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Fibroblast Growth Factor-1 (FGF-1) is a potent mitogen and angiogenic factor found in both normal and malignant tissues. FGF-1 mediates its diverse biological effects by binding to and activation of its high-affinity tyrosine kinase receptors, initiating a myriad of signaling cascades. Evidence indicates that the act of receptor engagement by the FGF ligand is insufficient for transduction of a full proliferative 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 that the FGF-1 mitogenic signal requires ligand endocytosis via the "classical" clathrin-mediated pathway and exposure to an acidic compartment. Furthermore, both a pepstatin- and leupeptin-sensitive event(s) appear critical for a full mitogenic response. Given the implicated role for a number of different proteases in contributing to the breast cancer pathology, this protease-sensitive activity in FGF-1 signaling is under investigation. FGF-1 has been reported to progress breast carcinomas from a hormone-dependent state to a hormone-independent state and from nonmetastatic to metastatic. Therefore, better understanding the mechanism of FGF-1 signaling could provide additional avenues for adjuvant therapies. An antimitogenic approach to treatment of breast cancer would have a broader efficacy, not limited by the hormone-responsive state of the tumor.

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Teri A Grieb 7/25/99

PI - Signature Date

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INTRODUCTION

The proteins comprising the fibroblast growth factor (FGF) family are functionally diverse and upon binding to and activation of their high-affinity tyrosine kinase receptors (FGFR) initiate a number of signaling cascades, none of which have been thoroughly characterized. Evidence indicates that the act of ligand binding to its high-affinity cell surface receptor is insufficient for transduction of a full proliferative signal. It is speculated that FGF-FGFR internalization and its subsequent processing may play an active role in completing the mitogenic signal. FGF-1 is a potent mitogen and angiogenic factor found in both normal and malignant tissues. However, the specific role(s) that FGF-1 and its cognate receptors (FGFRs) play in breast cancer has yet to be elucidated. A number of groups have reported that both the hormone-dependent breast cancer cell line MCF-7 and the hormone-independent breast cancer cell line MDA-MD-231 express FGF-1 mRNA, FGF-1 protein, and FGF receptors [1-3]. Studies using neutralizing anti-FGF-1 antibody [4] and FGF-1 transfected MCF-7 cells [5-7] indicate that proliferation of breast cancer cells may occur not only by a possible FGF paracrine signaling mechanism but also by an autocrine mechanism. Moreover, it has been proposed that FGF-1 signaling may be important in the transition of breast cancer cells from a hormone-dependent state to a hormone-independent state and from nonmetastatic to metastatic [5]. Additionally, it is speculated that FGF-1 plays a role in tumor vascularization, thus providing a putative route for metastasis [5,8,9]. Consequently, knowledge of how FGF-1 mediates its biological effects through an understanding of the FGF signal transduction pathway could lead to significant advances in its *in vivo* manipulation and use as an adjuvant treatment for breast cancer.

ORIGINAL WORKING HYPOTHESIS

The proposed hypothesis to be tested is that the mitogenic signaling pathway of FGF-1 is, in part, composed of a post-receptor, degradation-related event. There is increasing evidence that more than one distinct pathway exists for the mitogenic signal transduction of polypeptide growth factors. One pathway may require ligand binding to and activation of cell-surface tyrosine kinase receptors. An additional component(s) may require ligand internalization and its subsequent subjection to a specific proteolytic processing event, resulting in intracellular effectors that complete the mitogenic signal.

TECHNICAL OBJECTIVE 1:

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

RESULTS AND DISCUSSION

Mitogenic Response to and Proteolytic Processing of FGF in a "Nor mal" Cell Line

To further investigate the preliminary correlation between degradation and FGF mitogenicity, a possible role for FGF-1 internalization and proteolytic processing in growth factor-induced mitogenesis was evaluated. External ligands can be selectively endocytosed

by clathrin-dependent and clathrin-independent vesicular pathways. Therefore, I compared FGF-1 internalization via the clathrin-coated pathway characteristic of receptor-mediated endocytosis to the nonclathrin, caveolae-mediated pathway. To distinguish between these two pathways, cells were either treated with chlorpromazine or filipin complex. Chlorpromazine is a cationic amphilic drug that has been shown to disrupt receptor-mediated endocytosis by causing the recruitment of both the AP-2 adaptor protein and clathrin to uncoated, late endosomal vesicles [10-12]. Filipin is a sterol-binding agent with cholesterol binding properties that lead to the structural and functional abrogration of caveolae and glycolipid microdomains due to their cholesterol-rich nature [11,13,14].

Quiescent NIH 3T3 cells, a murine fibroblast cell line, were treated with increasing concentrations of either chlorpromazine or filipin and then stimulated with either calf serum or FGF-1. In the presence of chlorpromazine, the mitogenic activity induced by FGF-1 was progressively inhibited with increasing concentrations of the drug; whereas, no inhibitory effect was noted for serum-induced mitogenesis (Figure 1A). Neither serum-induced nor FGF-induced mitogenesis was altered in the presence of filipin at concentrations that did not affect cell viability (Figure 1B). When the degradation profiles of internalized ^{125}I-FGF were analyzed following drug treatment, filipin had no apparent affect on FGF catabolism, while the addition of chlorpromazine lead to slower processing (data not shown).

Receptor-mediated endocytosis subjects the receptor-ligand complex both to a gradually decreasing pH gradient and to an increasing spectrum of proteases as trafficking progresses from the early endosome to the lysosome. The FGF mitogenic pathway has a requirement for an acidic environment as was previously illustrated by the treatment of cells with chloroquine or monensin which act through different mechanisms to increase the endosomal/lysosomal pH. Chloroquine is a weak base that diffuses across cellular membranes in an uncharged form and accumulates in acidic compartments where it becomes protonated, raising the intracellular pH [15-18]. Monensin is a carboxylic ionophore that dissipates intracellular pH gradients by allowing protons to equilibrate across membranes by exchange with cations [15,19,20]

NIH 3T3 cells treated with 10 μM chloroquine or 0.1 μM monensin had an 82% and 89% decrease, respectively, in FGF-induced DNA synthesis; whereas, serum-stimulated cells showed essentially no diminition in DNA synthesis at the same concentrations of drugs (Figures 2A & 2B). Because significant loss of mitogenic activity in FGF-stimulated cells occurred at concentrations that did not significantly affect serum-stimulated cells, it is clear that the inhibition is not due to toxicity.

Treatment of FGFR-1 transfected NIH 3T3 cells with 10 μM chloroquine or 0.1 μM monensin prolonged the presence of internalized ^{125}I-FGF proteolytic fragments (Figure 2C). Higher concentrations of the drugs that also showed a reduction in serum-induced mitogenesis resulted in a complete inhibition of degradative processing of internalized FGF (data not shown). The reduction in degradation is consistent with the requirement of a low pH environment by most lysosomal proteases for optimal activity.

To ensure that the addition of the drugs was not impeding the interaction of FGF with its cell-surface receptors, the binding of ^{125}I-labeled FGF to both NIH 3T3 cells and FGFR-1 transfected NIH 3T3 cells was determined in the absence or presence of the drugs. As shown in Figure 3, none of the drugs significantly interfere with FGF binding to the cell-surface FGFRs. In the presence of 15 μM chlorpromazine, there is a modest reduction in cell-surface associated counts in both cell lines. Although the ~8% reduction in FGF binding to NIH 3T3

cells is likely to contribute to the inhibition of mitogenesis, it seems unlikely to account for the entire 82% decrease in activity. Moreover, it was determined that the presence of the drugs did not prevent FGFR activation. FGFR-1 was immunoprecipitated from FGF-activated NIH 3T3 cell lysates following a 30 hr serum-deprivation period that mimics the conditions of the mitogenic assays. The precipitated immune complexes were solubilized in Laemmli sample buffer, subjected to SDS-PAGE, and transferred for anti-phosphotyrosine immunoblot analysis. No discernible difference between the phosphorylation status of FGFR-1 in cells with or without drug treatment was noted when taking into consideration the quantity of receptors detected in each lane (data not shown).

The reduction in FGF-induced mitogenesis observed in the presence of chlorpromazine, chloroquine, and monensin indicates that trafficking to and processing within lysosomes may play a role in FGF signaling. Therefore, a number of protease inhibitors exhibiting specificities for three of the major classes of proteases—cysteine-, serine-, and aspartyl-proteases—were evaluated to determine whether FGF proteolytic processing contributes to the mitogenic signaling pathway. The fourth major class of proteases, the metallo proteases, has not presently been investigated because there has been no evidence to date for their existence in the endocytic/lysosomal pathway.

NIH 3T3 cells were stimulated with either FGF-1 or serum in the presence of the various protease inhibitors; the results of which are shown in Table 1. While the percentage of inhibition varied slightly from experiment to experiment, the general trend of inhibition remained consistent. Leupeptin, at 1 mM, inhibited approximately 76% of the mitogenic response induced by FGF, while only a ~12% reduction was observed in the serum-responding cells. The addition of TPCK resulted in a ~37% decrease in FGF activity with a minimal ~4% reduction in the serum-stimulated response. Because leupeptin and TPCK are reported to inhibit both serine and cysteine proteases, an effort was made to distinguish between the two classes of proteases using other inhibitors. However, no conclusions could be drawn as to the protease class, whether cysteine or serine, that participates in the mitogenic response to FGF. Neither the serine protease inhibitor aprotinin nor the cysteine protease inhibitor EST resulted in a decrease in FGF-induced mitogenesis; whereas, the naturally occurring cysteine protease inhibitor, cystatin, resulted in a modest reduction, 19%, in FGF-induced mitogenesis with no effect seen on serum-induced mitogenesis. The potent inhibitors N-acetyl-leu-leu-methioninal and 4-(2-aminoethyl)-benzenesulfonyl-fluoride that inhibit cysteine and serine proteases, respectively, both resulted in a ~60% decrease in the responsiveness of NIH 3T3 cells to FGF. The ~23% reduction in FGF mitogenic activity in the presence of 100 μM pepstatin suggests a possible role for an aspartyl-protese, such as cathepsin D, in FGF mitogenic signaling.

The degradation profiles of internalized ^{125}I-FGF-1 in either the absence or the presence of a number of the protease inhibitors was evaluated. As shown in Figure 4, FGF-1 remains intact at the 0.5 hr time point but is then processed to two distinct fragments that persist as long as 12 hr, and in some experiments are faintly seen at the 24 hr time point. In the presence of 1mM leupeptin, there is a complete absence of FGF proteolytic processing, thus strongly indicating a role for a cysteine and/or serine protease(s) in the catabolism of internalized FGF. Treatment with either 10 μM TPCK or 60 μM N-acetyl-leu-leu-methioninal, both of which resulted in a reduction in mitogenic activity, slowed the rate of FGF degradation. Treatment with 80 μM 4-(2-aminoethyl)-benzenesulfonyl-fluoride (BSF) had a less pronounced affect on FGF proteolysis. In the presence of the drug, the faster

migrating band of ~5 kD was not evident at the 1 hr time point and intact FGF was present through the 24 hr time point, although very faint at the later time points. Furthermore, the ~14 kD band can be detected as late as 24 hr. Aprotinin-treated cells exhibited a FGF degradation profile that did not seem to differ significantly from that of untreated, control FGF. The degradation profile of pepstatin-treated cells has been evaluated in the past and showed a profile essentially identical to that of FGF alone. However, experiments are underway to confirm that a lack of difference is indeed the case, as in the past year it was observed that a number of our frozen stocks of cells were contaminated by a bovine species of mycoplasm that likely came from a bad batch of serum. New cells were received from ATCC and are routinely screened for mycoplasm. I have been repeating all previous experiments in an effort to confirm their validity; however, the work is extensive and has not been completed. To date, there has been no apparent difference between the results obtained with the mycoplasm-infected cells and the mycoplasm-free cells. But, I prefer to finish repeating the experiments before making any definitive statements as to the affect of pepstatin on the degradation profile of FGF.

To better understand the inhibition of FGF mitogenic activity upon treatment with leupeptin, ^{125}I-FGF binding to and internalization within NIH 3T3 cells was followed over a 24 hr period (Figure 5). The presence of leupeptin neither hindered FGF binding to cell-surface receptors nor its internalization. However, leupeptin affected the intracellular catabolism of FGF. A greater number of intracellular counts remained for a longer duration in the presence of leupeptin. Also consistent with slowed degradation, a delay in counts released into the media was observed. Consequently, the inhibitory effect of leupeptin appears to be associated with its protease inhibitor activity and not due to a general antagonism of binding and endocytosis. Moreover, the diminished mitogenic response to FGF in leupeptin-treated cells is not likely to be attributed to a decrease in FGFR-1 activation, as FGFR-1 immunoprecipitated from leupeptin-treated NIH 3T3 cells actually showed a slight increase in tyrosine phosphorylation as compared to leupeptin-untreated, control cells (data not shown). However, there is no reason to believe that an increase in the phosphorylation state of the receptor would result in an increase in positive signaling to the cell, but rather may deliver a negative signal.

The impact on intracellular trafficking of FGF in the presence of leupeptin was evaluated. In order to mimic the conditions of the mitogenic assays, NIH 3T3 cells were treated comparably except that the cells were stimulated with ^{125}I-FGF. Subsequently, the cells were harvested at the time points indicated in Figure 6 and subjected to cellular fractionation into cytosolic and nuclear fractions. The radioactivity in the crude cytosolic and nuclear fractions was quantitated, and consistent with the previous results, 1 mM leupeptin resulted in an increase in intracellular FGF. In the absence of leupeptin, ^{125}I-FGF radioactivity associated with the cytosolic fraction peaked at 6 hr after growth factor addition; whereas, in the presence of leupeptin, ^{125}I-FGF counts continued to increase over the 24 hr period. A noticeable increase in FGF-associated counts was also seen in the nuclear fraction of leupeptin-treated cells, while near background counts were seen in FGF treated cells.

To ensure that the difference in nuclear counts observed between leupeptin-treated and -untreated cells was not due to cytosolic contamination, quantitation of acid phosphatase, a lysosomal component, was performed on all the fractions. The acid phosphatase activities of the fractions were compared at each time point to the activity level of whole,

unfractionated cell lysates that were harvested in parallel. The acid phosphatase levels of leupeptin-treated and -untreated cellular fractions were comparable at all time points. Acid phosphatase contamination in the nuclear fractions was ≤2.6% of the total cellular acid phosphatase activity (data not shown). Consequently, it cannot be ruled out that the low level of growth factor found in the nuclear fraction of cells stimulated with FGF alone is not due to cystosolic contamination as the nuclear counts consistently represented ~3% of the total cellular counts. However, the FGF in the nuclear fractions of cells treated with leupeptin ranged from 9-13% of the total cellular FGF and, therefore, less than one-third of the nuclear counts can be attributed to contamination

To visualize the FGF associated with the cytosolic and nuclear fractions at the various time points, the fractions were acid precipitated. The TCA-insoluble pellet was resuspended in Laemmli sample buffer and subjected to SDS-PAGE and autoradiography (Figure 7). The cytosolic fractions of cells treated with FGF-1 alone showed distinct, proteolytic fragments typical of the FGF degradation profile. In those cells treated with 1 mM leupeptin, FGF remained essentially intact in both the cytosolic and nuclear fractions. No data is shown for the nuclear fractions of FGF stimulated cells without leupeptin co-treatment because there were so few counts that even after an extended exposure time neither intact nor processed FGF was evident.

Mitogenic Response to and Proteolytic Processing of FGF in Breast Cancer Cell Lines

Much of the work described, thusfar, has been conducted in a NIH 3T3 cell system because the putative correlation between FGF-1 mitogenic activity and growth factor degradation was originally observed while using this cell line. The intent of the investigator in continuing studies in the NIH 3T3 cell line was to establish a basis for the link between internalization and mitogenesis in "normal" cells, thus establishing both a foundation and a reference point for the breast cancer cell lines. In the initial proposed work, I only mentioned the use of MCF-7 human breast cancer cells, which are well established as a hormone-dependent cell line. However, I have also decided to include the use of MDA-MD-231 human breast cancer cells, which are well characterized as a hormone-independent cell line. It is apparent to the investigator that pending the outcome of the studies conducted in the malignant cells, that a more appropriate reference point will be a "normal," human mammary cell line and such comparisons will be made.

The mitogenic responsiveness of the two breast cancer cell lines to FGF-1 was determined. As shown in Figure 8, MCF-7 cells respond to increasing concentrations of FGF-1. In the presence of 5 U/ml heparin, a well-known potentiator of FGF mitogenic activity, the mitogenic response was enhanced at the lower concentrations of FGF but less potentiation was noted at the higher FGF concentrations. When FGF-1-induced mitogenesis was evaluated in MDA-MD-231 cells, there was a modest increase in DNA synthesis over the untreated, serum-deprived control (Figure 8). However, it is questionable whether the increase is statistically significant especially at the higher concentrations of FGF. There was no apparent dose response with increasing concentrations of FGF as was seen in the MCF-7 cell line. Indeed, it appeared that the responsiveness decreased with increasing concentrations of FGF in the MDA-MB-231 cell line. In addition, there was no apparent difference in the mitogenic response in the absence or presence of heparin. The mitogenic assays were performed 4-5 days after serum withdrawal and in the case of MCF-7 cells in

phenol-red free media in order to limit stimulation by steroids and serum-associated growth factors. It is evident by the extent of [^{3}H]-thymidine incorporation in the serum-starved control of the MDA-MD-231 cells that they have a limited serum requirement and grow sufficiently in its absence. Yazidi and Biolly-Marer [4] reported a FGF-activity in the cell extracts and culture media of MDA-MD-231 cells that was sensitive to anti-FGF-1 neutralizing antibodies. Therefore, the limited response to exogenous FGF in MDA-MD-231 cells may not truly reflect their ability to respond to FGF. If MDA-MD-231 cells express levels of FGF as to sufficiently bind to and activate enough FGFRs to deliver a threshold biological signal, the addtion of more FGF would not necessarily further enhance the positive signal and may even diminish the signaling capability by interfering with receptor crosslinking. It has been speculated that the roll-over effect often seen in FGF mitogenic assays at high concentrations of FGF in the presence of heparin represent sufficient quantities of FGF binding to the receptor that receptor dimerization is hindered and a preponderance of FGFR monomers result.

The ability of FGF to sustain cell growth was also determined for both cell lines using a growth assay. Serum-free media supplemented with 10 ng/ml FGF was able to support an increase in MCF-7 cell number over an 8 day period over that of serum-free media alone (Figure 9). During the first 4 days, the number of FGF-treated cells increased comparably, although slightly less in the absence of heparin, to cells treated with phenol-red free growth media, containing 10% charcoal-stripped fetal bovine serum and 10 ug/ml insulin. However, in the last half of the study, treatment with growth media resulted in an increase in cell number that far exceeded that of the FGF-supplemented cells. When the ability of FGF-1 to sustain the growth of MDA-MB-231 cells was investigated, there was no evidence that FGF either supported or inhibited the growth of these cells (Figure 9).

In an effort to determine if the mitogenic potency of FGF correlated with its potential to be proteolytically processed, ^{125}I-FGF was added to MDA-MB-231 or MCF-7 cells and followed over 24 hr (Figure 10). The MDA-MB-231 cells internalized FGF to a greater extent than the MCF7 cells which is not surprising given that significantly more FGF bound to MDA-MB-231 cells in the absence of heparin, suggesting a greater number of low-affinity, heparan-sulphate proteoglycan (HSPG) binding sites on their cell surface (data not shown). It has been shown that HSPGs are capable of internalizing FGF independent of the FGFR. It was interesting to note that the MDA-MB-231 cells did not appear to significantly process the internalized FGF. The growth factor remained essential intact with the intensity growing progressively weaker over the time course, indicating its clearance. It is unclear whether intact FGF is recycled or whether once available for degradation, FGF is quickly processed such that the intermediary fragments are not witnessed. One way to address this will be to follow the radioactivity associated with the cell-surface, with the intracellular pool, and with the media over a 24 hr time course. Determining what percentage of each fraction is acid-precipitable will reveal whether the radioactive counts are associated with free amino acids, indicating complete processing, or larger forms of FGF, indicating modest-to-no processing. The lack of significant processing in MDA-MB-231 cells is of particular interest because it corresponds with a mutant form of FGF-1 studied in our laboratory (described in previous reports). The mutated FGF is capable of carrying out all the initial events of the mitogenic signal—equally as well as wild-type FGF--but is neither able to elicit a full mitogenic response in nor able to be degraded by NIH 3T3 cells. The degradation profile of the mutant FGF looks essentially identical to that of wild-type FGF in MDA-MB-231 cells.

Although much harder to see than the results of the MDA-MB-231 cells, the MCF-7 cells internalized FGF over the 24 hr period (Figure 10). However, the quantity of FGF internalized was so low that in order to visualize the counts in a reasonable time frame it was necessary to acid-precipitate the fractions. The TCA-insoluble pellet was solubilized in Laemmli sample buffer and subjected to SDS-PAGE and autoradiography. FGF was cleaved into 2 distinct fragments in the MCF-7 cells. However, a band consistent with being intact FGF was clearly present throughout the time course. The faster migrating forms of FGF were present in lesser quantities until the 24 hr time point when the intact form and ~14 kD form of FGF appeared to be equal.

Because 1 mM leupeptin showed such a marked decrease in the mitogenic potency of FGF on NIH 3T3 cells, the responsiveness of the breast cancer cell lines to FGF was examined in the presence of the drug (Figure 11). Treatment of MCF-7 cells with 1 mM leupeptin resulted in a decrease in both FGF-induced and serum-induced DNA synthesis, 31% and 20%, respectively. An increase in DNA synthesis was observed in MDA-MB-231 cells stimulated with FGF or serum in the presence of 1 mM leupeptin. Leupeptin treatment yielded an 8% increase in serum stimulation and a 15% increase in FGF stimulation. The relevance of this finding is unclear and it will be interesting to see if any of the other protease inhibitors have a similar effect.

Attempt to Find a Bioactive Fragment of Intracellular FGF-1

Implied in Technical Objective 1 is the premise that a bioactive fragment of FGF-1 might be generated upon internalization and proteolytic processing. The bioactive fragment would then act as an intracellular effector of the mitogenic signal and work in concert with the signaling events initiated by the activated FGFR at the cell-surface. If such a biologically active peptide was generated upon FGF enzymatic cleavage, one may envisage a need for the fragment to escape the endosome/lysosome and traffick to another cellular compartment to mediate its effect. Although a novel concept for FGF, this mechanism is employed by many of the toxins which are moderately processed following internalization, allowing for their translocation through the endosomal membrane to the cytosol where they carry out their toxic effect.

As outlined in the Statement of Work, subcellular fractionation and a purification scheme would be used to isolate and identify FGF-derived fragments. In order to sequence a protein by traditional Edman chemistry, it is critical that the sample to be sequenced is pure. Consequently, I explored the possibility of whether FGF's affinity for immobilized heparin-Sepharose could be exploited as an initial purification step to isolate the fragments. This affinity chromatography step would permit the separation of the fragments from all of the remaining cell lysate that did not also share an affinity for heparin, thus significantly reducing the number of contaminates. ^{125}I-FGF (10 ng/ml) and heparin (5 U/ml) were added to fifty 150-mm tissue culture dishes of FGFR-1 transfected NIH 3T3 cells and placed at 4°C for 90 minutes to establish equilibrium binding. After rinsing three times with PBS, the cells were placed at 37°C and the growth factor was allowed to internalized for 4 hours. Subsequently, the cells were harvested and subjected to a 100,000g spin to clarify the lysate. The supernatant from the high-speed spin was loaded onto a heparin-Sepharose column and following equilibration of the column, the column was eluted with a linear gradient of increasing salt concentration from 0.15 M to 2.0 M NaCl. Fractions were collected at a

volume of 1 mL and the radioactivity in each fraction was quantitated. An aliquot of each fraction was subjected to SDS-PAGE and autoradiography to determine the composition of the radioactive counts (data not shown).

It was determined that the proteolytic fragments of FGF did indeed bind the heparin-Sepharose; however, the elution pattern was broad. Coomassie blue staining of SDS-PAGE gels revealed that significant contamination by other proteins remained, and as expected additional purification schemes were necessary. Individually subjecting the heparin-Sepharose fractions to reversed-phase HPLC separated out some of the contaminants but again the elution of the fragments was broad and dispersed over several peaks. However, this preliminary experiment provided a basis for calculating the feasibility of obtaining sufficient sample to sequence using traditional Edman chemistry. Based on estimated calculations, to isolate enough sample to reach the lower sensitivity (1 picogram) of the protein sequencer would require greater than 500 150-mm culture dishes and a significant quantity of radio-labeled FGF. Therefore, scale-up of the purification process will not occur until sufficient evidence is provided that such efforts are warranted and likely to be fruitful.

Attempts were also made using cellular fractionation procedures to identify a fragment(s) of FGF outside of the endocytic pathway. To date, there has been no evidence during the fractionation procedures that would suggest an enzymatic product of FGF translocating to another cellular compartment. There is data indicating that exogenous, intact FGF may traffick to the nucleus and this observation may be explored if studies with the breast cancer cell lines support this finding.

An alternative approach to finding a bioactive fragment of FGF was explored. FGF was digested in a test tube with commercially available cathepsin D. Enzymatic cleavage by cathepsin D yielded two distinct products that ran on a SDS-PAGE gel similarly to the fragments generated *in vivo*. Following the cleavage reaction, the sample was subjected to reversed-phase HPLC separation which resulted in a more complex profile than was indicated by the gel. No mitogenic activity could be recovered in any of the HPLC peaks aside from a peak that contained undigested, intact FGF-1 (data not shown).

TECHNICAL OBJECTIVE 2:

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

Results and Discussion

From the studies described above and the literature base, a role for an internalization-related event in FGF-signaling is likely. What is unclear is whether the reduced mitogenic potency of FGF in the presence of the protease inhibitors is a direct or indirect effect that, coincidentally, can be monitored through the proteolytic processing of the mitogen. The results from studies in Objective 1 as to the mitogenic activity of FGF-1 in the presence of the various protease inhibitors will be used as a guide to approach Objective 2. Those protease inhibitors affecting FGF-1 mitogenic activity, and possibly degradation, will provide insight as to what proteases might be implicated and targeted for evaluation. For example, the data from Objective 1 indicates that in addition to an implicated role for an aspartyl-

protease in the mitogenic signaling pathway of FGF a possible role for a cysteine protease(s) exists. Of the cysteine proteases, cathepsin B and cathepsin L are the most abundant in the endocytic pathway and have implicated roles in breast cancer pathology. Therefore, the intracellular levels of cathepsin D and cathepsins B and L, as well as other possible candidate proteases, can be screened initially by immunoblotting. The activity levels of the proteases in the various cell lines can be screened by commercially available kits. The antibodies for immunoblotting have already been purchased and the studies are presently underway. A preliminary study in which the mitogenic response of cells treated with either anti-cathepsin D or anti-cathepsin B antibody in the presence or absence of FGF showed no difference in responsiveness compared to FGF-1 alone (data not shown). However, there is no evidence that the antibodies function to neutralize the activity of the protease in which case no effect would be expected. In addition, anti-cathepsin D antibody or anti-cathepsin B antibody was added to FGFR-1 transfected NIH 3T3 cells prior to stimulation with ^{125}I-FGF. The cells were incubated at 37°C for 4 hr to ensure sufficient internalization of FGF before harvesting to evaluate the degradation profiles (Figure 12). Neither antibody lead to a noticeable change in FGF catabolism. The degradation pattern in those cells treated with anti-cathepsin D antibody appeared more intense; however, it is presently unclear if this difference reflects enhanced or reduced processing or an artifact of cell number.

RESEARCH ACCOMPLISHMENTS

1. The mitogenic response to FGF-1 requires a receptor internalization event mediated by the "classical" endocytic pathway involving a clathrin-coated vesicle.
2. FGF-induced mitogenic response has a requirement for an acid compartment indicating that trafficking to and processing within lysosomes may play a role in FGF signaling.
3. A reduction in FGF mitogenic potency is consistent with an alteration in its proteolytic processing.
4. A leupeptin-inhibitable activity is implicated in both FGF mitogenic activity and FGF proteolytic processing.
5. Leupeptin treatment of NIH 3T3 cells results in an increase in nuclear trafficking of intact FGF; the significance of which is presently unclear.
6. FGF-1 is a mitogen for the hormone-dependent breast cancer cell line MCF-7 in the absence of estrogen, supporting both an increase in DNA synthesis and in cell number.
7. Exogenous FGF-1 does not appear to either support or inhibit cell growth in the hormone-independent cell line MDA-MB-231.
8. Proteolytic processing of internalized FGF by MCF-7 cells generates two degradation products consistent with that seen in NIH 3T3 cells; however, intact FGF is apparent even at the 24 hr time point.
9. FGF internalized by MDA-MB-231 cells remains essentially intact--with no evidence for significant proteolytic processing--but in less abundance over the 24 hr time course. This result is similar to the site-directed mutant of FGF-1, discussed in previous reports, that is capable of initiating the early events associated with the "classical" mitogenic signal transduction cascade equally as well as wild-type FGF but exhibits >100-fold less

potency in mitogenic activity. This mutant is not degraded upon internalization but remains in an intact form that progressively disappears over time.

10. Leupeptin treatment of MCF-7 cells results in a decrease in responsiveness to both serum and FGF.
11. Leupeptin treatment of MDA-MB-231 cells results in an increase in DNA synthesis stimulated by either serum or FGF.
12. Neither anti-cathepsin D nor anti-cathepsin B antibody significantly altered either the mitogenic responsiveness of or the ability to process FGF within NIH 3T3 cells.

REPORTABLE OUTCOMES

•A manuscript regarding the findings in the NIH 3T3 cell line is in preparation.

CONCLUSIONS

Although most of the degradation seen during FGF-1 internalization is likely due to general ligand turnover, the studies presented here, and highlighted under "Research Accomplishments," suggest a protease-sensitive activity associated with the FGF mitogenic pathway. There is substantial evidence in the FGF literature indicating that ligand binding to and the initial activation of the cell-surface FGF receptors is not sufficient for eliciting a complete biological response. Therefore, I have focused on studying those events subsequent to receptor engagement. All together, the data outlined above indicates that the FGF mitogenic signal requires ligand endocytosis via the "classical" clathrin pathway and exposure to an acidic compartment. Furthermore, a pepstatin- and leupeptin-sensitive event(s) appears critical for a full mitogenic response. What remains to be determined is whether the reduced mitogenic potential of FGF in the presence of the protease inhibitors is a direct or indirect result of altered ligand processing which coincidentally can be monitored by following FGF degradation. There is no evidence to date for a bioactive fragment of FGF being generated *in vivo*. However, coordinated roles exist for cathepsin D and cathepsin B--pepstatin-sensitive and leupeptin-sensitive proteases, respectively—in proteolytic modification and membrane translocation of internalized ligands. Therefore, the increase in nuclear localization of intact FGF in the leupeptin-treated cells may have significant relevance to the objective of understanding FGF mitogenic signaling.

Given that FGF is both a potent mitogen and an angiogenic factor and that it has been implicated in progressing breast carcinomas to a hormone-independent state, a better understanding of the signaling mechanism through which FGF mediates its biological effects is imperative. Such knowledge could provide additional avenues for adjuvant therapies; such as, neutralizing FGF's activity via enzyme inhibitors, antibodies, antagonist proteins, etc. In addition, it may provide insight into the mode of action of other angiogenic and growth modulators of human cancers. An antimitogenic treatment would be efficacious over a broader spectrum of cancers unlike antihormone therapeutics which have a limited application to hormone-dependent cancers. Consequently, my two main focuses will be on

(1) further characterizing the pepstatin- and leupeptin-sensitive aspect of FGF mitogenic signaling and (2) characterizing the differences between the hormone-dependent and hormone-independent breast cancer cell lines regarding FGF processing and the varied effects on mitogenesis in the presence of the protease inhibitors.

REFERENCES

1. Penault-Llorca, F.; Bertucci, F.; Adelaide, J.; Parc, P.; Coulier, F.; Jacquemier, J.; Birnbaum, D.; and DeLapeyriere, O. (1995) Expression of FGF and FGF receptor genes in human breast cancer. *Int. J. Cancer* **61**, 170-176.

2. Givol, D. and Yayon, A. (1992) Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.* **6**, 3362-3369.

3. Yoshimura, N.; Sano, H.; Hashiramoto, A.; Yamada, R.; Nakajima, H.; Kondo, M., and Oka, T. (1998) The expression and localization of fibroblast growth factor-1 (FGF-1) and FGF receptor-1 (FGFR-1) in human breast cancer. *Clin. Immunol. Immunopath.*, **89**, 28-34.

4. Yazidi, I.E. and Boilly-Marer, Y. (1995) Production of acidic and basic fibroblast growth factor by the hormone-independent breast cancer cell line MDA-MB-231. *Anticancer Res.* **15**, 783-790.

5. Zhang, L.; Kharbanda, S.; Chen, D.; Bullocks, J.; Miller, D.L.; Ding, I. YF.; Hanfelt, J.; McLeskey, S.W.; and Kern, F.G. (1997) MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice. *Oncogene* **15**, 2093-2108.

6. Zhang, L.; Kharbanda, S.; Hanfelt, J.; and Kern, F.G. (1998) Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. *Cancer Res.* **58**, 352-361.

7. McLeskey, S.W.; Zhang, L.; El-Ashry, D.; Trock, B.J.; Lopez, C.A.; Kharbanda, S.; Tobias, C.A.; Lorant, L.A.; Hannum, R.S.; Dickson, R.B.; and Kern, F.G. (1998) Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antietrogen ICI 182,780 and two aromatase inhibitors. *Clin. Cancer Res.* **4**, 697-711.

8. Duffy, M.J. (1997) Cancer metastasis: biological and clinical aspects. *Cancer Met.* **167**, 4-8.

9. Cross, M. and Dexter, T.M. (1991) Growth factors in development, transformation, and tumorigenesis. *Cell* **64**, 271-280.

10. Sofer, A. and Futerman, A.H. (1995) Cationic amphiphilic drugs inhibit the internalization of cholera toxin in the golgi apparatus and the susequent elevation of cyclic AMP. *J. Biol. Chem.* **270**, 12117-12122.

11. Orlandi, P.A. and Fishman, P.H. (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin intneralization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905-915.

12. Wang, L-H.; Rothberg, K.G.; and Anderson, R.G.W. (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* **123**, 1107-1117.

13. Rothberg, K.G.; heuser, J.E.; Donzell, W.C.; Ying, Y-S.; Glenney, J.R.; and Anderson, R.G.W. (1992) Caveolin, a protein component of caveolae membrane coats. *Cell* **68**,673-682.

14. Schnitzer, J.E.; Oh, P.; Pinney, E.; and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127**, 1217-1232.

15. Bottger, B.A.; Sjolund, M.; and Thyberg, J. (1988) Chloroquine and monensin inhibit induction of DNA synthesis in rat smooth muscle cells stimulated with platelet-derived growth factor. *Cell. Tissue Res.* **252**, 275-285.

16. King, A.C.; Hernaez-Davis, L.; and Cuatrecasas, P. (1981) Lysosomotropic amines inhibit mitogenesis induced by growth factors. *Proc. Natl. Acad. Sci.* **78**, 717-721.

17. Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci.* **75**, 3327-3331.

18. Draper, R.K. and Simon, M.I. (1980) The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. *J. Cell Biol.* **87**, 849-854.

19. King, A.C. (1984) Monensin, like methylamine, prevents degradation of ^{125}I-epidermal growth factor, causes intracellular accumulation of receptors, and blocks the mitogenic response. *Biochem. Biophys. Res. Comm.* **124**, 585-591.

20. Pressman, B.C. (1976) Biological applications of ionophores. *Annu. Rev. Biochem.* **45**, 501-530.

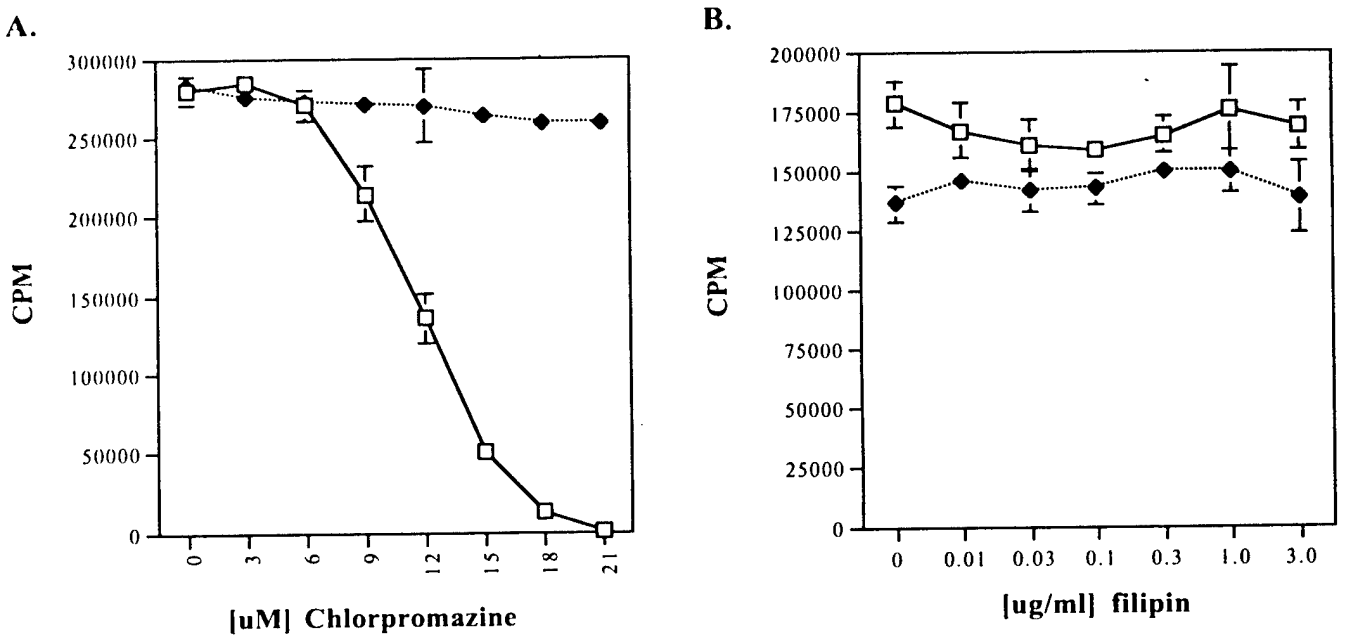

Figure 1. NIH 3T3 cells were either pretreated or not for 3 hr with increasing concentrations of chlorpromazine (**A**) or filipin complex (**B**) prior to stimulation with 3ng/ml FGF + 5U/ml heparin (□) or 10% calf serum (◆). DNA synthesis was measured as [^{3}H]-thymidine incorporated radioactivity and quantitated by scintillation counting. Values represent the means ± SE of triplicate wells.

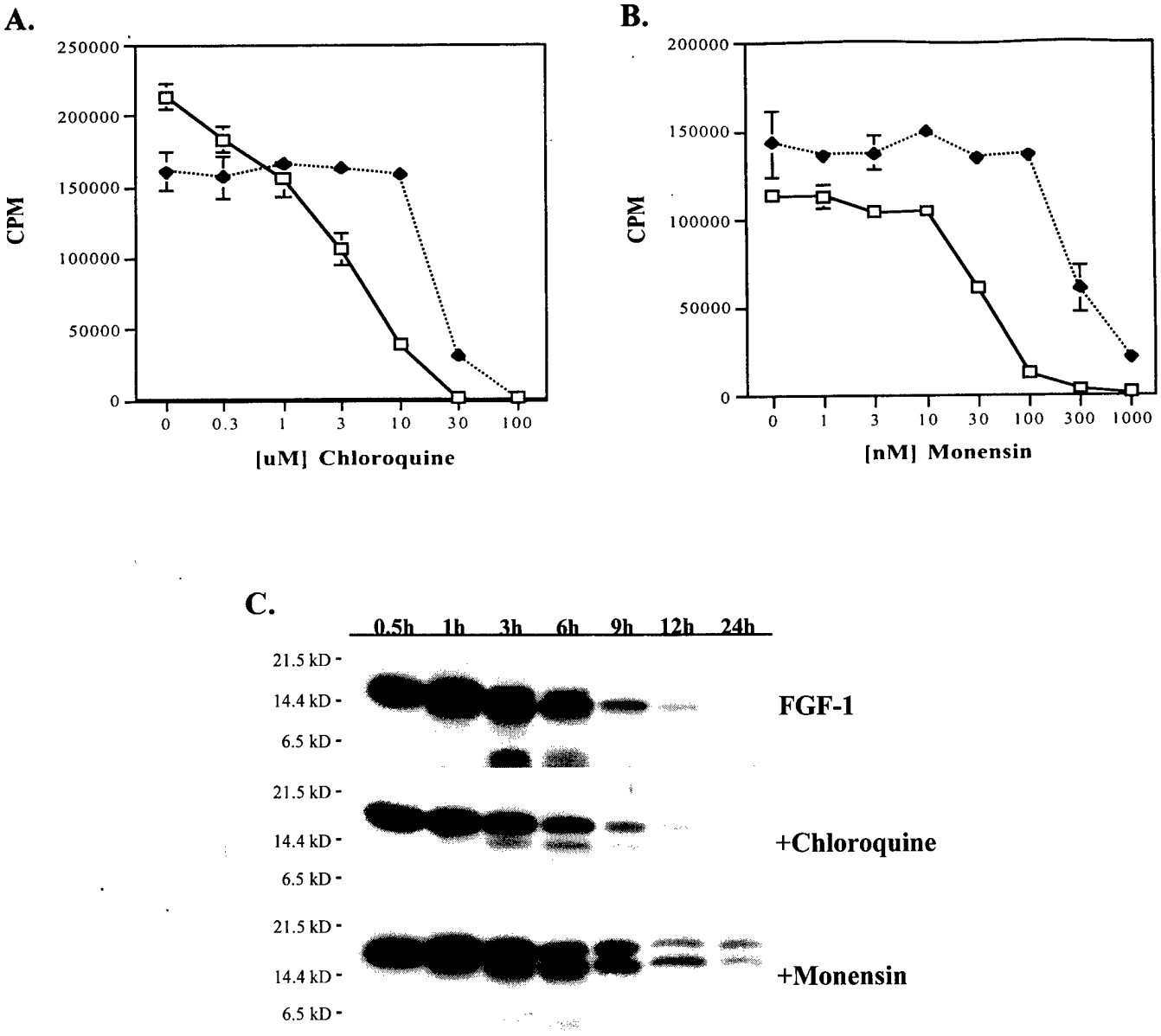

Figure 2. NIH 3T3 cells were either pretreated or not for 3 hr with increasing concentrations of chloroquine **(A)** or monensin **(B)** prior to stimulation with 3ng/ml FGF + 5U/ml heparin (□) or 10% calf serum (◆). DNA synthesis was measured as [^{3}H]-thymidine incorporated radioactivity and quantitated by scintillation counting. Values represent the means ± SE of triplicate wells. **(C)** FGFR-1 transfected NIH 3T3 cells were pretreatment with 10μM chloroquine or 0.1μM monensin for 3 hr at 37°C. Subsequently, ^{125}I-FGF-1 was added in the presence of the inhibitors to the cells and placed at 4°C for 90 min to allow equilibrium binding to occur. Cells were rinsed 3x with PBS and placed at 37°C for the length of time indicated. At the given time points, the cell-surface associated ^{125}I-FGF-1 was removed with an acid-salt wash and the cells were lysed. An equal volume of 2x Laemmli sample buffer was added to the clarified cell lysate followed by 15% SDS-PAGE and autoradiography.

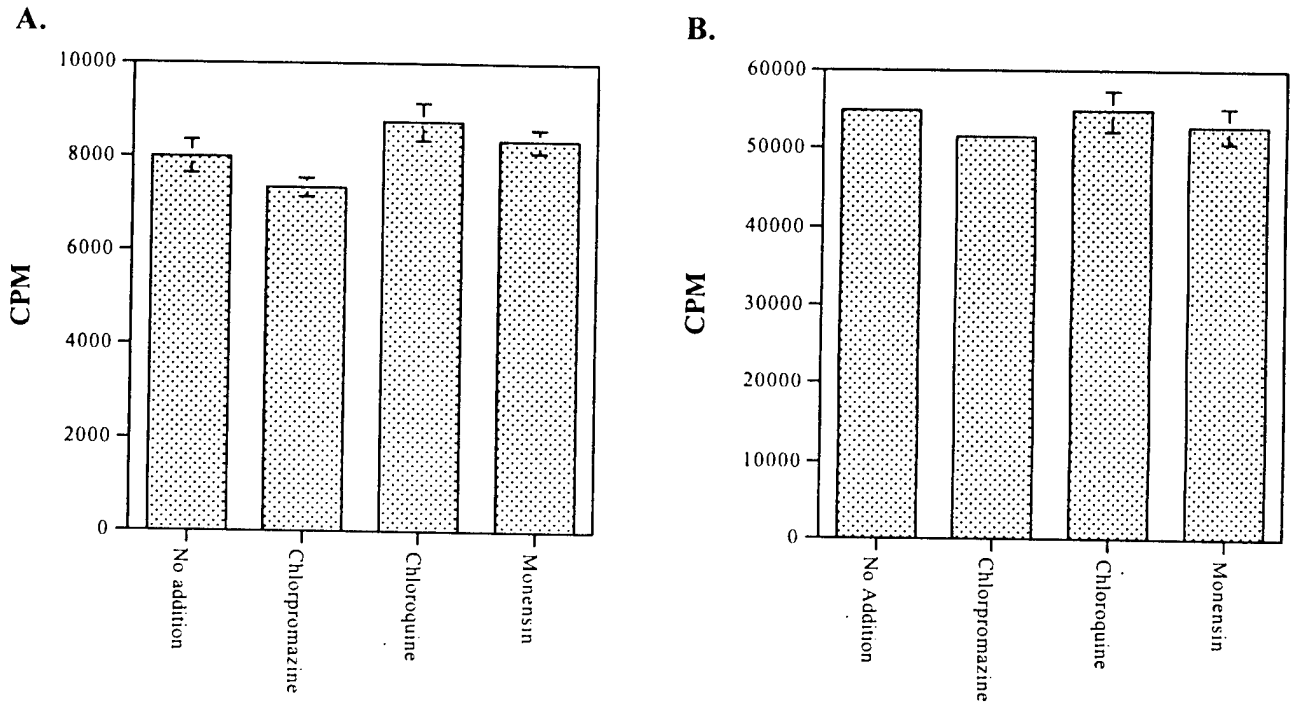

Figure 3. Cells were pretreated for 3 hr with 15μM chlorpromazine, 10μM chloroquine, or 0.1μM monensin. ^{125}I-FGF was added in the presence of the inhibitors to NIH 3T3 cells **(A)** or FGFR-1 transfected NIH 3T3 cells **(B)** in ice-cold binding buffer [DMEM; 20 mM HEPES, pH 7.4; 0.1% BSA] and placed at 4°C for 90 min to allow equilibrium binding to occur. Cells were rinsed 5x with PBS and then lysed in 0.5N NaOH. The radioactivity associated with the lysates was quantitated by gamma counting. Triplicate determinants were used.

Table I

INHIBITION (%)

Protease Inhibitor	Serum	FGF-1	Specificity
100 μM Aprotinin	13	3	Serine
3 μM Cathepsin Inhibitor	14	33	Cathepsins B & L
1 μg/ml Cystatin	0	19	Cysteine
10 μM EST (E-64d)	2	9	Cysteine; Calpain
1 mM Leupeptin	12	76	Serine & Cysteine
100 μM Pepstatin A	3	23	Aspartyl
10 μM TPCK	4	37	Serine & Cysteine
60 μM N-acetyl-L-L-methioninal	15	63	Calpain I & II; Cathepsins B & L
80 μM 4-(2-aminoethyl)-benzenesulfonyl-fluoride	2	57	Serine

A mitogenic response was induced in NIH 3T3 cells with either 10% calf serum or 3 ng/ml FGF-1 in the presence of the indicated protease inhibitors. The percent inhibition was calculated relative to the mitogenic response induced by either serum or FGF-1 in the absence of the protease inhibitor.

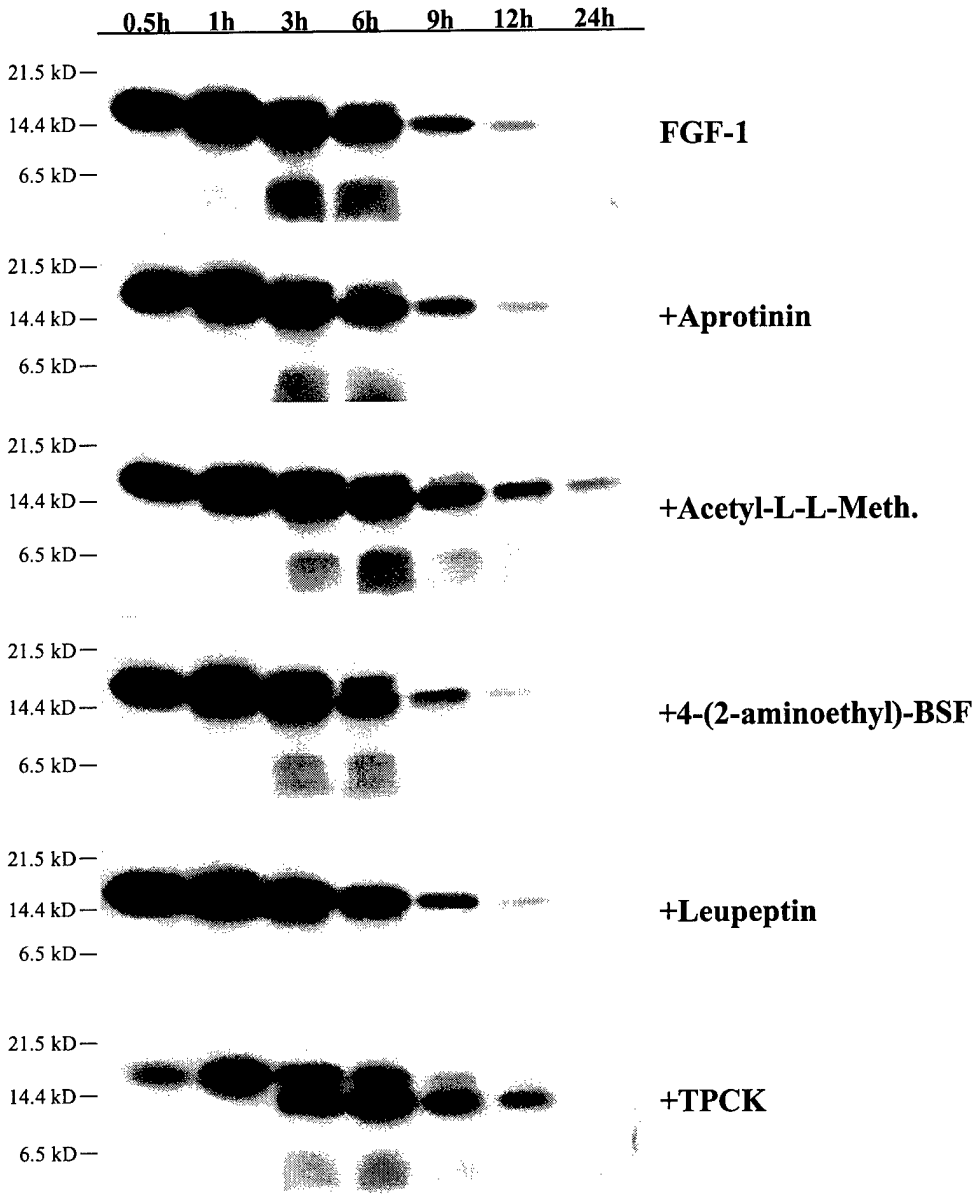

Figure 4. Degradation of ^{125}I-FGF in FGFR-1 transfected cells treated with the indicated protease inhibitors at the concentrations used in Table 1. FGFR-1 transfected NIH 3T3 cells were pretreatment with the protease inhibitors for 3 hr at 37(C. Subsequently, ^{125}I-FGF-1 was added in the presence of the inhibitors to the cells which were placed at 4(C for 90 min to allow equilibrium binding to occur. Cells were rinsed 3x with PBS and placed at 37(C for the length of time indicated. At the given time points, the cell-surface associated ^{125}I-FGF-1 was removed with an acid-salt wash and the cells were lysed. An equal volume of 2x Laemmli sample buffer was added to the clarified cell lysate followed by 15% SDS-PAGE and autoradiography.

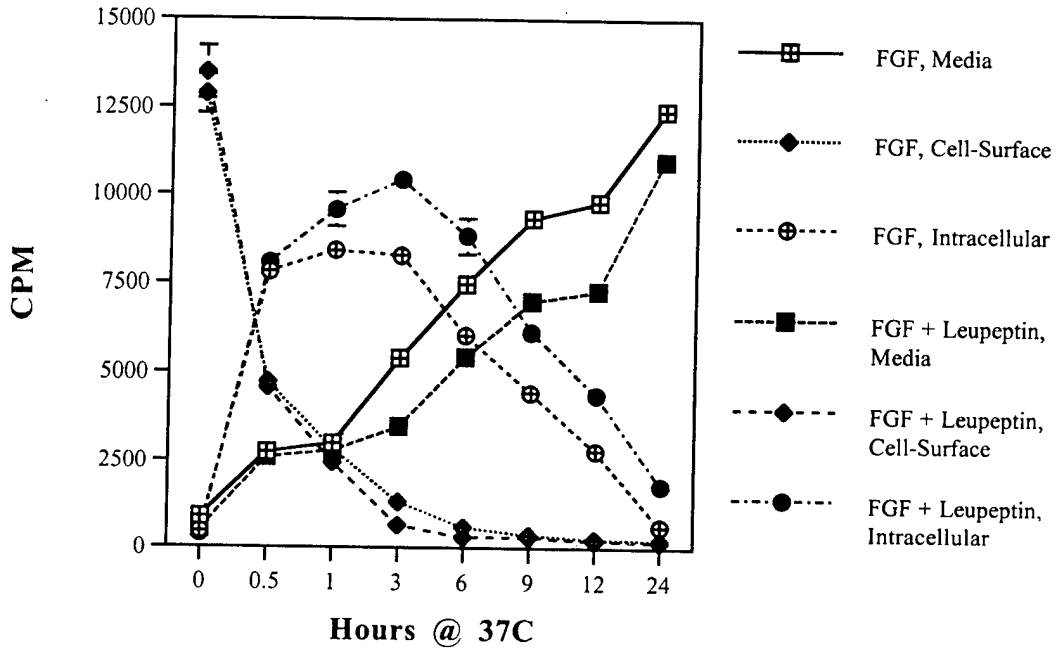

Figure 5. NIH 3T3 cells were either treated with 1mM leupeptin or left untreated. Subsequently, ^{125}I-FGF-1 was added either in the presence or absence of leupeptin to the cells which were then placed at 4°C for 90 min to allow equilibrium binding to occur. Cells were rinsed 3x with PBS and placed at 37°C for the length of time indicated. At the given time points, the media was removed and reserved to determine media-associated counts and the cell-surface associated ^{125}I-FGF-1 was removed with an acid-salt wash. The intracellular –associated counts were determined by that radioactivity retained within the cells. At each time point the respective fractions were subjected to gamma counting. Each data point represents the mean ± SE of triplicate determinants.

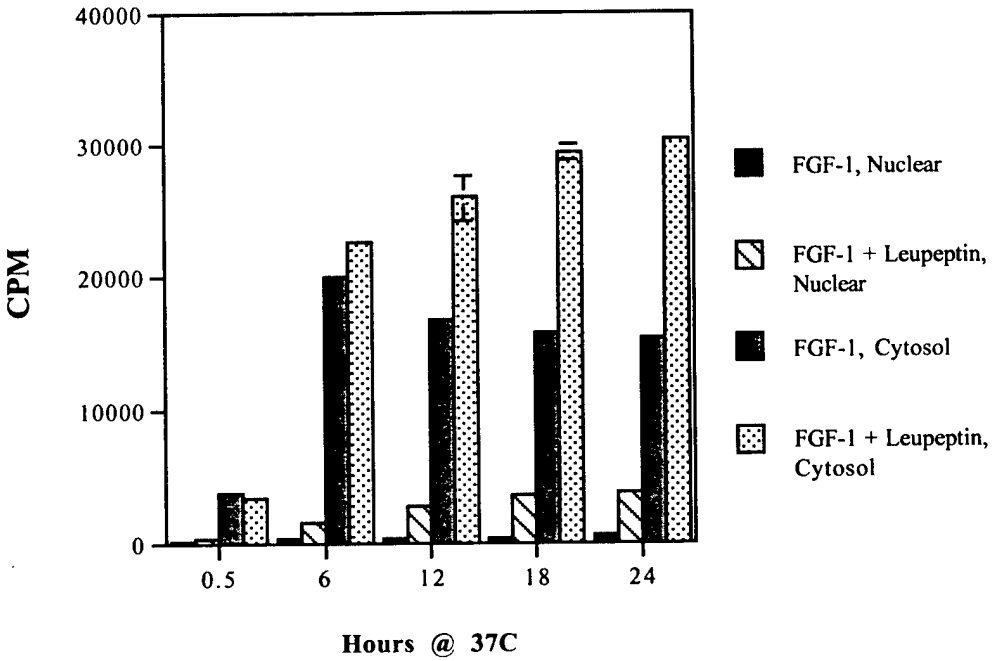

Figure 6. NIH 3T3 cells were grown to ~80% confluence then serum-deprived [0.5% calf serum] for 30 hr, consistent with conditions used in the mitogenic assays. The cells were either pretreated for 3 hr with 1mM leupeptin or left untreated. ^{125}I-FGF was added at 10 ng/ml along with 5 U/ml heparin to the leupeptin-treated or –untreated cells and returned to 37°C. At the indicated times, the cells were harvested and fractionated into crude cytosolic and nuclear fractions. The radioactivity for each fraction was quantitated by gamma counting. The values plotted represent the mean of triplicate determinants ± SE.

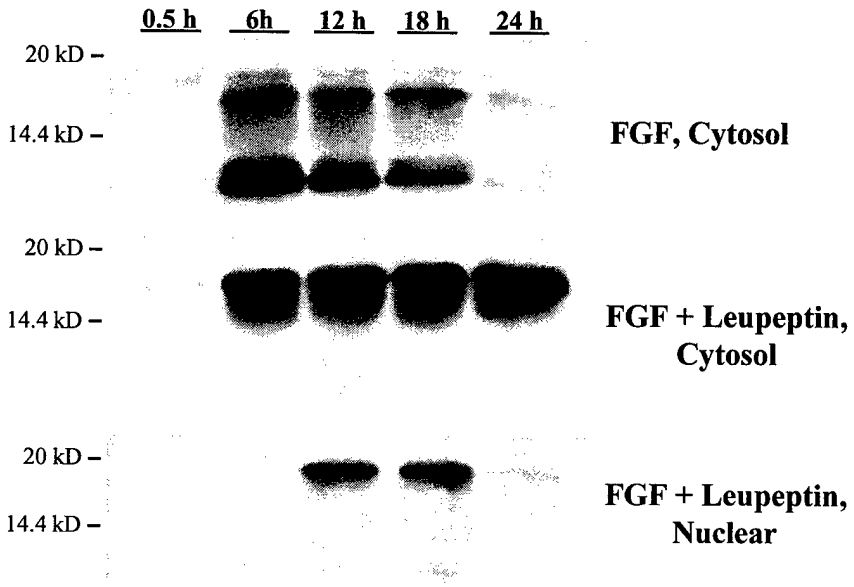

Figure 7. The crude cytosol and nuclear fractions from Fiugre 6 were subjected to TCA-precipitation. The TCA-insoluble pellet was solubilized in Laemmli sample buffer and then subjected to 15% SDS-PAGE and autoradiography.

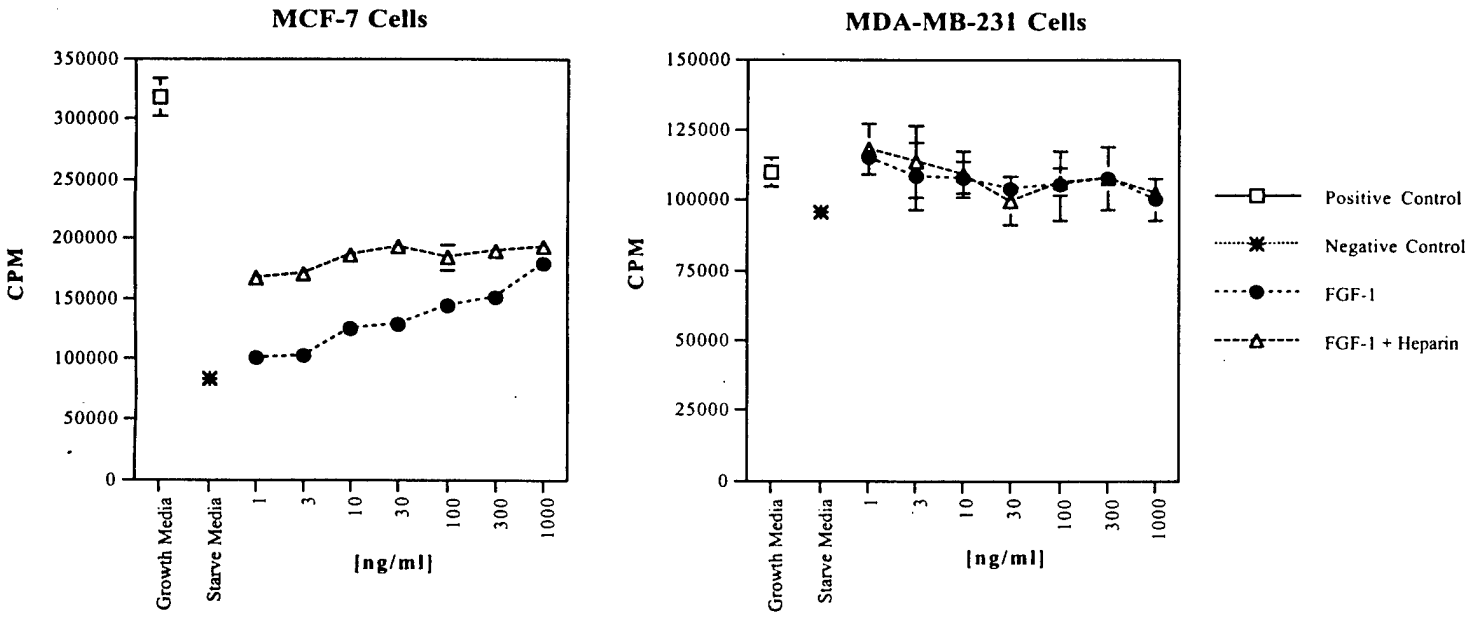

Figure 8. The ability of increasing concentrations of FGF ± 5 U/ml heparin to induce DNA synthesis in MCF-7 cells and MDA-MB-231 cells was determined.

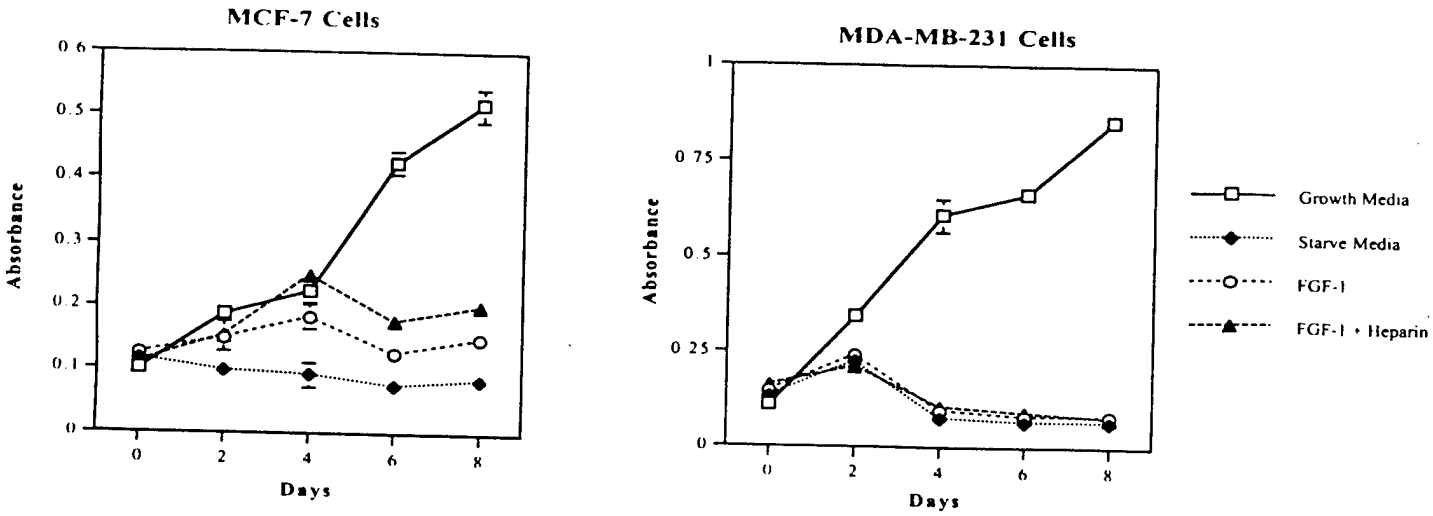

Figure 9. The ability of 10 ng/ml FGF ± 5 U/ml heparin to support cell growth in the hormone-dependent breast cancer cell line MCF-7 and in the hormone-independent breast cancer cell line MDA-MB-231 in the absence of serum or other stimulatory factors. Each data point represents the mean ± SE of triplicate determinants.

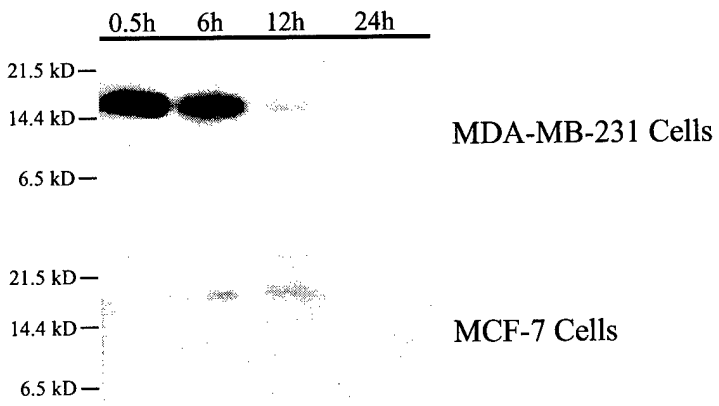

Figure 10. MDA-MB-231 cells and MCF-7 cells were stimulated with ^{125}I-FGF and then harvested at the times indicated in the figure. In the case of the MDA-MB-231 cells, the clarified cell lysates were subjected to 15% SDS-PAGE and autoradiography. Whereas, the MCF-7 cell lysates were TCA-precipitated and the acid-insoluble pellet was solubilized in sample buffer prior to SDS-PAGE and autoradiography.

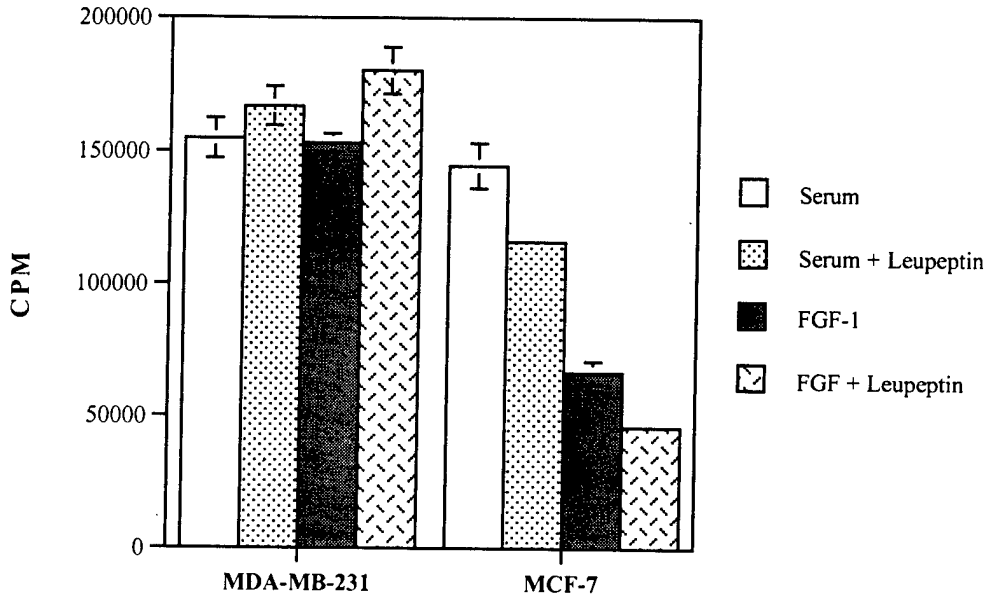

Figure 11. MDA-MB-231 cells and MCF-7 cells were treated ± 1 mM leupeptin for 3 hr prior to simulation of DNA synthesis with either 10% fetal bovine serum or 100 ng/ml FGF.

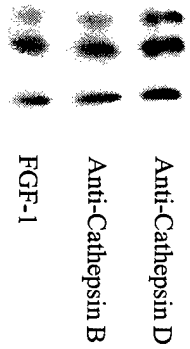

Figure 12. The degradation of ^{125}I-FGF in NIH 3T3 cells was evaluated in the absence or presence of either anti-cathepsin B or anti-cathepsin D antibody.