Expression and Activities of a Recombinant Basic Fibroblast Growth Factor-Saporin Fusion Protein*

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A fusion protein containing the full-length sequences of the mitogen, basic fibroblast growth factor (FGF-2), and the ribosome-inactivating protein, saporin (SAP), has been expressed in E. coli. As expected, it binds with high affinity to heparin-Sepharose like FGF-2 and can displace the binding of radiolabeled FGF-2 to its high affinity receptor. In contrast, the fusion protein only has much lower ribosome-inactivating activity than free saporin, although full ribosome-inactivating activity can be generated by proteolytic removal of the FGF-2 moiety. Cytotoxicity experiments with B16-F10 mouse melanoma cells establish that the fusion protein is active as a chemical conjugate against these intact cells. Presumably these cells have the ability to activate the SAP component of the fusion protein through an intracellular metabolism of the fusion protein. Because we also show the fusion protein has tumor growth inhibition properties and antimetastatic activity in in vivo models of melanoma, the findings support the hypothesis that FGF-based ligand-mediated cytotoxicity can serve to target cytotoxic agents in vivo.

The chemical conjugation of basic fibroblast growth factor (FGF-2)† with saporin (SAP) creates a molecule that is an extremely potent cytotoxic agent for cells that express surface high affinity FGFR receptors. This molecule (rFGF-SAP) has been used to eliminate contaminating FGF receptor-bearing cells from mixed cell populations (1, 2), in tumor models to inhibit solid tumor growth (3), in the inhibition of corneal re-epithelialization (4), and to inhibit smooth muscle cell proliferation in experimental models of restenosis (5, 6).

Over the course of the last few years, fusion proteins containing bacterial toxins and ligands (7–9) or antibody fragments (10) have led to a second generation of mitotoxins and immunotoxins. These recombinant proteins provide numerous advantages. First and foremost, proteins can be purified that are chemically homogeneous. Their structures can be engineered to enhance potency or to modulate their effects (11). In some instances, for example, the ligand moiety is modified to enhance its binding to high affinity receptors (12). In others, the toxin moiety is engineered for increased stability (13, 14), decreased intrinsic toxicity (15), or to enhance its translocation into the cellular cytoplasm after internalization (16).

In a recent study, we reported the expression of one of the four isoforms of saporin, a ribosome-inactivating protein isolated from the plant Saponaria officinalis in Escherichia coli (17). Saporin is a type I ribosome-inactivating protein that has been used in many applications as the targeted toxin moiety of chemically conjugated immunotoxins (18, 19) and mitotoxins (20). Recently, for example, SAP chemical conjugates have been evaluated in clinical trials as anti-tumor agents (21, 22) and in the development of experimental models of Alzheimer’s disease (23). Because the expression of saporin in E. coli produces a recombinant protein with the full intrinsic ability to inhibit protein synthesis (17), we constructed a cDNA encoding a fusion protein of FGF-2 and SAP. It was cloned into the pET11 expression system (24) and the expressed molecule (rFGF-SAP) was characterized.

**EXPERIMENTAL PROCEDURES**

Reagents—Restriction and modification enzymes were purchased from Life Technologies, Inc., Stratagene (La Jolla, CA), and New England Biolabs (Beverly, MA). Native SAP, chemically conjugated FGF-SAP, and rabbit polyclonal antisera to FGF-2 were prepared as described previously (25, 26). The pET System Induction Control was purchased from Novagen (Madison, WI). pFC80, containing the FGF-2 coding sequence (27), was a kind gift of Dr. Paolo Sarmientos of Farmitallia Carlo Erba (Milan, Italy). The SAP coding sequence Seq3 is described in Barthelemy et al. (17).

Plasmid Construction—An NcoI restriction site was introduced into the sequence of the SAP Seq3 by site-directed mutagenesis using the Amersham Corp. in vitro mutagenesis system 2.71. Oligonucleotides were synthesized using a 380B automatic DNA synthesizer (Applied Biosystems, Foster City, CA). The oligonucleotide used for creation of this site was CAA CCA CTG CCA TGG TCA CAT C. The resulting fragment was ligated to the NcoI site located at the beginning of the mature SAP coding sequence and is termed mpNG4. The stop codon of the FGF-2 coding sequence in the pHCl80 plasmid was also removed by site-directed mutagenesis. The FGF-2 sequence was isolated as a HindIII fragment and after filling its protruding ends, it was cloned in the Smal site of M13mp18. An insert in the origin of replication direction was mutagenized using the Amersham Corp. kit with the sequence GCT AAG AGC GCC ATG GAG A to replace the naturally occurring GCT AAG AOC TGA CAA TGG AGA. The resulting M13 replicative form was cut with NcoI and Scll to yield a fragment containing FGF-2 coding sequence with the stop codon replaced. An EcoRI-NcoI fragment from mpNG4 was ligated to a NcoI-SacI fragment from FGF13. The resulting fragment was inserted into M13mp18 opened with EcoRI and Scll to create mpFGSAP. mpFGSAP was digested with XbaI and EcoRI, and the resulting fragment that contains the

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† This paper is dedicated to the memory of our colleague, Gianpaolo Neri.

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§ The abbreviations used are: FGF-2, basic fibroblast growth factor; SAP, saporin; FGF, fibroblast growth factor; rFGF-SAP, recombinant basic fibroblast growth factor-saporin fusion protein; RPF, ribosome-inactivating protein; IL-4, interleukin 4; IL-6, interleukin 6; TGFα, transforming growth factor α; ELISA, enzyme-linked immunosorbent assay.
rFGF-SAP coding sequence was isolated and ligated into pET11a pre-treated with EcoRI and XbaI. The resulting plasmid (p7T-FS) contains a λ CI ribosome binding site from the pSC80 plasmid which replaces the ribosome binding site of the pET vector. It was transformed into host strain BL21(DE3)/pLysS according to manufacturer's instructions.

**Recombinant FGF-SAP**—4 liters of LB broth containing carbenicillin (Sigma, 50 μg/ml) and chloramphenicol (Sigma, 50 μg/ml) were inoculated with plated-containing bacterial cells from a culture grown to 1 OD₆₀₀ (100 dilution). Cells were grown at 28 °C in an incubator shaker to an OD₆₀₀ of 1.2, at which time cells were centrifuged. The pellet was resuspended in 10 mM Tris, 0.6 mM NaCl, pH 7.4. The solution was frozen and thawed five times and sonicated for 2 min. The suspension was centrifuged; the supernatant was saved and the pellet was resuspended for further analysis and saved. The supernatant was applied to a HiTrap heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 0.15 mM NaCl in 10 mM Tris, pH 7.4 (buffer A), and washed with equilibration buffer, 0.6 mM NaCl in buffer A, 1.0 mM NaCl in buffer A, and finally eluted with 2 mM NaCl in buffer A. The column fractions were analyzed by SDS-gel electrophoresis, Western blotting with anti-saporin antibodies, and the sandwich ELISA described below in the standard protocol. The SDS-polyacrylamide gel (Sigma) was added to a final concentration of 0.2 mM and growth continued to OD₆₀₀ of 1.2. The samples were centrifuged. The pellet was resuspended in 10 mM Tris, 0.5 mM NaCl, pH 7.4. The solution was frozen and thawed five times and sonicated for 2 min. The sample was centrifuged; the supernatant was saved and the pellet was resuspended for further analysis and saved. The supernatant was applied to HiTrap heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 0.15 mM NaCl in 10 mM Tris, pH 7.4 (buffer A), and washed with washing buffer, 0.6 mM NaCl in buffer A, 1.0 mM NaCl in buffer A, and finally eluted with 2 mM NaCl in buffer A. The column fractions were analyzed by SDS-gel electrophoresis, Western blotting with anti-saporin antibodies, and the sandwich ELISA described below in the standard protocol.

**Sodium Dodecyl Sulfate (SDS)-Gel Electrophoresis and Western Blotting**—SDS-gel electrophoresis was performed on a PhastSystem using the PhastSystem (Pharmacia), as described by the manufacturer. The antisera to FGF-SAP and FGF-2 were used at a 1:1000 dilution. Horseradish peroxidase-interpolated from the standard curve values.

**RESULTS**

**Expression of rFGF-SAP as a Fusion Protein**—Our first attempts to express and characterize a rFGF-SAP fusion protein from *E. coli* (30) resulted in the expression of a protein that had none of the binding properties of FGF-2, SAP's cell-free protein synthesis inhibition activity, or cytotoxicity to cells expressing the FGF-2 receptors. Because of these negative results, we revised our strategy and used the entire coding sequences of both saporin and FGF-2. New restriction sites were introduced by site-directed mutagenesis, and we used the pET expression system, which provides tight control of gene induction (24). This new construct is described in Fig. 1. An NcoI restriction site was introduced 5' to the SAP encoding sequence, leaving the EcoRI site derived from the genomic sequence intact. After removal of the stop signal in the FGF-2 sequence, it was ligated to SAP, resulting in a sequence encoding for the entire FGF-2 coding sequence plus an intervening 2-amino acid linker peptide (Ala-Met), and the entire SAP sequence. After excision from M13, the DNA was inserted into pET11a for expression.

**Purification of the Expressed Fusion Protein**—The induction of cells containing the plasmid p7T-FS results in the appearance of a 45-kDa protein detectable by Western blotting with anti-SAP and anti-bFGF antisera. Purification is straightforward by virtue of the heparin affinity of FGF-2. rFGF-SAP elutes from heparin-Sepharose with 2 mM NaCl just as native and recombinant FGF-2. This clearly indicates that the heparin affinity of FGF-2, one of its biochemical characteristics (31, 32), is retained in the fusion protein. The final step of purification was Mono S chromatography. Exploiting the strong positive charge of both FGF-2 (pI 9.6) and SAP (pI 10.5) yields an essentially pure protein, as judged by SDS-gel electrophoresis and sandwich ELISA.

As shown in Fig. 2, SDS gels were either stained with Coomassie Blue, or the proteins were transferred to nitrocellulose and blotted with anti-SAP or anti-FGF-2 antisera. Both antisera reveal rFGF-SAP as a band with molecular weight approximately 43,000, which is slightly lower than the sum of the molecular weights of SAP (30,000) and FGF-2 (18,000). The basic isoelectric points of the two proteins result in a faster migration and its appearance as a doublet. As expected, there is no rFGF-SAP in the fermentations of noninduced bacteria, establishing that there were punched out of the 96-well plate and counted by liquid scintillation techniques.

Cytotoxicity experiments were performed with the Promega CellTiter 96 Cell Proliferation/Cytotoxicity Assay using human (SR-Mel-28, ATCC HTB72) and mouse (B16-F10) melanoma cell lines. The procedures were optimized for 1500 cells plated per well.

**In Vivo Assay of Cytotoxic Activity**—Both a solid tumor and metastatic model of melanoma were examined. In the solid tumor model, on day 0, 5 x 10⁶ B16-F10 cells were injected subcutaneously in the flanks of each C57BL/6 mouse. By day 3, tumors were visible on all mice. The mice in Group C were given two injections at the LDI₅₀ (105 μg/kg), one on day 3, the other on day 7. Mice in Group D were injected on days 3, 6, and 9 with 26 μg/kg. Tumor size was measured on days 5 and 10. Saporin treatment was done on day 3 at 82 μg/kg, a dose equimolar to the LDI₅₀. Mice in the "no treatment" category were injected with Dulbecco's PBS at day 3. The mice were sacrificed on day 10, and the tumors were removed and weighed. The mean tumor weights and mean tumor volumes (tumor volume = ab²/2, where a is equal to the larger dimension measured and b is equal to the smaller dimension) were determined for each group, and statistical comparisons of the various treatment groups were performed using Student's t test.

For the metastatic model, on day 0 of the experiment, B16-F10 cells (5 x 10⁶ in 0.1 ml of Dulbecco's PBS) were injected via the tail vein into the circulation of C57BL/6 mice. On day 1, the mice were randomly divided into two groups and treated as follows: 12 mice were injected via the tail vein with 105 μg/kg rFGF-SAP, and 10 mice were injected as above with Dulbecco's PBS. On day 7, the mice were sacrificed, and the number of nodules on the lungs were counted using a dissecting microscope.
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**Fig. 1. Schematic of the construction of the rFGF-SAP fusion protein expression plasmid.** An NcoI restriction site was introduced into the 5' end of the saporin coding sequence as described under "Experimental Procedures." An EcoRI site remained from the native genomic DNA. A stop signal in the FGF-2 coding sequence was removed by site-directed mutagenesis. An EcoRI-NcoI fragment from mpNG4, containing the SAP coding sequence, was ligated to a NcoI-SacI fragment, containing the FGF-2 coding sequence, from FGFM13. The resulting fragment was inserted into M13mp18 opened with EcoRI and SacI to create mpFGFSAP. This fusion protein sequence, containing the entire coding sequence for FGF-2 and saporin, with a 2-amino acid spacer (Ala-Met), was excised from mpFGFSAP with EcoRI and XhoI and inserted by ligation into the pETlla vector that had been cut with the same restriction enzymes. This fusion protein, mpFGFSAP, was used for expression of rFGF-SAP.

**Fig. 2. SDS-gel electrophoresis analysis of rFGF-SAP production and purification.** Samples were electrophoresed with a PhastSystem (Pharmacia) in 12% PhastGels according to the manufacturer's instructions. Gels were either stained with Coomassie Blue or transferred to nitrocellulose by the PhastTransfer system and used in Western blotting with anti-saporin or anti-FGF-2 antisera. Lanes are the same for all three methods. See "Experimental Procedures" for details of the preparations. Lane A, resuspended pellet of extract of uninduced bacteria (0.5 ml); lane B, resuspended pellet of extract of induced bacteria (0.5 ml); lane C, supernatant of extract of uninduced bacteria (0.5 ml); lane D, supernatant of extract of induced bacteria (0.5 ml); lane E, pool of heparin-Sepharose chromatography peak fractions (1.1 pg for Coomassie staining, 11 ng for Westerns); lane F, pool of Mono S 5/5 chromatography peak fractions (7.5 ng for Coomassie staining, 7.5 ng for Westerns); lane G, FGF-SAP chemical conjugate (2.5 pg for Coomassie staining, 25 ng for Westerns). Molecular weight standards are given on the ends and correspond to the molecular weight in thousands. Lanes A-F are under reducing conditions; lane G is without reducing agent.

is tight control of rFGF-SAP expression in this expression system. Although some rFGF-SAP is also present in the pellet of the induced extract, no attempt was made to characterize this material. No immunoreactivity was detected in extracts of bacteria containing the pET11 plasmid without the rFGF-SAP coding sequence. Gas phase sequence analysis of the purified
nal methionine removed and begins with alanine, the second leucine incorporation into acid-precipitatable material (Fig. 3a). Surprisingly, the RIP activity of the fusion protein is significantly less than the RIP activity of chemically conjugated FGF-SAP or SAP alone. In contrast, the fusion protein's ability to compete with radiiodinated FGF-2 for FGF receptors, while somewhat less than FGF-2, is quite similar to the chemically conjugated FGF-SAP (Fig. 3b).

The cytotoxicity of rFGF-SAP was examined using B16-F10 cells, a subclone of a mouse melanoma which is widely used in anti-tumor screening procedures (34-38). These cells have been shown to be very sensitive to the chemical conjugate FGF-SAP, both in vitro and in vivo. As shown in Fig. 4, the chemical conjugate and the recombinant fusion protein have very similar cytotoxicity profiles in vitro. Similar results were seen with SK-MEL-28 cells, another melanoma cell line. The cytotoxicity is competed by free FGF-2, indicating that it is mediated through FGF receptors (data not shown). SAP alone is 100 times less cytotoxic to these cells than its targeted counterpart. This is particularly surprising in light of the results in the cell-free system described above, showing the SAP component of the fusion protein is less effective than SAP alone. We therefore examined whether its activity could be generated by degradation of the fusion protein.

**Generation of Biologically Active SAP from rFGF-SAP**—The full RIP activity of SAP can be detected from the fusion protein after proteolysis. Because saporin is protease-resistant (39, 40) and FGF-2 is not (41), we treated rFGF-SAP overnight at 37 °C with either chymotrypsin, trypsin, or cathepsin B. With chymotrypsin and trypsin, there was a complete and quantitative degradation of the 45-kDa fusion protein to a protein with a molecular weight similar to that expected for SAP. The protein was identified as SAP by Western blotting (Fig. 5a). Sequence analysis of the purified trypsin-treated material showed that it had been cleaved after the last lysine (Lys154) of FGF-2; this would leave 3 amino acids in addition to the NH₂ terminus of SAP. When assayed, it has RIP activity equivalent to that of native saporin (Fig. 5b). Because FGF-2 is rapidly degraded in target cells by cleavage at its COOH terminus shortly after internalization (41), we reasoned that the degradation of rFGF-SAP in B16-F10 and SK-Mel-28 cells activates the saporin moiety of the fusion protein to yield a molecule having full cytotoxicity.

**In Vitro Activities of the Fusion Protein**—The ability of FGF-SAP to inhibit protein synthesis was determined using rabbit reticulocyte lysates in a cell-free system which measures leucine incorporation into acid-precipitable material (Fig. 3a). Surprisingly, the RIP activity of the fusion protein is significantly less than the RIP activity of chemically conjugated FGF-SAP; □, chemically conjugated FGF-SAP; ●, rFGF-SAP; ▲, nonconjugated SAP.

**FIG. 4. Cytotoxicity of rFGF-SAP to melanoma cells expressing the FGF-2 receptor.** Incorporation and conversion of the dye 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide was used as a measure of living cells. Results indicate that rFGF-SAP has a similar cytotoxicity to B16-F10 melanoma cells as chemically conjugated FGF-SAP; □, chemically conjugated FGF-SAP; ●, rFGF-SAP; ▲, nonconjugated SAP.

**FIG. 3. a, inhibition of cell-free protein synthesis by rFGF-SAP, chemically conjugated FGF-SAP, and unconjugated SAP.** Leucine incorporation was used as a measure of protein synthesis. Experimentation was done as described under *Experimental Procedures.* Results indicate that the SAP moiety of rFGF-SAP is less active than free SAP or chemically conjugated FGF-SAP: ▲, SAP; ○, chemically conjugated FGF-SAP; ●, rFGF-SAP; □, chemically conjugated FGF-SAP. The capacity of rFGF-SAP to recognize FGF receptors was examined in baby hamster kidney cells by the method of Moscatelli (61). Briefly, cells were grown to confluence and incubated at 4 °C for 2 h with radiiodinated FGF-2 in the presence of unlabeled FGF-2 (●), chemically conjugated FGF-SAP (□), or rFGF-SAP (○). The cells were then washed twice with PBS and twice with 2% NaCl. Binding to high affinity receptor was determined by counting the membrane fraction that was solubilized with 0.5% Triton X-100 in PBS.

**b, binding of rFGF-SAP to FGF receptors on the cell surface.** The cells were then washed twice with PBS and twice with 2% NaCl. Binding to high affinity receptor was determined by counting the membrane fraction that was solubilized with 0.5% Triton X-100 in PBS.

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W. Ying, D. Martineau, J. Beitz, D. A. Lappi, and A. Baird, submitted for publication.
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FIG. 5. Proteolytic digestion of rFGF-SAP. 50-μg quantities of rFGF-SAP and 31 μg of SAP were incubated with 100-fold less (by weight) quantities of chymotrypsin, trypsin, or cathepsin B (Sigma) overnight at 37 °C. Samples were then removed and assayed for ribosome-inactivating protein activity or electrophoresed and stained with Coomassie stain or transferred to nitrocellulose and immunostained with anti-FGF-2 or anti-saporin. a, Coomassie staining and Western blotting of protease-treated rFGF-SAP. Lane A, rFGF-SAP, no protease, no 37 °C incubation overnight; lane B, rFGF-SAP, no protease, incubation at 37 °C overnight; lane C, rFGF-SAP, treated with chymotrypsin at 37 °C overnight; lane D, rFGF-SAP, treated with trypsin at 37 °C overnight; lane E, rFGF-SAP, treated with cathepsin B at 37 °C overnight; lane F, saporin standard (Coomassie and anti-saporin Western), FGF-2 (anti-FGF-2 Western). The double band of protease-treated rFGF-SAP. Inhibition of protein synthesis by a rabbit reticulocyte lysate system is measured as described under "Experimental Procedures." Samples were assayed at a final concentration of 45 μM. Error bars denote standard deviation. This saporin standard was incubated overnight at 37 °C without protease addition and is similar to untreated saporin standard and saporin that had been incubated with protease (data not shown). Samples containing proteases were assayed without removal of proteases; separate assays showed no effect of proteolytic enzymes at the same dilution on leucine incorporation.

In Vivo Activity of the Fusion Protein.—We tested the activities of the rFGF-SAP in both a solid tumor and a metastatic model of melanoma with B16-F10 cells in order to demonstrate its activity in vivo. In the solid tumor model, two different dosage regimens were used, one receiving two treatments (Group C) and the other receiving three (Group D), as described under "Experimental Procedures." For both groups, by day 5, there was a significant reduction in tumor volume (not shown) that increased in significance by day 10 (Fig. 6). For Group C, the mean tumor volume of the treated mice on day 10 was 28% that of the nontreated mice (p < 0.0005) and for Group D, the mean tumor size on day 10 was 29% of the mean tumor volume of the nontreated mice (p < 0.001). Tumor weight measurements gave similar results (no treatment: Group C, p < 0.001; no treatment: Group D, p < 0.005).

In the metastatic model, cells are injected in the tail vein of mice on day 0, and within 7 days, colonies of B16-F10 cells form in the lung. Because these colonies are black and on the lung surface, the extent of metastases can be quantitated by counting colonies under a dissecting microscope. The day after injecting cells, the mice were treated with a single intravenous dose (105 μg/kg) of rFGF-SAP. The mean number of colonies on the lungs of the vehicle-treated mice is 165 ± 23 (±S.E.). The mean number of colonies on the lungs of the animals treated with rFGF-SAP is 49 ± 14 (p < 0.001), a 70% reduction of the number of colonies in control animals. Treatment with the same dose of the chemically conjugated FGF-SAP resulted in a 60% decrease in the number of colonies in control animals (p < 0.002). Like its chemical conjugate counterpart, recombinant FGF-SAP possesses potent in vivo activity in this metastatic melanoma model system.

DISCUSSION

There are a number of ways in which the chemical heterogeneity of conjugated proteins can be minimized. For example, we have recently addressed this problem in FGF-SAP by performing site-directed mutagenesis of FGF-2, selectively removing reactive cysteines, and conjugating the protein to monomeric saporin. The reaction produces a chemical conjugate that appears as a single band in nonreducing sodium dodecyl sulfate-gel electrophoresis and has the same intrinsic activities of the wild type (unmutagenized) chemical conjugate (42). Because purified saporin preparations contain at least four isoforms of the protein that differ by several amino acids (17, 43, 44), there is still considerable heterogeneity in this material. For these reasons, we expressed the gene encoding the fusion protein in E. coli by transforming with a pRSET plasmid containing the saporin gene. The saporin was expressed as a single chain that retains the ability of FGF-2 to bind immobilized heparin, but that it also has greatly reduced ribosome-inactivating protein activity. When evaluated against an FGF receptor-bearing cell type B16-F10, however, the re-
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combinant material has cytotoxicity that is comparable with the chemical conjugate. This strongly implies that the saporin moiety of the fusion protein is cleaved from NH2-terminal of the saporin moiety by lysosomal proteases after internalization. Saporin activity, previously inhibited by the NH2-terminal extension, is then activated and has full intrinsic ribosome-inactivating activity. Indeed, proteolysis in vitro demonstrates that the degradation of rFGF-SAP activates the saporin moiety of the fusion protein. Because Walicke and Baird (41) have shown that the initial processing of FGF-2 after internalization occurs at a point seven amino acids from the carboxyl terminus of FGF-2, it is possible that cleavage at this point could activate saporin. This metabolism and activation could then mediate its cytotoxic actions on cells in culture and explain the observation that rFGF-SAP is also active as a single intravenous injection to inhibit tumor growth in a model of melanoma metastases. Whether cleavage at points that would leave longer residual portions of FGF-2 would still be active is under investigation. Preliminary results indicate that the ATF moiety, situated likewise at the NH2-terminal of SAP, is removed to obtain protein synthesis inhibition activity. Thus the inhibitory activity of the FGF-2 moiety is not peculiar to the FGF-2 sequence.

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