Expression and Secretion of Neuroleukin/Phosphohexose Isomerase/Maturation Factor as Autocrine Motility Factor by Tumor Cells

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ABSTRACT

The results obtained from fragmented protein microsequencing have suggested that autocline motility factor (AMF), a tumor-secreted M, 55,000 cytokine that regulates cell motility in vitro as well as invasion and metastasis in vivo, is the neuroleukin (NLK)/phosphohexose isomerase (PHI)/maturation factor (MF) polypeptide. Here, we cloned, sequenced, and studied the expression, secretion, and distribution of AMF/NLK/PHI/MF in neoplastic and their normal counterpart cells. Although both normal and neoplastic cells express the gene product, overexpression is associated with selective secretion of the protein. The results suggest that secretion by neoplastic cells is independent of mutation or alternative splicing. Immunohistochemical visualization has depicted AMF/NLK/PHI/MF to be localized in intracellular vesicles diffusely distributed throughout the cytoplasm and not colocalized with any particular cytoskeletal network. Confocal microscopic imaging had shown a partial colocalization between AMF and its receptor (M, 78,000 glycoprotein), especially on the malignant cell surface periphery. The results suggest that extracellular AMF activity may be a result of the product of intracellular cleavage of a precursor polypeptide, which is overexpressed and selectively secreted through a nonclassical secretory mechanism by neoplastic cells.

INTRODUCTION

Malignant tumors are characterized by their unregulated growth, invasion into the surrounding host tissue, and dissemination to distant organs (1), implying cell migration to be a prerequisite for tumor cell invasion. Therefore, studies that elucidate mechanisms of motile regulation and signaling should advance our understanding of tumor cell progression and be of clinical significance (2). In vivo cell motility may be initiated by a group of secreted cytokines having chemotactic motility induction properties (3). Among them, AMF is a molecule that was originally distinguished by its ability to stimulate directional motility (chemotaxis) and random motility (chemokinetics) of the AMF-producing cells (4), via binding to its receptor, a cell surface gp78 (5-7). This signal transduction pathway involves a pertussis toxin-sensitive G-protein activation (8), inositol phosphate production (9), receptor phosphorylation (7), and protein kinase C activation (10, 11).

In our recent study, partial amino acid sequencing of a purified murine AMF showed sequence identity to previously cloned gene products: the cytokine NLK and the enzyme PHI (12). NLK is a neurotrophic factor that promotes the survival of spinal neurons and sensory neurons (13). NLK is secreted by lectin-stimulated T cells and induces immunoglobulin secretion (14). PHI, which catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate (15), was found to be elevated in the serum of patients with malignant tumors, including colorectal, breast, lung, kidney, and gastrointestinal carcinomas (16-21), and its serum activity was found to be correlated with the development of metastases (21). Furthermore, AMF motile activity was detected in the urine of patients with bladder carcinoma. Overall, the presence of AMF/NLK/PHI/MF in the extracellular compartments of cancer patients suggests that, in these patients, the disease may have progressed, and it reflects the motile activity of invading and disseminating cells.

Previously, we showed that the secreted murine AMF exhibited a PHI enzymatic activity, whereas the commercial available rabbit PHI induces cell motility, supporting the initial assignment of AMF as NLK/PHI (12). Recently, the M, 54,300 MF, which mediates the differentiation of human myeloid leukemic cells to terminal monocytic cells, was also identified as NLK/PHI (22). Thus, AMF/NLK/PHI/MF exhibits multifunctional activities.

AMF lacks a consensus secretory signal peptide (13), which governs protein secretion via the classical endoplasmic reticulum (ER)-Golgi route, while functioning, at least in part, as a secreted soluble cytokine. It is of interest that AMF is not unique in this respect: several other proteins that lack a signal peptide are secreted from cells via an alternative, nonclassical pathway(s), such as IL-1B (23). Secretory mechanism may be controlled by mutation, alternative splicing, posttranslational cleavage, and glycosylation of the proteins. Mutations of lysosomal enzymes result in a change from cysotalic proteins to secretory proteins with modifications of their enzymatic activity (24). The alternatively spliced variable region of fibronectin is required for its secretion and fibronectin dimerization during biosynthesis (25). The posttranslational cleavage and glycosylation are required for secretion of Kaposi’s sarcoma FGF, in which inhibition of glycosylation impairs Kaposi’s sarcoma FGF secretion (26). In contrast, intracellular proteins such as M, 72,000 ER protein, protein disulfide isomerase, monomeric sulfatransferase, and rhodanase are secreted only when they are overexpressed in the cell (27, 28). Thus far, no evidence of phosphorylation or glycosylation of AMF has been reported.

There are at least two types of M, 55,000 AMF: acidic AMF and basic AMF, with isoelectric focusing points of pl 6.5 and pl 8.5, respectively (29). Furthermore, at least three variants of PHI subunit (M, 60,000, M, 57,000, and M, 56,000, with pl 9.1, pl 8.9, and pl 8.6, respectively) were reported in human gastrointestinal carcinoma cells (16-21), and its serum activity was found to be correlated with the development of metastases (21). In this report, we investigated the expression, secretion, and distribution of AMF using an AMF-specific antibody, cloned, and sequenced human tumor and normal cDNAs that encode AMF.

MATERIALS AND METHODS

Cell Culture. The human lung diploid fibroblast cell strain IMR90 (ATCC CCL 186) and the human fibrosarcoma cell line HT1080 (ATCC CCL 121) were obtained from the American Type Culture Collection (Manassas, VA).
The BALB/c 3T3-A31 cell line was obtained originally from Dr. A. Ben-Ze’ev, Weizmann Institute (Rehovot, Israel), and the sublines were derived as described previously (30). Briefly, the transformed variant A31-TR was subcloned from cells that were capable of anchorage-independent growth. The tumorigenic A31-TU subline arose spontaneously in irradiated mice injected s.c. with the transformed A31-TR cells. A31-M subline was derived from a pulmonary metastasis of a mouse injected i.v. with the A31-TU cells. The A31-TU and A31-M cells were histologically classified as angiosarcoma (30).

All cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum supplemented with nonessential amino acids and penicillin-streptomycin antibiotic mixture (Life Technologies, Inc., Grand Island, NY). Cultures were maintained at 37°C in an air-7% CO2 incubator at constant humidity. Cells were harvested and passaged for experiments with 0.25% trypsin and 0.025% EDTA, and viability was monitored by trypan blue exclusion. To ensure maximal reproducibility, cultures were grown for no longer than six passages after recovery from frozen stocks and monitored to prevent mycoplasma contamination.

Antibodies and Chemicals. In advance, we synthesized two independent oligo peptides identical to partial sequences for human AMF (NLK). One of them was effective to be an antigen for immunization. Monospecific polyclonal antibody directed against AMF was generated by immunization with a synthetic peptide YFQQGDMESNGKYITK, corresponding to 351–366 amino acid sequence of the human NLK (GenBank accession no. K03515). Monoclonal antibody against gp78 (3F3A) was used either in the form of ascites fluid or concentrated hybridoma supernatant (5–7). Monoclonal antibodies AE1 and AE3 to the human cytokeratins (DAKO, Carpinteria, CA), monoclonal antibody TUB 2.1 to the human β-tubulin, monoclonal antibody VIM-13.2 to the human vimentin, rhodamine-labeled antiactin phalloidin,HRPO-conjugated goat antirabbit IgG antibody, HRPO-conjugated goat antirat IgG + M antibody, and -conjugated anteriactin phalloidin were all purchased from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated goat antirabbit IgG (H+L) antibody was purchased from Zymed (South San Francisco, CA). TXRD-conjugated goat antirat immunoglobulin (H+L) antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). TXRD-conjugated goat antimmunoglobulin (H+L) antibody was purchased from Cappel Organon Teknika (Durham, NC).

SDS-PAGE and Western Blotting. Aliquots of the cell lysates and conditioned media were separated by 8% or 10% SDS-PAGE and blotted onto polyvinylidene fluoride membranes (PVDF-plus; MSI, Westborough, MA) or a nitrocellulose membrane (Zetaprobe, Bio-Rad, Hercules, CA). The blots were blocked with 5% nonfat dry milk in PBS for overnight, incubated with the anti-AMF polyclonal antibody (1:1000) or with the antiggp78 monoclonal antibody (1:500) for 1 h at room temperature and then with HRP-conjugated antirabbit (1:1000) or with antirat antibody (1:1000) for 1 h at room temperature. The labeled bands were revealed by chemiluminescence using ECL Western blotting detection reagents (Amersham, Arlington Heights, IL) and exposure to Kodak X-OMAT film.

Cloning of Human AMF cDNA. Total cellular RNA was isolated from 70–80% subconfluent IMR90 and HT1080 cells using RNA isolator (Gentex, the Woodlands, TX) according to the procedure provided by the manufacturer. The cDNA was synthesized by a reverse transcriptase from the total RNA using random hexamers of first-stranded cDNA synthesis kit (Pharmacia, Uppsala, Sweden). Oligonucleotide primers for PCR were designed to contain the open reading frame of the AMF (NLK) mRNA sequence reported in 1987 (GenBank accession no. K03515). The sequences were as follows: sense (5'-TATTCCTGGAATTGCTATGCCTGCTC) and antisense (5'-GAGGGACTTAAGCGAGAAGAGAAAGGGGAG), which corresponded to nucleotides +4 to +15 and +1708 to +1726, respectively. Each of these primers was designed to contain an added EcoRI restriction site. The PCR conditions were as follows: 94°C for 10 min as an initial denaturation; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and 72°C for 10 min as a final extension. The amplified AMF/NLK/PHI/MF cDNA was digested with EcoRI, ligated to pGEM7 (Promega, Madison, WI) or pCR1 (Invitrogen, San Diego, CA), and transformed into JM109 (Promega, Madison, WI). The plasmid DNA was subcloned, amplified, purified, and subjected to automated sequence analysis using an ABI-373 automated sequencer (Perkin-Elmer Corp., Norwalk, CT). Alternatively, HT1080 Agt11 cDNA library was screened with an AMF-specific DNA probe, which was prepared from EcoRI fragment DNA of the cDNA described above. Positive clones were subcloned into pGEM7, amplified, purified, and subjected to sequence analysis by GENETYX-MAC software (Software Development Co., Ltd., Tokyo, Japan). Sequence analysis and homology search were done using GENETYX-MAC and BLAST searches in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Northern Blot Analysis. The plasmid DNA, which carries the AMF cDNA sequence, was digested with EcoRI. The released DNA fragment was purified by Qiaex II Gel Extraction Kit (Qiagen, Chatsworth, CA) and labeled with 32p using random primed Ready-To-Go DNA labeling kit (Pharmacia, Uppsala, Sweden). A portion (20 μg) of total RNA was denatured at 65°C for 5 min with formamide and formamide, electrophoresed on a 1% agarose-formamide gel, and blotted onto a nylon membrane (Micronech, MA) with 10X SSC (1X SSC containing 0.15 M NaCl and 0.015 M sodium citrate). Prehybridization was performed overnight at 42°C in 50% formamide, 5% dextran sulfate, 5X Denhardt’s solution, 0.05 M Na2HPO4 (pH 7.0), 5X SSC, and 0.3 mg/ml denatured salmon sperm DNA. Then, the blot was hybridized overnight with a 32P-labeled DNA probe at 42°C. The membrane was washed by vigorous agitation at room temperature for 30 min in each of the following solutions: 2X SSC-0.1% SDS, 0.5X SSC-0.1% SDS, and 0.1X SSC-0.1% SDS. After washing, the membrane was exposed to film (Kodak Biomax MR) with an intensifying screen at ~80°C.

FACSscan Analysis. All of the following procedures were performed at 4°C or on ice. Cells (1X106 cells/ml) were suspended in PBS containing 0.9 mm Ca2+, 0.5 mm Mg2+, 0.2% sodium azide, and 1% BSA and rocked for 30 min. Then, anti-AMF polyclonal antibody (1:50) was added and incubated for 1 h with rocking. After the cells were washed twice with PBS containing 0.9 mm Ca2+, 0.5 mm Mg2+, 0.2% sodium azide, and 1% BSA, the cells were incubated with FITC-conjugated antibodirabbit antibody (1:10) as the secondary antibody, with rocking. After 1 h of incubation, the cells were washed twice in PBS containing 0.9 mm Ca2+ and 0.5 mm Mg2+, and cell surface fluorescence was analyzed by flow cytometry using a FACSscan (Becton Dickinson, Mountain View, CA). The control cells were incubated identically, except without the first antibody. A scatter window was set to eliminate dead cells and cell debris. The frequency and fluorescence profiles of the stained cells were determined using a laser output of 125 mV.

Double Indirect Immunofluorescence and Confocal Imaging. Cells (1X106 cells/well) were seeded on 18-mm glass coverslips in six-well plates and cultured overnight and washed twice with PBS containing 0.9 mm Ca2+ and 0.5 mm Mg2+, followed by fixation and permeabilization with prechilled methanol, 3.3% paraformaldehyde, and 0.5% Triton X-100. The cells were washed briefly with PBS and blocked with 1% FCS in PBS for 30 min, followed by incubation at 4°C with rabbit anti-AMF (1:50) and rat anti-gp78 (1:25), mouse anticytokeratin (1:50), mouse anti-β-tubulin (1:100), mouse antivimentin (1:50), or rhodamine-conjugated antianticin phalloidin (1:100). After 1 h of incubation with primary antibodies, the cells were washed with PBS and incubated with FITC-conjugated goat antirabbit antibody (1:75) and either TXRD-conjugated goat antirat antibody (1:100) or TXRD-conjugated goat antimouse antibody (1:100) for 45 min at 4°C, followed by extensive wash with PBS. The coverslips were then mounted on slides in 13% polyvinyl alcohol, 30% glycerol and 0.6X PBS and visualized using a Nikon Optiphot fluorescence microscope. Alternatively, the cells were processed as described above, except that they were fixed with 3.3% paraformaldehyde in PBS for 30 min after completion of staining. The latter procedure was used to detect cell surface antigens.

For the confocal imaging, the cells were processed as described above, except that the coverslips were mounted in SlowFade (Molecular Probes, Eugene, OR) and visualized by Zeiss 310 Laser scanning microscope. Controls receiving no primary antibody, a nonspecific rabbit IgG, a nonspecific rat IgG, or a nonspecific mouse IgG exhibited no background labeling. No cross-reaction of antirabbit antibody with antimouse or antirat antibody was observed. Controls showed significant low signals compared with positive staining.

RESULTS

AMF Cellular Identification and Localization. Previously, it was suggested that malignant cells might overexpress AMF (2, 6, 7, 31). However, direct quantitative evidence was lacking, due to the
Expression and Secretion of AMF by Normal and Neoplastic Cells. To quantitative the relative AMF protein expression levels in normal and transformed cells, cell lysates of A31, A31M, IMR90, and HT1080 cells were subjected to Western blot analysis. We found that the M, 57,000 AMF expression level in the murine A31M angiosarcoma cells was 2-fold higher than that in normal counterpart A31 fibroblasts (Fig. 2A), whereas the HT1080 fibrosarcoma cells expressed approximately the same level of M, 57,000 AMF as did IMR90 embryonal fibroblasts. However, AMF secretion into the conditioned media could be observed only in the A31M and HT1080 tumors cells, as compared with the A31 and IMR90 normal cell counterparts, respectively. This differential cellular pattern between the tumor and the normal cells of gp78 expression was similar to the up-regulated level of secretion of AMF by these cells (Fig. 2B).

To test whether the AMF expression and secretion correspond to its mRNA level, Northern blot analysis was performed on RNA extracted from HT1080 and IMR90 cells, using the newly cloned human AMF cDNA as a probe (see "Materials and Methods"). Unexpectedly, we found that the expression of AMF mRNA was markedly higher (4-fold) in the HT1080 cells than in IMR90 cells (Fig. 3), which did not directly correspond to the differences in the total cellular levels of the protein (Fig. 2A). This result, however, was concomitant with the
differential secretion, the result of M, 57,000 AMF by these cells (Fig. 2B). Thus, it may be suggested that AMF protein, when overexpressed, is probably secreted and not stored in an intracellular pool.

**Sequence Analysis of cDNA for AMF mRNA.** The above data indicated that total AMF mRNA expression level was enhanced in tumor cells as compared with normal cells (Fig. 3), whereas the intracellular protein levels were similar (Figs. 1 and 2A), and that the overexpressed protein fraction was secreted (Fig. 2B) and associated with the up-regulation of gp78 (Fig. 2C). AMF is a tumor-secreted motility factor, NLK is a neutrophic factor (13), PHI is a ubiquitous phosphoenzyme that catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate (15), and MF mediates the differentiation of human myeloid leukemic cells to terminal monocytic cells (22). Although these molecules have different assigned functions, they are the product of a single gene (13, 15). The observation that normal cells did not secrete AMF raised the question concerning the reason for AMF/NLK/PHI/MF secretion by cancer cells and other activated cells because secretion efficiency is known to be augmented by modification, alternative splicing, posttranslational cleavage, and glycosylation (24, 25). Previously, three variants of PHI (M, 60,000, M, 57,000, and M, 56,000, with pl 9.1, pl 8.9, and pl 8.6, respectively) have been reported to be present in gastrointestinal carcinoma (16), which may reflect a specific intracellular cleavage of the mature molecule (15, 16). It is, however, unknown why only the M, 57,000 species are selectively secreted, although several variant forms of AMF molecules were detected in the cell extracts. To address this, we initially cloned PCR products AMF from HT1080 and IMR90 cells. Consequently, four independent clones from HT1080 RNA and two independent clones from IMR90 RNA were sequenced. We further sequenced two independent clones from screening of HT1080 agt11 cDNA library. All of the above clones carried the inserts completely identical to known sequence of the human NLK (Fig. 4). Thus, we can exclude the possibility that the selective secretion of AMF by cancer cells was due to a mutation in the coding sequence.

**Cellular Localization of AMF.** The binding of AMF to cell surface receptors modulates a signal transduction pathway, resulting in cell motility or cell growth (5–11). Similarly, NLK has been shown to bind to a surface component of the sensory neuron (14). The total cellular level of AMF in A31M cells was 2-fold higher than that of the normal A31 counterpart cells (Fig. 2A). We questioned whether this overexpression is localized to or associated with a particular cell’s compartment. To address this, a quantitation of AMF expression on the cell surface in A31 and A31M cells was established by flow cytometry using FACSscan analysis. As seen in Fig. 5, A31M cells expressed a higher amount of cell surface AMF than A31 cells, indicating the excess of AMF observed in A31M over the parental A31 is due, in part, to its localization on the cell membrane, probably in a bound form, to gp78.

Next, to examine in detail the intracellular distribution of AMF in the cytoplasm, which might hint at the route of its secretion, A31, A31M, IMR90, and HT1080 cells were sparsely seeded onto coverslips. After 24 h of culturing at 37°C, they were fixed, permeabilized, and labeled by immunofluorescence with anti-AMF. We focused our analysis on single cells, especially semicircular shaped cells, because this cell shape was associated with the gliding mode of locomotion (32). AMF was found to be localized predominantly to perinuclear and nuclear region (Fig. 6, A, C, and F), with tubular formation extending to the short cellular axis. A possible colocalization of these AMF tubular structures and cytoskeleton proteins was investigated by double indirect immunofluorescence labeling of AMF and β-tubulin, vimentin, or actin. β-Tubulin was diffusely expressed in the cytoplasm, forming a clear visible meshwork that was well polarized in A31M cells (Fig. 6B). Vimentin was predominantly localized to the perinuclear region, forming fine tubules extending to the cell periphery, some of which overlapped with AMF-containing tubules with no obvious colocalization (Fig. 6D). Actin fibers were extending along the long cellular axis, which was different from the AMF distribution pattern (Fig. 6F).

**Colocalization of AMF with gp78.** Flow cytometry analysis have shown AMF to be present on the cell surface of transformed cells at a higher density, as compared with their normal counterpart cells (Fig. 5), which led us to question its possible colocalization with gp78 on the cell surface. To examine this, A31, A31M, IMR90, and HT1080 cells were sparsely seeded on coverslips and consecutively labeled with 12P-labeled AMF cDNA probe. Then, the membrane was washed and hybridized with 32P-labeled β-actin cDNA probe (β-actin). The expression of AMF gene transcripts is much higher (4-fold) in HT1080 cells than in IMR90 cells.

Fig. 3. Northern blot of AMF expression in human lung diploid IMR90 fibroblasts and the human fibrosarcoma HT1080 cells grown under subconfluent condition. Total RNA was isolated from each cell line after 70–80% confluency was reached. A portion (20 µg) of denatured total RNA was electrophoresed on a 1% agarose-formaldehyde gel and blotted onto a nylon membrane. After prehybridization, the blot was hybridized with 32P-labeled AMF cDNA probe. Then, the membrane was washed and hybridized with 32P-labeled β-actin cDNA probe (β-actin). The expression of AMF gene transcripts is much higher (4-fold) in HT1080 cells than in IMR90 cells.
gp78 on the cell surface was strong and likely to be restricted or polarized (Fig. 7A), whereas the colocalization of intracellular AMF with gp78 was a little weak but much more diffusely observed (Fig. 7C). These results suggested that AMF interaction with its receptor might predominantly occur on the cell surface and be incorporated into the cytoplasm.

**DISCUSSION**

Cell motility is an integral aspect of tumor invasion and metastasis, suggesting that differential exploitation of migration-associated processes might endow a tumor cell with a correspondingly greater malignant potential (33, 34). Correlation of overexpression of AMF receptor with cell motility and experimental metastasis has been reported (2, 5–8), suggesting that gp78 is involved in invasion during tumor cell metastasis (8). This thesis has been supported by many clinical observations in patients with bladder carcinoma (35), colorectal cancer (36), esophageal squamous cell carcinoma (37), cutaneous malignant melanoma (38), and gastric cancer (39). In those clinical studies, it was found that overexpression of gp78 was correlated with poor patient survival. Similarly and independently of the gp78 studies, it has been reported that the expression and activity of AMF may be of prognostic value in cancer patients (2, 6–8, 31). A direct analysis of the relationship between AMF and gp78 was not possible in the current study, but our findings support the hypothesis that gp78 is a useful marker for assessing the potential for tumor cell invasion and metastasis.
A31 and A31M cells (1 × 10⁶ cells/ml) grown under subconfluent conditions were incubated with anti-AMF antibody (1:50) for 1 h and with FITC-conjugated antirabbit antibody (1:10) for 1 h (shaded peaks). Then, cell-surface fluorescence was analyzed by FACSscan. The control cells were incubated identically without the primary antibody (left solid peak). A scatter window was set to eliminate dead cells and cell debris. The frequency and fluorescence profiles of the stained cells were determined using a laser output of 125 mW. A31M cells (right shaded peak) express more of AMF on their cell surface than do A31 cells (left shaded peak).

feasible until now, due to lack of immunological and molecular probes. Here, for the first time, total cellular AMF protein expression and secretion were evaluated by quantitative Western blot analyses, and the AMF gene products were identified and quantitated by Northern blot analysis. Northern blot analysis revealed a significant AMF mRNA overexpression by HT1080 fibrosarcoma, as compared with the IMR90 normal fibroblasts (Fig. 3). However, the total cellular AMF protein levels did not correspond to the mRNA levels (Figs. 1 and 2A). To the contrary, secretion level of AMF/NLK protein into the conditioned medium was up-regulated only by A31M angiosarcoma cells and HT1080 fibrosarcoma cells, suggesting that the secretion of AMF was up-regulated by malignant cells (Fig. 2B). Furthermore, the secretion of AMF was seen in accordance with up-regulation of its receptor expression (Fig. 2C). The results shown here report that the overexpression and secretion of AMF were restricted to cancer cells. Serum PHI was shown to serve as a tumor marker for monitoring patients with malignant progressing tumors (16–21). The results reported here give credence to the above PHI studies and together suggest that monitoring of AMF in tissue, serum, and urine might be an important tool for clinical prognosis.

In this study, we showed AMF to be colocalized with gp78 on the cell surface (Fig. 7). Similarly, NLK has been shown to bind to a cell surface component of the sensory neuron (14). NLK, which is partially homologous to the envelope protein gp120 of HIV, is a neurotrophic factor, which promotes the survival of spinal neurons and sensory neurons (13). This activity is blocked by gp120 (40). Lectin-stimulated T cells secrete NLK, which induces the secretion of immunoglobulin by human monolayer cells (14). A maturation inducer has been shown to mediate the differentiation of human myeloid leukemic cells to terminal monocytic cells (22). It is possible that the receptor(s) for NLK or MF of cell lineages may be different from gp78 because these factors exert functions different from motile stimulation. Alternatively, these factors may interact with gp78, followed by a different cascade(s) for individual cell lineage signaling pathways, because AMF, NLK, and MF are derived from the same gene, representing

![Cell number vs. Relative fluorescence](image)

Fig. 5. Flow cytometry of AMF expression on the cell surface analyzed by FACSscan. A31 and A31M cells (1 × 10⁶ cells/ml) grown under subconfluent conditions were incubated with anti-AMF antibody (1:50) for 1 h and with FITC-conjugated antirabbit antibody (1:10) for 1 h (shaded peaks). Then, cell-surface fluorescence was analyzed by FACSscan. The control cells were incubated identically without the primary antibody (left solid peak). A scatter window was set to eliminate dead cells and cell debris. The frequency and fluorescence profiles of the stained cells were determined using a laser output of 125 mW. A31M cells (right shaded peak) express more of AMF on their cell surface than do A31 cells (left shaded peak).

![Image with A31 and A31M cells](image)

Fig. 6. Expression and localization of AMF (A, C, and E) compared with β-tubulin (B), vimentin (D), or actin (F). Sparsely seeded A31 and A31M cells were fixed and permeabilized and then subjected to double indirect immunofluorescence. AMF/NLK/PHI/MF was visualized with FITC (A, C, and E), β-tubulin (B), and vimentin (D) with TIMD and actin (F) with rhodamine isothiocyanate, as detailed in “Materials and Methods.” AMF/NLK/PHI/MF is diffusely expressed throughout the cytoplasm with tubular formation in the periphery, which partially overlaps with other proteins. However, no colocalization with any other cytoskeleton proteins is observed.
of signal peptide were thought to be released by death and lysis of a fraction of the cultures cell population or by transient membrane weight variants, which were observed in Fig. 1, were the result of an alteration in its sequence. These results suggested that the molecular modifications of the same protein products, as determined by molecular weight on SDS-PAGE (12, 15, 22). Thus far, the receptor(s) for NLK or MF on their corresponding cells have not been identified.

AMF/NLK/PHI/MF protein, as shown here and previously, is found in a variety of different sizes. Here, at least five variant forms were detected intracellularly, under reduced conditions with molecular weights of $M_r$ 65,000, $M_r$ 57,000, $M_r$ 46,000, $M_r$ 38,000, and $M_r$ 31,000. The only difference in expression between tumor and normal cells was the up-regulation of the $M_r$ 46,000 intracellular form in malignant cells. Although its function is unknown, it is probably a truncated nonsecreted form of PHI. The secreted extracellular form of AMF is detected as a single band, the estimated size of which was $M_r$ 57,000 (Fig. 1). No other variant form was found in the conditioned medium at a detectable level, except for a $M_r$ 110,000 dimer form of the $M_r$ 57,000 subunit under nonreduced conditions, which was dissociated by reducing conditions (Fig. 1). This observation is in accordance with the previously reported molecular weights of AMF [$M_r$ 55,000 (4)], NLK [$M_r$ 56,000 (13)], and MF [$M_r$ 54,300 (22)]. Although the intracellular expression of $M_r$ 57,000 form was preferentially up-regulated in tumor cells, the secretion of this form was found to be enhanced only in tumor cells (Fig. 2, A and B).

The secretory efficiency of proteins by cells may be altered by mutation (24), alternative splicing (25), posttranslational cleavage (26), or glycosylation (27). In contrast, intracellular proteins, such as ER protein, protein disulfide isomerase, monomeric sulfatransferase, and rhodanase, are secreted only when they are overexpressed (27, 28). Thus far, no phosphorylation or glycosylation of AMF has been reported. We investigated the possible genetic alterations, such as mutation, deletion, insertion, or alternative splicing of AMF, that might affect or explain its secretion, but we could not find any genetic alteration in its sequence. These results suggested that the molecular weight variants, which were observed in Fig. 1, were the result of an intracellular cleavage, as predicted previously (15, 16).

AMF peptide lacks a secretory signal peptide (13), which is critical to the secretion via the classical ER-Golgi route. The proteins devoid of signal peptide were thought to be released by death and lysis of a small fraction of the cultures cell population or by transient membrane disruption (41). However, the selective release of $M_r$ 57,000 AMF into the conditioned media cannot be explained by this manner. Therefore, $M_r$ 57,000 AMF is thought to be actively secreted via a novel alternative pathway, which has been indicated in the secretion of the proteins lack of secretory signal sequence, such as IL-1 (23, 42), FGF (43), galectin-1 (44), galectin-3 (45, 46), and others. An enhancement of secretion by calcium ionophore A23187 or by heat shock is commonly observed, and the secretion is not inhibited by drugs that block ER-Golgi transport or by multidrug-resistant proteins (23, 42–46). The calcium ionophore A23187 enhances this alternative secretory pathway and exocytosis; however, it does not convert nonsecretory cells into secretory cells (42). Thus far, a consensus sequence (if any) shared by proteins using the alternative secretory pathway is not known. Therefore, there must be certain cascades of regulation steps, which may be controlled by various molecules and mechanisms. For example, the secretion of FGF-2 is inhibited by serum starvation (43), whereas the secretion of FGF-1 is induced by serum starvation (47), suggesting the existence of reciprocal regulatory mechanisms. The results of deletion and mutation experiments have shown that the precursor peptide sequence controls the secretion of IL-1β, which differs depending on the cell type (48). This regulation is thought to be controlled by a conformational change, which affects the interaction with the regulatory molecules (48). Together, the current hypothesis is that the secretion of AMF might be initiated by a specific cleavage of the precursor protein, which results in a specific conformational change, leading preferentially to a selective secretion. It is possible, but not yet tested, that other members of the ectoenzyme/exoenzyme group (49), including other phosphoenzymes like thymidine phosphorylase (50) and phosphodiesterase-homologous autotaxin (49) shown to induce motility, undergo specific cleavage prior to secretion. In addition, it was reported that the platelet basic proteins, the cysteine-X-cysteine chemokines connective tissue activating peptide-III and neutrophil activating peptide-2, are also heparin/heparan sulfate-degrading enzymes (heparanases; Ref. 51) that can exert mitogenic activities (52). It was, therefore, suggested that there may be a complex relationship in which each activity could alter the activity or bioavailability of the other function(s) in various pathological situations (51). Thus, the multifunctional activities that AMF/PHI/ NLK/MF ascribes to a single polypeptide are not unique. Whether the
PHI activity is critical to AMF, NLK, and MF activities or they function independently of the enzymatic activities under normal and pathological situations as is yet unknown.

In summary, the results suggest that extracellular AMF activity may be a resulting product of intracellular cleavage of a precursor polypeptide, which is overexpressed and selectively secreted through a non-classical secretory mechanism by neoplastic cells and exerts its activity following cell surface binding. Experiments aimed at unveiling the regulation of AMF expression and mode of secretion are presently underway.

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