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Autocrine motility factor and its receptor: Role in cell locomotion and metastasis

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Summary

The ability to locomote and migrate is fundamental to the acquisition of invasive and metastatic properties by tumor cells. Autocrine motility factor (AMF) is a 55 kD cytokine produced by various tumor cells which stimulates their *in vitro* motility and *in vivo* lung colonizing ability. AMF stimulates cell motility via a receptor-mediated signalling pathway. Signal transduction following binding of AMF to its receptor, a cell surface glycoprotein of 78 kD (gp78) homologous to p53, is mediated by a pertussis toxin sensitive G protein, inositol phosphate production and the phosphorylation of gp78. Cell surface gp78 is localized to the leading and trailing edges of motile cells but following cell permeabilization is found within an extended network of intracellular tubulovesicles. Gp78 tubulovesicles colocalize with microtubules and extension of the tubulovesicular network to the cell periphery is dependent on the presence of intact microtubules. Gp78 labeled vasicles can be induced to translocate between the cell center and periphery by altering intracellular pH as previously described for tubulovesicles labeled by fluid phase uptake. Anti-gp78 mAb added to viable motile cells is localized to large multivesicular bodies which, with time, relocate to the leading edge. Binding of AMF to its receptor induces signal transduction, similar to chemotactic stimulation of neutrophil mobility, as well as the internalization and transport of its receptor to the leading edge stimulating pseudopodial protrusion and cell motility.

Introduction

The introduction and regulation of active locomotion in eukaryotic cells is central to the development and well-being of higher organisms. The directed migration of cells is crucial to diverse biological processes including embryogenesis, morphogenesis, wound healing and immunity as well as enabling the most devastating aspect of neoplastic disease, metastasis [1, 2]. However, despite many studies the mechanisms which regulate and direct cell migration are largely unknown.

To understand the role that active cell locomotion plays in various biological processes, efforts have focused on chemotactic and motility factor induction of cell locomotion. Host serum proteins and extracellular matrix breakdown products were found to exert a chemotactic effect on various tumor cells [3–5]. A group of secreted cytokines which specifically induce cell motility has been identified with molecular weights between 55 and 92 kD which appear to be specific inducers of cell motility. Rat ascites hepatoma AH109A cells secrete a chemotactic polypeptide thought to facil-

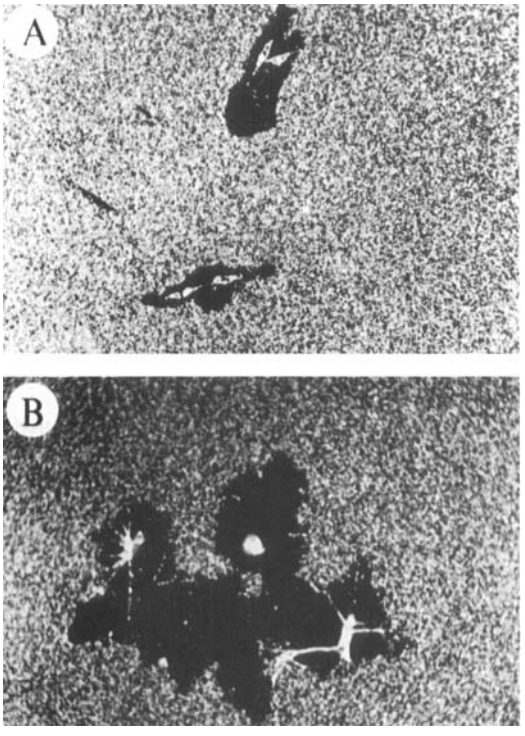

Fig. 1. Induced motility of 31 fibroblasts. Uniform carpets of gold particles were prepared on cover slips coated with BSA according to Albrecht-Buehler [73]. Colloidal gold coated cover slips were placed in 35 mm tissue culture dishes containing 2 ml CMEM (DMEM containing 10% heat-inactivated fetal bovine serum, glutamine, non-essential amino acids, antibiotics and vitamins) as is (A) or supplemented with 25 μl/ml anti-gp78 ascites fluid (B) and 2000 A31 fibroblasts were added to each plate. After 24 hrs at 37° C, phagokinetic tracks were visualized using dark field illumination. In dark field microscopy the gold particles reflect light and are seen as bright dots while the area cleared of gold particles by cell movement is dark. Addition of the anti-gp78 mAb to the culture medium induced a two-fold increase in their motility from $24 \pm 4\ \mu^2$/hr to $44 \pm 7\ \mu^2$/hr in the presence of the anti-gp78 mAb (data from more than 50 isolated cells per group) as previously described for other cell lines [14, 16] (390X).

itate malignant invasion [6]. Fetal and tumor-derived fibroblasts exhibit similar modes of migratory behavior and were found to secrete migration stimulating factor (MSF) which stimulated cellular penetration through a collagen matrix [7]. A fibroblast-produced scatter factor (SF) was reported to exert paracrine activity on epithelial-mesenchymal cell interaction and to induce invasiveness of tumor epithelial cells [8–10]. The human gF was recently found to be identical to the human hepatocyte growth factor [11]. A tumor secreted cytokine was implicated in the induction of both random and directed cell migration of producing cells and has therefore been denoted autocrine motility factor (AMF) [12, 13]. These factors, and others, may represent a family of cytokines whose regulated expression induces cell motility in non-cancerous situations such as wound healing (scatter factor) and embryogenesis (MSF) and whose constitutive autocrine expression (AMF) may confer metastatic capabilities on neoplastic cells.

Autocrine motility factor

AMF motility stimulating activity has been identified in the conditioned media of various cultured tumor cell lines as well as from an adenocarcinoma tumor [12–15]. While AMF production and secretion is restricted to transformed cells, AMF stimulates the motility of both transformed and untransformed cells including human A2058 melanoma, HeLa cervical carcinoma, MCF-7 breast carcinoma, HT-1080 lung carcinoma and murine B16 melanoma, UV-2237 fibrosarcoma, K-1735 melanoma as well as NIH-3T3 and Balb/A31 fibroblast cells [12, 14, 16] (Fig. 1). Whereas untransformed NIH-3T3 fibroblasts do not secrete AMF, three different ras transformed clones of NIH-3T3 fibroblasts produce large amounts of AMF [12]. Interestingly, both the ras transformed and untransformed fibroblasts exhibit an equivalent motile response to AMF [12]. AMF could therefore play a paracrine role for normal cells whereas transformed cells, which both produce AMF and express the receptor, could stimulate their own motility in an autocrine fashion.

AMF has been purified from human A2058 melanoma [12], murine B16-F1 melanoma [15] and human HT-1080 fibrosarcoma cells [16]. AMF is a protein with an apparent molecular weight of 55 kD under non-reducing and 64 kD under reducing gel electrophoresis reflecting the presence of disulfide bonds. These bonds are essential to its function since treatment with dithiothreitol eliminates the ability of AMF to stimulate cell motility

[12]. Under non-reducing conditions mouse AMF resolves into two polypeptides of pI 6.35 and 6.4 [15] while human AMF resolved into four polypeptides, two minor ones identical to those found in mouse, as well as two major polypeptides of pI 6.1 and 6.2 [16]. The presence of AMF's of different isoelectric points is most likely due to posttranslational modification such as differential glycosylation. However, AMF glycosylation could not be detected by either neuraminidase cleavage or ^{125}I-WGA blotting of AMF from B16-F1 or HT-1080 cells. The multiple AMF isoforms could rather be due to the presence of more than one homologous gene for AMF or to posttranslational modification of the AMF protein core. The absence of glycosylation distinguishes AMF from other motility inducing factors such as the 70 kD MSF [7, 17], the 32–92 kD scatter factors [9, 10, 18]. the acidic and basic fibroblasts growth factors [19, 20] and the insulin-like growth factor [20]. AMF, like scatter factor, migrates differently in SDS-PAGE in the presence of a reducing agent [9, 10, 18]. However, unlike scatter factor, which dissociates under reducing conditions into several polypeptides, mouse B16-F1 melanoma, human A2058 melanoma and HT-1080 fibrosarcoma AMF remain as intact polypeptides under reducing conditions [12, 15, 16].

The signalling pathway of AMF motility stimulation

AMF does not stimulate neutrophil motility and the neutrophil and leukocyte chemoattractant, f-met-leu-phe, does not stimulate a motile response in cells responsive to AMF, however inhibitors of f-met-leu-phe stimulated chemotaxis also inhibit AMF stimulated motility, suggesting that the signalling response to the two factors is mediated by different receptors but follows a similar pathway [12]. Binding of f-met-leu-phe to the chemotactic receptor on leukocytes results in activation of a guanine regulatory (G) protein which stimulates a polyphosphoinositide specific phopholipase C resulting in the cleavage of phosphatidylinositol, 4,5-biphosphate to produce two second messengers, inositol triphosphate and diacylglycerol [21]. Inactivation of certain G proteins by *Bordella* pertussis toxin (PT) blocks the chemotactic response of neutrophils and leukocytes to f-met-leu-phe as well as other associated responses such as Ca^{++} mobilization, O^{2-} production, actin polymerization, lysosomal enzyme secretion and inositol phosphate production [22–24]. The chemotactic response is not inhibited by cholera toxin indicating that it is independent of a CAMP mediated pathway [23].

The signalling pathway of the AMF stimulated motile response in melanoma cells is specifically sensitive to PT. Neither cholera toxin nor other inhibitors of the CAMP metabolic pathway affect AMF motility stimulation [25]. Furthermore, AMF stimulates the increased incorporation of inositol into cellular lipids and inositol phosphates, in particular inositol triphosphates [21, 26]. AMF stimulated production of inositol phosphates is directly correlated with induced cell motility in response to different levels of AMF and is partially inhibited by pretreatment of the cells with PT [26].

In neutrophils, low levels of stimulation with f-met-leu-phe can be used to restrict the cellular responses to the chemotactic stimulus; two responses which persist under these conditions are actin polymerization and production of phosphatidyl inositol triphosphate (PIP_3), a precursor of inositol triphosphate, suggesting that PIP_3 production may regulate actin polymerization in neutrophil chemotaxis [27]. In moving fibroblasts, actin polymerizes in a meshwork of filaments perpendicular to the direction of movement at the base of the extending leading edge as well as in microspikes aligned longitudinally in extending lamellipodia [28, 29]. AMF stimulates the extension of cell pseudopodia which exhibit prominent axial actin filament bundles; pseudopodia isolated following AMF stimulation contain 20 times more laminin and fibronectin receptors than are found in the plasma membrane of unstimulated cells [30]. AMF activity is therefore receptor mediated and binding of AMF to its receptor initiates a signalling cascade, similar in many respects to the chemotactic response of neutrophils and leukocytes, which induces pseudopodial extension and translocation of extracellular matrix receptors to the leading edge.

The AMF receptor

The receptor for AMF, Gp78, was first identified by the direct correlation between its increased O-linked glycosylation and the increased metastatic ability of B16-F1 cells grown in a spherical configuration [31–33]. Evidence demonstrating the involvement of O-linked glycosylation of gp78 in metastasis was obtained from studies of a selected adhesive B16-F1 variant; following growth in suspension culture it did not express increased O-linked glycosylation of gp78 and was not more metastasic [33]. The causal involvement of gp78 in metastatis was demonstrated by the ability of $F_{\alpha\beta}$ fragments from polyclonal anti-gp78 antibodies to enhance the lung colonizing ability of B16-F1 melanoma cells following i.v. inoculation into the tail vein of syngeneic mice [32]. Similarly, cells treated with a monoclonal anti-gp78 antibody exhibited enhanced *in vitro* phagokinetic motility on colloidal gold coated cover slides as well as increased *in vivo* lung colonizing ability [14, 16]. B16-F1 melanoma AMF containing conditioned media stimulated cell motility in an identical fashion to anti-gp78 mAb and both antibody and AMF stimulated motility of B16-F1 cells were inhibited by PT.

Identification of gp78 as a motility factor receptor was initially obtained from the inhibition of binding of anti-gp78 mAb to gp78 in immunoblots by AMF-containing conditioned media; heat inactivated AMF was incapable of blocking recognition of gp78 by the antibody suggesting that AMF and the anti-gp78 Mab bind to the same motility-activating epitope on gp78 [14]. Confirmation of the identity of gp78 as the receptor for AMF has been obtained from studies of gp78 immunopurified from B16-F1 melanoma cells and AMF purified from B16-F1 conditioned media by Sephacryl S-200 chromatography. Iodinated AMF bound quantitatively to purified gp78 immobilized on nitrocellulose demonstrating the direct interaction between AMF and gp78. Furthermore, the addition of small amounts of soluble gp78 (0.05 μg) to the culture medium was able to inhibit the stimulation of the phagokinetic motility of B16-F1 cells by both AMF and anti-gp78 mAb [15].

Cloning of gp78 from a human fibrosarcoma cDNA library has revealed that gp78 is a protein of 323 amino acids consisting of an 11 amino acid extracellular domain, a hydrophobic 25 amino acid transmembrane domain and a 186 amino acid intracellular region [34]. A single postulated N-linked glycosylation site was found in the extracellular domain as well as several potential O-linked glycosylation sites. 12 cysteine residues are found in both the extra and intracellular domains. The cytoplasmic domain of gp78 contains a SER/THR-Xaa-ARG/LYS motif, the consensus sequence for a phosphorylation site [35], as well as a nucleotide binding consensus sequence found in nucleotide-binding proteins including several serine/threonine kinases [36]. Interestingly, a computer search of several sequence databases revealed partial homology of gp78 to only one previously reported DNA sequence, the human tumor suppressor/oncogene gene p53 [34, 37].

Expression of gp78 in motile cells

On the surface of motile cells gp78 is localized to distinct surface areas at the leading edge, trailing edge and to a region proximal to the nucleus (Fig. 2 A, B) [14]. The temperature and energy dependent redistribution of cell surface proteins on motile cells has been previously described and can be explained by either the attachment of cell surface proteins to a submembrane cytoskeleton restricting their movement or to the rearward flow of surface membrane and component proteins due to insertion of new membrane at the leading edge [38–42]. Recently, using sensitive microscopy of living cells two types of motion by surface attached particles is observed; some particles remain stationary with respect to the cell while others migrate rearward in a centripetal fashion to the nuclear region traversing the length of the cell as the cell moves forward [42, 43]. It has been suggested that the different behaviors might be due to the stronger attachment of cell surface glycoproteins to the submembrane cytoskeleton at the leading edge which then move to the rear of the cell in coordination with the cytoskeleton [44].

The polarized surface distribution of gp78 is not

a consequence of cell movement as gp78 is localized to one side of round cells attached for only two hours to the substrate [14]. The distinctive localization of gp78 to both the leading and trailing edges of the motile cell most closely resembles that described for the fibronectin receptor in CHO cells and for galactosyl transferase in mesenchymal cells plated specifically on laminin with which the galactosyl transferase was shown to interact [45, 46]. This pattern of surface expression may be characteristic of proteins specifically involved in the motile process and suggests the active relocation to the leading edge of such proteins during cell locomotion.

Exocytosis of proteins occurs at the cell periphery in non-motile cells and at the leading edge in motile cells. In virally infected HeLa cells, newly synthesized hemagglutinin first appears at the cell periphery in round cells and at protrusions in irregularly shaped cells [47]. In giant HeLa cells, in which the sites of exocytosis and endocytosis on the cell surface are sufficiently distant from one another so that surface localization reflects the site of exocytosis, endocytosing proteins are similarly localized to cell protrusions and the cell periphery while non-endocytosing surface proteins are uniformly distributed over the entire surface of the cell [48]. It has been proposed that exocytosis of membrane vesicles at the leading edge supplies membrane for extension of the leading edge as well as generating force for cell locomotion [1, 49].

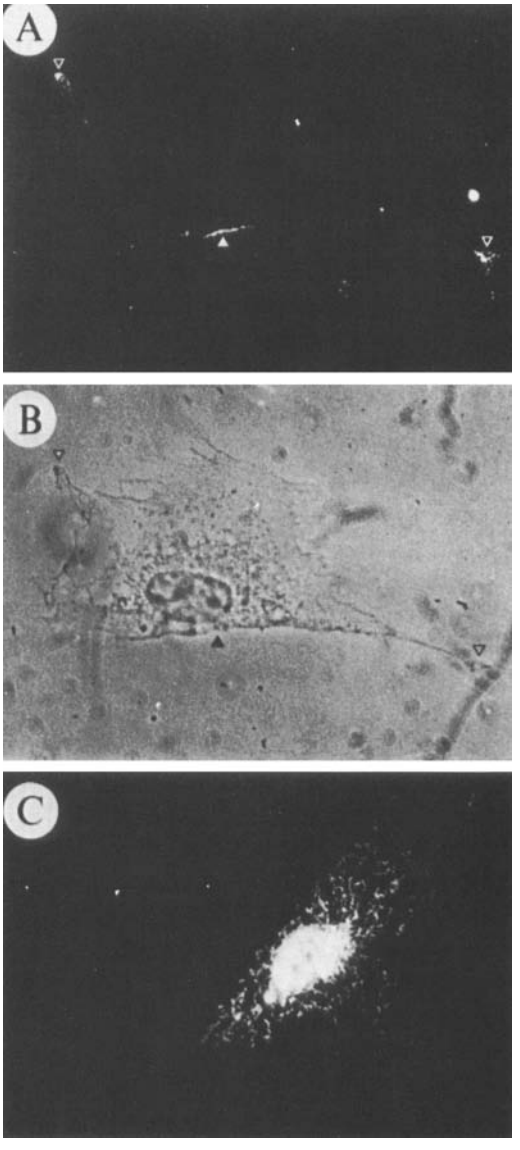

Fig. 2. Immunofluorescent labeling of gp78. Surface immunofluorescence of gp78 was performed by incubating viable A31 fibroblasts with anti-gp78 mAb (1:10) in ice cold CMEM. The cells were rinsed three times with ice cold PBS and then fixed with cold 3% paraformaldehyde for 15 min after which they were incubated with TRITC conjugated anti-rat IgG (Zymed, CA). Arrowheads indicate corresponding locations in the fluorescent (A) and phase (B) images. To visualize the intracellular distribution of gp78 (C), cells were fixed and permeabilized by immersion of the cover slip in precooled (−80°C) for 30 min at −20AG C. Identical images were seen in cells permeabilized for 2 min in 0.5% Triton X-100 in a cytoskeleton preserving buffer consisting of 60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 1 mM $MgCl_2$ (pH 7.4) [74] and then fixed in 3% paraformaldehyde in the same buffer. The cover slides were incubated with anti-gp78 mAb (1:50) for 30 min and then with TRITC conjugated anti-rat IgG. There is a significantly larger amount of gp78 within the cell than on the surface such that at the mAb dilution used to visualize the intracellular labeling (1:50) no surface labeling is detectable. (1200X).

Intracellular localization of gp78 to tubulovesicles

Immunofluorescent labeling of permeabilized A31/BALBc fibroblasts, whose spread morphology enables better cytoplasmic visualization in fluorescent microscopy than B16-F1 cells, reveals the intracellular localization of gp78 to elongated beaded structures extending to the periphery of the cell (Fig. 2C). In the cell periphery, the network of gp78 containing vesicles highly resembled the extended tubular networks of vesicles labeled by fluid phase uptake previously described in macrophages and in chick fibroblasts [50, 51]. Tubular lysosomes in macrophages were identified as lysosomes by

acid phosphatase histochemistry and colocalization with cathepsin L [50]. However, gp78 exhibits only partial colocalization with the lysosomal membrane glycoprotein, LAMP-1, as was previously reported in B16-F1 melanoma cells (Fig. 3) [16]. Lysosomes labeled with both gp78 and LAMP-1 are positioned near the nucleus while tubular vesicles labeled only with gp78 are predominantly located near the cell periphery. Therefore, while gp78 is present in lysosomes it is also a component of non-lysosomal vesicles.

The morphology of tubular lysosomes in macrophages is dependent on the presence of intact microtubules; treatment of cells with microtubule disrupting drugs results in the collapse of the tubular network to the cell center [50]. Similarly, gp78 vesicles exhibit significant colocalization with microtubules (Fig. 4) and disruption of microtubules with colchicine results in the disappearance of gp78 labeled vesicles from the periphery of the cell and their clustering around the nucleus (Fig. 5). Gp78 vesicles are therefore associated with microtubules and the distribution of these vesicles throughout the cell is dependent on microtubule network integrity. The ability of gp78 vesicles to translocate between the cell center and the periphery was demonstrated by their redistribution in response to changes in cytoplasmic pH. Exactly as described by Heuser [51], cytoplasmic acidification results in the relocation of densely stained vesicles, resulting from tubule fission, to the cell periphery and corresponding to outward movement (Fig. 6). Realkalinization induces the formation after five minutes of elongated, inward moving, gp78 containing tubules which, after fifteen minutes, results in the concentration of gp78 around the cell nucleus. The tubular morphology of gp78 vesicles, their colocalization with microtubules and their distinctive relocation in response to changes in cytoplasmic pH leads us to label them tubulovesicles. Gp78, the receptor for AMF, may be a fundamental membrane component of tubulovesicles.

A 78 kD lysosomal membrane glycoprotein, endolyn-78, has been described which bears striking resemblance to gp78 [52]. Both contain O-linked oligosaccharide residues and endolyn-78 was found in the plasma membrane and lysosomes as well as in morphologically diverse compartments such as peripheral tubular endosomes, vacuolar endosomes and multivesicular bodies. It was suggested that it may be a component of early endosomal compartments which other lysosomal membrane glycoproteins, such as the family of heavily glycosylated lysosomal associated membrane proteins (LAMP), are present only in mature lysosomes [52, 53]. LEP100, the avian LAMP-1, is predominantly localized to lysosomes but can also be detected at low levels in endosomes and the plasma membrane of chick fibroblasts [54, 55]. In MDCK epithelial cells, following biosynthesis an endogenous LAMP is targeted to the basolateral surface domain prior to delivery to lysosomes [56]. Interestingly, changes in oligosaccharide branching of a mouse LAMP-1 homologue, the P2B protein, results in its increased cell surface expression and is associated with efficient tumor cell metastasis [57]. Increased O-linked glycosylation of gp78 is similarly associated with the increased metastatic ability of B16-F1 cells cultured in a spherical configuration [32, 33]. Another lysosomal membrane glycoprotein, the CD63 antigen, is identical to the melanoma associated antigen, ME491 [58, 59]. Altered glycosylation and cell surface expression of proteins normally found within intracellular compartments may play a role in acquisition of enhanced metastatic and motile properties by tumor cells.

Endocytosed gp78-antibody complexes relocate to the leading edge

To determine whether gp78 is involved in membrane flow to the leading edge, A31 fibroblasts were incubated with anti-gp78 mAb in the cold and then warmed to 37° C and the endocytosis of the gp78-antibody complex monitored after different times (Fig. 7). Ten minutes following antibody binding, gp78-antibody complexes were diffusely distributed throughout the cell corresponding to antibody uptake to early endosomes and indicating that, in contrast to its localized surface distribution, gp78 endocytosis is not restricted to distinct surface domains. After twenty minutes large perinuclear

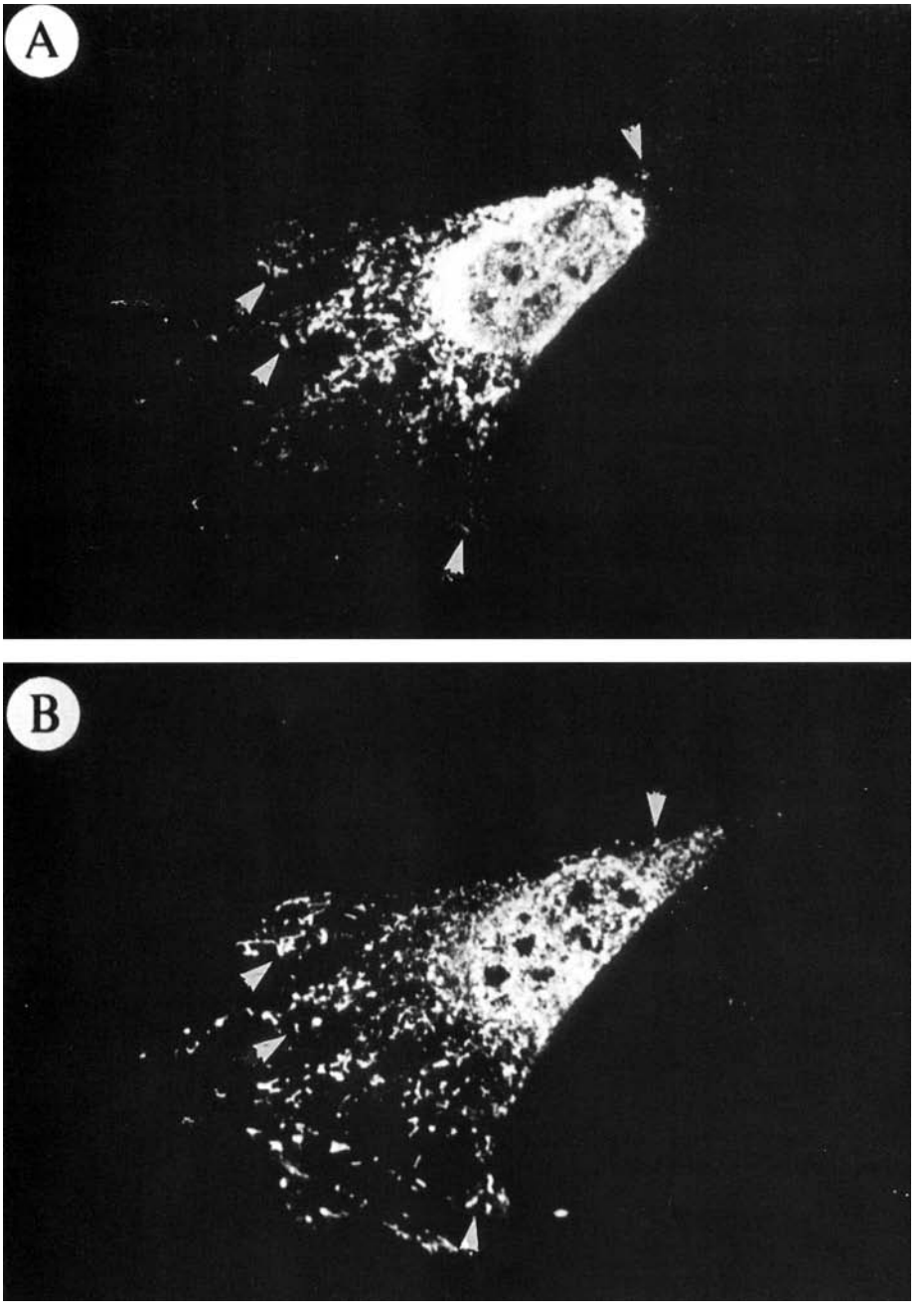

Fig. 3. Double indirect immunofluorescent labeling for LAMP-1 (A) and gp78 (B). A31 fibroblasts were fixed and permeabilized prior to treatment with anti-P2B/LAMP-1 antisera (kindly provided by J. Dennis) and fluorescein anti-rabbit IgG followed by anti-gp78 mAb and rhodamine anti-rat IgG. No crossreactivity of the two secondary antibodies to cells labeled with either of the two primary antibodies was detected. Partial colocalization is observed indicating that gp78 is found in lysosomes but is also present in non-lysosomal vesicles (2143X).

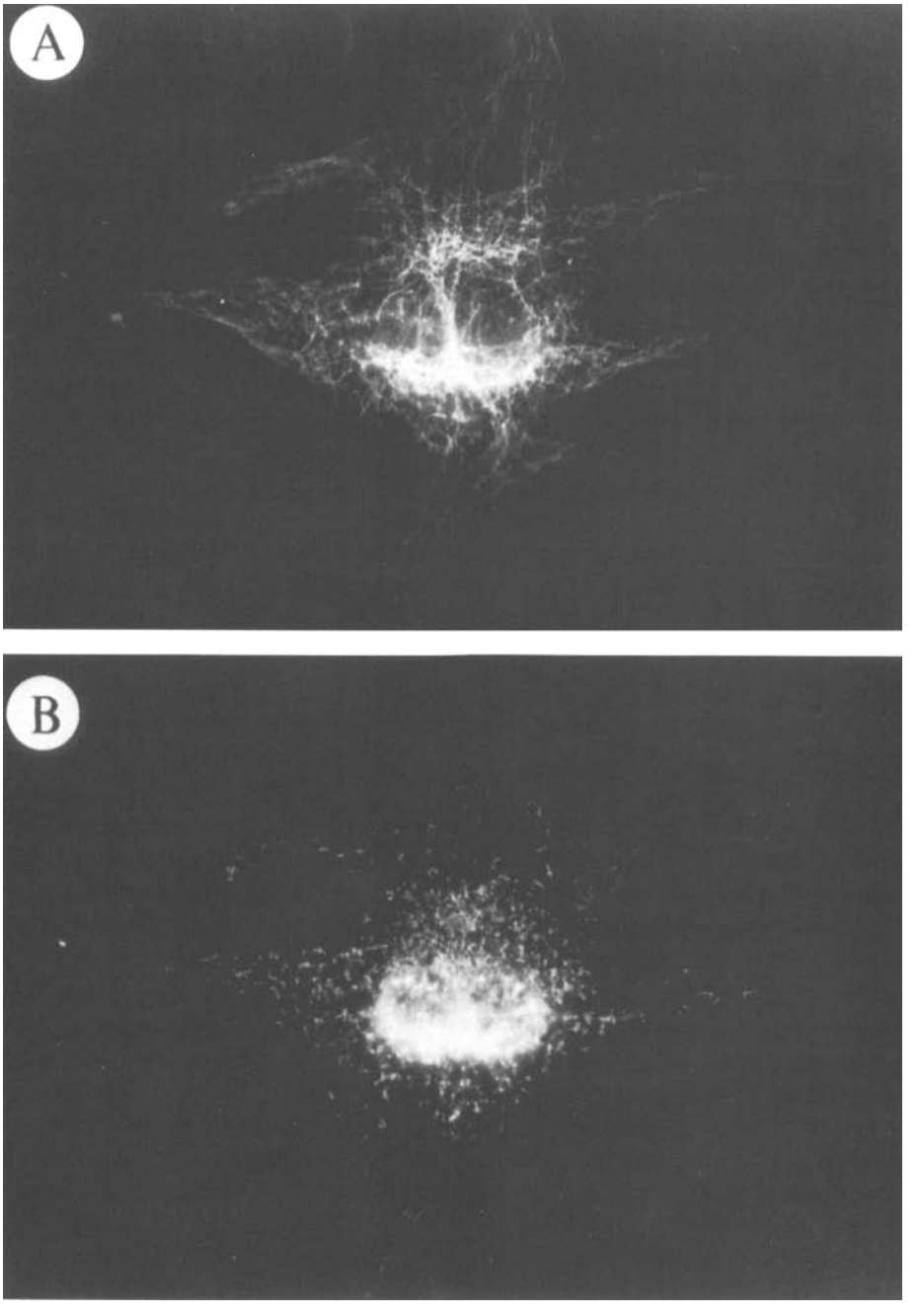

Fig. 4. Double indirect immunofluorescent labeling for microtubules (A) and gp78 (B). A31 fibroblasts were fixed and permeabilized prior to treatment with anti-tubulin mAb and fluorescein anti-mouse IgG followed by labeling with anti-gp78 mAb and rhodamine anti-rat IgG. No cross reactivity of the two secondary antibodies to cells labeled with either of the two primary antibodies was observed. Extensive colocalization of gp78 tubulovesicles with microtubules is observed. (2143X).

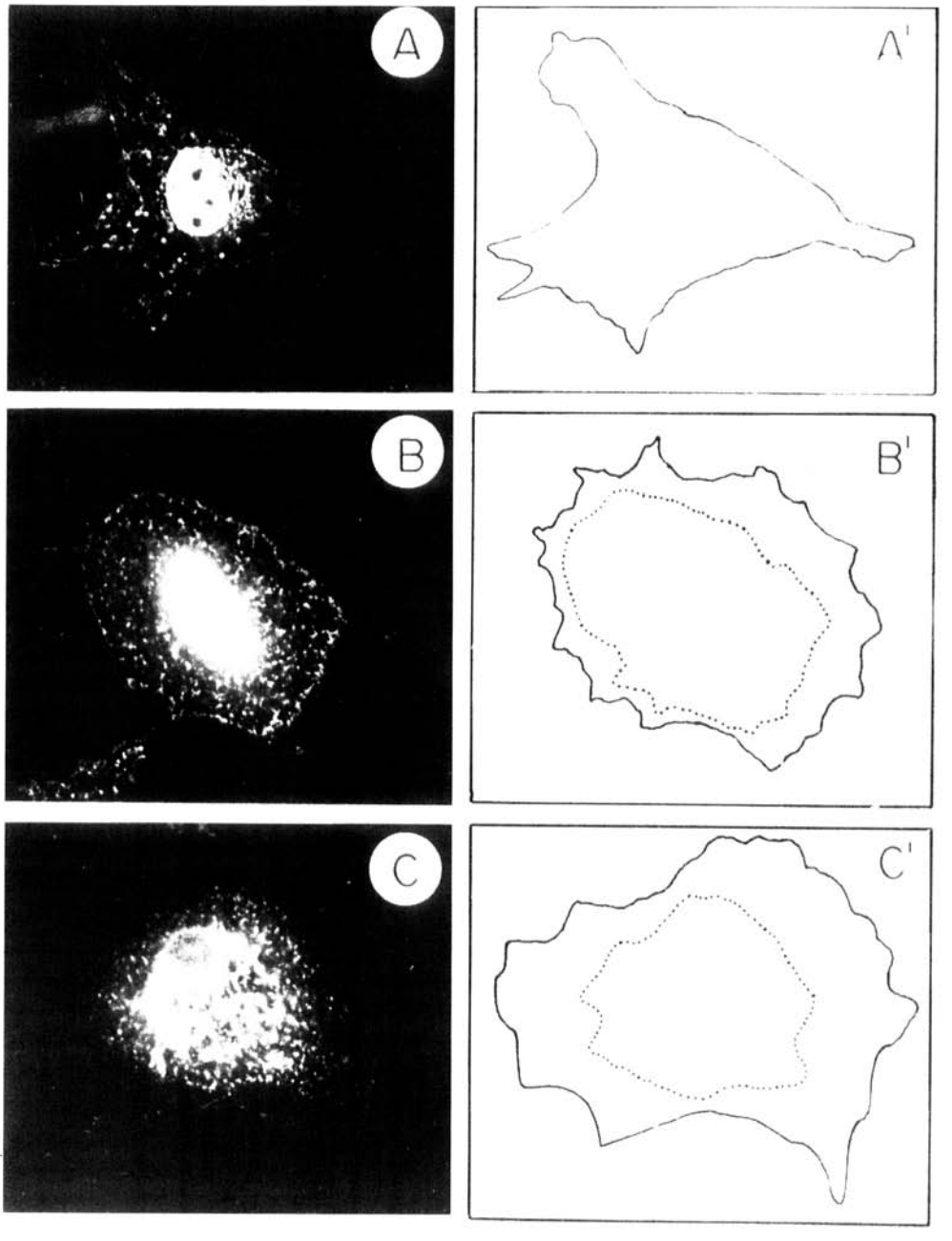

Fig. 5. Gp78 distribution following colchicine and monensin treatment. A31 fibroblasts (A) treated with 25 mM colchicine for 1 h (B) or 10^{-5} M monensin for 2 hrs (C) were labeled for immunofluorescence with anti-gp78 mAb. Cells treated with other drug revealed an almost identical pattern of staining; no peripheral localization of gp78 was observed with gp78 restricted to a central region of the cell around the nucleus, schematically represented (primes) in which solid lines represent the cell perimeter and dotted lines the region of gp78 fluorescence. Tubulin labeling of colchicine treated cells revealed total dispersal of tubulin fibers (977X).

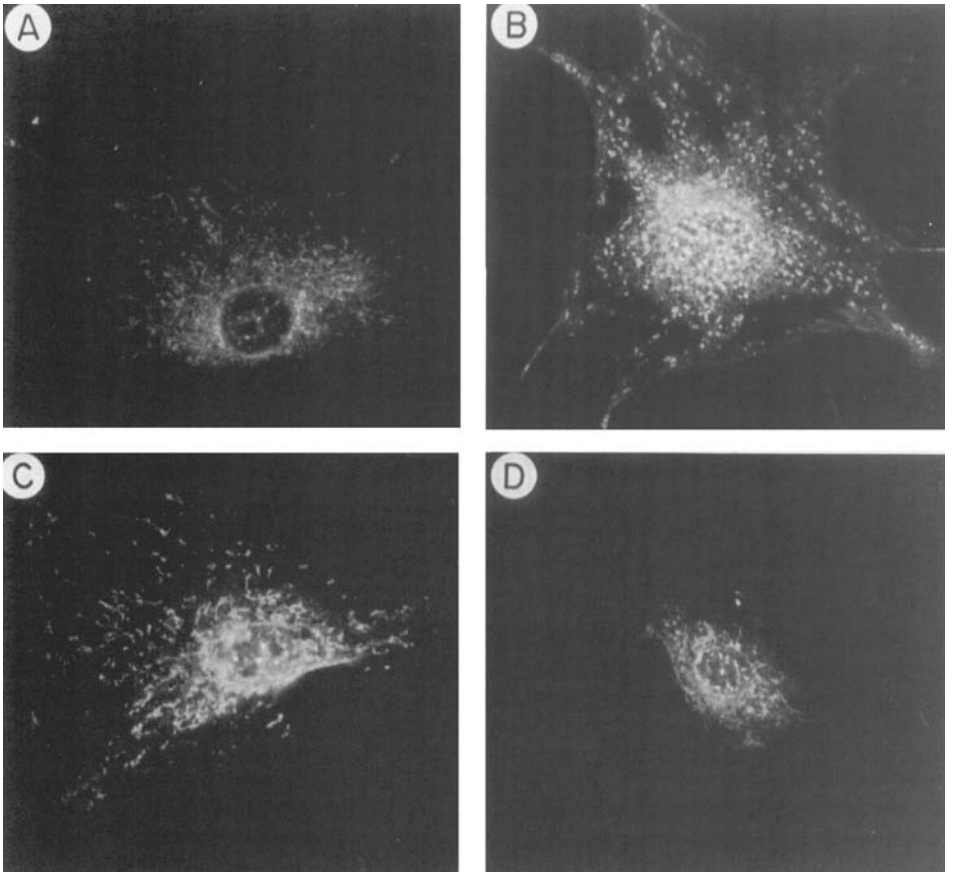

Fig. 6. Distribution of gp78 after changes in cytoplasmic pH. Manipulation of cytoplasmic pH was performed exactly as described [51]. Cells were rinsed for fifteen min (3X) with Ringers solution in a 37° C incubator without CO_2 flow (A). The cells were acidified by the addition for fifteen min of Ringers acetate solution in which 70 mM sodium acetate replaced an equivalent amount of sodium chloride (B). The cells were realkalinized by the replacement of Ringers acetate with normal Ringers solution for five (C) or fifteen (D) min. At each point, the cells were fixed by the rapid addition of precooled methanol prior to immunofluorescent labeling for gp78. As shown in fibroblasts labeled by horseradish peroxidase uptake [51], cytoplasmic acidification causes fission of gp78 tubulovesicular arrays to generate distinct vesicular structures located preferentially at the cell periphery. Realkalinization induces vesicular fusion to form elongated tubules after five min resulting in gp78 concentration at the cell center after fifteen min directly corresponding to the inward migration of gp78 vesicles in response to changes in cytoplasmic pH. (1080X).

fluorescent-labeled complexes formed similar to multivesicular bodies labeled by endocytosed transferrin receptor [60]. With time (40–60 min) the gp78 labeled complexes polarized, relocated and moved towards the leading edge of the cell (Fig. 7C, D).

Comparable fluorescent images to those we have seen for endocytosed gp78 have been described for endocytosed transferrin receptor. Using low light confocal video microscopy, fluorescent swellings or varicosities containing endocytosed fluorescent marker were seen along tubular cisternae at irregular intervals; the labeled structures were shown to be large multivesicular bodies (MVB) and could be seen moving along a fluorescent filamentous network described as a continuous endosomal reticulum [61]. The movement of MVBs along a filamentous network supports our observations of the colocalization of gp78 tubulovesicles with microtubules in permeabilized cells and the endocytosis of gp78 into large complex structures which move to the leading edge. Other tubulovesicular pathways

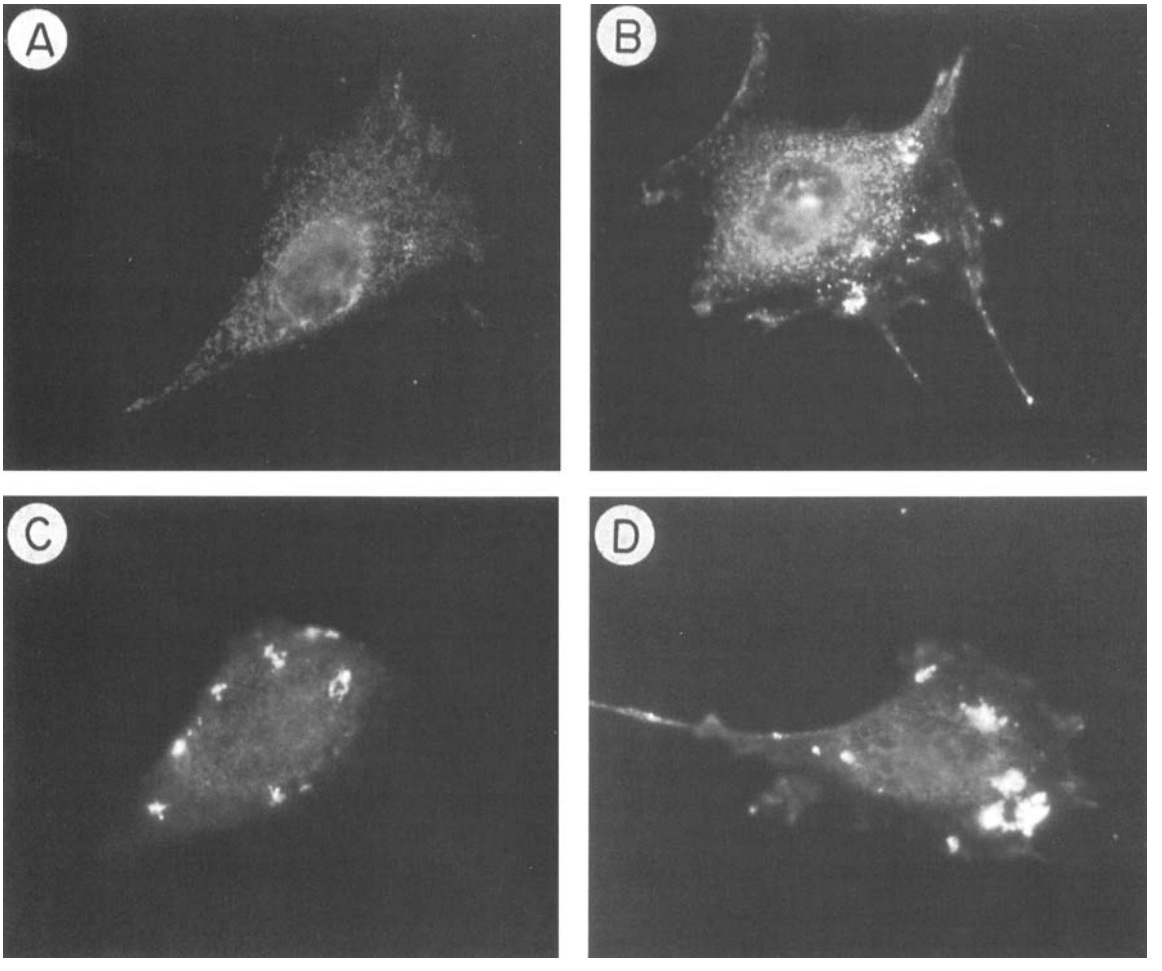

Fig. 7. Internalization and transport of endocytosed gp78-antibody complexes to the leading edge. A31 fibroblasts were rinsed with cold CMEM and incubated with anti-gp78 mAb diluted 1:1 in CMEM on ice for 20 min. The cells were rinsed with cold CMEM and then placed in warm CMEM at 37° C for 10 (A), 20 (B), 40 (C) or 60 (D) min after which they were fixed with precooled methanol. Internalized gp78-antibody complexes were visualized with TRITC conjugated anti-rat IgG (Zymed, CA). Cells fixed without permeabilization revealed no surface labeling at any time point. 10 min following transfer to 37° C gp78 is barely detectable and is diffusely distributed within the cell. Commencing at 20 min large strongly labeled complexes form which after 40 min are grouped non-directionally around the cell nucleus but after 60 min are oriented towards the leading edge of the cell.

of membrane traffic have been described. Tubulovesicular processes have been observed to interlink Golgi cisternae and to emerge from the trans-Golgi and migrate along microtubules [62]. Similarly, treatment of cells with brefeldin A induces the formation of microtubule-dependent tubular processes extending from the salvage compartment, intermediate between the endoplasmic reticulum and the Golgi, back to the endoplasmic reticulum [63]. Certain pathways of membrane traffic within the cell may be tubulovesicular in nature.

The distinctive surface expression, intracellular localization to tubulovesicles and internalization and transport to the leading edge of the receptor for AMF suggests the existence of a specific motility factor stimulated pathway of membrane flow. Translocation of gp78 membrane vesicles to the front of the cell with supply membrane for extension of the leading edge and result in the exocytosis

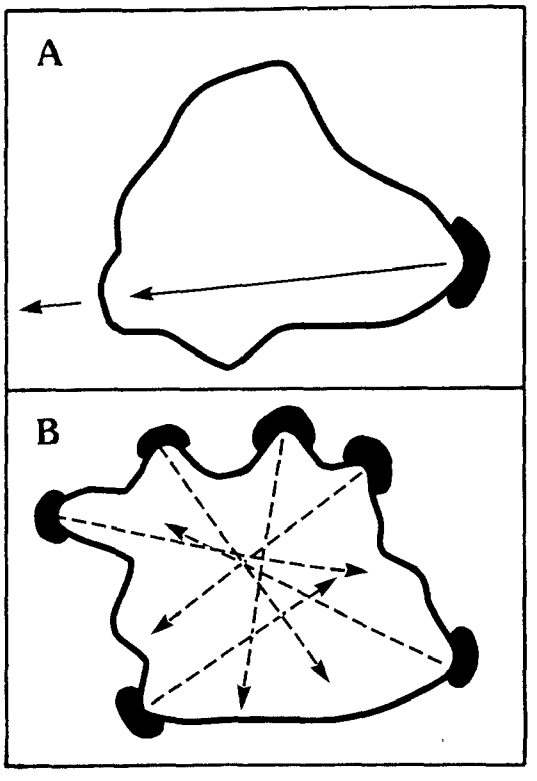

Fig. 8. Schematic representation of gp78 expression and trafficking in motile cells. In a motile cell (A) gp78 is internalized and then transported to the leading edge from the perinuclear region. Unendocytosed gp78 accumulates at the rear of the cell and at the site of exocytosis at the leading edge. In a less motile cell (B), the internalization and transport of gp78 to the leading edge are reduced resulting in a greater accumulation of gp78 on the cell surface at the rear of the cell. The direction of movement of the cell is shown by the arrow to the left of the cells.

of gp78 at the leading edge. Subsequent endocytosis of gp78 must not necessarily occur at the trailing edge of the cell but might occur on the whole cell surface such that only the accumulation of unendocytosed gp78 at the rear of the cell due to forward cell movement can be visualized on the cell surface by immunofluorescent labeling. Ten minutes following warming of cells incubated with anti-gp78 mAb, gp78 is in fact distributed in early endosomes throughout the cell (Fig. 7A). Our studies of the surface expression of gp78 in differentially motile metastatic variants suggest that the amount of gp78 on the cell surface is inversely proportional to the motility of the cells [16]. On highly motile cells a majority of gp78 might be endocytosed soon after exocytosis at the leading edge and might even travel within the cell to the perinuclear region before being translocated forward to the leading edge (Fig. 8). Provision of membrane to the leading edge by vesicular transport and exocytosis may induce but is not dependent on the existence of a rearward surface membrane flow.

Directed flow of gp78 and cell locomotion

Singer and coworkers have demonstrated the necessity of the polarized delivery of membrane vesicles to the cell periphery to generate a leading edge and direct cell movement [11]. Following wounding of a confluent monolayer of NRK fibroblasts, the microtubule organizing center (MTOC) and the Golgi apparatus coordinately relocate to a position forward of the nucleus in the direction of the extending lamella of the cell [64]. In VSV infected fibroblasts, viral G protein is transported to the leading edge of motile cells [65]. Disruption of microtubules results in the loss of direction of transport to the leading edge, extension of multiple leading edges and loss of cell motility indicating that microtubules play a crucial role in directing extension of the leading edge [66]. The colocalization of gp78 vesicles with microtubules and the necessity of the presence of an intact microtubule network for their distribution to the cell periphery implicates gp78 in the directed endocytosis and movement of membrane vesicles along microtubules to the leading edge.

Studies in our laboratory on the surface distribution of gp78 in low and high metastatic melanoma variants which exhibit differential *in vitro* motility support these predictions [16, 67]. High-metastatic B16-F10 and K1735-M1 melanoma cells exhibit substantially increased lung colonization ability and *in vitro* motility compared to their low-metastatic counterparts B16-LR^6 and K-1735-C1-11. The low metastatic clones express increased levels of surface gp78 compared to the high-metastatic cells by fluorescent activated cell sorter (FACS) analysis; indirect immunofluorescence labelling of unpermeabilized cells reveals that the low-metastatic cells have multiple gp78 surface domains

whereas the more motile high-metastatic cells have only one [16]. We postulate that the multiple stained areas on the surface of the slower cells are due to extension of multiple leading edges generating multiple routes of membrane flow and decreasing the net forward displacement of the cell. The single intense area visualized on the more motile cells may be the consequence of extension of a single leading edge and a unidirectional flow of membrane.

Addition of anti-gp78 mAb or AMF could act by inducing gp78 endocytosis, enhancing the rate of membrane flow and inducing extension of the leading edge. On motile cells extending a single leading edge, motility factor stimulation would increase the directional motility of the cell. However, on non-motile cells extending multiple leading edges in opposing directions, motility factor stimulation would increase phagocytosis but not necessarily cell motility. Corroboration of this interpretation was obtained from studies of the *in vitro* motility and *in vivo* metastatic response of high and low-metastatic clones to anti-gp78 antibody. Whereas both high and low metastatic cells exhibited a similar 2–2.5 fold increase in phagokinetic motility, only the high metastatic variants responded to the anti-gp78 antibody with increased lung colonization [16]. The phagokinetic track assay measures non-directional motility; extension of multiple leading edges will result in the phagocytosis of gold particles all around the circumference of the cell generating an area cleared of gold particles which may not be representative of directional cell movement. The experimental metastasis assay, in which cells are inoculated i.v. into the tail vein of syngeneic mice and resulting lung colonies counted, requires the cell to exhibit directional motility to extravasate into the parenchyma. Since only the high-metastatic variants responded to the anti-gp78 antibody with increased lung colonization, it may be only in those cells that motility factor stimulation induces the unidirectional transport of endocytosed gp78 to a single extending surface.

AMF receptor activation

AMF acts to stimulate cell motility not only by generating a cellular response via second messenger signal transduction but also by inducing internalization and translocation of its receptor, gp78, stimulating cell motility via membrane flow. Ligand-induced receptor phosphorylation is a well-characterized signal for receptor internalization and signal transduction [68]. The cDNA sequence of gp78 reveals consensus sequences for nucleotide binding and a phosphorylation site within the gp78 cytoplasmic domain. Stimulation of HT-1080 fibrosarcoma cells with AMF induces the phosphorylation of gp78 within four minutes suggesting a relationship between AMF binding to cell surface gp78, gp78 phosphorylation and gp78 internalization [34].

AMF motility signal transduction is mediated by a pertussis toxin sensitive G protein [14, 25, 34]. Interestingly, while pretreatment of cells with PT blocks their *in vitro* motility [14, 25], overnight treatment of B16-F1 melanoma cells with anti-gp78 mAb in the presence of PT significantly enhances their *in vivo* lung colonizing ability above the effect of the antibody alone [16]. PT alone had no effect on the lung colonizing ability of the cells. The ability of PT to enhance the stimulatory effect of anti-gp78 mAb may be due to inhibition by PT of gp78 internalization such that cells incubated overnight would accumulate gp78-antibody complexes on the cell surface. Removal of PT by washing and inoculation into the mouse would enable recruitment to the cell surface of newly synthesized G protein resulting in internalization of the gp78-antibody complexes and stimulation of cell motility. Supporting this interpretation, treatment of B16-F1 melanoma with PT for 18 hours does not alter the cell surface expression of gp78; however, following replacement of the medium with PT-free medium, allowing recruitment of uninhibited G proteins to the plasma membrane, downregulation of gp78 occurs within one hour [16]. AMF might therefore stimulate cell motility by inducing the internalization of its receptor to microtubule aligned tubulovesicles generating a flow of membrane to the leading edge of motile cells. AMF stimulation of A375

melanoma cells also induces extension of pseudopodia which exhibit prominent axial bundles and contain twenty times more laminin and fibronectin receptors than found on the membrane of non stimulated cells [30]. Microtubules penetrate into the leading edge and actin microspikes are characteristic of the leading lamella [27, 69, 70] and coordinated action between these two cytoskeletal networks, one supplying material for leading edge extension and the other actively extending pseudopodia, may serve to regulate the directed locomotion of eukaryotic cells. Which of the two cytoskeletal networks serves to generate the force required for cell locomotion is not clear. Interestingly, injection of anti-myosin antibodies to chicken fibroblasts induced the breakdown of stress fibers and the development of extensive lamellae as well as enhanced cell motility [71]. Furthermore, loss of actin cytoskeletal organization, stress fibers and focal contacts, is correlated with increased motility and metastatic ability in a range of metastatic melanoma variants [72]. The absence of an organized actin cytoskeleton may release highly metastatic cells from cell-cell and cell-substrate contacts such that supply of membrane to the leading edge becomes the major determinant of cell motility.

Conclusion

The cellular response to AMF is mediated hy a PT-sensitive G protein, inositol triphosphate production and gp78 phosphorylation. These signalling responses in turn lead to the internalization of gp78 to tubulovesicles, vesicular transport to the leading edge and pseudopodial extension resulting in enhanced cell locomotion. The study of the cellular responses to AMF motility induction should provide insight into the mechanics and regulatory mechanisms which control the motility of mammalian cells. Such an understanding may lead to methods and approaches to inhibit cell motility *in vitro* and invasion and metastasis *in vivo*.

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