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Cathepsin D was initially thought to be an individual prognostic indictor for the disease state of human breast cancers. Over the years, the data substantiating such a role for cathepsin D has been quite conflicting. However, there is strong evidence that cathepsin D plays a role in the degradation of the extracellular matrix (ECM) and basement membrane surrounding tumors. In doing so, cathepsin D is an active participant in releasing ECM-bound growth factors, one of which is FGF-1.

FGF-1 is a potent mitogen and angiogenic factor found in both normal and malignant tissues. FGF-1 mediates its effects by binding to its cognate receptor. Evidence indicates that the act of ligand biding to the receptor is insufficient for transduction of the mitogenic signal. It is speculated that FGF-FGFR internalization and its subsequent processing may play an active role in completing the mitogenic signal. The reported studies indicate FGF-1 is internalized into clathrin-coated vesicles by the "classical," receptor-mediated endocytic pathway. Upon disruption of internalization and processing pharmacological inhibitors, FGF-1 mitogenic activity is hindered. Furthermore, pretreatment of FGF-1-stimulated cells with an inhibitor of the major lysosomal proteases, cathepsin D, results in a reduction in the mitogenic response. Therefore, a cathepsin-D-like activity and possibly other cellular proteases may be participants in the FGF-1 signal transduction pathway. A small, linear fragment of FGF-1 has been identified that mimics, in some instances, the mitogenic bioactivity of the intact growth factor, supporting the notion that a processed form of FGF may have an intracellular role in signaling.

Breast carcinomas are characterized by the overexpression and secretion of various proteases, one of which is cathepsin D. Consequently, cathepsin D may play a coordinated role with FGF-1 in tumor progression and invasion. The secreted protease may actively release FGFs form the ECM, freeing them for subsequent internalization where cathepsin D may act in a later event necessary for FGF-1 signaling.

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INTRODUCTION

Cathepsin D levels in human breast cancers were initially believed to be an independent prognostic marker for metastasis and survival (1-3). The overall validity of this correlation has been called into question and the usefulness of cathepsin D as a prognostic indicator remains controversial. However, many human breast cancer cells are characterized by high-expression levels of cathepsin D, and other proteases, such that intracellular shuttling to the lysosome is overwhelmed, and a portion is constituitively secreted (1,4). This ubiquitous, lysosomal, aspartyl protease has been implicated in tumor invasiveness due to its ability to degrade extracellular matrix, ECM (4-6). Evidence indicates that cathepsin D is involved in the liberation of fibroblast growth factors (FGFs) from the ECM by proteolytic release of the heparan sulphate proteoglycans (HSPGs) which bind FGFs (7,8). Normal cells, as well as many malignant, mammary epithelial cells, will proliferate in response to FGFs.

FGF expression has been detected in both normal and malignant breast tissue. However, the specific role(s) that FGFs and their cognate receptors (FGFRs) play in breast cancer has yet to be elucidated. An exhaustive analysis of the gene expression of FGF family members and the FGF tyrosine-kinase receptors was undertaken, and it showed that RNA transcripts for FGF-1 were detected in all 103 malignant breast samples tested but were not evident in all the evaluated tumor cell lines (9). Messenger RNA for all four of the FGFRs has been detected in both breast cancer cell lines and primary tissue (10). Furthermore, FGFR-1 and FGFR-2 have been reported to be amplified in 20% of human breast cancers (11). *In vivo* studies utilizing MCF-7 cells overexpressing FGF-1 and FGF-4 implicate FGFs as both autocrine and paracrine modulators of breast cancer progression (12). A recent study by Zhang *et al* (13) indicated that FGF-1 may have a role in the transition of breast cancer cells to a hormone-independent, metastatic state. Additionally, it is speculated that FGF plays a role in tumor vascularization, thus providing a putative route for metastasis (14,15).

The FGF proteins are functionally diverse and upon receptor activation initiate a number of signaling cascades, none of which have been thoroughly characterized. In addition, there has been no determination to date as to what signaling cascades are essential in eliciting given biological responses. While undertaking studies to elucidate the structure-function relationship of FGF-1, our laboratory generated a site-directed mutant that indicated that receptor binding and activation could be dissociated from mitogenic activity. Burgess *et al* (16) mutated lysine residue at position 132 of human FGF-1 to a glutamic acid residue. The mutant, 132E, is able to compete with wild-type FGF-1 for high-affinity receptor binding, to stimulate tyrosine kinase phosphorylation, and to initiate transcription of immediate-early genes but is not capable of eliciting a mitogenic response (17). A second mutant was generated in which the cysteine residue at position 131 of the 132E mutant was changed to a serine residue, consistent with that of bovine FGF-1. This double mutant, 131C-132E has a mitogenic activity intermediate to the full response seen with wild-type FGF and the at least 100-fold reduction in response seen with the 132E (Figure 1A). The degradation profiles of the mutants were compared to that of wild-type FGF using ^{125}I-labeled ligands (Figure 1B). The wild-type FGF-1 degradation profile reveals distinct, relatively large fragments being generated; in contrasts, 132E remains entirely intact and disappears more rapidly over the time course, and the double mutant displays a profile that appears intermediate to that of wild type and the single mutant. The degradation profiles parallel the mitogenic activity of each protein as measured by [^{3}H]-thymidine incorporation assays. The extent to which the mitogenic activity of 131C-132E is restore qualitatively

corresponds to its degree of degradation as compared to 132E which is neither degraded nor mitogenic. In addition, Wiedlocha and colleagues (18,19) have demonstrated that a fusion protein of FGF-1 and diphtheria A is capable of stimulating DNA synthesis independent of FGFR activation when added exogenously to FGFR-deficient cells. The FGF-diphtheria A fusion protein is internalized by a diphtheria-mediated pathway with no evidence of an increase in intracellular protein phosphorylation. Moreover, Lin *et al* (20) reported that a seven-residue fragment of FGF-1 could function as a mitogen. The FGF-1 fragment, NYKKPKL, comprises the nuclear localization (NLS) of the full-length polypeptide. Using the secretion-signal peptide of FGF-4 linked to the FGF-1 NLS sequence, cells were able to import this synthesized peptide, also referred to as SA, in an apparent receptor-independent manner. Consequently, FGF binding to its tyrosine kinase receptor is likely necessary but is not solely sufficient for the cell to commit to proliferation, suggesting an as yet identified component in FGF mitogenic signaling.

The preliminary data and supportive literature discussed above led to the hypothesis that FGF-1 mitogenic signaling is mediated, in part, by a post-receptor, degradation-related event. There is increasing evidence that mitogenic signal transduction by polypeptide growth factors is multifaceted. It has been suggested that the internalized ligand, receptor, or ligand-receptor complex would guarantee the specificity of a response to a particular ligand amidst the complex, redundant signaling mechanisms initiated at the plasma membrane by a myriad of growth factors (21). Therefore, one component of the signaling pathway requires ligand binding to and activation of cell-surface tyrosine kinase receptors, while another aspect may require ligand internalization and its subsequent modification by a specific proteolytic processing event. Preliminary studies in our laboratory indicate that specific proteolytic processing upon ligand internalization may play a role in FGF mitogenic signaling and that a cathepsin-D-like activity may play a role in this processing.

In summary, many breast malignancies have been reported to express various FGF and FGFR family members (9). Both autocrine and paracrine effects of FGF-1 have been implicated in the estrogen-independent and tamoxifen-resistant growth of MCF-7 breast cancer cells both *in vitro* and *in vivo* (22). Additionally, FGF-2 has been shown to increase the steady-state levels of cathepsin D in this same cell line (23). And, given that FGFs are commonly found associated with the extracellular matrix and basement membrane, secretion of cathepsin D into the extracellular milieu may assist in FGF release and activation (4,5). Thus, an ideal environment is created for proliferation, neovascularization, and metastasis of breast carcinomas. Consequently, knowledge of how FGF-1 mediates its biological effects through an understanding of the FGF signal transduction pathway could lead to its possible *in vivo* manipulation as an adjuvant treatment for breast cancer.

METHODS AND PROCEDURES

Recombinant Protein Purification: Recombinant wild-type and mutant FGF were purified as described previously (16). The proteins were purified to >95% using reversed-phase HPLC, and concentrations were determined by amino acid analysis.

Amino Acid Analysis: Amino acid analysis was performed as outlined by Waters Associates (Milford, MA). Briefly, samples were hydrolyzed in vacuo in 6 N HCl/0.1% phenol (Pierce, Rockford, IL) at 150°C for 1 hour. Amino acid compositions were determined based on reversed phase separation of the

phenylthiocarbamyl derivatives using a PICO•TAG amino acid analysis system (Waters Associates, Milford, MA).

^{125}I-FGF Iodination: Recombinant, human FGF-1 was iodinated using the chloramine T method. Briefly, 1 mCi ^{125}I-Na was added to a mixture of 5 µg FGF-1 and 10 µl of 0.1 mg/ml chloramine T (Sigma Chemical Company, St. Louis, MO) and was allowed to react for one minute. The reaction was quenched with 10 µl of 1 mg/ml sodium metabisulfate (Sigma). The iodinated FGF-1 was isolated by heparin-Sepharose chromatography.

Internalization and Degradation: Murine NIH 3T3 cells or NIH 3T3 cells transfected with FGFR-1 were grown to ~90% confluence. The media was changed to binding buffer [DMEM, 25 mM HEPES (pH 7.4), 0.5% bovine serum albumin] containing ^{125}I-FGF-1 in the absence or presence of the other factors at the given concentrations as indicated in the figures. Equilibrium binding to cell-surface receptors was achieved by incubating the cells at 4°C for 90 minutes. Those cells receiving inhibitors, pepstatin A (Sigma) or chloroquine (Sigma), were pretreated for 1 hour at 37°C prior to growth factor addition and shifting to 4°C. The cells were rinsed three times with ice-cold binding buffer and then returned to 37°C in binding buffer in either the absence or presence of inhibitors. At the various time points, the cells were rinsed once with ice-cold PBS, twice with 1.5 M NaCl, 0.2 M acetic acid (pH 3.0), and then again once with ice-cold PBS. Cells were lysed in the dish with RIPA buffer [20 mM Tris (pH 7.4), 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, and 1x protease inhibitor cocktail (Calbiochem-Novabiochem Corp., La Jolla, CA)] and immediately scraped using a rubber policeman into microcentrifuge tubes. The tubes were placed on ice for 10 minutes prior to centrifugation at 10,000 x g for 10 minutes at 4°C. The supernatant was transferred to a new tube and an equal volume of Laemmli 2x sample buffer was added. Equal volumes of the samples were subjected to SDS-PAGE and proteins were visualized with Coomassie Brilliant Blue (BDH Laboratory Supplies, Poole, England). The gels were dried using a vacuum gel dryer and then were subjected to autoradiography.

Mitogenic Assays: Murine NIH 3T3 cells were seeded into either 48-well or 96-well plates and grown to ~80% confluence in DMEM (Biofluids Inc., Rockville, MD) containing 10% calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mcg/ml streptomycin (Biofluids Inc.) at 37°C. The cells were serum starved [0.5% calf serum] for 30 hours. Varying concentrations of growth factor in either the absence or the presence of 5.0 U/ml heparin (Upjohn Company, Kalamazoo, MI) were added to the serum-starved cells. Those cells receiving inhibitor treatment were pretreated for either 1 or 2 hours with the respective inhibitor prior to growth factor addition. After 18 hours, the cells were pulsed with 0.2 µCi/ml of [^{3}H]-thymidine (Amersham Life Science, Arlington Heights, IL) for 4 hours. The 48-well-plated cells were fixed with 10% TCA, rinsed with PBS, and then solubilized in 0.5 N NaOH. The amount of [^{3}H]-thymidine incorporated into acid-insoluble material was determined by scintillation counting. The 96-well-plated cells were either harvested essentially as described for 48-well plates or, alternatively, cells were harvested onto a glass fiber filter (Packard Instrument Co., Inc., Meriden, CT) using a FilterMate Cell Harvester (Packard Instrument Co., Inc.) and were analyzed by a Matrix 9600 Direct Beta Counter (Packard Instrument Co., Inc.), counting time of 3 minutes. All conditions were performed in triplicate.

Proliferation Assays: Human umbilical vein endothelial cells (HUVEC) were maintained on fibronectin-coated plates in Medium 199 (Biofluids Inc.) supplemented with 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 mcg/ml streptomycin, 10 U/ml heparin, and 10 ng/ml recombinant FGF-1. For proliferation assays, the cells were seeded in 6-well plates at ≈2,000 cells/well in the previously described medium in the absence of FGF-1. The indicated factors were added to the wells, and the media was changed every other day for seven days. On day eight, the cultured cells were trypsinized and counted using a Coulter counter (Coulter Electronics Inc., Hialeah, FL).

^{35}S-labeled Recombinant FGF: Recombinant, full-length FGF-1 was ^{35}S-labeled *in vivo*. Briefly, a single colony of BL21 *E. coli* transformed with a ßFGF-1 recombinant plasmid was picked from a Luria broth streak plate containing 35 µg/ml chloramphenicol and 100 µg/ml carbenicillin and was grown in Circlegrow medium containing the aforementioned antibiotics at 37°C until the OD_{600} reached 0.7. The bacterial cells were pelleted by centrifugation at 6,000 x g for 10 minutes. L-cysteine and L-methionine deficient MEM medium was made using the MEM Select-Amine Kit per the directions provided by Gibco BRL (Grand Island, NY). The pelleted cells were resuspended in MEM-deficient media supplemented with 2 mM L-glutamine, 25 mM HEPES (pH 7.4), and 7.5 mCi ^{35}S-Promix (Amersham Life Science) for *in vivo* cell labeling. Recombinant protein expression was induced upon addition of IPTG to 0.4 mM and incubation at 37°C was continued for an additional 2.5 hours. *E. coli* cell pellets were collected by centrifugation at 6,000 x g for 10 minutes at 4°C, and the metabolically labeled FGF-1 was purified by heparin-Sepharose chromatography as described under "Recombinant Protein Purification."

Anti-phosphotyrosine Immunoblots: NIH 3T3 cells were grown to near confluence in 60 mm dishes at which time the cells were serum starved (0.5% calf serum) for ~48 hours. Cells were exposed to the concentrations of factors and for the lengths of time as indicated. The cells were rinsed once in cold PBS containing 1 mM sodium orthovanadate. The cells were lysed in a lysis buffer [10 mM Tris (pH 7.4), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 µM sodium orthovanadate, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail] and then immediately scraped from the plates with a rubber policeman. The cell lysates were vortexed for 10 seconds and centrifuged at 10,000 x g for 10 minutes at 4°C. Total protein content of the lysate was determined by BCA protein assay (Pierce Chemical Corp., Rockford, IL). Laemmli sample buffer was added to each lysate, and an equal concentration of protein was separated on 7.5% SDS-PAGE gels. Proteins were transferred electrophoretically from the gels to nitrocellulose in transfer buffer [50 mM Tris, 384 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS] for 1 hour at 28 volts and then 14 hours at 84 volts. Immunoblotting was performed by using the murine, anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology) at 1 µg/ml and horseradish peroxidase-conjugated, goat anti-mouse secondary antibody (Bio-Rad Laboratories) at 1:30,000. The blots were visualized using the ECL chemiluminescence method per the manufacture's instructions (Amersham Life Science).

Peptide Synthesis: Peptides were synthesized using an Applied Biosystems (Foster City, CA) model 431A peptide synthesizer and small scale F-moc cycles supplied by the manufacturer. Peptides were purified by reversed-phase HPLC and were analyzed and quantitated by amino acid analysis.

SPECIFIC AIM 1:

To determine whether a unique FGF-1 derived fragment(s) that correlates with mitogenic signaling can be identified.

Results and Discussion

Degradation and Mitogenic Analysis of FGF

To further investigate the correlation between degradation and mitogenicity, FGF-1 degradation profiles were evaluated in the presence of two inhibitors, chloroquine and pepstatin A, which are known to target different aspects of ligand processing. As shown in Figure 2A,

FGF-1 degradation is sensitive to the disruption of normal lysosomal functions by chloroquine, a weak base that accumulates in acid compartments of the cell by permeation, thus increasing the pH by proton trapping. Upon treatment of cells with exogenous FGF-1 in the presence of 0.01 mM chloroquine, the degradation fragments normally generated are either absent or significantly reduced (Figure 2A), and the mitogenic activity of FGF-1 is inhibited (Figure 2B). In addition, a major endosomal and lysosomal protease, cathepsin D, was targeted by the addition of pepstatin. The addition of 0.01 mM pepstatin A resulted in a reduction in the mitogenic activity of FGF-1 (Figure 2B); whereas, no inhibitory effect was seen on serum-treated cells (Figure 2C). The lack of effect seen on the control, serum-treated cells at the given concentration indicates that the inhibition in the mitogenic assay is not due to general toxicity. Likewise, the inhibitors did not alter basal-level DNA synthesis when added alone to serum-deprived cells (Figure 2C). No obvious difference was noted when the degradation profiles of ^{125}I-FGF-1 in the absence or presence of 0.01 mM pepstatin A were compared (Figure 2A). However, the lack of difference may be due to limitations in detection and/or to the use of pepstatin A at a suboptimal dose as compared to reported use at ≥0.5 mM (24). Additionally, it is possible that pepstatin A may inhibit the internalization of FGF-1. Briozzo *et al* (8) reported that addition of pepstatin A inhibited the internalization of ^{125}I-FGF-2 as a result of preventing extracellular cathepsin D from releasing the ECM-bound FGF. They too reported not seeing an effect on the degradation of FGF-2. However, it is not likely that pepstatin A affected the internalization of FGF-1 in this study because Briozzo *et al* (8) did not observe an effect on internalization when FGF-2 was in solution and not experimentally incorporated into the ECM. Besides, it is unlikely that NIH3T3 cells are secreting significant quantities of cathepsin D into the extracellular milieu. Experiments are currently underway, however, to address the question of whether pepstatin A affects FGF-1 internalization through extracellular cathepsin D inhibition.

To ensure that the reduction in mitogenesis was the result of modifying degradation and not due to an alteration in the receptor-mediated signaling pathway, the phosphorylation pattern of NIH3T3 cells under mitogenic conditions (i.e., FGF-1) and reduced mitogenic conditions (i.e., FGF-1 with inhibitors) was compared. Neither chloroquine nor pepstatin A appeared to have an effect on the phosphorylation cascade of FGF-1-stimulated NIH3T3 cells (data not shown). This observation suggests that the receptor-signaling component, as viewed by phosphorylation patterns, is not altered. Therefore, the reduction in mitogenic activity may be associated with the degradation pathway.

Additional protease inhibitors were recently tested to determine their effect on mitogenesis in an effort to provide additional insight into the possible relationship between degradation and mitogenesis. Presently, the chloroquine data suggests that an acidic environment is a necessary component in FGF-1 mitogenesis. However, a caveat to the use of chloroquine is that it retards receptor recycling, although FGFRs are believed to traffic to and be degraded in the lysosome. Monensin, a carboxylic ionophore, was found to efficiently inhibit both degradation and mitogenesis at 1μM (data not shown). However, at this concentration significant inhibition of DNA synthesis was observed in the serum-treated, positive control. Therefore, the inhibition noted cannot be attributed to a FGF-related effect. An initial examination of leupeptin, a thiol-protease inhibitor known to act on cathepsin B, indicates that it may have a stronger inhibitory effect than pepstatin A on FGF-induced DNA synthesis in NIH3T3 cells. This inhibitor has only been evaluated in one experiment currently; therefore, the results are too preliminary to make any determinations. Both chlorpromazine, a cationic amphilic drug that acts on the clathrin-dependent pathway and inhibits receptor-mediated endocytosis, and filipin complex, a sterol-binding drug that disrupts caveolar structure and

function, have undergone an initial screening in order to determine the potential importance of these different endocytic vesicles in FGF signaling. An ultrastructural study of FGF-2 internalization indicated that endocytosis was consistent with a caveolae-like vesicle structure (25). Treatment of NIH3T3 cells with filipin complex did not affect the mitogenic activity of FGF-1 (data not shown). However, treatment with 10 μM chlorpromazine significantly inhibited the stimulatory effect of FGF (Figure 3). This dose of chlorpromazine had no inhibitory effect on serum-treated cells. In addition, the dose range used to inhibit the FGF activity was reasonable, as 25 μM chlorpromazine was shown to partially inhibit cholera toxin activity in hippocampal neurons (26). This result is consistent with FGF-1 signaling requiring a receptor internalization event mediated by the "classical" endocytic pathway involving a clathrin-coated vesicle.

Role of Heparin in Degradation

Using ^{125}I-labeled ligand limits the detection of fragments to only those containing an ^{125}I-tyrosyl residue; therefore, an alternative labeling approach was employed in an attempt to identify additional fragments. Recombinant FGF-1 was metabolically labeled *in vivo* using ^{35}S-cysteine and ^{35}S-methionine supplemented media during the induction of *E. coli* transformed with a FGF-1 plasmid (see Material and Methods). As shown in Figure 4A, a difference was observed when FGFR-1 transfected NIH3T3 cells were treated with either ^{125}I-FGF-1 or ^{35}S-FGF-1 and the FGF degradation profiles were compared. Metabolically ^{35}S-labeled FGF-1 results in the visualization of an additional degradation product. Consequently, ^{35}S-FGF-1 was used in a subsequent internalization experiment to determine whether heparin, a known protector of FGF proteolytic inactivation *in vitro*, had an effect on intracellular proteolytic processing of FGF.

The degradation profile resulting from ^{35}S-FGF-1 treatment of FGFR-1 transfected NIH3T3 cells was evaluated both in the presence and in the absence of heparin at a concentration, 5.0 U/ml, known to potentiate FGF-1-induced mitogenesis. Although the degradation products look similar at the earlier time points, the presence of heparin appears either to protect or to assist in the generation of the fragments such that they are present at the longer time points, persisting even at 24 hours (Figure 4B). However, unlike co-treatment of NIH3T3 cells with either chloroquine or pepstatin, the phosphorylation pattern of proteins in quiescent NIH3T3 cells stimulated with FGF in the absence or presence of heparin was different. Heparin co-treatment increased the duration in which several proteins were phosphorylated, particularly a phosphotyrosyl-containing protein with an apparent molecular weight of ≈40 kDa. Heparin treatment alone had a protein phosphorylation pattern, at all time points, that was indistinguishable from that of the unstimulated control. Due to these observed differences in the phosphorylation-signaling cascade upon FGF-heparin stimulation, it is unclear whether the FGF-derived fragments that persist in the presence of heparin play a role in the ability of heparin to act as a potentiator of mitogenesis.

Mitogenic Activity of a Small Fragment of FGF-1

In accordance with the hypothesis that an internalization, degradation-related event may play a role in FGF-1 mitogenic signaling, the SA peptide, which consists of the NLS of FGF-1 and the signal sequence of FGF-4, reported by Lin *et al* (20) was synthesized (Figure 5). In addition, the signal sequence of FGF-4 alone (cellular uptake peptide, CUP) was synthesized as a control. It was confirmed that the SA peptide was indeed capable of inducing a mitogenic

response in NIH3T3 cells; however, in extension to the authors' work, it was determined that this mitogenic response was completely abrogated in the presence of heparin (Figure 6). The heparin inhibition was alleviated upon reducing the heparin concentration to 0.5 U/ml. These results lead to the speculation that the SA peptide might be interacting with and internalized by cell-surface HSPGs and that the addition of heparin was competing for this interaction. However, upon testing the ability of the peptide to bind heparin-Sepharose, there was no evidence for such an association (data not shown). This observation is consistent with previous studies in our laboratory that have shown that although the amino acid sequence KKPK of the NLS is in agreement with a heparin-binding motif, this region of FGF-1 is not involved in heparin interactions (27). In addition, heparinase treatment of NIH3T3 cells to remove cell-surface HSPGs, which have been characterized as the "low"-affinity binding sites for FGFs, had no effect on the cells' responsiveness to the SA peptide (data not shown). It should be noted that the control, CUP peptide, tested in parallel during all the studies, did not display any biological activity.

Entry into the S-phase of the cell cycle as measured by [^{3}H]-thymidine incorporation does not necessarily imply that the cell is capable of undergoing mitotic division and, thus, proliferating. Therefore, the ability of the SA peptide to induce cell proliferation as determined by an increase in cell number was evaluated. The SA peptide was capable of supporting an increase in NIH3T3 cell number in a serum-free growth assay, although the peptide did not display as potent of an activity as was initially indicated by the thymidine-incorporation assay (Figure 7A). The SA peptide, however, was not able to maintain growth of HUVECs which are absolutely dependent on the presence of FGF for normal growth in tissue culture (Figure 7B).

In an attempt to better understand the signaling capability of the SA peptide and the role heparin played in inhibiting the SA peptide activity, anti-phosphotyrosine immunoblots of NIH3T3 cells treated with SA in either the absence or presence of heparin were analyzed (Figure 8). Noticeable differences were detected between the SA and the SA-heparin stimulated cells; whereas, again, heparin treatment alone had no observable effect. Quiescent NIH3T3 cells treated with the SA peptide alone had a more intense pattern of tyrosine phosphorylation as compared to cells co-treated with SA peptide and 5 U/ml heparin over the same time course. Furthermore, a phosphotyrosyl-containing polypeptide with the apparent molecular weight of $\approx$60.2 kDa, present at the zero time point and throughout the time course of the SA-heparin treated cells, was absent from the cells treated with the SA peptide alone. Therefore, treatment of quiescent NIH3T3 cells with the SA peptide results in both phosphorylation- and dephosphorylation-signaling events.

Although the mechanism by which the SA peptide is mediating cell growth is unclear, the data supports the notion that a relatively small fragment of FGF-1 is capable of mimicking certain of the intact protein's biological activity. It is possible that the biological effects observed are not a direct result of the activity of the SA peptide but rather an indirect result of releasing and/or competing for another intracellular factor possessing a NLS sequence. However, the data, as a whole, indicate that the view of a single, linear signaling cascade being responsible for the various biological responses associated with FGF is likely too simplistic.

SPECIFIC AIM 2:

To determine whether the mitogenic potency of FGF-1 for particular cell types correlates with their level of cathepsin D expression.

Results and Discussion

Studies under this specific aim have not yet been initiated but are a priority in this coming year. Currently, studies on this specific aim are pending the outcome of the results of the studies on additional protease inhibitors and their effects on the degradation and mitogenic activity of FGF-1. The initial findings that pepstatin A is able to reduce the mitogenic potential of FGF-1 on NIH3T3 cells is a key component to this study; however, additional evidence indicates that a leupeptin-inhibitable activity, suggestive of cathepsin B, may also play a role in mitogenic signaling. Therefore, this aim may be expanded to include the evaluation of both cathepsin D and cathepsin B levels in different cell lines and their possible correlation with FGF-1 mitogenic potency. Because cathepsin B has also been shown to be overexpressed in many breast malignancies and have implicated roles in progression to metastasis (15.33), expanding the study does not detract from the significance of the initial findings with cathepsin D or deviate from the overall theme of the proposed investigation.

STATEMENT OF WORK--READDRESSED

Specific Aim 1: The original Statement of Work called for determining the subcellular localization of FGF-1-derived fragments. This study is still a strong priority and initial gradients using marker beads have been attempted to find the appropriate conditions for adequate separation of endosomal and lysosomal fractions. However, if a proteolytically-modified form of FGF-1 has an important role in mitogensis, it would not likely remain in the endosomal or lysosomal compartment but rather would be trafficked elsewhere to mediate its effect. Therefore, the fractionation experiments will be expanded to look for exogenous FGF-1 in other subcellular locales also. Attempts will be made to isolate, sequence, and characterize the identified fragments. Investigation of subcellular trafficking of FGF-1 and FGF-1-derived fragments will be determined in MCF-7 cells also. The subcellular fractionation work will be supplemented with immunofluorescence work using exogenous, biotinylated FGF-1 which will not only distinguish it from endogenous FGF but should also allow for continued detection upon proteolytic cleavage of full-length FGF.

Specific Aim 2: Given the results obtained under Specific Aim 1 with the different drug treatments and their corresponding relationship to mitogenesis and degradation in a "normal" cell line, these treatments will now be applied to MCF-7 breast cancer cells which are known to overexpress both cathepsin D and cathepsin B. The protease levels of MCF-7 cells will be compared by Western blot analysis to the levels found in NIH3T3 cells. Consistent with the work of many others, I have been able to establish a FGF-1 mitogenic dose response in MCF-7 cells. Therefore, if administration of pepstatin A and leupeptin is able to inhibit the FGF-1-

induced mitogenic response in MCF-7 cells also, studies will be expanded to additional cell lines within the laboratory to substantiate the proteases' involvement in FGF-1 mitogenesis.

The original Statement of Work indicates that in year 2 the responsiveness of MCF-7 cells to FGF-1 will be tested in the presence of estrogen and anti-estrogen drugs. It has been reported that FGF-1 is capable of supporting estrogen-independent and antiestrogen-resistant growth in MCF-7 cells (22). And, although the cathepsin D gene has an estrogen-responsive promoter, estrogen treatment of MCF-7 cells induces a number of effects in addition to the possible upregulation of cathepsin D. Hence, the initially proposed studies are not likely to produce any clear results. Therefore, at this time, such studies will not be attempted unless a more defined, straight-forward need for the use of estrogen and anti-estrogen treatment arises.

CONCLUSIONS

During the internalization process of receptor-ligand complexes, there are several possible fates for the receptor and ligand. Proteases are found within endocytic vesicles along the entire pathway; hence proteolytic processing can occur at any point. Although most of the degradation seen during FGF-1 internalization is likely the result of ligand turnover, the data suggests that an unidentified, later event plays a role in FGF-1 signaling. It is hypothesized that this later event may involve a processed form of FGF-1. Although presently a novel concept for FGF-1, there is precedence for proteolytic modification leading to the activation of internalized ligands. The limited number of proteases and gradually decreasing pH gradient associated with the endocytic pathway provide a controlled environment for selective processing. Diment *et al* (28) reported that bovine parathyroid hormone is cleaved within endosomes of macrophages by the aspartyl-protease cathepsin D, thus generating a biologically active peptide that is recycled to the cell surface and released. In addition, the cytolytic activity of tumor necrosis factor (TNF) corresponds to its internalization and degradation in TNF-sensitive cells; whereas, TNF-insensitive cells appear incapable of degrading this cytokine (29). Similarly, the cytotoxicity of the plant toxin ricin A-chain requires an activation or membrane translocation event that is dependent on the endosomal proteases cathepsin B and cathepsin D (24). An obligatory role for both endosomal processing and membrane translocation have been described in mediating the action of both cholera toxin (30) and diphtheria toxin (31). This supportive literature and the recent report that FGF-1 is capable of interacting with and inducing leakiness of plasma membranes in a low pH environment (32) suggests a possible mechanism by which FGF-1 can be selectively modified and then traverse the endocytic vesicle membrane, localizing to other cellular compartments. And, given that a short, linear sequence of FGF-1 is capable of mimicking some of the biological functions of the intact polypeptide as was shown by the SA peptide studies, it is possible that upon receptor internalization FGF or the FGF-FGFR complex is modified such that it is able to transduce signals within the cell.

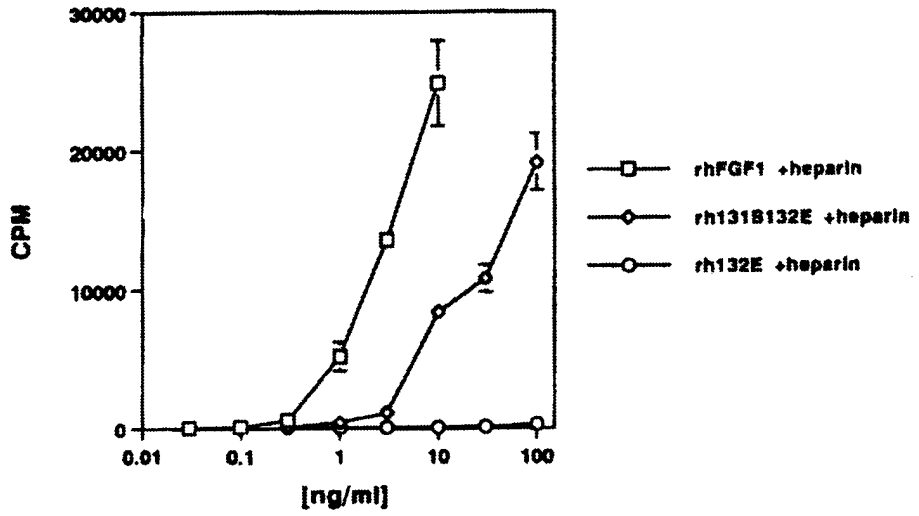
Treatment of cells with the lysosomotrophic drug chloroquine indicated that processing of FGF-1 or the ligand-receptor complex in an acidic compartment may play a role in mitogenesis. Endocytosis of the FGF-FGFR complex appears to follow the "classical" clathrin-coated, endosomal pathway, unlike the report of FGF-2 being internalized by a caveolae-like structure (25). Furthermore, evidence suggests that cathepsin D and/or cathepsin B may have an active role in FGF-1-signaling. Additional studies are underway to better characterize their putative role. The addition of pepstatin A and leupeptin, cathepsin D and cathepsin B inhibitors,

respectively, resulted in a reduction in FGF-1-induced mitogenesis on NIH3T3 cells. As mentioned above, there are reports of coordinated roles for cathepsin D and cathepsin B in proteolytic modification and membrane translocation of internalized ligands. It has not yet been determined whether inhibition of the proteases results in a reduced mitogenic activity due to an alteration in the proteolytic-processing of a necessary FGF-1 fragment or an alteration in the ability of FGF-1, intact or otherwise, to traffic to other cellular compartments. Exogenous FGFs have been reported to translocate to the nucleus by an as yet identified pathway. However, both the putative nuclear localization of FGFs and its functional role in the nucleus are controversial and an active area of investigation.

Although the work described in this progress report appears to have limited relevance to breast cancer research at this stage, the hopes of the investigator is to establish a basis for the link between internalization and mitogenesis in a "normal" cell line, thus establishing a foundation and reference point prior to moving on to studies with MCF-7 human breast cancer cells. A post-receptor event in FGF-1 signaling is becoming well accepted within the FGF field. Therefore, although much of the data presented at this time is circumstantial, it is building the foundation to better understand this post-receptor event. The present focus of identifying the subcellular location of exogenously added FGF-1 by a coordinated approach, employing both cellular fractionation and immunocytochemical techniques, in the presence and absence of the various inhibitors should provide a more concrete understanding of the internalization component of FGF-1 signal transduction. Because FGF-1 is well recognized both as a potent mitogen and as an angiogenic factor, it may have a multifunctional role in the progression of human breast cancers and the development of tumors at secondary sites. Therefore, a better understand of the mechanism through which FGF is mediating its biological effects may provide additional avenues for putative adjuvant therapies for breast cancer and, perhaps, provide insight into the mode of action of other angiogenic and growth modulators of human carcinomas.

FIGURES

A.



B.

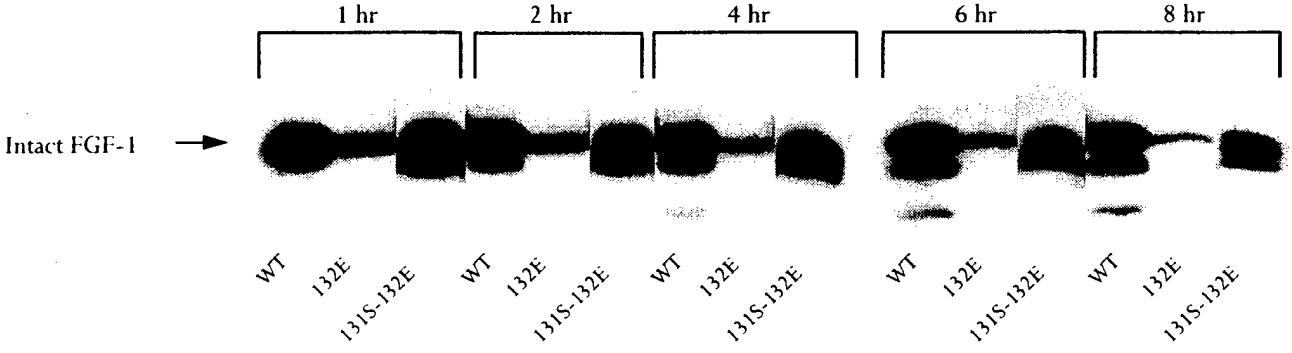


FIGURE 1. **(A)** Ability of wild-type and mutant forms of FGF-1 to stimulate DNA synthesis in NIH3T3 cells. **(B)** SDS-PAGE analysis of the degradation of ^{125}I-labeled wild type (WT), 132E, and131S-132E following internalization by FGFR-1 transfected NIH3T3 cells for the lengths of time indicated.

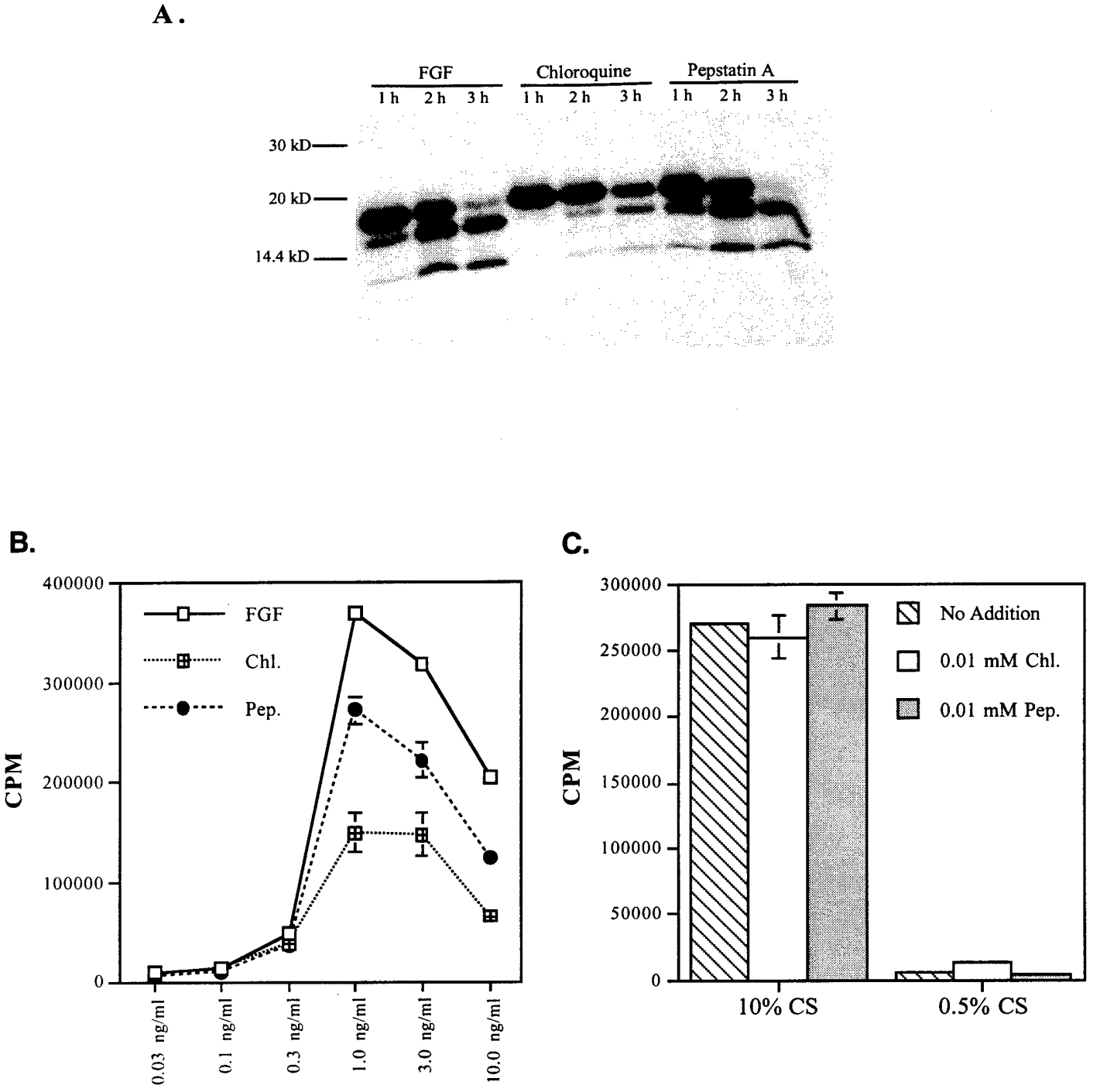
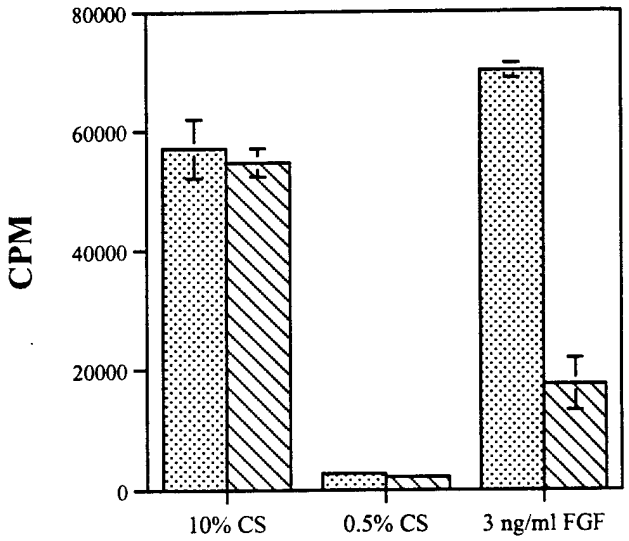
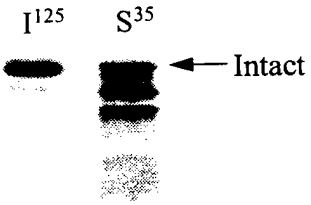


FIGURE 2. **(A)** Analysis of the degradation profiles of ^{125}I-FGF-1 in the absence or the presence of the lysosomotrophic agent chloroquine (0.01 mM) or the cathepsin-D inhibitor, pepstatin (0.01 mM) for 1, 2, or 3 hours at 37°C. **(B)** Ability of 0.01 mM chloroquine (Chl.) and 0.01 mM pepstatin A (Pep.) to inhibit FGF-1-stimulated DNA synthesis in NIH3T3 cells. **(C)** DNA synthesis of control, serum-treated (10% calf serum) or serum-starved (0.5% calf serum) NIH3T3 cells in the absence or presence of inhibitors.

FIGURE 3. The effect of 10μM Chlorpromazine on serum-stimulated (10% calf serum), serum-starved (0.5% calf serum), or FGF-stimulated NIH3T3 cells.



A.



B.

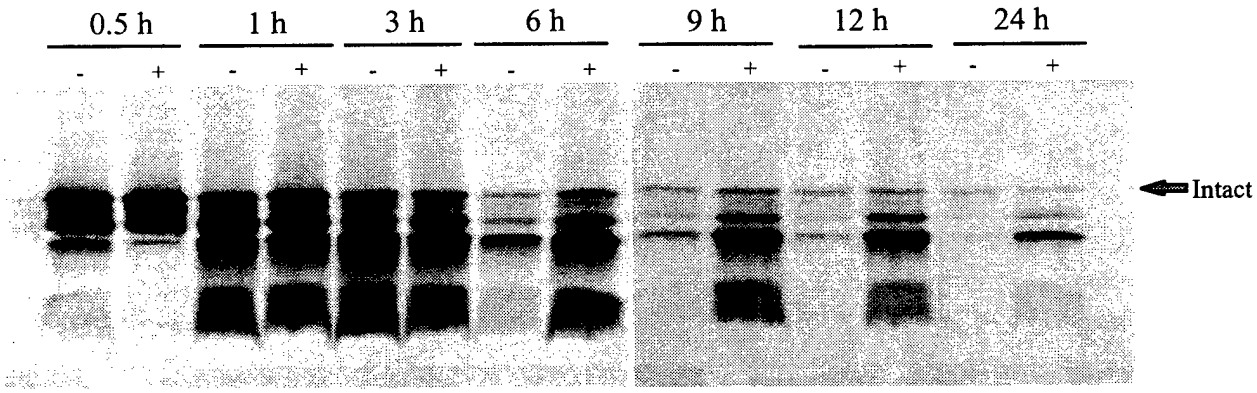


FIGURE 4. **(A)** The different degradation profiles generated when either ^{125}I- or ^{35}S-labled FGF is subjected to internalization and catabolism by FGFR-1 transfected NIH3T3 cells for 2 hours. **(B)** A comparison of the degradation profiles of metabolically ^{35}S-labled FGF in either the absence (-) or presence (+) 5.0 U/ml heparin. Internalization of exogenous ^{35}S-FGF by FGFR-1 transfected NIH3T3 cells occurred at 37°C for the lengths of time indicated.

SA Peptide	A A V A L L P A V L L A L L A P A A A **N Y K K P K L**
CUP Peptide	A A V A L L P A V L L A L L A P E

FIGURE 5. Amino acid sequence of the peptides synthesized.

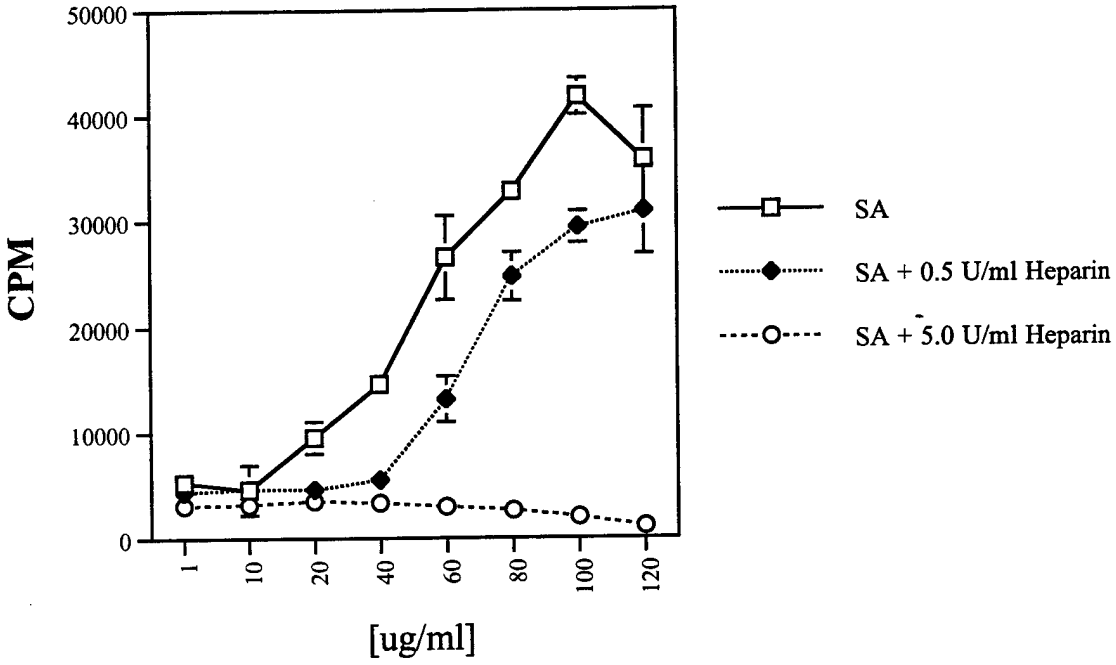


FIGURE 6. The ability of the SA peptide to stimulate DNA synthesis in NIH3T3 cells in the absence or presence of the given concentrations of heparin.

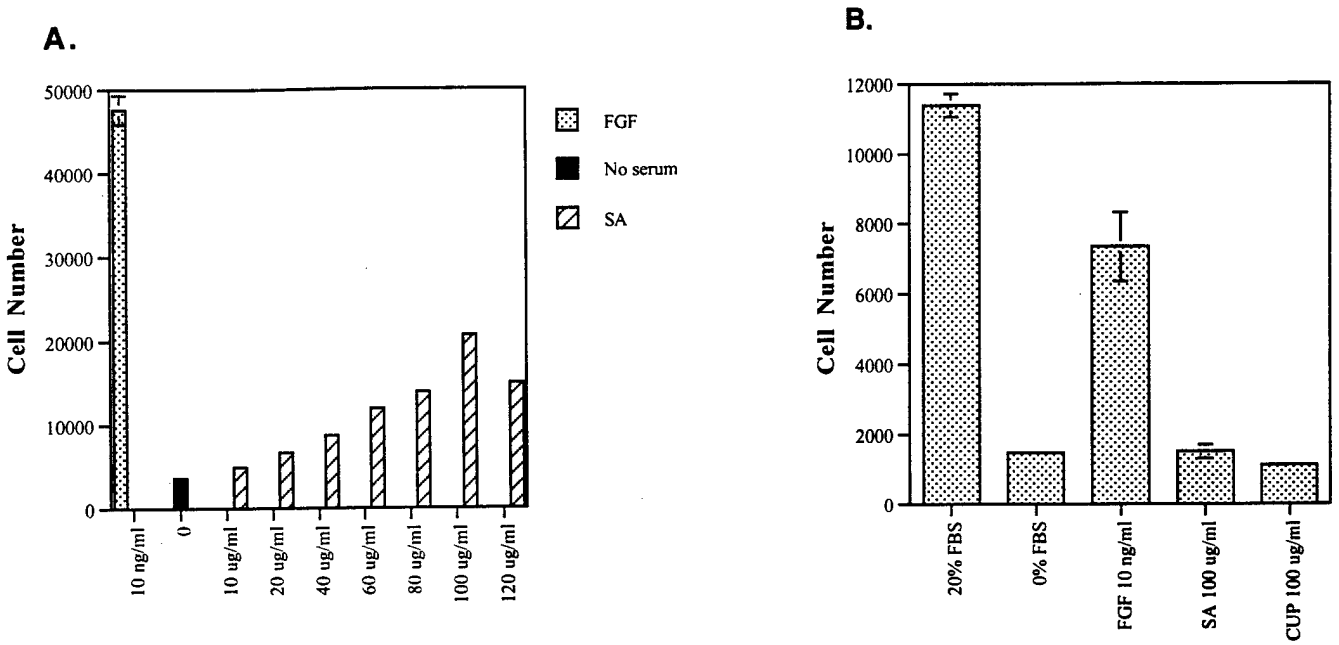


FIGURE 7. (A) The ability of the SA peptide to support NIH3T3 cell proliferation in serum-free medium as compared to that of an optimal concentration, 10 ng/ml, of FGF-1. **(B)** A comparison of the ability of FGF-1, 10 ng/ml, and the SA peptide, 100 µg/ml, to support HUVEC growth in the absence of normal growth-supporting factors. The CUP peptide is included as a control to show that the FGF-4 signal peptide does not contribute to the activity of theSA peptide.

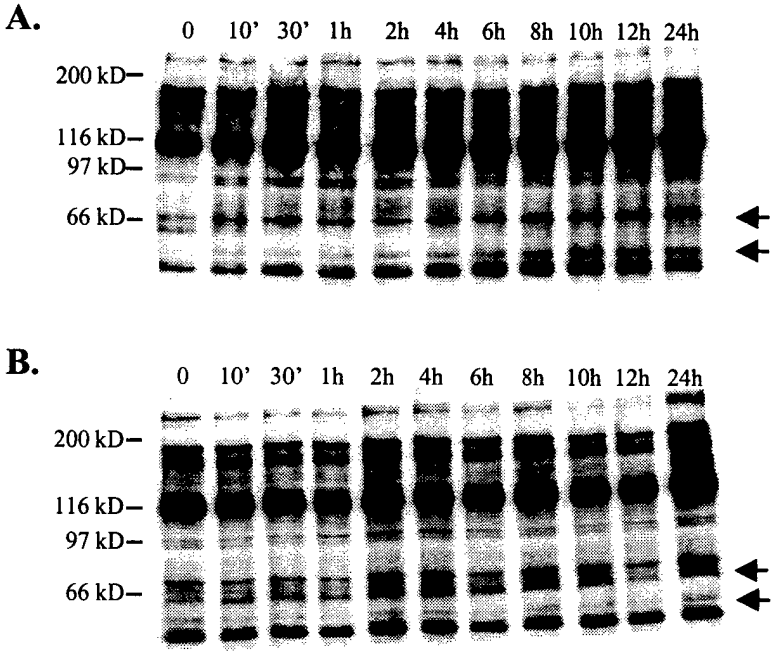


FIGURE 8. Anti-phosphotyrosine analysis of NIH3T3 cells treated with 100 µg/ml of the SA peptide in the absence **(A)** or the presence **(B)** of 5.0 U/ml heparin. The presence of the heparin abrogates all mitogenic activity of the SA peptide. The arrows denote obvious difference between the phosphorylation patterns, showing both phosphorylation- and dephosphorylation signaling-events.

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