JOURNAL OF VIROLOGY, Feb. 1997, p. 1576–1582 Vol. 71, No. 2
0022-538X/97/$04.00+0

Interaction of an Adenovirus 14.7-Kilodalton Protein Inhibitor of Tumor Necrosis Factor Alpha Cytolysis with a New Member of the GTPase Superfamily of Signal Transducers

YONGAN LI, JIAN KANG, AND MARSHALL S. HORWITZ*

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461

Received 3 July 1996/Accepted 14 October 1996

The adenovirus (Ad) 14.7-kDa E3 protein (E3-14.7K), which can inhibit tumor necrosis factor alpha (TNF-α) cytolysis, was used to screen HeLa cell cDNA libraries for interacting proteins in the yeast two-hybrid system. A new member of the low-molecular-weight (LMW) GTP-binding protein family with Ras and ADP-ribosylation factor homology was discovered by this selection and has been named FIP-1 (14.7K-interacting protein). FIP-1 colocalized with Ad E3-14.7K in the cytoplasm especially near the nuclear membrane and in discrete foci on or near the plasma membrane. Its interaction with E3-14.7K was dependent on the FIP-1 GTP-binding domain. The stable expression of FIP-1 antisense message partially protected the cells from TNF-α cytolysis. FIP-1 was associated transiently with several unknown phosphorylated cellular proteins within 15 min after treatment with TNF-α. FIP-1 mRNA was expressed ubiquitously but at higher levels in human skeletal muscle, heart, and brain. In addition to homology to other LMW GTP-binding proteins, FIP-1 has regions of homology to two prokaryotic metalloproteases. However, there was no homology between FIP-1 and any of the recently isolated death proteins in the TNF-α or Fas/APO1 cytolytic pathway and no interaction with several members of the Bcl-2 family of inhibitors of apoptosis. These data suggest that FIP-1, as a cellular target for Ad E3-14.7K, is either a new intermediate on a previously described pathway or part of a novel TNF-α-induced cell death pathway. FIP-1 has two consensus sequences for myristoylation which would be expected to facilitate membrane association and also has sequences for Ser/Thr as well as Tyr phosphorylation that could affect its function.

Viruses code for proteins that regulate the activity of cytokines and are thought to facilitate acute infection or promote persistence in animal hosts (32). Human adenovirus (Ad) types 2 and 5 contain an early transcription region 3 (E3) that codes for three proteins that inhibit the cytolytic effects of tumor necrosis factor alpha (TNF-α) (48). In some cells, TNF-α cytolysis requires cofactors such as cycloheximide or the presence of another early Ad protein, E1A. In all of these conditions, the action of TNF-α is inhibited by an Ad 14.7-kDa E3 protein (E3-14.7K) expressed either after viral infection or from transfected plasmids (20, 22, 24). There are two other Ad E3 proteins, of 10.4 and 14.5 kDa (E3-10.4K and E3-14.5K, respectively), which function as a heterotrimer to inhibit TNF-α-mediated cytolysis (21). E3-10.4K can also accelerate the internalization of the epidermal growth factor receptor (8), which belongs to the TNF-α receptor (TNFR) family. The control of TNF-α cytolysis presumably is important to Ads because there is yet another region, Ad E1B, that codes for a 19-kDa protein (E1B-19K) that inhibits cell death induced by TNF-α (47). Ad E1B-19K is a structural and functional homolog of Bcl-2, a cellular protein that inhibits apoptosis (12).

Ad E3-14.7K has an effect in vivo, as shown by using viral deletion mutants of the Ad E3-14.7K (plus E3-10.4K and E3-14.5K) anti-TNF proteins which increased polymorphonuclear leukocytic infiltration in a cotton rat model of pneumonia induced by Ad type 5 (19). The effects of isolated Ad E3-14.7K were also demonstrated when its gene was cloned into vaccinia virus (VV) in various combinations with TNF-α. In this model of VV-induced pneumonia in mice, it was shown that E3-14.7K antagonized the effects of TNF-α, measured by an increase in pulmonary inflammation, viral titers in lung tissue, and mortality (44). The enhancing effects of Ad E3-14.7K on VV disease were observed even in SCID mice, which indicated that neither B nor T cells were necessary for these effects (43).

TNF-α is a proinflammatory cytokine which has a number of biologic functions, including the control of viral infection (reviewed in reference 49). All of the functions of TNF-α are mediated through specific receptors on the cell surface. There are two TNFRs, which contain 55- and 70-kDa polypeptides, respectively (42). The 55-kDa TNFR undergoes interactions to form a trimer that has an extracellular, transmembrane, and short intracytoplasmic domain (39), and the molecules that interact with the cytoplasmic domain are being elucidated. A death-promoting molecule, called TRADD, was recently isolated by using the 55-kDa TNFR intracellular domain in the yeast two-hybrid system (27). Two other proteins, MORT1 (also named FADD) and RIP (3, 11, 41), which have death domain homology with TRADD, were identified initially by their interactions with Fas/APO1, another member of the TNFR family (15). The interactions among these death proteins and the their functional significance have been recently demonstrated (25, 26). Several other molecules, such as TRAF1 and TRAF2 (34), TRAF3 (10, 28, 33), and TRAP1 and TRAP2 (40), have also been shown to interact with TNF-α receptors and are thought to be important for transmitting signals from the receptor to downstream targets. Although the identification of these molecules has greatly enhanced our understanding of the early steps of TNF-α signal transduction, less is known about downstream steps that appear to activate

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2083 or 2230. E-mail: horwitz@aecom.yu.edu.

specific proteases that are postulated to be the proximal effectors of cell death (45).

The mechanism of Ad E3-14.7K inhibition of TNF-α cytolysis is unknown; however, this viral protein can prevent the TNF-α-stimulated release of arachidonic acid by the action of phospholipase A2 (PLA2) (51). The effect is indirect, as E3-14.7K does not inhibit the enzymatic activity of PLA2. There is also evidence that PLA2 may not be directly involved in TNF-α cytolysis in some cases (1). Ad E3-14.7K does not affect either of the two TNFRs (24), nor does it inhibit any of the TNF-α transcriptional effects mediated through NFκB (16). Available data as discussed above suggested that the anti-TNF function of E3-14.7K is independent of other viral proteins; therefore, E3-14.7K most probably inhibits TNF cytolysis by interacting with cellular proteins involved in TNF signaling pathways.

The goal of this study was to define the host cell protein target(s) that binds to Ad E3-14.7K and eventually to determine its mechanism of action. The Ad E3-14.7K protein was used successfully in the yeast two-hybrid system to find four host cell-interacting proteins; one of these, called FIP-1, which is homologous to a new family of low-molecular-weight (LMW) GTPase proteins, is the subject of this report.

(The data in this paper are from a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.)

MATERIALS AND METHODS

Cell lines. The human 293 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. The mouse fibroblast C3HA cell lines (obtained from Linda Gooding, Emory University) with or without constitutively expressed Ad E3-14.7K were maintained in Dulbecco's modified Eagle's medium (DMEM) with the supplements specified above.

To make a stable cell line which expressed the FIP-1 antisense message from the cytomegalovirus promoter, mouse C3HA cells were transfected by using LipofectAmine as described by the manufacturer. Forty-eight hours after transfection, the cells were trypsinized and replated in DMEM containing 1 mM G418 (Gibco-BRL). When the cells became confluent, they were trypsinized again and replated at fivefold dilution in DMEM with 1 mM G418. This trypsinization and replating process was repeated three more times. For controls, pcDNA3 without the FIP-1 sequence was transfected into the same cells, which underwent the same selection process. As a control for successful selection, the untransfected cells were processed similarly and were completely killed by this selection process.

Plasmid constructs. pBMT-14.7K, the bait vector containing Ad E3-14.7K, was constructed by cloning Ad2 E3-14.7K cDNA in frame into the C terminus of the LexA DNA-binding domain of the bait vector pBTM116 (obtained from R. Sternglanz, State University of New York at Stony Brook). pcDNA-T7 was constructed by inserting the T7 tag expressed from complementary oligonucleotides into pcDNA-3 (InVitrogen) linearized with restriction endonucleases *Hin*dIII and *Eco*RI. pcDNA-T7-FIP-1 was produced by inserting the FIP-1 cDNA sequence between the *Bam*HI and *Xho*I sites 3′ to the T7 tag. The fidelity of the constructs was confirmed by sequencing. The vector expressing the FIP-1 antisense message was constructed as follows. The FIP-1 antisense DNA message was amplified by PCR using primers 5′ GCTCTAGAGCTCTAGAACTA GTGGATCCC 3′ and 5′ GCCTCGAGCCGTCTTTGCAATCTGATACG 3′. The PCR product was purified and digested with *Xba*I and *Xho*I and cloned into the *Xba*I/*Xho*I-digested plasmid pcDNA3 (InVitrogen). The GST-14.7K expression plasmid was constructed by cloning Ad E3-14.7K into the C terminus of the glutathione-*S*-transferase (GST) vector pGEX5X-1 (Pharmacia) at the *Eco*RI/*Xho*I sites. For in vitro transcription and translation of FIP-1, the *Bam*HI/*Xho*I-released FIP-1 from the target vector was cloned into pCITE-4b (Novagen) at the corresponding sites.

The deletion mutants of FIP-1 were constructed by using clone H7a, which lacks the first seven amino acids (FIP-1Δ7). FIP-1Δ35 was constructed by digesting the clone with *Nru*I and *Sma*I and religating the larger fragment. FIP-1CΔ271 was constructed by digesting the clone with *Sun*I/*Xho*I, which was blunt ended with Klenow enzyme and religated.

Yeast two-hybrid screening and the specificity test. The pBMT-14.7K construct was used to screen the Gal4 activation domain-tagged target HeLa library as described previously (36, 37). The screening was done in *Saccharomyces cerevisiae* L40, which is selectable by both His auxotrophy and β-galactosidase (β-Gal) activity in the presence of 60 mM 3-aminotriazole. From approximately 10^7 colonies screened, 12 were found to contain the FIP-1 insert. As a test for specificity, the isolated clones were cotransformed with various heterologous baits which were cloned into the same bait vector. After transformation, their β-Gal activities and abilities to grow on His-deficient medium were assayed. The heterologous baits included human lamin (hLamin), the transactivation domain (TAD) or basic helix-loop-helix (bHLH) domain of mouse c-*myc* (mMyc), and mouse MaxI (mMaxI) (37). To test whether the FIP-1 interacted with Bcl-2 or Ad E1B-19K, FIP-1 was cotransformed with either Bcl-2 or E1B-19K and tested by the β-Gal filter assay as described previously (6). BIK-1, which was previously shown to interact with Ad E1B-19K and Bcl-2, was used as a positive control. The HeLa cDNA library was a gift from Greg Hannon and David Beach, Cold Spring Harbor Laboratory. hLamin, TAD, bHLH, and mMaxI were kindly provided by Ron DePinho and are described in reference (37). Bcl-2, E1B-19K, and BIK-1 were generously made available to us by R. Chinnaduri, St. Louis University, St. Louis, Mo.

Coimmunofluorescent labeling of mouse cells containing a constitutively expressed Ad E3-14.7K and a transiently transfected T7/FIP-1 fusion protein. C3HA cells containing the Ad E3-14.7K gene behind a metal-inducible promoter were grown on chamber slides (Novagen) and were transfected with 1 μg of pcDNA-T7-FIP-1 DNA per well, using the LipofectAmine technique. Patterns of expression of FIPs in spatial relation to Ad E3-14.7K were observed by analysis of double immunofluorescence (rhodamine and fluorescein) on a confocal microscope. The antibody to E3-14.7K was a generous gift from William Wold, St. Louis University, St. Louis, Mo.

Preparation and use of an Ad E3-14.7K/GST fusion protein. The expression and absorption of the fusion protein to GST-beads were previously described (14). The FIP-1 protein was labeled with [^{35}S]methionine (Amersham) with the Single-Tube Protein System (Novagen) as described by the manufacturer. An aliquot of labeled FIP-1 was incubated at 4°C for 2 h with either GST alone or GST-14.7K preabsorbed on GST-beads. Following the incubation, the beads were washed three times with 150 mM NaCl–NETN buffer (14), twice with 500 mM NaCl–NETN buffer, and three times with 150 mM 150 mM NaCl–NETN buffer. After the washes, the beads were resuspended in Laemmli buffer, and equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Coimmunoprecipitation of FIP-1 and cellular phosphoproteins. Human 293 cells on 60-mm-diameter dishes were transfected with 2 μg of pcDNA-T7-FIP-1 plasmid DNA by using the LipofectAmine technique as described by the manufacturer (Gibco-BRL). Forty hours after transfection, cells were washed three times with phosphate-free RPMI 1640 medium and incubated at 37°C for 2 h in phosphate-free RPMI 1640 supplemented with 5% dialyzed fetal bovine serum. Cells were washed three times again with phosphate-free RPMI 1640 and labeled with carrier-free [^{32}P]phosphate (1 mCi/ml) for 2 h at 37°C. Cells were treated for 15 or 60 min with 400 U of TNF-α (Gibco-BRL) per ml. Treated cells were washed with ice-cold 0.9% (wt/vol) NaCl once and disrupted with ice-cold Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8], 1% NP-40, 150 μg of phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml). The lysate was cleared by centrifugation at maximum speed in a microcentrifuge, and 300 μl of each lysate was subjected to immunoprecipitation with 2 μg of T7 monoclonal antibody. After rocking for 1 h at 4°C, 30 μl of 50% (vol/vol) protein A-beads (Sigma) was added to the lysate, which was rocked for another hour. The beads were washed five times with NP-40 lysis buffer and resuspended in 30 μl of Laemmli buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris [pH 6.8], 0.001% bromphenol blue). After boiling, the samples were subjected to SDS-PAGE. The gel was dried and exposed to X-ray film for 2 h at room temperature. The dried gel was rehydrated and transblotted to nitrocellulose. The blot was preblocked with 1× phosphate-buffered saline–5% nonfat dry milk and interacted with T7 monoclonal antibody. After four 15-min washes with 1× phosphate-buffered saline–0.1% Tween 20, the blot was exposed to an anti-mouse secondary antibody for 1 h at room temperature and washed four times. Immunoreactive proteins were detected with a chemiluminescence reagent (Boehringer).

TNF-α cytolysis assays in a mouse cell line containing antisense FIP-1 RNA. To assay the inhibitory effect of the antisense FIP-1 on TNF-α-induced cytolysis, C3HA cells stably transfected with antisense FIP-1 were trypsinized and counted. Each of 12 wells of a 96-well plate was seeded with 8.2×10^4 cells. Twenty-four hours later, the cells were treated with the indicated concentration of TNF-α (Gibco-BRL) plus cycloheximide (25 μg/ml). Eighteen hours after treatment, the cells were fixed for 30 min with formal saline (10% formaldehyde, 0.8% NaCl), washed, and stained with crystal violet solution (0.1% crystal violet, 20% methanol). Stained cells were washed with tap water, dried, photographed, and dissolved in 100 μl of methanol. The number of cells remaining was quantified from the intensity of the stain determined by an enzyme-linked immunosorbent assay reader at 600 nm. The percentage of cells surviving (N) at each TNF-α concentration was calculated by using numbers obtained from wells treated with cycloheximide alone (Y), which were regarded as 100% surviving, and a number (A_0) from the vector-transfected cells treated with the highest concentration of TNF-α (500 U/ml) which killed all cells and was regarded as a blank: [$N = (X - A_0)/(Y - A_0)$, where X is the A_{600} reading from each TNF-α concentration].

TABLE 1. Specificity of FIP-1 protein interactions in the yeast two-hybrid system[a]

Lex A Hybrid (bait)	Gal4 Hybrid (target)	β-Gal activity (colony color)	His growth
Ad E3-14.7K	FIP-1	Blue	+
hLamin-C	FIP-1	White	−
mMyc-TAD	FIP-1	White	−
mMyc-bHLH	FIP-1	White	−
mMaxI	FIP-1	White	−
Ad E1B-19K	BIK-1	Blue	NS[b]
Bcl-2	BIK-1	Blue	NS
Ad E1B-19K	FIP-1	White	NS
Bcl-2	FIP-1	White	NS

[a] Two-hybrid screening and specificity tests were performed with FIP-1 and various baits as described previously (36, 37). BIK was described previously (5) and included as a positive control for the interaction with Ad E1B-19K and Bcl-2.
[b] NS, the combination is not selectable by His auxotrophy.

RESULTS

Ad E3-14.7K interacts with a novel human cell protein, FIP-1. Specific interactions between human cell proteins and the Ad E3-14.7K protein were detected by using a yeast (*S. cerevisiae*-based) complementation assay originally developed by Fields and Song (18). FIP-1, which interacted with Ad E3-14.7K, was isolated as a family of multiple overlapping clones which were identical at their 3′ ends but extended for various lengths toward the 5′ end of the gene. FIP-1 did not interact with a series of heterologous baits (hLamin-C, mMyc-TAD, mMyc-bHLH, or mMaxI) or with the Ad E1B-19K or Bcl-2 proteins, indicating that the cell proteins interacting specifically with the E3-14.7K did not overlap with the targets selected by interaction with the Ad E1B or Bcl-2 proteins (Table 1).

The sequence of FIP-1 is shown in Fig. 1A. Its amino terminus is highly homologous to yeast GTP-binding protein GTR1 (45% identity), a protein presumably involved in phosphate transport (7), with decreasing homology toward the carboxyl end. FIP-1 is also highly homologous to a *Caenorhabditis elegans* protein (ceT24F1) of unknown function (61% identity). The *C. elegans* protein has been postulated from sequencing the genome, and no function has yet been assigned to it. FIP-1 has various homologies within four regions that are contained in the LMW GTPase family of proteins such as the transforming protein p21/K-Ras-2 or in ADP-ribosylation factor family members (Fig. 1B), with the highest homology in the G-1 and G-3 domains. The G-2 region of FIP-1 is more similar to that contained in the G-protein elongation factor, α-subunit signal transduction protein. FIP-1 has a region of homology with two bacterial proteases (Fig. 1C) but not with proteases such as the interleukin-1β-converting enzyme (ICE) or its *C. elegans* homolog, CED3, which have been postulated to be effectors of cell death by apoptosis (50). Further sequence comparison of FIP-1 and other small GTP-binding proteins has suggested that FIP-1 belongs to a new family of small GTP-binding proteins (Fig. 2). Since each family of small GTP-binding proteins often has a specific function, we anticipate that the FIP-1 protein found in our studies may have some unique functions.

Ad E3-14.7K colocalizes in vivo and interacts in vitro with FIP-1. In addition to protein-protein interaction in the yeast

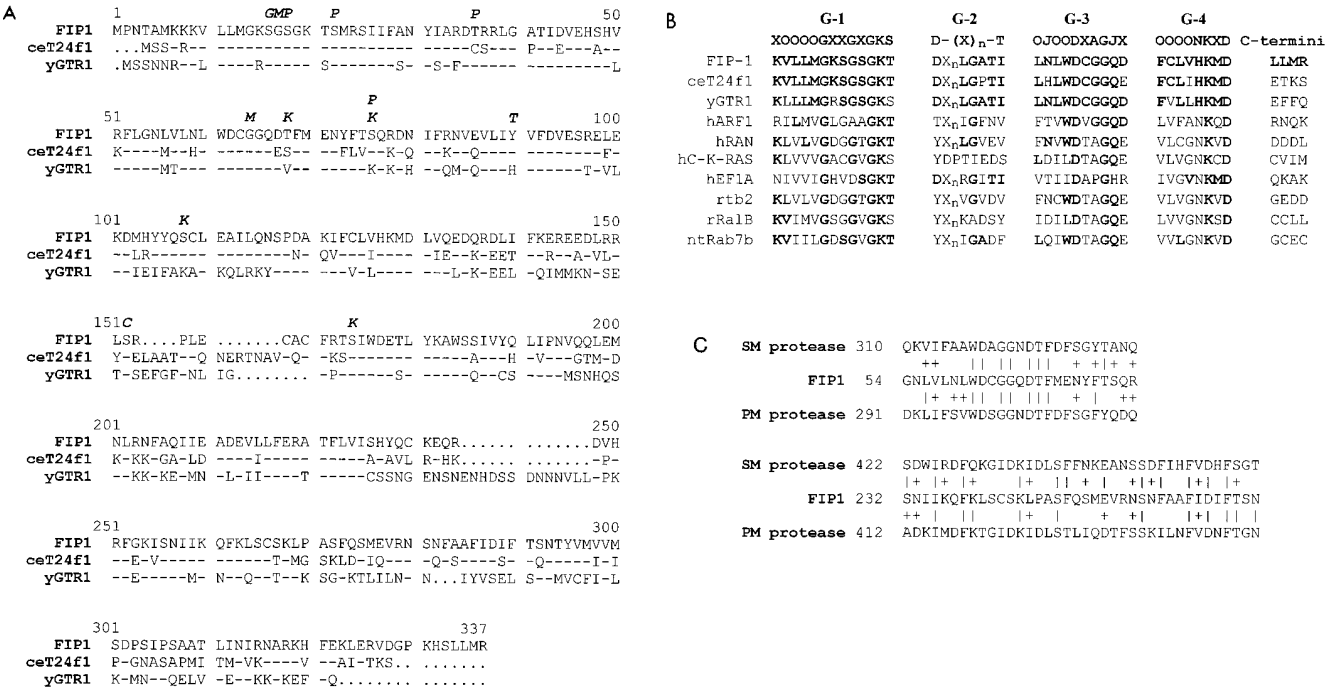

FIG. 1. Homology of FIP-1 to GTP-binding proteins and bacterial proteases. (A) Sequence comparison of FIP-1 to *S. cerevisiae* GTP-binding protein GTR1 (yGTR1) and a *C. elegans* putative protein (ceT24F1) proposed from genomic sequencing. Dashes indicate identical amino acids, and dots indicate gaps in the alignment. Putative posttranslational modification sites are marked above the residues: M, myristoylation; P, protein kinase c phosphorylation site; C, cyclic AMP- and cyclic GMP-dependent protein phosphorylation site; K, casein kinase II phosphorylation site; G, glycosaminoglycan attachment site; T, tyrosine kinase phosphorylation site. (B) Regions of homology in the consensus G-1 to G-4 domains defined by Bourne et al. (4) and the carboxyl terminus of the GTPase family of proteins compared to FIP-1. X, any amino acid; O, hydrophobic amino acid; J, hydrophilic amino acid. (C) Homology between FIP-1 and -2 bacterial metalloproteases. SM protease, *Serratia marcescens* protease (GenBank accession number S12164); PM protease, *Proteus mirabilis* protease (GenBank accession number U25950). Identical (|) and conservative (+) substitutions of amino acids are indicated. All sequence comparisons were done by using programs in the Genetic Computer Group package (University of Wisconsin) unless otherwise indicated.

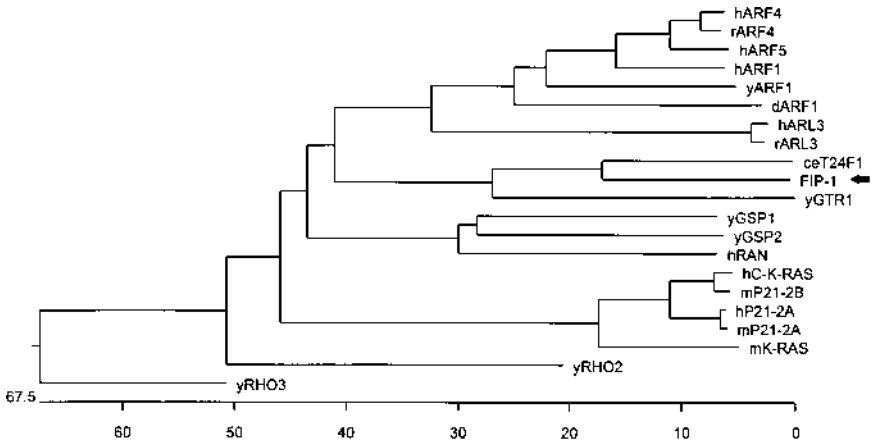

FIG. 2. Dendrogram of an alignment of FIP-1 with other small GTP-binding proteins. All sequences were retrieved from GenBank. The alignment was done by using the Clustal method included in the Megalign program of the DNAStar sequence analysis program package (gap penalty, 10; gap length penalty, 10). Prefixes: m, mouse; h, human; r, rat; y, yeast; ce, *C. elegans*.

two-hybrid selection system, evidence of FIP-1 interactions with Ad E3-14.7K was demonstrated both by in vivo colocalization by immunocytochemistry and by in vitro protein binding assays. Figure 3A demonstrates the localization of the Ad E3-14.7K in cells of a C3HA murine cell line, stably transfected with this Ad gene (24). Some of these cells also contain FIP-1, as shown by staining for transiently transfected expression in identical sections (Fig. 3B). The colocalization of Ad E3-14.7K and FIP-1 is primarily cytoplasmic, with accentuation around the nuclear membrane and in discrete foci near or within the plasma membrane. There also appears to be FIP-1 (Fig. 3B) in the nucleoplasm without apparent accompanying Ad E3-14.7K staining (Fig. 3A). FIP-1–14.7K interaction was also shown by creating a GST-14.7K fusion protein that was bound to GST-beads. The GST-14.7K protein selectively bound to FIP-1, allowing the latter to absorb to the glutathione-beads, whereas the protein derived from the GST vector alone failed to retain FIP-1 (Fig. 3C).

TNF-α signaling promotes the association of phosphoproteins with FIP-1. FIP-1 probably plays a direct role in TNF-α signaling, because it was shown to associate with several unknown phosphorylated cellular proteins upon treatment of the cells with TNF-α, and the association was transient. FIP-1, which itself appears to be minimally phosphorylated, formed a complex with other cellular phosphoproteins within 15 min after TNF-α addition (Fig. 4). One hour after TNF-α treatment, the amount of phosphate on FIP-1 had returned to the untreated state, and no association with other phosphoproteins could be detected by coimmunoprecipitation. The rapid and transient activation of FIP-1 by TNF-α suggests its direct role

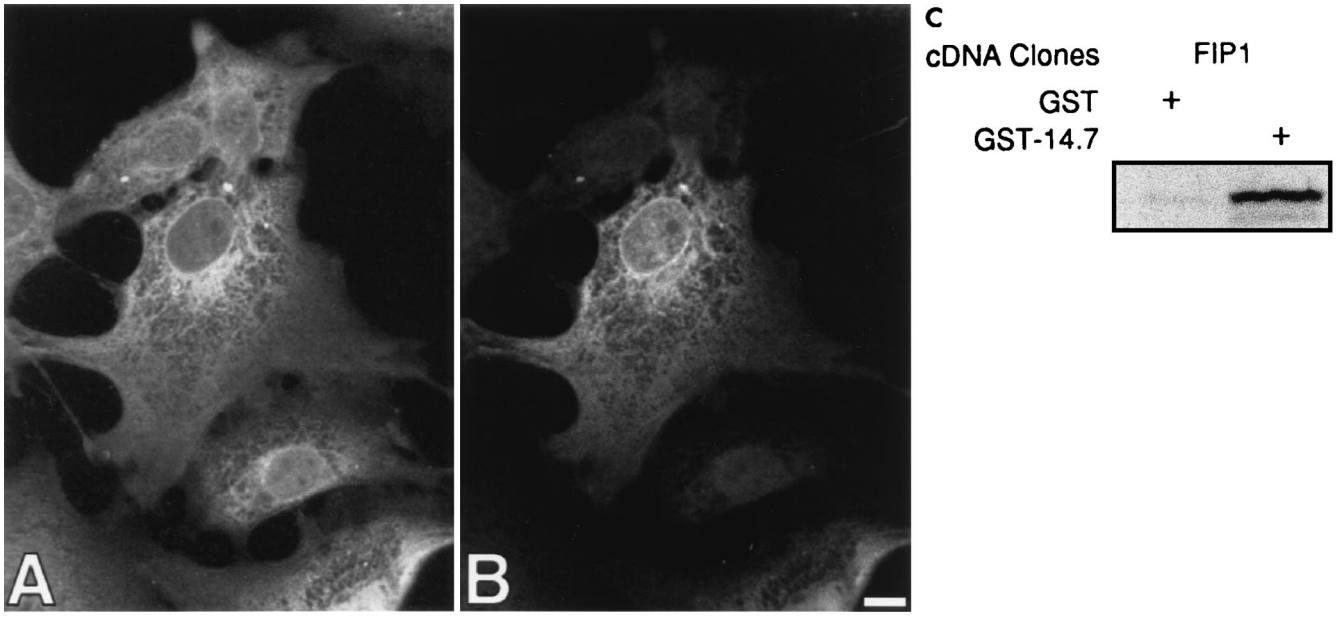

FIG. 3. FIP-1 interaction with Ad E3-14.7K both in vivo and in vitro. FIP-1 was cloned behind the cytomegalovirus promoter and coexpressed as a fusion protein with a T7 tag in the murine C3HA cell line constitutively expressing Ad E3-14.7K (24). (A) Ad E3-14.7K was visualized with a polyclonal antibody. (B) FIP-1 was visualized on identical cells with an antibody to T7. The bar indicates 10 μm. (C) The interaction of radiolabeled FIP-1 with the GST-14.7K fusion protein is described in Materials and Methods and is shown in lane 2; the GST protein alone as a negative control is shown in lane 1.

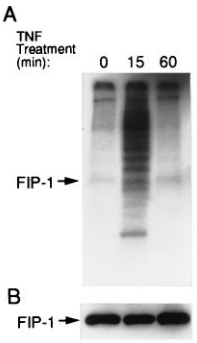

FIG. 4. FIP-1 association with cellular phosphoproteins induced by TNF-α treatment. (A) Forty hours after transfection, the cells were labeled with carrier-free [^{32}P]phosphate for 2 h, after which TNF-α (400 U/ml) was added. Cells were harvested either at the time of addition of TNF-α or 15 or 60 min thereafter and lysed. Cleared lysates were subjected to immunoprecipitation with an anti-T7 monoclonal antibody and analyzed by autoradiography after SDS-PAGE. (B) After exposure to X-ray film, the dried gel was rehydrated and transblotted. The amount of FIP-1 in each lane was detected by an anti-T7 monoclonal antibody.

as a TNF-α signal transducer and its involvement in the TNF-α cytolysis pathway.

Antisense FIP-1 RNA inhibits TNF-α-induced cytolysis. Further evidence supporting FIP-1's role in the TNF-α cytolysis pathway was provided by stably transfecting antisense FIP-1 into C3HA, a murine cell line which also could be protected against TNF-α cytolysis by E3-14.7K (Fig. 5) (24). The expression of antisense FIP-1 partially protected cells from TNF-α cytolysis compared with control cells stably transfected with vector alone.

FIP-1 is expressed in many human tissues. FIP-1 is ubiquitously expressed in a variety of human tissues assayed. A Northern blot of eight normal human tissues was analyzed by using a FIP-1 cDNA probe. The amount of FIP-1 mRNA detected in skeletal muscle was highest, and the least quantity appeared in lung tissue (Fig. 6). A single band of approximately 1.7 kb was detected. This size is consistent with the longest cDNAs selected from the HeLa library used in the yeast two-hybrid system. The ubiquitous expression of FIP-1 in many tissues is consistent with the expectation that proteins

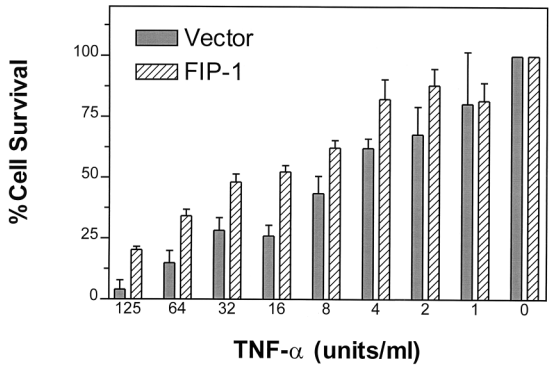

FIG. 5. Inhibitory effect of antisense FIP-1 on TNF-α cytolysis. To assay the inhibitory effect of the antisense FIP-1 RNA on TNF-α-induced cytolysis, stably transfected C3HA cells were seeded onto a 96-well plate and treated with the indicated concentrations of TNF-α plus cycloheximide (25 μg/ml). Eighteen hours after treatment, the cells were fixed with formalin-saline and stained with crystal violet solution. Stained cells were dissolved in methanol and quantified in an enzyme-linked immunosorbent assay reader at 600 nm. The percentage of cell survival was calculated as described in Materials and Methods. Averages of four identical samples are shown.

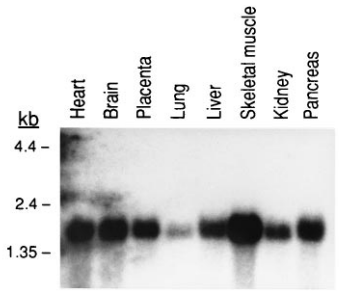

FIG. 6. Expression of FIP-1 mRNA in human tissues. A blot of mRNAs obtained from eight human organs as indicated was purchased from Clontech and hybridized under stringent conditions as described by the supplier. The FIP-1 cDNA used as probe was labeled with [^{32}P]dCTP (Amersham).

involved in cytolysis would be constitutively expressed in many cell types.

Functional domains of FIP-1 determined by deletion mutations. By deletion mapping, the GTP-binding domain in the G-1 region and the C terminus domain were found to be indispensable for the interaction of FIP-1 with Ad E3-14.7K (Fig. 7). Deletion of the first 34 residues at the N terminus or last 43 residues at the C terminus totally abolished their ability to interact with E3-14.7K in the yeast two-hybrid system, and the colonies did not turn blue. In addition, all of the clones identified in the two-hybrid screening contained these critical domains (data not shown). These data suggest either that GTP binding is required to maintain a correct conformation to facilitate the interaction of the C terminus with E3-14.7K or that both domains are directly involved in the Ad E3-14.7K–FIP-1 interaction.

DISCUSSION

By using the yeast two-hybrid system, we have identified a cellular protein, FIP-1, which interacts with the Ad anti-TNF-α protein E3-14.7K. The evidence for a complex containing these two proteins is derived from (i) a strong interaction in the yeast two-hybrid system with 12 independent and various-length FIP-1 isolates from a HeLa cDNA library, (ii) interaction between a GST-14.7K fusion protein and the FIP-1 protein, and (iii) colocalization of FIP-1 and Ad E3-14.7K intracellularly as assayed by confocal microscopy. FIP-1 also appears to be a component of the TNF-α signaling pathway because transiently expressed FIP-1 associates with a series of phosphoproteins upon the addition of TNF-α to cells. In addition, antisense FIP-1 RNA stably expressed in C3HA cells partially

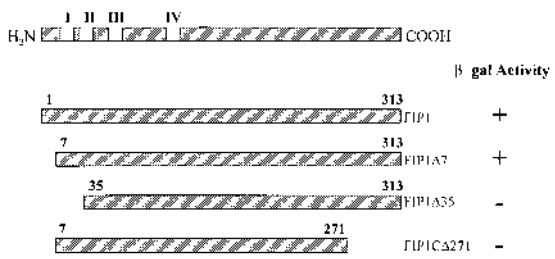

FIG. 7. Deletion mapping of FIP-1. All of the deletion mutants were derived from clone H7a, which lacks the first six amino acid residues (FIP-1Δ7) as described in Materials and Methods. The interactions between FIP-1 deletion mutants and E3-14.7K were assayed by using the yeast two-hybrid system as described in Table 1, footnote *a*. The positions of the four G boxes described are indicated by the roman numerals.

prevents cytolysis upon the addition of TNF-α to these cells. The partial protection against TNF-induced cytolysis achieved by expressing antisense FIP-1 RNA may be due to low levels of the inhibitory RNA or to the requirement for simultaneous inhibition of the synthesis of other host cell proteins in the TNF-α-induced complex. We already know that there are four FIPs, and the protective effects of inhibiting their synthesis may be additive.

FIP-1 appears to belong to the LMW GTPase superfamily. The major difference in FIP-1 from Ras and many other members of the GTPase family is that FIP-1 has a longer C terminus but lacks a common Cys within the last four amino acids at the carboxyl terminus. Sequence comparison has suggested that FIP-1 belongs to a new family of small GTP-binding proteins. Since each of these families often has its own unique function, it is expected that the FIP-1 family also may have some functional uniqueness. Although the absence of a Cys near the carboxyl terminus of FIP-1 precludes posttranslational modification with a prenylated or geranylgeranyl lipid at that site, the presence of putative myristoylation sites at residues 17 and 64 could still allow the addition of myristylate (*M* in Fig. 1A) to the molecule. These modifications would result in hydrophobic domains that may promote protein attachment to membranes (9). Some of the previously described membrane associations of LMW GTPases are on the cell surface, while others are in compartments such as the endoplasmic reticulum or Golgi complex. Further morphologic studies will be required to identify the perinuclear organelle that contains the FIP-1/14.7K complexes. The FIP-1/14.7K also appears to colocalize at discrete areas in or near the plasma membrane. The nucleoplasm also appears to contain FIP-1; however, there does not seem to be significant amounts of E3-14.7K in this compartment by morphologic criteria, although there is a report identifying Ad E3-14.7K in the nucleus by cell fractionation (22).

Although FIP-1 has a region of homology with two bacterial proteases, it has not yet been examined for any intrinsic protease activity. Since FIP-1 contains two domains which are homologous to bacterial metalloproteases, it is intriguing to postulate that FIP-1 may contain an intrinsic protease activity. This degradative function could be activated by cell surface signals and could further activate the downstream targets that lead to cell death. Proteases appear to be important in the induction of cell death. A number of CED3/ICE-like proteases have been identified recently, and they have been implicated in cell death (reviewed in reference 23). Furthermore, it has been suggested that cell death results from a cascade of protease activation (reviewed in reference 31). Recently, a novel protease, MACH/FLICE, that contains regions of homology with the CED3/ICE protease family was isolated by its binding to MORT1 (FADD) in the yeast two-hybrid system. MACH/FLICE is a key protein that bridges the molecules that bind to the intracytoplasmic domains of receptors with those that have protease function (2, 33a).

It has been observed that GTPγS affects the activity of PLA2 on zymogen granules in a calcium-dependent process (35). These observations suggest that different GTP-binding proteins might regulate PLA2 activity, an enzyme which has been postulated to be on the TNF-α pathway inducing cell death (30). Since Ad E3-14.7K can block the PLA2 activation by TNF-α (51), it is possible that FIP-1 is one of the GTP-binding proteins involved in regulating PLA2 activity.

FIP-1 does not appear to be homologous to any of the recently characterized proteins that bind to the cytoplasmic domains of either TNF-α or the Fas/APO1 receptors. Likewise, there is no homology to any members of the Bcl-2 family of activators or inhibitors of TNF-α-induced apoptosis. The FIP-1 GTPase either affects a different step of the putative pathway of TNF-α-induced cytolysis or perhaps interacts with one of the recently discovered proteins on this or the Fas/APO1 pathways. Very likely, FIP-1 is involved in steps downstream from the proteins directly interacting with TNFRs. Recently, an LMW GTPase named RagA, with a structure identical to that of FIP-1, was selected from a human fetal brain library screened by using a PCR product of degenerate primers conserved in Ras-related small GTPases (38). RagA was shown to bind GTPγS. The study described in this report links this new GTPase to the TNF-α-induced cytolytic pathway and its viral inhibitor, Ad E3-14.7K.

ACKNOWLEDGMENTS

We acknowledge the invaluable assistance of Michael Cammer of the Analytical Imaging Facility, a shared facility of the Albert Einstein College of Medicine Institutional Cancer Center.

This research was supported by grant P30-CA13330 (M.S.H.) and National Institutes of Health training grant 5T32 CA09060 (Y.L.).

REFERENCES

1. **Beyaert, R., and W. Fiers.** 1994. Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we do understand and what we do not. FEBS Lett. **340:**9–16. (Review.)
2. **Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach.** 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell **85:**803–815.
3. **Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Camonis, and D. Wallach.** 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J. Biol. Chem. **270:**7795–7798.
4. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117–127. (Review.)
5. **Boyd, J. M., G. J. Gallo, B. Elangovan, A. B. Houghton, S. Malstrom, B. J. Avery, R. G. Ebb, T. Subramanian, T. Chittenden, R. J. Lutz, and G. Chinnadurai.** 1995. Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene **11:**1921–1928.
6. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell **79:**341–351.
7. **Bun-Ya, M., S. Harashima, and Y. Oshima.** 1992. Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**2958–2966.
8. **Carlin, C. R., A. E. Tollefson, H. A. Brady, B. L. Hoffman, and W. S. M. Wold.** 1989. Epidermal growth factor receptor is down-regulated by a 10,400 mw protein encoded by the E3 region of adenovirus. Cell **57:**135–144.
9. **Casey, P. J.** 1994. Lipid modifications of G proteins. Curr. Opin. Cell Biol. **6:**219–225. (Review.)
10. **Cheng, G., A. M. Cleary, Z. S. Ye, D. I. Hong, S. Lederman, and D. Baltimore.** 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science **267:**1494–1498.
11. **Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit.** 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell **81:**505–512.
12. **Chiou, S. K., C. C. Tseng, L. Rao, and E. White.** 1994. Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J. Virol. **68:**6553–6566.
13. **Chittenden, T., E. A. Harrington, R. O'Connor, C. Flemington, R. J. Lutz, G. I. Evan, and B. C. Guild.** 1995. Induction of apoptosis by the Bcl-2 homologue Bak. Nature **374:**733–736.
14. **Chittenden, T., D. M. Livingston, and W. G. Kaelin, Jr.** 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell **65:**1073–1082.
15. **Cleveland, J. L., and J. N. Ihle.** 1995. Contenders in FasL/TNF death signaling. Cell **81:**479–482. (Review.)
16. **Dimitrov, T., M. Hannick, and W. S. Wold.** 1995. Personal communication.
17. **Farrow, S. N., J. H. White, I. Martinou, T. Raven, K. T. Pun, C. J. Grinham, J. C. Martinou, and R. Brown.** 1995. Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature **374:**731–733.
18. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
19. **Ginsberg, H. S., U. Lundholm-Beauchamp, R. L. Horswood, B. Pernis, W. S. M. Wold, R. M. Chanock, and G. A. Prince.** 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. Proc. Natl. Acad. Sci. USA **86:**3823–3827.

20. **Gooding, L. R., L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold.** 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53:**341–346.
21. **Gooding, L. R., T. S. Ranheim, A. E. Tollefson, L. Aquino, P. J. Duerksen-Hughes, T. M. Horton, and W. S. M. Wold.** 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. **65:**4114–4123.
22. **Gooding, L. R., I. O. Sofola, A. E. Tollefson, P. J. Duerksen-Hughes, and W. S. M. Wold.** 1990. The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. J. Immunol. **145:**3080–3086.
23. **Henkart, P. A.** 1996. ICE family proteases: mediators of all apoptotic cell death? Immunity **4:**195–201.
24. **Horton, T. M., T. S. Ranheim, L. Aquino, D. I. Kusher, S. K. Saha, C. F. Ware, W. S. M. Wold, and L. R. Gooding.** 1991. Adenovirus E3 14.7K protein functions in the absence of other adenovirus proteins to protect transfected cells from tumor necrosis factor cytolysis. J. Virol. **65:**2629–2639.
25. **Hsu, H., J. Huang, H.-B. Shu, V. Baichwal, and D. V. Goeddel.** 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity **4:**387–396.
26. **Hsu, H., B. H. Shu, M. G. Pan, and D. V. Goeddel.** 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell **84:**299–308.
27. **Hsu, H., J. Xiong, and D. V. Goeddel.** 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell **81:**495–504.
28. **Hu, H. M., K. O'Rourke, M. S. Boguski, and V. M. Dixit.** 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. J. Biol. Chem. **269:**30069–30072.
29. **Kiefer, M. C., M. J. Brauer, V. C. Powers, J. J. Wu, S. R. Umansky, L. D. Tomei, and P. J. Barr.** 1995. Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature **374:**736–739.
30. **Kolesnick, R., and D. W. Golde.** 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell **77:**325–328. (Review.)
31. **Martin, S. J. and D. R. Green.** 1995. Protease activation during apoptosis: death by a thousand cuts? Cell **82:**349–352. (Review.)
32. **McFadden, G.** 1995. Getting to know you: viruses meet CD40 ligand. Nat. Med. **1:**408–409. (Comment.)
33. **Mosialos, G., M. Birkenbach, R. Yalamanchili, T. VanArsdale, C. Ware, and E. Kieff.** 1995. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell **80:**389–399.

33a. **Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell **85:**817–827.

34. **Rothe, M., S. C. Wong, W. J. Henzel, and D. V. Goeddel.** 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell **78:**681–692.
35. **Rubin, R. P., M. Withiam-Leitch, and S. G. Laychock.** 1991. Modulation of phospholipase A2 activity in zymogen granule membranes by GTP[S]; evidence for GTP-binding protein regulation. Biochem. Biophys. Res. Commun. **177:**22–26.
36. **Schreiber-Agus, N., L. Chin, K. Chen, R. Torres, G. Rao, P. Guida, A. I. Skoultchi, and R. A. DePinho.** 1995. An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell **80:**777–786.
37. **Schreiber-Agus, N., L. Chin, K. Chen, R. Torres, C. T. Thomson, J. C. Sacchettini, and R. A. DePinho.** 1994. Evolutionary relationships and functional conservation among vertebrate Max-associated proteins: the zebra fish homolog of Mxi1. Oncogene **9:**3167–3177.
38. **Schurmann, A., A. Brauers, S. Mabmann, W. Becker, and H. G. Joost.** 1995. Cloning of a novel family of mammalian GTP-binding proteins (RagA, RagB, RagB′) with remote similarity to the ras-related GTPases. J. Biol. Chem. **270:**28982–28988.
39. **Smith, C. A., T. Farrah, and R. G. Goodwin.** 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell **76:**959–962. (Review.)
40. **Song, H. Y., J. D. Dunbar, Y. X. Zhang, D. Guo, and D. B. Donner.** 1995. Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor. J. Biol. Chem. **270:**3574–3581.
41. **Stanger, B. Z., P. Leder, T. H. Lee, E. Kim, and B. Seed.** 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell **81:**513–523.
42. **Tartaglia, L. A., and D. V. Goeddel.** 1992. Two TNF receptors. Immunol. Today **13:**151–153. (Review.)
43. **Tufariello, J., S. Cho, and M. S. Horwitz.** 1994. The adenovirus E3 14.7K protein, an antagonist of tumor necrosis factor cytolysis, increases the virulence of vaccinia virus in SCID mice. Proc. Natl. Acad. Sci. USA **91:**10987–10991.
44. **Tufariello, J., S. Cho, and M. S. Horwitz.** 1994. The adenovirus E3 14.7-kilodalton protein which inhibits cytolysis by tumor necrosis factor increases the virulence of vaccinia virus in a murine pneumonia model. J. Virol. **68:**453–462.
45. **Vandenabeele, P., W. Declercq, R. Beyaert, and W. Fiers.** 1995. Two tumour necrosis factor receptors: structure and function. Trends Cell Biol. **5:**392–399.
46. **White, E.** 1996. Life, death, and the pursuit of apoptosis. Genes Dev. **10:**1–15.
47. **White, E., P. Sabbatini, M. Debbas, W. S. M. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol. Cell. Biol. **12:**2570–2580.
48. **Wold, W. S. M., T. W. Hermiston, and A. E. Tollefson.** 1994. Adenovirus proteins that subvert host defenses. Trends Microbiol. **2:**437–443. (Review.)
49. **Wong, G. H. W., A. Kamb, and D. V. Goeddel.** 1992. Antiviral properties of TNF, p. 371–381. *In* B. Beutler (ed.), Tumor necrosis factors: the molecules and their emerging role in medicine. Raven Press, New York, N.Y.
50. **Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz.** 1993. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell **75:**641–652.
51. **Zilli, D., C. Voelkel-Johnson, T. Skinner, and S. M. Laster.** 1992. The adenovirus E3 region 14.7 kDa protein, heat and sodium arsinate inhibit the TNF-induced release of arachidonic acid. Biochem. Biophys. Res. Commun. **188:**177–183.