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Expression of Autocrine Motility Factor/Phosphohexose Isomerase in Cos7 Cells

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Autocrine motility factor (AMF) is identical to the glycolytic enzyme phosphohexose isomerase (PHI) and overexpression of AMF/PHI is associated with tumor malignancy. In order to study the overexpression of AMF/PHI, an HA-tagged AMF construct was transiently transfected into Cos7 cells. Expression of a tagged AMF-HA allowed us to determine that over a period of 16 hours only a small amount (0.1–1%) of total cellular AMF-HA was secreted into the cell medium. Cell-associated AMF-HA was exclusively cytosolic as it could be completely extracted with Triton X-100 and concentrated within actin rich pseudopodial domains. Treatment of the cells with the glycolysis inhibitor oxamate disrupted the association of AMF-HA with actin concentrations demonstrating that glycolysis regulates the formation of these AMF/PHI-associated actin-rich protrusions. AMF/PHI is a well-characterized tumor cell secreted cytokine and we identify here an alternate intracellular function for this glycolytic enzyme/cytokine in cell motility. © 2000 Academic Press

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The cytosolic glycolytic enzyme, phosphohexose isomerase (PHI), is identical to the tumor cell secreted cytokine autocrine motility factor (AMF) (1), to neuroleukin (NLK), a neurotrophic factor for spinal and sensory neurons and a lymphokine (2, 3), and to maturation factor (MF), which mediates the differentiation of human myeloid leukemic cells to terminal monocytic cells (4). The cDNA sequences of AMF, NLK, and MF found in both human cancer and normal cells were found to be identical to that of PHI suggesting that under select cellular conditions PHI is secreted into the extracellular milieu (1–5). AMF purified from tumor cell conditioned medium exhibits isomerase activity and PHI purified from rabbit muscle stimulates cell motility and binds to the AMF receptor, AMF-R, demonstrating that the same protein exhibits two distinct functions (1, 6). PHI is therefore a cytosolic enzyme that catalyzes the reversible isomerization of D-glucose-6-phosphate to D-fructose-6-phosphate, an essential reaction in glycolysis and gluconeogenesis, and that upon secretion acts as a cytokine variously referred to as AMF, NLK or MF.

Increased expression of AMF/PHI in the urine and serum of patients is associated with malignant colorectal, breast, lung, kidney and gastrointestinal carcinomas (7–13). However, exogenous introduction of high levels of AMF/PHI into cells has yet to be performed. Here, we transfected Cos7 cells with a fusion protein of AMF containing a C-terminal hemagglutinin epitope tag, AMF-HA, cloned into the high copy pCDNA3-RSV expression vector in order to study the overexpression of AMF-HA. Transiently transfected AMF-HA is localized to the cytosol of Cos7 cells where it concentrates within actin-rich protrusive domains in a glycolysis-dependent manner. Interaction between actin and glycolytic enzymes is therefore proposed to provide an immediate and localized energy supply for actin filament assembly which drives pseudopod formation. Our data therefore demonstrate that the function of AMF/PHI in cell motility is not limited to its activity as a secreted cytokine.

MATERIAL AND METHODS

Antibodies and reagents. Immunopurified monoclonal antibody to the 10 amino acid hemagglutinin tag (HA) kindly provided by Michel Bouvier (Department of Biochemistry, Université de Montréal). Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Texas Red conjugated phalloidin and Alexa 488 conjugated goat anti-mouse secondary antibody were purchased from Molecular Probes (Eugene, OR). Oxamate, pyruvate, trypsin-chymotrypsin inhibitor, luminol, PMSF, *p*-nitrophenyl *p′*-guanidino benzoate, leupeptin, pepstatin A, aprotinin, and sodium butyrate were purchased from Sigma.

Preparation of AMF-HA cDNA. The human nucleotide sequence of AMF cDNA (5) (GenBank accession no. KO3515) inserted at the EcoRI site of the pBK-CMV phagemid was amplified by PCR with Vent DNA polymerase (New England BioLabs), using as primers 5′-ATCAAGCTTTCCGCCATGGCCGCTCTCAC-3′, corresponding to

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the 5′ sequence of AMF and including a Hind III site upstream of the start codon, and 5′-GATTCTAGATTAGCGGCCGCTTTGGAC-TCTGGCCTCGCGCTG-3′, corresponding to the 3′ sequence of AMF and including a Not I site upstream and a Xba I site downstream of the stop codon. The resulting PCR fragment was cloned into EcoR I site of pBluescript (Stratagen) by blunt end cloning, then excised by digestion with Hind III and XbaI, and the resulting fragment subcloned into the pCDNA3/RSV vector (14) provided by Michel Bouvier (Department of Biochemistry, Université de Montréal). The Not I restriction enzyme site permitted the introduction of a Not I cassette containing three copies of the HA tag (Pierre Belhumeur, Department of Microbiology and Immunology, Université de Montréal) at the 3′ extremity of the AMF cDNA.

Cell culture, transfection and drug treatments. Cos7 cells were cultured in Dulbecco minimum essential medium containing 25 mM $NaHCO_3$ (DMEM-$NaHCO_3$), 10% fetal calf serum, glutamine, essential amino acids, vitamins, penicillin and streptomycin (Gibco; Burlington, Ontario, Canada) under 5% CO_2 atmosphere at 37°C. Transient transfection of Cos7 cells was performed with lipofectin (Gibco BRL). Cells were plated sparsely on glass coverslip for 1 day, and then incubated in lipofectin mixed with 5 μg/ml AMF-HA cDNA or pCDNA3/RSV vector in serum free medium for 6 hours, then incubated in regular medium for 24 hours. Oxamate and pyruvate were added at a concentration of 100 mM directly to the cell culture medium for 1 hour. To prepare conditioned medium, cells were incubated in medium supplemented with 1 μg/ml BSA, 10 μg/ml aprotinin, 2 mM butyrate, 100 μg/ml trypsin-chymotrypsin inhibitor for 16 hours.

Cell lysates, conditioned medium and Western blots. To prepare cell lysates, cell monolayers were washed three times with ice-cold PBS/CM and harvested by scraping. Cell pellets were resuspended in 100 μl of lysis buffer consisting of PBS containing 1% SDS, 1 mM EDTA and protease inhibitors (1 mM PMSF, 0.05 mM *p*-nitrophenyl *p*′-guanidino benzoate, 10 μg/ml leupeptin, pepstatin A and aprotinin). Cells were lysed for 20 min on ice and then DNA was broken by sonication. The cell lysates were centrifuged at 15,000 rpm for 5 minutes and the supernatant recovered and assayed for protein concentration using the BCA protein assay (Pierce, Rockford, IL). 20 μg of protein were analyzed by SDS–PAGE.

To analyze secreted proteins, conditioned medium was centrifuged at 3000 rpm and filtered with a 0.2 μm filter to eliminate detached and dead cells. Proteins were precipitated with 4 volumes of precooled (−20°C) acetone, placed for 1 hour at −80°C, and then pelleted by centrifugation at 6,000 rpm for 20 min. The pellet was resuspended to 300 μl with sample buffer. Total cell lysates were prepared from the same dishes from which conditioned medium was collected and the cell pellet resuspended in an equivalent 300 μl volume of sample buffer. For the lanes labeled 1:1, 25 μg of protein in the cell lysate and an equivalent volume of the conditioned medium sample were loaded for SDS–PAGE. For the lanes labeled 1:10, 1:100 and 1:1000, each well was loaded with 2.5, 0.25, and 0.025 μg protein of the cell lysate, respectively.

Samples were separated on 9% SDS–PAGE gels and blotted to nitrocellulose membranes using a Mini-Protean apparatus (BioRad Labs, Mississuaga, ON). The blot was blocked with 5% skim milk in PBS/CM, incubated with anti-HA primary antibody and then with the appropriate secondary conjugated to horseradish peroxidase. The labeled bands were revealed by chemiluminescence using preflashed Kodak X-Omat film. Prestained Kaleidoscope molecular weight markers were purchased from BioRad.

Indirect immunofluorescence. Cells were fixed with 3% paraformaldehyde for 15 min at room temperature, rinsed extensively with PBS (pH 7.4) supplemented with 0.1 mM Ca^{2+} and 1 mM Mg^{2+} (PBS/CM), permeabilized with 0.2% Triton X-100 in PBS/CM for 10 min, and then incubated for 30 min with PBS/CM containing 0.5% BSA (PBS/CM/BSA) to reduce nonspecific binding. Where indicated, cells were extracted with 0.2% Triton X-100 in PBS/CM for 1 min before fixation (15). All washings and incubations with both primary and secondary antibodies were done with PBS/CM/BSA. AMF-HA was revealed by incubation with mouse anti-HA monoclonal antibody for 30 min followed by Alexa 488 coupled anti-mouse antibody for 30 min. Actin was labelled by incubation with Texas Red phalloidin for 30 min. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals, Allentown, PA) and viewed in a Zeiss Axioskop fluorescent microscope equipped with a 63 X Plan Apochromat objective and selective filters. Images were photographed using Kodak T-Max 400 film. Confocal microscopy was performed with the 60X Nikon plan Apochromat objective of a dual-channel Bio-Rad 600 laser scanning confocal microscope equipped with a krypton-argon laser and the appropriate dichroic reflectors to distinguish Alexa 488 and Texas Red labeling. The number of colocalizations between actin and AMF-HA concentrations were counted per transfected cell from double labeled merged confocal images.

RESULTS

Cos7 cells transiently transfected with a cDNA coding for the AMF-HA fusion protein were analyzed by western blot using the anti-HA monoclonal antibody (Fig. 1A). As controls, untransfected cells and cells transfected with vector alone or with untagged AMF were also analyzed. In the cell lysates, the anti-HA antibody recognized two bands of ~120 and 80 kD in a nonspecific fashion however a specific band of 56 kD was observed under reducing conditions only in AMF-HA transfected Cos7 cells corresponding to the established molecular weight of the protein. Under non-reducing conditions, two specific bands were observed at 64 and 130 kD correspond to the monomeric nondenatured and dimeric forms of AMF-HA respectively (Fig. 1A). The migration of AMF-HA at 56 kD under reducing conditions and at 64 kD under non-reducing condition has been previously reported for AMF (1, 16) and together with the presence of a 130 kD form under non-reducing conditions corresponding to the glycolytically active dimer form of the enzyme, these data indicate that the C-terminal HA tag has not affected the proper folding of the protein. In concentrated conditioned medium prepared from AMF-HA transfected Cos7 cells, the anti-HA antibody recognized a band that migrated slightly below the AMF-HA found in cell lysates (Fig. 1B). The protein precipitate of conditioned medium and the cell pellet obtained from the same culture dish were resuspended in the same volume of sample buffer in order to determine quantitatively the amount of AMF-HA that was secreted relative to cell expressed AMF-HA. The intensity of the AMF-HA band was less than that of a sample of the cell lysate diluted 100× but greater than that of a sample diluted 1000× indicating that small quantities of AMF-HA equivalent to 0.1–1% of total cellular AMF-HA are secreted over a 16 hour period (Fig. 1B).

By immunofluorescence, AMF-HA is diffusely localized within the cytoplasm of Cos7 cells and there is a marked accumulation of AMF-HA in F-actin rich sites

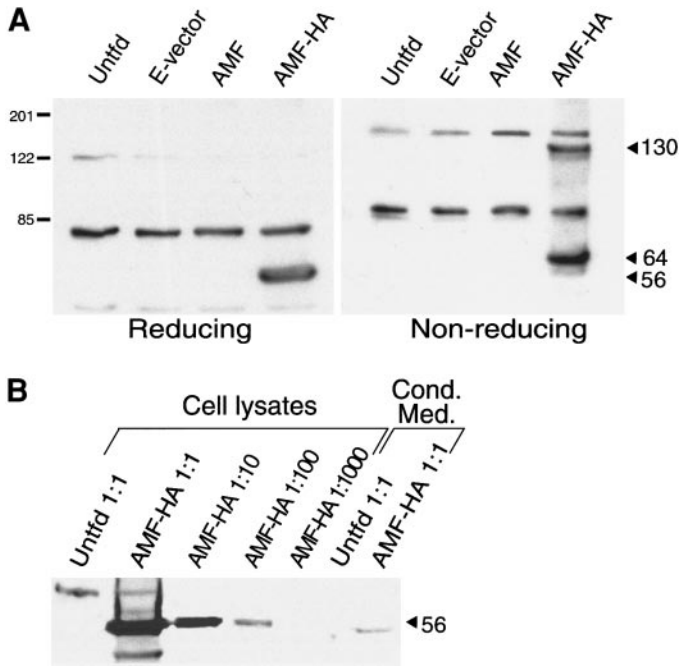

FIG. 1. AMF-HA expression of transiently transfected Cos7 cells. (A) Cell lysates of untransfected Cos7 cells (Untfd), or Cos7 cells transfected with empty pCDNA3/RSV vector (E vector), pCDNA3/RSV vector containing AMF cDNA (AMF) or pCDNA3/RSV vector containing AMF-HA cDNA (AMF-HA), were loaded under reducing and nonreducing conditions, as indicated, on SDS–polyacrylamide gels and immunoblotted with antibody to HA. Specific bands corresponding to AMF-HA migrated at 56 Kd under reducing conditions and at 64 Kd and 130Kd, corresponding to the monomeric and dimeric forms of AMF-HA, respectively, under non-reducing conditions. Molecular weight markers are indicated on the lefthand side of the gels and calculated molecular sizes of AMF-HA under non-reducing conditions are indicated with arrowheads on the righthand side of the gels. (B) Conditioned medium (Cond. Med.) was prepared from untransfected (Untfd) and AMF-HA transfected Cos7 cells as described in the Materials and Methods. From the same plate, a cell lysate was prepared in an equivalent volume of sample buffer. Undiluted (1:1) samples of the cell lysate and conditioned medium and diluted samples (1:10, 1:100, and 1:1000 as indicated) of the AMF-HA cell lysate were loaded under reducing conditions on SDS-polyacrylamide gels and immunoblotted with antibody to HA. Migration of the 56 kD AMF-HA in cell lysates is indicated by the arrowhead.

(Figs. 2A and 2B). Untransfected cells are not labeled indicating that the nonspecific binding of the anti-HA antibody by Western blot is not detected by immunofluorescence and that the labeling is specific. Incubation of cells with 0.2% Triton-X100 prior to fixation resulted in the complete extraction of the labeling demonstrating the cytosolic distribution of AMF-HA (Fig. 2C). The phalloidin labeled actin cytoskeleton was not completely extracted reflecting a weak association between AMF-HA and actin.

By confocal microscopy, the clear colocalization of AMF-HA with actin concentrations at cellular protrusions can be seen (Figs. 3A, 3B, and 3C). PHI is a glycolytic enzyme that catalyzes the reversible isomerisation of D-glucose-6-phosphate to D-fructose-6-phosphate. In order to determine whether the glycolytic activity of PHI regulates its association with actin-rich protrusive domains, we treated the cells for one hour with 100 mM oxamate, an inhibitor of anaerobic glycolysis which blocks the activity of lactate dehydrogenase (17, 18). Treatment with oxamate results in the clear disruption of AMF-HA accumulation in actin-rich domains and a diffuse labeling of AMF-HA throughout the cell (Figs. 3D, 3E, and 3F). Treatment of the cells with oxamate in the presence of an equivalent concentration of pyruvate, the natural substrate for lactic dehydrogenase, restored the colocalization of AMF-HA concentrations with actin-rich domains (Figs. 3G, 3H, and 3I). Essentially all actin-rich domains exhibited a concentration of AMF-HA. Quantification of the number of actin-rich domains per cell to which AMF-HA was concentrated revealed that untreated cells or cells treated with oxamate in the presence of pyruvate contained 7–8 actin rich domains per AMF-HA expressing cell (Fig. 4). Oxamate treated cells exhibited only 1–2 actin-rich domains to which AMF-HA was concentrated demonstrated that active glycolysis regulates the formation of actin-rich protrusions.

DISCUSSION

Epitope tagged AMF/PHI is shown here to associate with actin-rich domains of transiently transfected Cos7 cells. The addition of the HA tag to the AMF/PHI C-terminal domain did not perturb PHI folding because the dimeric form of AMF-HA was detected in nonreducing SDS-PAGE (Fig. 1A). Furthermore, the association of AMF-HA with actin-rich cellular protrusions as well as the ability of an inhibitor of glycolysis to alter the cellular distribution of AMF-HA further indicates that the transfected enzyme has retained its glycolysis-related activity (Figs. 3 and 4). Increased expression of AMF in tumor cells is associated with its secretion and function as an extracellular cytokine (19). Small quantities of AMF-HA, equivalent to 0.1–1% of total cellular AMF-HA, were released into the conditioned medium of AMF-HA transfected Cos7 cells over a period of 16 hours (Fig. 1B). AMF-HA was not detected in the conditioned medium of untransfected cells and the conditioned medium was centrifuged and filtered to eliminate any contribution from dissociated cells. The minimal levels of secreted AMF-HA that we have detected may correspond to cytokine levels released normally by AMF/PHI secreting cells such that the amount of cytokine released represents only a minor fraction of cellular expression of this cytosolic glycolytic enzyme. Due to the minimal amounts of AMF-HA secreted it is possible that the AMF-HA detected was released from dead or lysed cells and that Cos7 cells do not actively secrete AMF/

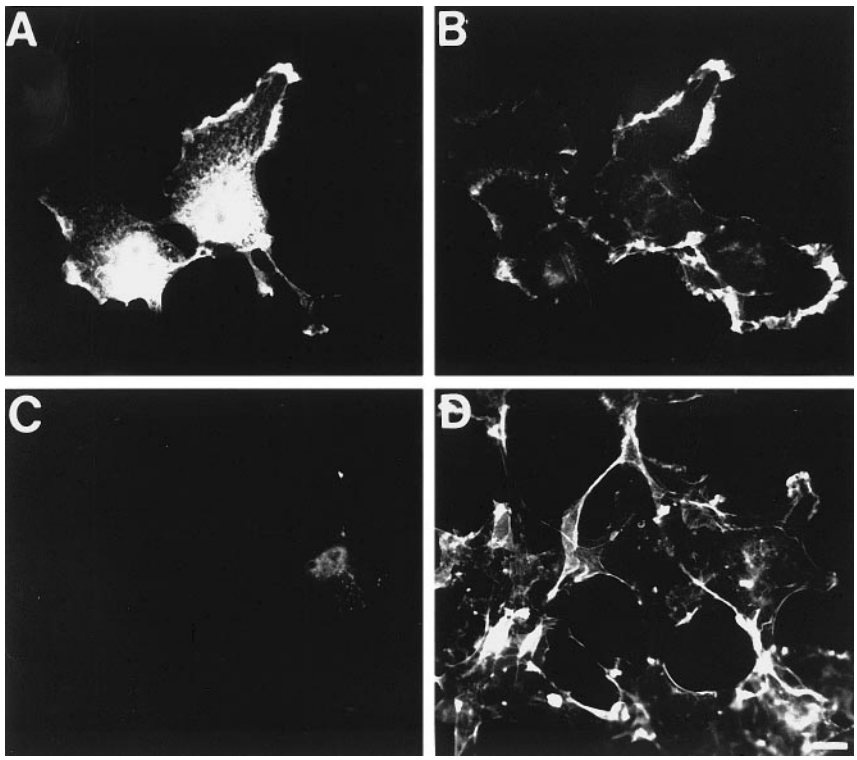

FIG. 2. Subcellular localization of the AMF-HA fusion protein. Cos7 cells were transiently transfected with cDNA coding for AMF-HA and fixed with paraformaldehyde prior to (A, B) or after (C, D) Triton X-100 permeabilization. The cells were then immunofluorescently double labeled with anti-HA antibody and Alexa 488 conjugated anti-mouse secondary antibodies (A, C) and Texas Red phalloidin to reveal F-actin (B, D). AMF-HA is diffusely localized within the cytoplasm and concentrates at peripheral actin densities (A, B). Prior treatment with Triton-X100 extracts AMF-HA (C) but not F-actin (D) demonstrating the cytosolic distribution of AMF-HA and its weak association with actin rich protrusions. Bar = 10 μm.

PHI. However, the fact that secreted AMF-HA migrated slightly faster than cell associated AMF-HA suggests that it may have undergone post-translational modification and was therefore specifically secreted by the cells. AMF/PHI is phosphorylated by casein kinase II in HT-1080 cells and it has been proposed that AMF/PHI phosphorylation may regulate its secretion (20). Whether the amount of AMF-HA secreted relative to total cellular expression by metastatic tumor cells is equivalent to the minimal levels secreted by Cos7 cells remains to be determined.

Glycolytic enzymes have long been described to associate with the actin cytoskeleton enabling the compartmentalization of these energy-generating enzymes to select cytosolic locations (21). Specific binding of glycolytic enzymes to actin in vitro has been described (22–24). In vivo, glyceraldehyde-3-phosphate dehydrogenase has been shown to be associated with F-actin in synaptosomes and at post-synaptic sites (25). In very confluent cultures of epithelial MDCK cells, glyceraldehyde-3-phosphate dehydrogenase was Triton X-100 insoluble and exhibited ATP-dependent cytoskeleton association (26). Glycolytic activity correlates with cell-cycle associated changes in the F-actin cytoskeleton suggesting that glycolytic activity may regulate actin cytoskeleton organization (27). Aldolase exists in equilibrium between a soluble and actin associated form; the ability to disrupt interaction with the actin cytoskeleton with the glycolysis inhibitor, 2-deoxyglucose, demonstrates that glycolytic activity can regulate the association of glycolytic enzymes with the actin cytoskeleton (28, 29). In Cos7 cells, the association of AMF-HA with actin-rich domains is completely detergent extractable (Fig. 2) reflecting its weak cytoskeletal association, as described previously for other glycolytic enzymes (30). In the presence of the glycolytic inhibitor oxamate, AMF-HA exhibits a more diffuse distribution than in untreated cells (Figs. 3D, 3E, and 3F) and exhibits a significantly reduced association with actin densities (Fig. 4). Due to the disruptive effect of glycolysis inhibition on the expression of actin-rich pseudopodia shown here in Cos7 cells, as described previously in an invasive epithelial-derived cell line (31), it is not clear whether inhibition of glycolysis in these cells affects the formation of actin-rich pseudopodia or specifically the association of AMF/PHI with these cellular domains. Nevertheless, glycolysis is shown to be required for the formation of protrusive cellular domains rich in both actin and the glycolytic enzyme PHI.

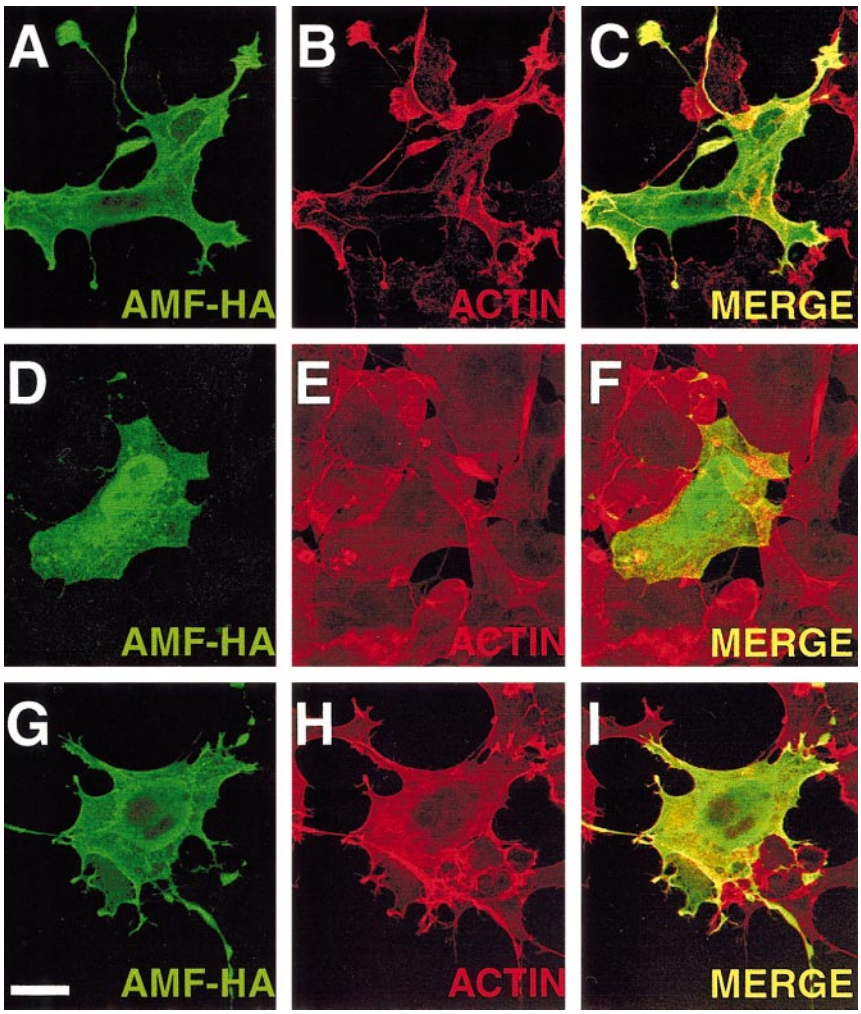

FIG. 3. Glycolysis regulates AMF-HA association with actin-rich protrusions. Cos7 cells transiently transfected with cDNA coding for AMF-HA were left untreated (A, B, C), treated with 100 mM oxamate for 1 hour (D, E, F) or with 100 mM oxamate and 100 mM pyruvate for 1 hour (G, H, I) before fixation with paraformaldehyde and immunofluorescent labeling with anti-HA antibody and Alexa 488 conjugated anti-mouse secondary antibodies (A, D, G) and Texas Red phalloidin to reveal F-actin (B, E, H). Confocal images from both fluorescent channels were superimposed (C, F, I) and colocalization appears in yellow. Bar = 10 μm.

AMF/PHI specifically associates with peripheral actin-rich protrusions corresponding to the lamellipodia or pseudopodia of a motile cell. Localized actin polymerization drives the protrusion of motility-associated cellular structures and pseudopodial protrusion is a critical element of cell movement (32–36). For the tumor cell, up-regulation of multiple glycolytic enzymes is associated with tumor malignancy (37, 38) and glycolysis is the principal supply of energy for cell motility (39). Glyceraldehyde 3-phosphate dehydrogenase has recently been localized to the actin-rich pseudopodia of an invasive variant of Moloney sarcoma transformed MDCK cells and glycolysis shown to regulate the protrusion of the multiple pseudopodia, and thereby the motility, of those cells (31). The localization of AMF/PHI to actin rich domains therefore identifies another glycolytic enzyme that is associated with actin-rich pseudopodia and suggests that multiple glycolytic enzymes should be expected to be

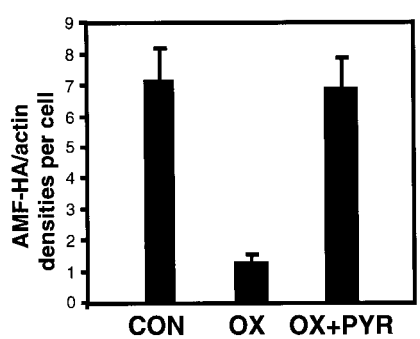

FIG. 4. Quantification of AMF-HA association with actin-rich protrusions. For at least 15 Cos7 cells transiently transfected with cDNA coding for AMF-HA, the number of AMF-HA concentrations which colocalized with peripheral actin densities (as in Figs. 2 and 3) were counted per cell for cells left untreated (CTL) or treated with 100 mM oxamate for 1 hour (OX) or with 100 mM oxamate and 100 mM pyruvate for 1 hour (OX + PYR).

associated with motile actin-rich pseudopodial domains. The reduction of actin densities in oxamate treated Cos7 cells observed here further supports a role for glycolysis in pseudopodial protrusion in cell motility. Formation of a localized glycolytic cytomatrix at the site of pseudopodial protrusion is therefore involved in active actin-mediated pseudopodial protrusion. Increased expression of AMF/PHI in tumor cells may act to enhance tumor malignancy in two ways: (1) by enhancing glycolytic activity and stimulating pseudopodial activity; and (2) via its secretion and activity as an extracellular motility stimulating cytokine.

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