be identified, because no significant differences were seen when either the wild-type ore the deletion mutants were coexpressed (data not shown). Therefore, possibly more than one distinct domain of qFAF are involved in the association with FAS.

Another hint into the possible role of FAF comes from homology searches of protein databases which have revealed remarkable sequence homologies between FAF and proteins which are involved in the ubiquitination pathway (Becker et al., 1997; and our own results). One of these homologues is a putative ubiquitin conjugating enzyme (Ubc) from *C. elegans*. Indeed, members of the Ubc family have recently been demonstrated to be associated with the FAS receptor (Becker et al., 1997; Wright et al., 1996). It was speculated that Ubc family proteins are involved in regulating the half-life time of the receptor/ligand complex, thus limiting the extent and duration of intracellular signalling. Thus, FAF may be involved in the ubiquitination cascade, which comprises activation of ubiquitin by an ubiquitin activating enzyme, transfer to a cysteine residue of an Ubc, and, finally, the ligation to a target sequence of a protein destined to be degraded by a proteasome (for review see Hochstrasser, 1995). qFAF/FAF1, however, does not include the Ubc core domain containing the conserved cysteine group that serves as the ubiquitin acceptor/donor (Tong et al., 1997). This domain is present in its entirety in the translation product of the *C. elegans* C28G1 cosmid, to which

qFAF/FAF1 shares homology and which is likely to represent a new member of the Ubc family (Wilson et al., 1994). The relation to another translation product from the *C. elegans* cosmid clone F48A11.5 is more extensive: the entire carboxy-terminal halves of both molecules show extensive sequence homology. From these data it is proposed that the F48A11.5 translation product might be the FAF homologue from *C. elegans* (Wilson et al., 1994). The degree of homology of the aligned carboxy-terminal part of qFAF is even higher for this molecule than for the putative *C. elegans* Ubc, and both *C. elegans* molecules are more closely related to qFAF/FAF1 than to each other.

Overexpression of the wild-type qFAF protein resulted in a homogeneous distribution over interphase nuclei, but exclusion from nucleoli. The nuclear distribution of qFAF is dependent on a distinct α-helical domain of 35 amino acids in the amino-terminal half of the protein (S275 to V309) which we have found to be both necessary and sufficient for nuclear translocation. Although a variety of nuclear import mechanisms may exist, only two of them have been elucidated in detail: The classical, importin mediated pathway depends on clusters of basic amino acids found in a large variety of nuclear proteins which are subsumed under the term 'classical nuclear localization signal' (NLS), and the M9 dependent import pathway which requires the M9 domain, a stretch of hydrophobic amino acids, principally found in hnRNPs (for review see Görlich, 1997; Yoneda, 1997). No homologies with either the classical NLS or the characteristics of the M9 domain were found in the qFAF nuclear translocation domain. Therefore, there are at least three possibilities, which may explain, how qFAF is transported into the nucleus: (1) it is either transported 'piggy-back' by interacting with an unidentified protein that uses one of the known nuclear transport mechanisms; specific protein-protein interactions of this kind have been described to contribute to the nuclear localization of several proteins (Sommer et al., 1991; Zhao and Padmanabhan, 1988); (2) the NLS of qFAF is an unknown target of one of the known transport mechanisms, or (3) the NLS of qFAF employs a novel, as yet undescribed transport mechanism.

The nuclear localization of qFAF raises the question whether the function of FAF in the nucleus is modulated by FAS. Data from our anti-phosphoserine blots support the conclusion that the function of qFAF might be regulated by serine phosphorylation and therefore might be targeted by an unknown signalling event. Unexpectedly, overexpression of a qFAF deletion mutant ($FqFAF^{M1\text{-}G467}$) which lacks the carboxy terminus of the molecule including the α-helix 2, led to apoptosis of transfected cells. Together with the nuclear localization of qFAF this supports the concept that qFAF has a more general cellular function than just regulating FAS signal transduction. The apoptosis inducing effect of overexpressed $FqFAF^{M1\text{-}G467}$ may be mediated by a dominant negative effect of this mutant protein on endogenously expressed qFAF. In contrast, overexpression of all amino-terminal mutants as well as of a shorter qFAF deletion mutant, $FqFAF^{M1\text{-}V309}$, which lacked the complete carboxy-terminal half from A310 to E648 (including the C28G1 homology domain and the α-helix 2), had no effect on the viability of transfected cells. We conclude from these results that the intra-nuclear function of qFAF is executed via the cooperation of functional domains located in the carboxy-terminal half of the protein. The main difference between $FqFAF^{M1\text{-}V309}$ and $FqFAF^{M1\text{-}G467}$ is that the latter still contains part of the C28G1-like domain from which it can be inferred that this domain mediates a key function which is compromised in the absence of the carboxy terminus. With regard to the possible function of FAF in FAS mediated apoptosis signalling, it is conceivable that the C28G1 homology domain exerts an apoptosis-inducing function upon FAS signalling which is otherwise repressed by interaction with the downstream domain.

The peptide sequence deleted in the $FqFAF^{M1\text{-}G467}$ mutant contains a broad, acidic, α-helical region which shares remarkable sequence similarity with the p150 subunit of chromatin assembly factor (CAF). In the p150 subunit of CAF the clusters of amino acids with acidic side chains are thought to play a role in histone binding (Kaufman et al., 1995).

Whether qFAF/FAF1, like CAF, binds to basic nuclear proteins via its hydrophilic α-helical domain 2, remains to be examined.

In conclusion, we have characterized novel functional domains in FAF that confer nuclear targeting and induce apoptosis. Early embryonic inducibility, ubiquitous expression, FAS-association, nuclear location, serine phosphorylation, structural relationship to proteins of the ubiquitination pathway and induction of apoptosis implicate this protein in complex and essential cellular functions.

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