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The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family

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ABSTRACT **Exoenzyme S (ExoS), which has been implicated as a virulence factor of *Pseudomonas aeruginosa*, catalyzes transfer of the ADP-ribose moiety of NAD^+ to many eukaryotic cellular proteins. Its preferred substrates include Ras and several other 21- to 25-kDa GTP-binding proteins. ExoS absolutely requires a ubiquitous eukaryotic protein factor, termed FAS (factor activating ExoS), for enzymatic activity. Here we describe the cloning and expression of a gene encoding FAS from a bovine brain cDNA library and demonstrate that purified recombinant FAS produced in *Escherichia coli* activates ExoS in a defined cell-free system. The deduced amino acid sequence of FAS shows that the protein (245 residues, calculated molecular mass 27,743 Da) belongs to a highly conserved, widely distributed eukaryotic protein family, collectively designated as 14-3-3 proteins. Various functions have been reported for members of the 14-3-3 family, including phospholipase A_2 activity and regulation of tyrosine hydroxylase, tryptophan hydroxylase, and, possibly, protein kinase C activities. Identification of FAS as a 14-3-3 protein establishes an additional function for this family of proteins—the activation of an exogenous ADP-ribosyltransferase. Elucidation of the precise role of FAS in activating ExoS will contribute to understanding the molecular mechanisms by which *P. aeruginosa* causes disease.**

Exoenzyme S (ExoS) is a mono-ADP-ribosyltransferase secreted by the opportunistic pathogen *Pseudomonas aeruginosa* (1, 2). This enzyme has been associated with the establishment of infection and with tissue damage, although its precise role in pathogenesis has not been elucidated. *In vitro*, ExoS catalyzes the transfer of the ADP-ribose moiety of NAD^+ to many eukaryotic cellular proteins, but its preferred substrates are a subset of the small GTP-binding proteins of 21–25 kDa, including the products of the c-Ha-*ras*, *ral*, *rap1A*, *rab3*, and *rab4* genes (3, 4). These small GTP-binding proteins are thought to function as central components of the signaling pathways regulating intracellular vesicle transport, cell proliferation, and differentiation (5). ADP-ribosylation of the small GTP-binding proteins by ExoS is not known to alter their nucleotide interactions but may affect their interactions with other cellular proteins (3).

A eukaryotic cellular factor was found to be absolutely required for ADP-ribosylation of Ras and all other substrates by ExoS *in vitro* (6). The factor, designated FAS (*f*actor *a*ctivating Exo*S*), appeared to have a monomeric molecular mass of ≈29 kDa and an isoelectric point of 4.3–4.5. Activity was detected in all eukaryotic cells and tissues tested, but activity was absent from bacterial species.

The requirement of a eukaryotic protein cofactor for ADP-ribosyltransferase activity has also been found for certain other bacterial toxins. The intrinsically low ADP-ribosyltransferase activities of cholera toxin and *Escherichia coli* heat-labile toxin are stimulated by several orders of magnitude by a 21-kDa eukaryotic GTP-binding protein, termed ADP-ribosylation factor (ARF; refs. 7 and 8). ARFs appear to be abundant and ubiquitous and represent a subfamily of the highly conserved GTP-binding proteins of the Ras superfamily. Evidence is accumulating that ARF is associated with Golgi membranes and may be directly involved in the regulation of vesicle trafficking (9). It has also been reported that the ADP-ribosyltransferase activity of exoenzyme C3, secreted from *Clostridium botulinum*, is enhanced by a bovine brain cytosolic protein (10), but this activator protein has not been well characterized.

Like ARF, FAS is abundant and widely distributed throughout the eukaryotes. To determine the molecular identity and structural characteristics of FAS, we undertook the molecular cloning, expression, and characterization of its gene from a bovine brain cDNA library.§ Our purified recombinant FAS protein was as active as that purified from bovine testes in a defined assay system for ADP-ribosylation by ExoS. Sequence homology comparisons have revealed that FAS is a member of the multifunctional 14-3-3 protein family.

MATERIALS AND METHODS

Strains, Plasmids, and General Methods. *E. coli* strain XL1-Blue (Stratagene) was used for the propagation of plasmids, and *E. coli* BL21(DE3) was used for expression of proteins using bacteriophage T7 promoter-based overexpression vectors (11). Plasmid pET-15b was used as a parent expression vector (Novagen, Madison, WI). Restriction-enzyme mapping, subcloning, Southern hybridization, and gel electrophoresis procedures were done essentially as described (12) or as indicated.

Preparation of FAS-Specific Probe. To obtain amino acid sequence information, proteins in the partially purified FAS preparation were separated by SDS/11.25% PAGE and transferred to a nitrocellulose membrane (13). After brief staining with Ponceau S and washing with 1% acetic acid, the putative FAS protein band was excised for peptide sequencing. Because the N terminus of FAS is blocked (data not shown), three internal peptide sequences were obtained after *in situ* trypsin digestion and separation by reverse-phase HPLC (Harvard MicroChem Facility). These peptide sequences are underlined in Fig. 1. For PCR amplification of FAS DNA, two degenerate primers were designed based on the peptide sequences (5′ primer: 5′-CAAGAATTCGAGCTCTTYCTNATHCCNAAYGCNTC-3′; 3′ primer: 5′-TCAAGC TTCTGCAGCYTCRTCRAANGCNGT-3′). The underlined sequences indicate the restriction sites introduced,

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Abbreviations: ExoS, exoenzyme S; FAS, factor activating ExoS; PLA_2, phospholipase A_2; SBTI, soybean trypsin inhibitor; ARF, ADP-ribosylation factor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07955).

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                                                                                5'...TGCTGAGCCCCGTCCGTCCGCCG CCACCTACTCCGGACACAGAACATCCAGTC

ATGGATAAAAACGAGCTGGTACAGAAGGCC AAACTGGCCGAGCAGGCTGAGCGATATGAT GACATGGCAGCCTGCATGAAGTCTGTAACT GAGCAAGGAGCTGAATTATCCAATGAGGAG
MetAspLysAsnGluLeuValGlnLysAla LysLeuAlaGluGlnAlaGluArgTyrAsp AspMetAlaAlaCysMetLysSerValThr GluGlnGlyAlaGluLeuSerAsnGluGlu
                        10                                  20                                  30                                  40

AGGAATCTTCTCTCAGTTGCTTATAAAAAT GTTGTAGGAGCCCGTAGGTCATCTTGGAGG GTCGTCTCCAGTATTGAGCAAAAGACGGAA GGTGCTGAGAAAAAACAGCAGATGGCTCGA
ArgAsnLeuLeuSerValAlaTyrLysAsn ValValGlyAlaArgArgSerSerTrpArg ValValSerSerIleGluGlnLysThrGlu GlyAlaGluLysLysGlnGlnMetAlaArg
                        50                                  60                                  70                                  80
                                                                    PCR-5'primer
GAATACAGAGAGAAAATAGAGACCGAGCTA AGAGATATCTGCAATGATGTACTGTCTCTT TTGGAAAAGTTCTTGATCCCCAACGCTTCA CAAGCAGAGAGCAAAGTCTTCTATTTGAAA
GluTyrArgGluLysIleGluThrGluLeu ArgAspIleCysAsnAspValLeuSerLeu LeuGluLysPheLeuIleProAsnAlaSer GlnAlaGluSerLysValPheTyrLeuLys
                        90                                  100                 pep1            110                                 120
                                                                                                                                    <-
ATGAAAGGAGACTACTACCGCTACTTGGCT GAGGTTGCAGCTGGTGATGACAAGAAAGGG ATTGTGGACCAGTCACAGCAAGCATACCAA GAAGCTTTTGAAATCAGCAAAAAGGAAATG
MetLysGlyAspTyrTyrArgTyrLeuAla GluValAlaAlaGlyAspAspLysLysGly IleValAspGlnSerGlnGlnAlaTyrGln GluAlaPheGluIleSerLysLysGluMet
                        130                                 140  pep2                           150                                 160
sequencing primer R1                                                                                        PCR-3'primer
CAACCAACACATCCTATCAGACTGGGTCTG GCCCTTAACTTCTCTGTGTTCTATTATGAG ATTCTGAACTCCCCTGAGAAAGCCTGCTCT CTTGCAAAGACAGCATTTGATGAAGCCATT
GlnProThrHisProIleArgLeuGlyLeu AlaLeuAsnPheSerValPheTyrTyrGlu IleLeuAsnSerProGluLysAlaCysSer LeuAlaLysThrAlaPheAspGluAlaIle
                        170                                 180                                 190         pep3                    200

GCTGAACTTGATACATTAAGTGAAGAGTCA TACAAAGACAGCACGCTAATAATGCAGTTA CTGAGAGATAACTTGACATTGTGGACATCG GATACCCAAGGAGACGAAGCTGAAGCAGGA
AlaGluLeuAspThrLeuSerGluGluSer TyrLysAspSerThrLeuIleMetGlnLeu LeuArgAspAsnLeuThrLeuTrpThrSer AspThrGlnGlyAspGluAlaGluAlaGly
                        210                                 220                                 230                                 240

GAAGGAGGGGAAAATTAACCTGCCTTCCAA CTTTTGTCTGCCTCATTCTAAAATTTACACAGTAGACCCCGGAATTC...3'
GluGlyGlyGluAsnEND
```

FIG. 1. Nucleotide sequence of the cDNA encoding bovine brain FAS and predicted primary structure of FAS. Amino acid residues are numbered. Three peptide sequences obtained from the tryptic digestion of native FAS (pep1, pep2, and pep3) are indicated by a single underline. Location of the PCR primers and a sequencing primer, R1, are indicated by arrows. Amino acid residues identical among all known members of 14-3-3 family are in boldface type (see Fig. 5). The two residues of FAS that differ from that of the rat or sheep homologues are double underlined.

*Eco*RI and *Sac* I for the 5′ primer and *Hin*dIII and *Pst* I for the 3′ primer.

cDNA was synthesized from bovine brain total RNA (Clontech) using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) and used as a template to amplify the FAS-specific fragment by PCR (Perkin–Elmer/Cetus), according to Lee and Caskey (14). The resultant PCR product (≈0.29 kb) was purified with Mermaid kit (Bio 101, La Jolla, CA), subcloned into pUC19 (replacing *Sac* I–*Pst* I fragment, New England Biolabs), and sequenced with Sequenase version 2.0 (United States Biochemical).

cDNA Cloning and Sequencing. The 0.29-kb *Sac* I–*Pst* I fragment carrying FAS-specific sequence was purified and labeled with [α-^{32}P]dCTP (New England Nuclear; ref. 12) and used to probe a λgt10 bovine brain cDNA library (Clontech). High-stringency conditions were followed for library screening by plaque hybridization, according to the manufacturer's specifications. λ DNA from the positive plaques was isolated by the Lambda-Trap protocol (Clontech). After restriction mapping and Southern hybridization to localize the FAS-encoding gene in λ DNA, the inserts containing the FAS gene were subcloned into pUC19 at the *Eco*RI site. One of these clones, carrying a complete FAS sequence, is pHAF603. The DNA sequence of FAS was obtained by sequencing both strands in plasmid pHAF603.

Construction of Expression Vector for FAS. The cDNA insert encoding FAS in pHAF603 was amplified by PCR with the following primers: 5′ primer, 5′-GATTCAAGAATTCATATGGATAAGAACGAGC-3′; and 3′ primer, 5′-CGTATAGAATTCGTCGACGGTCATTAATTTTCCCCTCC-3′. In this way, two restriction sites (underlined) flanking each end were introduced (5′ end, *Eco*RI–*Nde* I; 3′ end, *Sal* I–*Eco*RI) into the final PCR product for ease of subsequent manipulations. For the expression of FAS, the 0.75-kb *Nde* I–*Eco*RI fragment of the PCR product containing the complete coding sequence of FAS was purified and ligated into the *Nde* I and *Eco*RI sites of pET-15b, generating pHAF612. The integrity of the coding sequence was verified by sequencing. This construct generated a translational fusion of FAS to the hexa-His tag present in pET-15b (Fig. 2).

Protein Purification. ExoS was purified from the culture supernatant of *P. aeruginosa* strain 388, and native FAS was partially purified from bovine testes, as described (6). The recombinant FAS was purified by affinity chromatography on a Ni^{2+}-charged histidine-binding column (Novagen). Briefly, the cell lysate, prepared from *E. coli* BL21(DE3)/pHAF612 by sonication, was cleared by centrifugation (16,000 × *g*, 20 min) and loaded onto the Ni^{2+}-charged column. The buffers and resins used were as specified by the manufacturer. After the column was washed with 100 mM imidazole (in 20 mM Tris·HCl, pH 7.9/500 mM NaCl), FAS was eluted with a 500 mM imidazole solution. The hexa-His tag at the N terminus of FAS was removed by incubating the FAS fusion with human thrombin (1 unit/mg of FAS) at 25°C

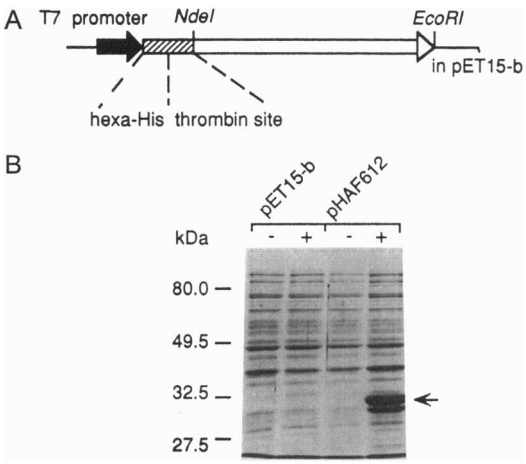

FIG. 2. Expression of bovine FAS in *E. coli*. (*A*) Expression vector for FAS, pHAF612, showing the T7 promoter region (black arrow), the region encoding the hexa-His tag and the thrombin site, and the 0.75-kb *Nde* I–*Eco*RI PCR fragment carrying FAS gene. (*B*) Expression of FAS in *E. coli* BL21(DE3) carrying pET-15b or pHAF612. *E. coli* samples were grown in LB liquid medium at 37°C. Isopropyl β-D-thiogalactoside (1 mM) was added when OD_{600} reached ≈1.0, and culture samples (40 μl) were collected for SDS/10% PAGE analysis after 4 hr of induction. The gel was stained with Coomassie brilliant blue for 30 min and destained overnight. Molecular mass markers are shown at left, and the location of FAS protein is indicated by the arrow.

overnight. The cleavage was monitored on SDS/PAGE by observing the mobility shift after removal of the 2-kDa tag. Free hexa-His tag was removed from the solution with a Centricon-10 centrifugal microconcentrator (Amicon). Any undigested hexa-His tag and thrombin in the reaction solutions were absorbed by Ni^{2+}-charged resin and benzamidine Sepharose-6B resin (Pharmacia). The purified FAS protein, with Gly-Ser-His remaining at its N terminus from the vector, was stored in HBS buffer (10 mM Hepes, pH 7.3/130 mM NaCl/aprotinin at 0.01 trypsin inhibitor unit/ml; ref. 6).

Assays. The FAS- and ExoS-dependent ADP-ribosyltransferase assay was done essentially as described (6). The reaction mixture contained [*adenylate*-^{32}P]NAD^+ (5 μM; New England Nuclear), either soybean trypsin inhibitor (SBTI; 100 μg/ml, Sigma) or Ras (100 μg/ml, provided by L. Feig and C. Farnsworth, Tufts University; ref. 15), ExoS (1 μg/ml or variable for individual experiments), and FAS, in a total vol of 20 μl. The reactions were incubated for 20 min at 25°C and stopped by spotting 10 μl of the mixture onto a trichloroacetic acid-saturated Whatman paper (16) and adding 2× SDS/PAGE sample buffer (12) into the remaining mixture for the gel assay. [^{32}P]ADP-ribose incorporation into trichloroacetic acid-precipitable material was quantified in a liquid scintillation counter after extensive washing with 5% trichloroacetic acid. The specific incorporation of [^{32}P]ADP-ribose into SBTI or Ras was verified by SDS/PAGE and autoradiography.

Protein concentration was estimated by the bicinchoninic acid assay (17) with the Micro BCA protein assay reagent (Pierce).

RESULTS

Isolation of a Bovine cDNA Clone Encoding FAS. FAS, partially purified from bovine testes, showed two predominant bands on SDS/PAGE (29 kDa and 31 kDa; ref. 6). On the basis of activity assays, the 29-kDa protein was proposed to be FAS (6). From partial amino acid sequences obtained from this 29-kDa protein band, we designed two degenerate primers and used them to amplify putative FAS-specific sequences from bovine brain cDNA by PCR. A 0.29-kb DNA fragment was obtained, and the deduced amino acid sequence from one reading frame of this fragment was identical to the peptide sequence derived from the 29-kDa protein from bovine testes (pep2 in Fig. 1), thereby confirming the identity of the amplified PCR product. This 0.29-kb FAS-specific fragment was labeled with ^{32}P and used to probe a bovine brain λgt10 cDNA library. Screening of 1.4×10^5 plaques by Southern hybridization led to the identification of 23 positive clones. Subsequent to restriction-enzyme mapping, 10 of the positive candidates were subcloned into pUC19 for sequence analysis. We found that 9 of 10 clones in pUC19 had the identical sequences in the region ≈200 nt from primer R1 (Fig. 1). We sequenced both strands of FAS-insert DNA in pHAF603 and, guided by the peptide sequences from FAS protein, identified a 738-bp open reading frame.

The complete nucleotide sequence of the FAS cDNA and the deduced amino acid sequence of the cloned gene are shown in Fig. 1. We assumed that Met-1, encoded by one of three methionine codons preceding the pep1-coding sequence, was the initiating methionine because the sequence AGTCATGG best fit the consensus sequence for eukaryotic initiation sites. Also, the derived amino acid sequence predicted a 245-residue polypeptide of 27,743 Da, which is the expected size (6). The three-peptide sequences of FAS obtained from the partially purified bovine protein corresponded to the amino acid sequence predicted from the cloned gene (Fig. 1). The protein is highly charged, containing 45 acidic (18%) and 32 basic (13%) amino acids, and its estimated isoelectric point is 4.5, consistent with the data from the native protein. A hydropathy plot revealed neither a typical leader sequence nor other prominent hydrophobic regions (18). Thus, it is likely that FAS is an intracellular cytosolic protein.

Expression of Recombinant FAS in *E. coli*. Functional properties of the recombinant protein were studied after expression of the cloned gene in *E. coli*, an organism with no endogenous FAS activity (6). The coding sequence of FAS was amplified by PCR and cloned into pET-15b, generating pHAF612, in which the FAS gene was under the transcriptional control of the T7 promoter (Fig. 2*A*). The protein product encoded by pHAF612 contained FAS fused to a hexa-His sequence, to aid in purification, and an intervening thrombin cleavage site, to permit the hexa-His peptide to be removed easily. *E. coli* BL21(DE3) was transformed with pHAF612, and expression of FAS was induced by the activation of the T7 RNA polymerase with isopropyl β-D-thiogalactoside (1 mM; ref. 11). As shown in Fig. 2*B*, concomitant with the addition of isopropyl β-D-thiogalactoside(+), an intense protein band of ≈30 kDa appeared (arrow), which was absent from the uninduced culture (lane 3) or the control culture carrying pET-15b without the FAS gene insert. Crude extracts prepared from *E. coli* BL21(DE3)/pHAF612 activated ExoS to catalyze ADP-ribosylation of SBTI and other *E. coli* cellular substrates present in the assay (data not shown).

Purified FAS Activates the ADP-Ribosyltransferase Activity of ExoS. Recombinant FAS was purified from crude *E. coli* extracts by affinity chromatography (Fig. 3). After passage through a single Ni^{2+}-charged histidine-binding column, the purified FAS fusion protein was obtained, which gave a single major band on Coomassie blue-stained SDS/PAGE (lane 2). The hexa-His affinity tag was removed by thrombin digestion (lane 3), and during this step, several thrombin-sensitive contaminants were also digested, increasing the purity of the isolated FAS. The hexa-His tag and the thrombin in the preparation were then removed by a mixture of Ni^{2+}-charged and benzamidine-conjugated resins (lane 4).

The purified recombinant FAS activated the ADP-ribosyltransferase activity of ExoS in a defined cell-free system containing ExoS, FAS, ^{32}P-labeled NAD^+, and SBTI, in HBS buffer (Fig. 4). No ADP-ribosylation activity was detectable in the absence of FAS, and the recombinant FAS, like native FAS from bovine testes, activated ExoS to ADP-ribosylate SBTI in a dose-dependent manner. Incorporation of [^{32}P]ADP-ribose into SBTI increased linearly with FAS at low concentrations (0.01–0.25 μg/ml) and reached a plateau at higher FAS concentrations. Label was incorporated specifically into SBTI (Fig. 4*B*, lane 2); no label was detected in FAS or ExoS. The recombinant FAS, like native

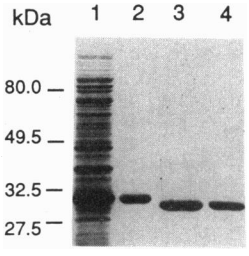

FIG. 3. Purification of recombinant FAS by affinity chromatography. Protein samples from various purification steps were loaded onto SDS/10% PAGE, and the gel was stained, as described for Fig. 2. Lanes: 1, crude *E. coli* BL21(DE3)/pHAF612 extracts prepared by sonication and centrifugation (15 μg of supernatant); 2, FAS protein (2 μg) eluted from a Ni^{2+}-charged histidine-binding column with 500 mM imidazole solution; 3, thrombin-digested FAS showing decreased size (2.5 μg); 4, FAS after removing hexa-His tag and thrombin (2 μg). Molecular mass markers are shown at left.

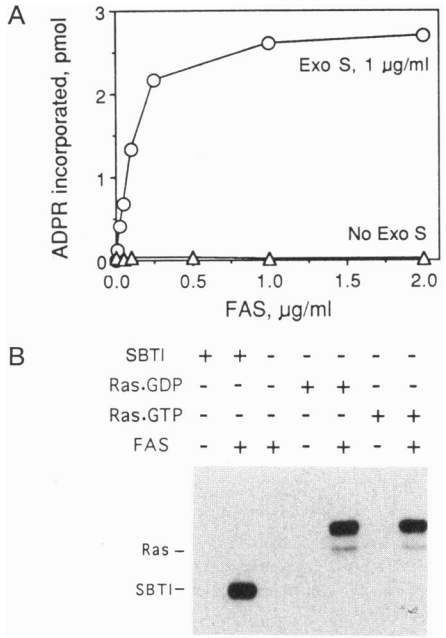

FIG. 4. FAS-dependent ADP-ribosylation of SBTI and Ras by ExoS. The ADP-ribosylation assays were done at 25°C for 20 min, as described. The reaction mixture contained 5 μM [^{32}P]NAD$^+$, affinity-purified FAS, purified ExoS (1 μg/ml), and SBTI or Ras (100 μg/ml). (*A*) ADP-ribosylation of SBTI ([^{32}P]ADPR-labeled SBTI) by ExoS with various FAS concentrations. (*B*) Requirements for ExoS to ADP-ribosylate substrates, SBTI or Ras. The reaction mixtures were electrophoresed on SDS/13.5% PAGE, and incorporation of [^{32}P]ADP-ribose into SBTI or Ras was revealed by autoradiography.

FAS, also activated ExoS toward Ras protein. The GTP- and GDP-bound forms of Ras were equally modified, implying that the nucleotide-bound state of Ras is not important for ADP-ribosylation by ExoS *in vitro* (Fig. 4). However, ExoS was unable to modify another small GTP-binding protein, Rho A, even in the presence of recombinant FAS, thereby illustrating the substrate selectivity of the enzyme (data not shown). Consistent with previous reports (19), we showed that Rho A was modified by the C3 ADP-ribosyltransferase of *C. botulinum* and that this reaction was independent of FAS (data not shown; Rho A and C3 proteins were provided by L. Feig and S. Dillon, Tufts University). These results, taken together, show that FAS is necessary and sufficient to activate ExoS.

FAS Is a Member of the 14-3-3 Protein Family. When the amino acid sequence of FAS was scanned against various data bases with the Genetics Computer Group programs (18), extensive sequence similarities were detected to a group of proteins collectively termed 14-3-3 proteins (Fig. 5). The 14-3-3 proteins were originally identified on the two-dimensional "protein maps" of extracts of brain and other organs and isolated as brain-specific proteins by Moore and Perez (34). They compose a family of acidic proteins of ≈30 kDa, which can be resolved by reverse-phase HPLC into seven to eight peaks, representing different isoforms (α–η, according to Ichimura *et al.*, ref. 20). These proteins are present in most mammalian tissues (21, 35) but are abundant in the brain (≈1% of cytosolic proteins) and especially so in neurons (36). The 14-3-3 proteins or genes have also been identified in amphibians, insects, plants, and yeast, implying that this family is widely distributed among eukaryotic organisms (30–33).

FAS shares extensive protein sequence homology (>60% identity) with all members of the 14-3-3 family (Fig. 5). The amino acid sequence of FAS was found to be identical to that of the bovine ζ protein derived from peptide sequencing (22)

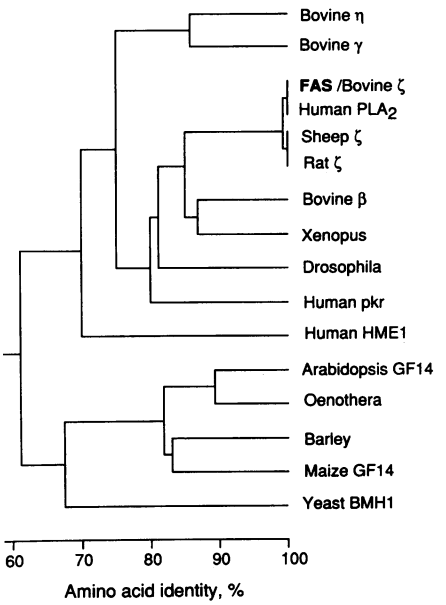

FIG. 5. Sequence relationships between FAS and other known 14-3-3 proteins. The amino acid sequence of FAS was aligned with those of other known 14-3-3 proteins with PILEUP and GAP programs of the Genetics Computer Group package (18). The sources of the 14-3-3 sequences are as follows: bovine FAS (this work), bovine β, γ, ζ, and η (20–22), human phospholipase A_2 (PLA_2) (23), protein kinase regulator (pkr; ref. 24), epithelial cell marker (HME1; ref. 25), sheep ζ (26), rat ζ (26), barley 14-3-3 homologue (27), G-box binding complex-associated protein GF14 from maize (28) and *Arabidopsis* (29), *Oenothera* protein kinase C inhibitor homologue (30), *Xenopus* 14-3-3 homologue (31), *Drosophila* 14-3-3 (32), and *Saccharomyces cerevisiae* brain modulosignalin homologue 1 (BMH1; ref. 33). Among those, bovine β and γ, and the ζ from bovine, sheep, and rat were obtained by peptide sequencing. The published partial sequences of 14-3-3 homologues are excluded from this alignment.

and to a human intracellular PLA_2 sequence (30), the human homologue of the bovine ζ polypeptide. At the nucleotide level, there are only 14 differences between this human PLA_2 and the bovine FAS genes within the coding region. Similarly, the published peptide sequences of ζ isoform from sheep and rat are identical (26), and these isoforms differ from the deduced bovine FAS sequence only at two positions (Ala-109 → Arg and Ala-112 → Pro, double-underlined in Fig. 1). FAS showed 75% identity with η and γ polypeptides from bovine sources and 87% identity with the β protein.

DISCUSSION

By cloning and sequencing the gene for FAS, we have identified this protein as a member of the highly conserved 14-3-3 protein family. This work establishes a specific function for a 14-3-3 protein, activation of a ADP-ribosyltransferase of bacterial origin; and it defines a 14-3-3 protein as a eukaryotic host factor relevant to infection by *P. aeruginosa*.

At least 16 members of the 14-3-3 protein family are currently known, including representatives of mammalian, amphibian, insect, plant, and yeast origin. There is remarkable conservation of primary structure throughout this family of proteins. Bovine FAS and a homologue from yeast show 64% identity, both at the amino acid and nucleotide levels. Such conservation of structure must reflect the fundamental importance of 14-3-3 proteins in cellular physiology.

Several activities have been reported for members of 14-3-3 protein family. FAS (ζ) from bovine brain has been reported to activate tyrosine and tryptophan hydroxylases (the rate-limiting enzymes involved in catecholamine and serotonin biosynthesis, respectively) in the presence of Ca^{2+}/

calmodulin-dependent kinase II (21, 37). A human placental protein identical in sequence to FAS has been shown to have cytosolic PLA_2 activity (23). Purified 14-3-3 proteins were found to activate Ca^{2+}-dependent exocytosis by digitonin-permeabilized adrenal chromaffin cells (38). In addition, there is a report of down-regulation of a 14-3-3 protein in neoplastic mammary cells (25), and there are conflicting reports regarding possible regulation of protein kinase C activity by 14-3-3 proteins (22, 39, 40). Moreover, at least one of the 14-3-3 proteins is proposed to play a role in signal transduction in response to a fungal pathogen in barley (27). How such diverse activities interrelate remains unclear. Isolation and further analysis of genes encoding various isoforms of 14-3-3 proteins should aid in the elucidation of their physiological roles in eukaryotic cells and the structural basis of their diverse biochemical activities.

The fact that purified recombinant FAS protein activates ExoS demonstrates that a single eukaryotic gene product is sufficient for activation. Preliminary evidence suggests that the 31-kDa protein that copurifies with FAS from bovine testes (6) also activates ExoS, but further studies will be necessary to determine whether this is a 14-3-3 protein and how widespread FAS activity is within the 14-3-3 family. The specific activity of the recombinant FAS was comparable to that of native FAS from bovine testes (data not shown), implying that co- or posttranslational processing of FAS is not necessary for FAS activity *in vitro*. Although the primary structure of FAS from bovine brain is identical to that of a human placental PLA_2, as indicated above, we believe it unlikely that PLA_2 activity plays a role in the activation of ExoS for two reasons. (*i*) No phospholipids were present in our assays (except for possible trace contaminants), and (*ii*) there is evidence that FAS activates ExoS in a stoichiometric manner, rather than via an enzymatic pathway. Thus, one can reversibly activate or deactivate ExoS by adding or removing FAS (6).

There is evidence that ExoS promotes dissemination of *P. aeruginosa* from the site of inoculation (41). It has been postulated that the ADP-ribosylation of small GTP-binding proteins by this enzyme may disrupt normal vesicle trafficking and thereby inhibit the antimicrobial functions of cells such as neutrophils and macrophages, which normally prevent dissemination (4). Recognition of 14-3-3 proteins as host factors for ExoS may aid in determining the role of this enzyme in *P. aeruginosa* infection. For instance, the localizations of 14-3-3 proteins within cells may provide clues to the targets of ExoS and the consequences of its action. The 14-3-3 proteins may also represent a device that *P. aeruginosa* exploits to restrict the action of ExoS to host cells (6).

Is there any relationship between ExoS and the physiological function of FAS *in vivo*? Recently, a 20-kDa endogenous ADP-ribosyltransferase from bovine brain cytosol similar to botulinum C3 was reported (42). This bovine ADP-ribosyltransferase shares substrate specificity (Rho and Rac) and the activator requirement with the bacterial C3 protein. In a similar vein, it is conceivable that FAS might interact with and, thus, regulate the activity of an endogenous protein with some structural similarity to ExoS. The hexa-His tag–FAS fusion protein reported here may be useful as a probe to search for intracellular protein(s) that interact(s) with FAS, including potential ADP-ribosyltransferases.

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