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Amphoterin, the 30-kDa Protein in a Family of HMG1-type Polypeptides

ENHANCED EXPRESSION IN TRANSFORMED CELLS, LEADING EDGE LOCALIZATION, AND INTERACTIONS WITH PLASMINOGEN ACTIVATION*

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Amphoterin is a heparin-binding protein that is developmentally regulated in brain and functionally involved in neurite outgrowth. Unexpectedly, amphoterin has a high mobility group 1 (HMG1)-type sequence. In the present study we have expressed amphoterin cDNA in a baculovirus vector and produced antibodies against the recombinant protein and several synthetic peptides. It was found that the amphoterin cDNA encodes the 30-kDa form of the protein isolated from tissues, whereas the co-purifying 28- and 29-kDa proteins (p28 and p29) have closely related but distinct primary structures. Partial amino acid sequencing shows several local changes in the sequences of p28 and p29 compared with amphoterin, suggesting the occurrence of a multigene family that encodes at least three different HMG1-type sequences in the rat. Studies using the probes that discern amphoterin from the other HMG1-type proteins indicate a high level expression in various transformed cell lines. Immunostaining of cells with the amphoterin-specific antibodies indicates a cytoplasmic localization that becomes remarkably enriched at the leading edges in spreading and motile cells. An extracellular localization is suggested by immunostaining of nonpermeabilized cells and by a plasminogen-dependent degradation of amphoterin in the substratum-attached material of cells. Tissue-derived and recombinant amphoterins strongly enhance the rate of plasminogen activation and promote the generation of surface-bound plasmin both by tissue-type and urokinase-type plasminogen activators. The results suggest an extracellular function for amphoterin in the leading edge of various invasive cells.

Amphoterin is a heparin-binding protein that is abundantly expressed in the developing brain (Rauvala and Pihlaskari 1987; Merenmies *et al.*, 1991). It has a highly dipolar sequence, hence the designation amphoterin, that is identical to a sequence cloned in studies of the high mobility group 1 (HMG1)[1] protein (Bianchi *et al.*, 1989; Merenmies *et al.*, 1991). Amphoterin is specifically localized to filopodia of neural cells. The finding that several anti-amphoterin antibodies in the culture medium inhibited neurite outgrowth in a reversible, non-toxic manner indicates that extracellular amphoterin is required for process extension (Merenmies *et al.*, 1991). Amphoterin has also been suggested to be involved in neuron-glia interactions (Daston and Ratner, 1991).

Amphoterin was recently shown to bind plasminogen and tissue-type plasminogen activator (t-PA) and effectively enhance plasmin generation (Parkkinen and Rauvala, 1991). The plasminogen activator/plasmin system plays a crucial part in extracellular proteolysis during cell invasion and tissue remodeling (for a review, see Vassalli *et al.* (1991)). Most invasive cells employ urokinase-type plasminogen activator (u-PA) that binds to a specific cell surface receptor localized to the leading edge in migrating cells (Estreicher *et al.*, 1990). The binding of both plasminogen and its activator to the cell surface is a prerequisite for effective plasmin generation, and the binding protects the generated plasmin from its inhibitors (Ellis and Danø, 1991; Vaheri *et al.*, 1990). The binding may also generate a gradient of active plasmin into the direction of cell migration. However, it is not known what type of molecules serve as plasminogen binding sites at the leading edge. As amphoterin co-localizes with t-PA in the filopodia of neuroblastoma cells, it was suggested that one of the functions of amphoterin at the advancing membrane of neural cells is to bind plasminogen and promote its activation (Parkkinen and Rauvala, 1991). Due to the lysine-rich structure (20% of all amino acids), amphoterin is rapidly degraded by plasmin.

There is an apparent discrepancy between the proposed function of amphoterin as a filopodia-associated protein and its HMG1-type sequence that is currently considered to define a DNA-binding nuclear protein (see Johns (1982) for a review). On the other hand, the tissue-derived amphoterin/HMG1 protein is heterogeneous containing additional 28–29-kDa component(s) that co-purify with the major 30-kDa component. To study the reason for this heterogeneity, we have in the present study produced antibodies against several synthetic peptides covering the amphoterin/HMG-1 sequence and against a recombinant protein (rec-Atn) obtained by

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[1] The abbreviations used are: HMG, high mobility group; BSA, bovine serum albumin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; PBS, phosphate buffered saline; rec-Atn, recombinant amphoterin; t-PA, tissue plasminogen activator; scu-PA, single chain urokinase; u-PA, urokinase-type plasminogen activator; PAGE, polyacrylamide gel electrophoresis; kb, kilobase.

expressing the amphoterin cDNA in a baculovirus vector. It was found that the 28- and 29-kDa components (p28 and p29) are closely homologous but distinct polypeptides.

To gain insight into the functions of amphoterin in different types of cells, we studied its expression and localization in various normal and transformed cell lines by using the probes that discern it from the other homologous proteins. All transformed cell lines studied showed a high level expression of amphoterin that was at the mRNA level one to two orders higher than in the corresponding normal cells. Immunostaining of spreading and motile cells indicated that amphoterin becomes accumulated at the leading edges in different types of cells, and it was detected on the surface of cells at these sites. Amphoterin was also present in the substratum-attached material of cells, where its amount was strongly decreased if the cells were cultured in the presence of plasminogen. These findings suggest that amphoterin has an extracellular function at the leading edge of migrating cells and that it is degraded by a plasminogen-dependent mechanism.

The strong enhancing effect on plasminogen activation observed before with tissue-derived amphoterin was confirmed with the recombinant protein. Furthermore, surface-bound rec-Atn effectively promoted the generation of bound plasmin activity, both in the presence of t-PA or single chain u-PA (scu-PA), and the amphoterin-associated plasmin was protected from plasma inhibitors. These findings suggest that amphoterin may serve as a spatially and temporally regulated plasminogen binding site at the leading membrane and in the extracellular matrix.

EXPERIMENTAL PROCEDURES

Materials—Human Glu-plasminogen, single chain u-PA (scu-PA), and CNBr-fragmented fibrinogen were from Technoclone (Vienna, Austria). Single-chain t-PA from human melanoma cells and S-2251 were from Kabi (Stockholm, Sweden). Z-Lysine thiobenzyl ester was from Peninsula Laboratories (San Carlos, CA). Laminin, aprotinin, aprotinin-agarose, and *p*-aminobenzamidine-Sepharose 6B were from Sigma. Matrigel was from Collaborative Research Inc. (Bedford, MA). Heparin-Sepharose 6B, lysine-Sepharose 4B, and CNBr-Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. Alkaline phosphatase-conjugated anti-chicken rabbit IgG was from Sigma, alkaline phosphatase-conjugated anti-rabbit goat IgG from Bio-Rad, horseradish peroxidase-conjugated anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit swine Ig from DAKO (Glostrup, Denmark), and FITC-conjugated anti-chicken rabbit IgG from Zymed (San Francisco, CA). Synthetic peptides were purchased from Multiple Peptide Systems (San Diego, CA). HMG2 protein was kindly donated by Dr. J. Palvimo (University of Kuopio, Finland).

Purification and Amino Acid Sequencing of Amphoterin (p30), p28, and p29—Amphoterin, p28, and p29 were purified from early postnatal rat brains by heparin-Sepharose and Affi-Gel blue as described before (Rauvala and Pihlaskari, 1987). Separation of p29, p30, and p28 was carried out by HPLC on a Vydac C18 column eluted with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid in 30 min. Elution of the polypeptides was monitored at 218 nm. Tryptic peptides were prepared by using sequencing grade trypsin (Boehringer Mannheim) according to the instructions of the manufacturer. The peptides were separated by reverse phase HPLC for the amino acid sequence analysis. Amino acid sequencing was performed by automated Edman degradation with a Applied Biosystems sequencer 477A/120A in the gas phase mode as described before (Baumann, 1990).

Construction of the Recombinant Baculovirus Vector—Synthetic oligonucleotide primers were used to generate *Bam*HI restriction sites to both ends of the coding region of amphoterin cDNA in the polymerase chain reaction (PCR, GeneAmp®, Perkin-Elmer Cetus). The primers used were 5′-CGGGATCCAACTAAACATGGGCAAAGGAGA-3′ (sense) and 5′-CGGGATCCATGCGTAGAACCAACTTATTCA-3′ (antisense). The PCR product was isolated by gel electrophoresis on 1% agarose (GTG SeaPlague®, FMC BioProducts Rockland, ME) and cloned into the unique *Bam*HI cloning site of the pAcYM1 vector (Matsuura *et al.*, 1987). Sequencing of the insert by the dideoxy chain termination method (Sanger *et al.*, 1977) revealed the same sequence as found before for amphoterin cDNA (Merenmies *et al.*, 1991). The plasmid pAcYM1/amphoterin containing the entire coding region of amphoterin with nine leading nucleotides under the AcNPV polyhedrin promoter sequence was co-transfected with wild-type (E2 strain) baculovirus using the Lipofectin® system (Life Technologies, Inc.) to *Spodoptera frugiperda* (SF9) cells. Supernatant from the transfected cells was subjected to plaque purification as described previously (Summers and Smith, 1986). Occlusion-negative plaques were identified by their morphology under a dissecting microscope and the isolates were screened by Western blotting with anti-peptide antibodies against amphoterin. One positive clone was amplified to a high titer stock virus and subsequently used for large scale production of the recombinant protein.

Production and Purification of the Recombinant Protein—SF9 cells were grown in 1000-ml spinner flasks in TNM-FH insect medium (Sigma) containing 10% fetal calf serum (FCS) to an approximate density of 2×10^6/ml. Cells were harvested by centrifugation and infected with 5–10 pfu/cell. After a 16-h adsorption with occasional agitation the volume was raised back to original 1000 ml with the culture medium. The cells were grown for 72 h, harvested by centrifugation, and suspended on ice in lysis buffer containing 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 50 mM octyl glucoside, and 2 mg/liter aprotinin. The sample was centrifuged for 45 min at 20,000 rpm in a Sorval SS-34 rotor and the supernatant was applied to a 5-ml heparin-Sepharose Hi-Trap-column (Pharmacia). The column was eluted with a linear gradient of 0.15–1.5 M NaCl in 50 mM Tris-HCl, pH 8.5. Fractions containing recombinant amphoterin were pooled and subjected to a second round of purification on heparin-Sepharose under the same conditions.

Production of Antibodies—Hens were immunized with rec-Atn and IgG was purified from egg yolk as described before (Jensenius *et al.*, 1981). Anti-rec-Atn antibodies were purified on a column (0.25 ml) of rec-Atn-Sepharose prepared by coupling 1 mg of rec-Atn to CNBr-activated Sepharose. Anti-rec-Atn antibodies were eluted from the column with 0.1 M glycine buffer, pH 2.5, followed by immediate neutralization with 1 M Tris-HCl, pH 8.5. Production and affinity purification of the rabbit anti-synthetic peptide antibodies that bind the amino-terminal sequence of amphoterin (GKGDPKKPRGK; the antibody has been designated as anti-peptide I) and a sequence corresponding to amino acids 166–175 (KPDAAKKGVV; anti-peptide II) has been described before (Rauvala *et al.*, 1988; Merenmies *et al.*, 1991). Antibodies against the amphoterin sequences representing the amino acids 73–83 (EMKTYIPPKGE; anti-peptide III), 87–100 (KFKDPNAPKRPPSA; anti-peptide IV), and 176–180 (KAEKSKKKKEE; anti-peptide V) were produced in rabbits using the peptides coupled to keyhole limpet hemocyanin (Green *et al.*, 1982). HMG2-specific antibodies were produced using the peptide KSEAGKKGPGC (Walker *et al.*, 1980b). The sequences of the peptides used as immunogens were confirmed by amino acid sequencing. Immunization of the rabbits and affinity purification of the antibodies were carried out as described previously (Rauvala *et al.*, 1988). Affinity-purified rabbit antibodies against tissue-derived amphoterin were available from a previous study (Rauvala *et al.*, 1988).

Electrophoretic Methods—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried in gradient gels with or without reduction of the samples with 5% mercaptoethanol. For immunoblotting the samples were transferred to nitrocellulose (Towbin *et al.*, 1979) and the membrane was incubated overnight with 1% bovine serum albumin (BSA) in 20 mM Tris-HCl, pH 7.8, 500 mM NaCl (TBS). Immunostaining was carried out with affinity-purified anti-peptide antibodies at 100–250 ng/ml or anti-rec-Atn at 10 ng/ml in TBS containing 0.5% BSA and 0.05% Tween 20 for 1–3 h at 23 °C. The bound antibodies were detected with alkaline phosphatase-conjugated second antibodies and the 5-bromo-4-chloro-3-endoyl phosphate-nitro blue tetrazolium reaction (Bio-Rad) or with horseradish peroxidase-conjugated second antibodies and the enhanced chemiluminescence reaction (Amersham Corp.).

RNA was isolated from the cells by the guanidine thiocyanate method (Chomczynsky and Sacchi, 1987). For Northern blotting RNA was separated on 1.2% agarose gels containing 7% formaldehyde and transferred to a nylon filter (Hybond-N, Amersham Corp.) (Sambrook *et al.*, 1989). An amphoterin cDNA clone containing nucleotides 42–746 was labeled with ^{32}P (Quick Prime, Pharmacia) and was used to probe the filters.

Cells and Cell Culture—IMR90, WI38, WI38/Va4, WI38/Va13, HT1080, and A375 cells were obtained from American Type Culture Collection (Rockville, MD). B16-F1 cells were obtained from Dr.

Jukka Finne (University of Turku, Finland). Human embryonic skin fibroblasts initiated by standard methods were kindly provided by Dr. Ismo Virtanen (Department of Anatomy, University of Helsinki). Fibroblast lines were cultured in RPMI 1640, other cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin at 37 °C in humidified 95% air and 5% CO_2. In short term cultures without serum, cells were cultured in the appropriate medium supplemented with 0.1% BSA on glass coverslips or microwells coated with laminin, 20 μg/ml in PBS, or Matrigel, 50 μg/ml, for 2 h at 37 °C. To obtain the substratum-attached material, the cells were removed as described by Avnur and Geiger (1981).

Fluorescence Microscopy—The cells were fixed with 4% paraformaldehyde-0.05% glutaraldehyde, washed with PBS, and the excess aldehydes were destroyed by reduction with 1 mg/ml of $NaBH_4$ (two times for 5 min in PBS). Fixation with paraformaldehyde only gave essentially similar results, but paraformaldehyde-glutaraldehyde was routinely used to ensure irreversible fixation and to prevent redistribution of the protein after the fixation procedure. The fixed cells were permeabilized with methanol at −20 °C for 5 min. Permeabilization of the cells with Triton X-100 instead of methanol did not change the results. Nonspecific binding sites were blocked with 10% normal serum (from the animal species in which the secondary antibodies were produced) or with 1% BSA and 0.12% glycine in PBS for 0.5 h at room temperature. The antibodies were incubated in 0.5% BSA in PBS for 1 h at 23 °C followed by six washes with 0.12% glycine-PBS. For surface labeling, the cells were cooled and incubated with the antibodies for 30 min on ice bath, washed, and fixed with 4% paraformaldehyde for 30 min. Alternatively, the cells were first fixed with 4% paraformaldehyde for 30 min, washed, and blocked with BSA, and then incubated with the antibodies.

The bound antibodies were detected with FITC-conjugated swine anti-rabbit Ig or with FITC-conjugated rabbit anti-chicken IgG. Control incubations in which the primary antibodies were substituted with nonimmune IgG from the corresponding animal were analyzed in parallel in all experiments. The samples were studied with a Zeiss microscope (model IM35) equipped for epifluorescence with appropriate filters to specifically detect the FITC. The Planapo 63x oil immersion objective (Zeiss) was used in all figures shown.

Immunoelectron Microscopy—The cells were cultured on gold grids in complete medium for 16 h and fixed with 4% paraformaldehyde, 0.05% glutaraldehyde, reduced, and permeabilized with methanol as described for immunofluorescence staining. Nonspecific binding sites were blocked with BSA, and the cells were incubated with antibodies and washed as above. The bound antibodies were detected by incubating the grids with anti-rabbit Ig-coated colloidal gold particles (AuroProbe EM GAR G5, 1:25; Janssen Biochimica, Beerse, Belgium) for 20 min. The grids were then washed by dipping 20 times in 0.12% glycine-PBS followed by washing six times for 10 min in the same buffer. The samples were treated with 2.5% glutaraldehyde and 0.1% osmium tetroxide. They were then dehydrated in a graded ethanol series and critical-point dried. The grids were examined using a Jeol 1200 EX electron microscope.

Plasminogen Activation Assays—Activation of plasminogen in solution was followed by the plasmin-catalyzed hydrolysis of S-2251. Initial activation rates at different plasminogen concentrations were determined as described before (Zamarron *et al.*, 1984). Activation of plasminogen on protein surfaces was studied in microwells (Microstrip, Labsystems, Helsinki, Finland) that were coated with the proteins studied at 10 μg/ml in 100 μl of PBS for 2 h at 37 °C, washed, and saturated with 1% BSA for 1 h at 37 °C. scu-PA was treated with benzamidine-Sepharose in 0.1% BSA-PBS for 30 min in order to remove any contaminating u-PA (Stump *et al.*, 1986). Plasminogen, t-PA, or scu-PA were added in 100 μl of 0.1% BSA, PBS and incubated for varying time periods at 37 °C. The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). The solid-phase bound plasmin was measured either by S-2251 (100 μl of 0.4 mM solution in PBS-Tween) or by thioesterase activity (Green and Shaw, 1979) by adding 100 μl of PBS containing 0.1% Triton X-100, 200 μM Z-lysine thiobenzyl ester, and 220 μM 5,5′-dithiobis(2-nitrobenzoic acid). After incubation for 0.5–1 h at 37 °C the absorbance at 405 nm was measured in a Titertek Multiscan Fotometer (Labsystems). An estimate of the amount of active plasmin in the wells was made from a calibration curve with plasmin.

Rec-Atn was iodinated and purified by heparin-Sepharose as described for the tissue protein (Parkkinen and Rauvala, 1991). Radiolabeled amphoterin surfaces were prepared to microwells by incubating in 100 μl of PBS, 0.5 μg of rec-Atn, and 5×10^3 cpm ^{125}I-labeled rec-Atn overnight at 4 °C. The wells were washed four times with PBS, saturated with 1% BSA for 2 h at 37 °C, and incubated with plasminogen and t-PA or scu-PA in 0.1% BSA or in 10% EDTA-anticoagulated human plasma. Plasminogen-depleted plasma was prepared by running 5 ml of plasma through a 1-ml column of lysine-Sepharose at 4 °C.

RESULTS

Production of Recombinant Amphoterin and Anti-Recombinant Amphoterin Antibodies—To produce a homogeneous preparation of amphoterin for immunization and for functional studies, we inserted the coding region of amphoterin (Merenmies *et al.*, 1991), including nine leading nucleotides, into the pAcYM1 vector. Sequencing of the inserted DNA of the pAcYM1/amphoterin construct revealed an identical sequence as compared to the coding region of amphoterin cDNA, showing that the sequence had not been changed during the PCR or other procedures used in the construction and purification of the vector. Insect cells infected with the recombinant virus produced a major protein with a molecular mass of 30 kDa that was released by lysis of the cells with octyl glucoside. Non-infected insect cells did not produce any amphoterin-like protein as revealed by immunoblotting of solubilized cells with anti-peptide antibodies (not shown). The recombinant protein was obtained in a highly purified form by repeated affinity chromatography on heparin-Sepharose (Fig. 1). The yield of the recombinant protein was 50–100 mg/l of cell suspension culture indicating a high level expression. Sequencing of 38 amino-terminal amino acids suggested a homogeneous polypeptide structure, the sequence of which was identical to that reported for amphoterin (Merenmies *et al.*, 1991).

Immunization of rabbits with the recombinant protein did not induce antibodies that would detect the protein in immunoblots. Instead, immunization of hens induced antibodies that specifically detected rec-Atn in enzyme-linked immunosorbent assay and, after affinity purification on a rec-Atn-Sepharose column, specifically recognized the 30-kDa amphoterin band in immunoblotting of crude cell extracts (Fig. 2).

Biochemical Characteristics of the Recombinant Amphoterin Compared with the Tissue-derived Protein—In the salt gradient elution, the recombinant amphoterin was detached from heparin-Sepharose at 0.8–0.95 M NaCl which is very similar to the behavior of the tissue-derived protein (Rauvala and Pihlaskari, 1987). The purified recombinant protein co-migrated in SDS-PAGE in reduced and nonreduced form with the major band of the tissue amphoterin preparation, 30 kDa in a reduced form (Fig. 1*A*). Both the tissue-derived and recombinant amphoterins migrated somewhat faster under non-reducing conditions than under reducing conditions, which suggests the presence of intra-chain disulfide bond(s) in both forms of the protein (Fig. 1*A*). The apparently identical gel electrophoretic behavior and the finding that the tissue-derived 30-kDa amphoterin and the recombinant amphoterin were detected in the same way by five anti-synthetic peptide antibodies (Fig. 1*B*), which cover the amphoterin sequence from the amino terminus close to the carboxyl terminus, indicated that a full-length recombinant was obtained.

In addition to the major 30-kDa band, the tissue protein contained components with a slightly lower apparent molecular mass (Fig. 1*A*). This indicates that the tissue protein might be however different from the recombinant protein, for example due to post-translational modifications. Alternatively, the tissue protein might contain co-purifying proteins that are excluded in the expression system used. In order to resolve between the different possibilities, further fractiona-

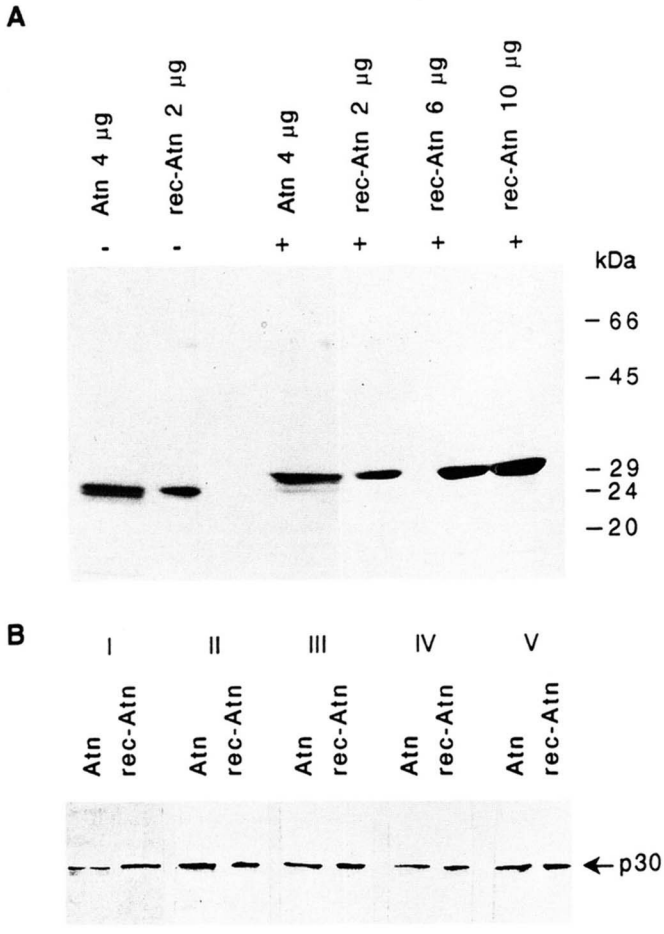

FIG. 1. **Comparison of tissue-derived amphoterin (*Atn*) and recombinant amphoterin (*rec-Atn*) in SDS-PAGE and immunoblotting with anti-synthetic peptide antibodies.** *A*, samples of Atn and rec-Atn were run either without (−) or with (+) reducing agent in 5–20% SDS-PAGE and stained with Coomassie Brilliant Blue. *B*, immunoblotting of Atn, 200 ng/lane, and rec-Atn, 100 ng/lane, with anti-synthetic peptide antibodies I–V (*I–V*, respectively).

tion of tissue-derived samples was carried out. We observed that the slightly lower molecular mass proteins (p28 and p29) can be separated by reverse phase HPLC from the major peak that corresponds to the recombinant amphoterin (Fig. 3*A*). In addition to the three proteins shown in Fig. 3, trace amounts of protein that apparently corresponds to the HMG2 (the amino-terminal sequence was read as GKGDPNKPRG; see Shirakawa *et al.* (1990) for the HMG2 sequence) was observed in fractions eluting close to p29.

To study the possible differences in the primary structures of amphoterin (p30), p28, and p29, the proteins were studied by immunoblotting with the five different anti-peptide antibodies covering the amphoterin sequence. Only the 30-kDa tissue amphoterin and the recombinant amphoterin (Fig. 1*B*) were detected by all five sequence-specific antibodies, whereas p28 and p29 were both detected by two of the five antibodies (Fig. 3*B*). The detection patterns of p28 and p29 were also different from each other. Sequence-specific antibodies to the HMG2 protein (Walker *et al.*, 1980a; Shirakawa *et al.*, 1990), which is homologous with amphoterin, did not detect any of the proteins studied but strongly detected the HMG2 protein (not shown). Of the peptide antibodies, only the anti-peptides II and V were specific for amphoterin within the protein family studied (Fig. 3*B*). The anti-protein antibodies (anti-Atn and anti-rec-Atn) clearly detected amphoterin, but they detected p29 only weakly and did not detect p28 (Fig. 3*B*).

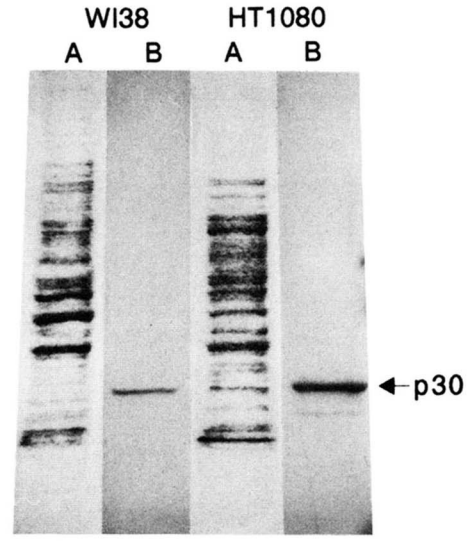

FIG. 2. **Specificity of anti-recombinant amphoterin antibodies.** Samples of WI38 and HT1080 cells solubilized in 1% SDS were run in SDS-PAGE and blotted to nitrocellulose. The filter was stained with Ponceau S (*A*) and subsequently incubated with affinity purified anti-rec-Atn antibodies at 10 ng/ml (*B*). The bound antibodies were detected with alkaline phophatase-conjugated anti-chicken IgG.

On the other hand, only one of the antibodies (anti-peptide IV) detected all three proteins. Anti-peptide IV also detected the HMG2 protein (not shown).

The above results suggest that p28 and p29 are novel proteins, the structures of which are distinct from amphoterin. Partial amino acid sequencing confirmed that p28 and p29 have homologous but distinct sequences as compared to the sequence of amphoterin. For example, there are several single amino acid differences in the amino-terminal regions of the three proteins (Fig. 4). In contrast, the sequences containing the hydrophobic regions (YAFFV and AFFLF) are conserved in p28 and p29 (Fig. 4). The inference that the central region containing the hydrophobic sequence AFFLF is the same or very similar in amphoterin, p28 and p29, is also supported by the finding that the peptide antibodies produced against this region (anti-peptide IV) detect all three proteins. Peptide maps generated by digestion with trypsin and with endoproteinase Lys-C (not shown) also indicated that amphoterin, p28 and p29 are structurally distinct proteins although some common fragments also exist. These results call for caution in the interpretation of studies concerning amphoterin-type proteins since several homologous but clearly distinct proteins, which are difficult to discern from each other, may exist in the same tissue of one animal species.

Amphoterin Protein and mRNA Levels in Normal and Transformed Cells—The occurrence of amphoterin protein in various cell lines was studied by immunoblotting with the anti-peptide antibodies. Amphoterin was found at relatively high levels in all transformed cell lines studied (C6 glioma, HL-60 promyelocytes, U937 promonocytes, HT1080 fibrosarcoma, B16 melanoma cells). The expression levels found were comparable to the high expression level of amphoterin found in early brain and in neuroblastoma cells (Rauvala and Pihlaskari, 1987; Rauvala *et al.*, 1988). Amphoterin is thus expressed by cells of various origins.

To compare amphoterin levels in differentiated cells and in corresponding transformed cells, we studied human embryonic fibroblast lines IMR90 and WI38, SV40-transformants of the latter, WI38/Va4 and WI38/Va13, and HT1080 fibrosarcoma cells. Immunoblotting with anti-peptide I, II, and III

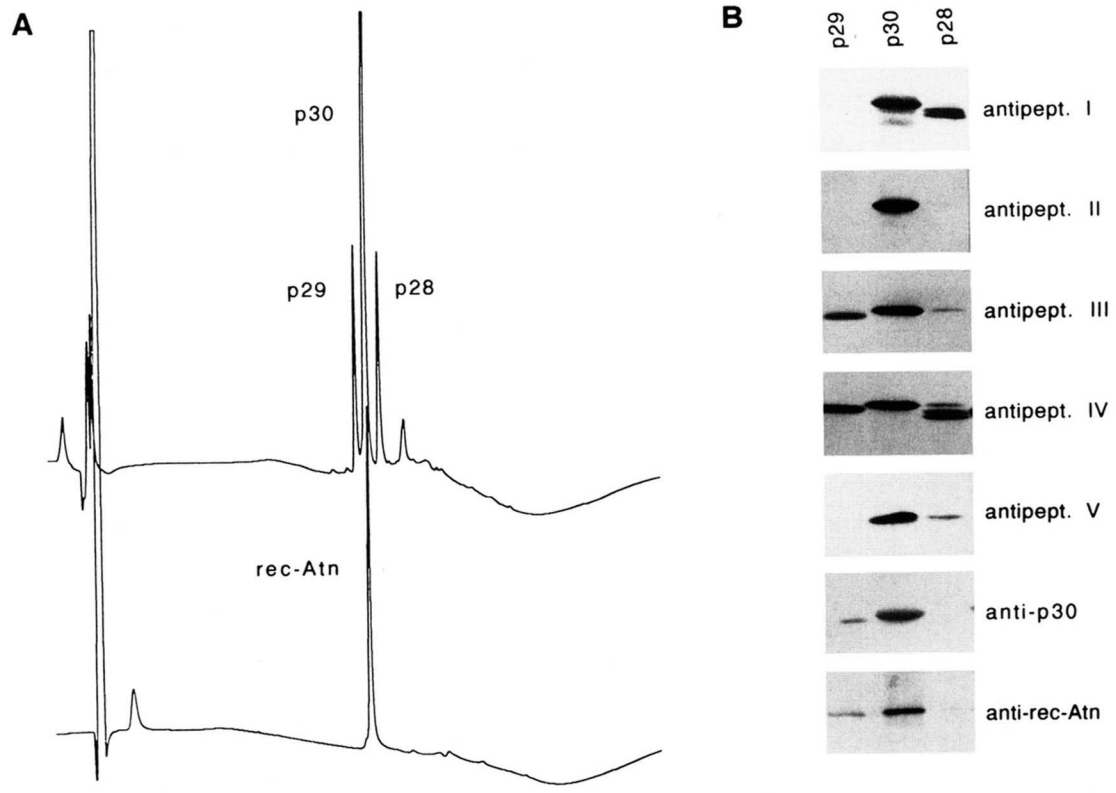

FIG. 3. **Separation of amphoterin (*p30*) and the co-purifying polypeptides (*p28* and *p29*) by reversed-phase HPLC and immunoblotting with anti-synthetic peptide and anti-amphoterin antibodies.** *A*, tissue-derived (*top*) and recombinant (*bottom*) amphoterins were separated on a Vydac C18 column as described under "Experimental Procedures." *B*, similar amounts of the isolated proteins were run in SDS-PAGE, transferred to nitrocellulose, and immunostained with the antibodies indicated.

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Atn  1G K G D P K K P R G K M S S Y A F F V Q T C R E E H K K K H P D A S V N F
p28  1A K G D P K K P K G K M S A Y A F F V Q T X R E E
p29  BX               G K M S A Y A F F V Q T

Atn  S E F S K K C S E R W K T M S A K E K G K F E D M A K A D K A R Y E R E M
p28
p29                                                  Y D K

Atn K T Y I P P K G E T K K K F K D P N A P K R P P S A F F L F C S E Y R P K
p28                                                  P P S A F F L F X S E Y R P K
p29   D Y G P A K                                    P P S A F F L F X S E X R P

Atn I K G E H P G L S I G D V A K K L G E M W N N T A A D D K Q P Y E K K A147
p28                                                        D K Q P Y E Q K K
p29     S A N P G I S I G D V A X X L G E M
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FIG. 4. **Analysis of the amino-terminal sequences and tryptic peptides of p29 and p28.** Amino-terminal sequencing of p28 indicated one homogeneous sequence whereas p29 was amino-terminally blocked (^{B}X). The amino-terminal sequences and the sequences obtained from tryptic peptides are compared to the sequence (amino acids 1–147) of amphoterin. Identical residues are *underlined*.

antibodies (shown for anti-peptide I in Fig. 5) revealed a faint 30-kDa amphoterin band in the embryonic fibroblast lines whereas an intense band was visualized in the SV40-transformants and HT1080 cells. Quantification of the amphoterin band with the peptide antibodies and ^{125}I-protein A binding indicated 3–10-fold higher amounts of amphoterin in the transformed cell lines than in the normal fibroblasts. The amount of amphoterin in the transformed cells was on the average 0.1% of total cellular protein as estimated from a calibration curve with rec-Atn.

Northern blot hybridization of total cellular RNA with amphoterin cDNA indicated a remarkable difference in amphoterin mRNA levels between the normal and transformed cell lines. The most abundant mRNA species with 1.3 kb was visible in all cell lines. Additionally, the 2.6-kb band and a less intensive 1.1-kb band were visible in the transformed

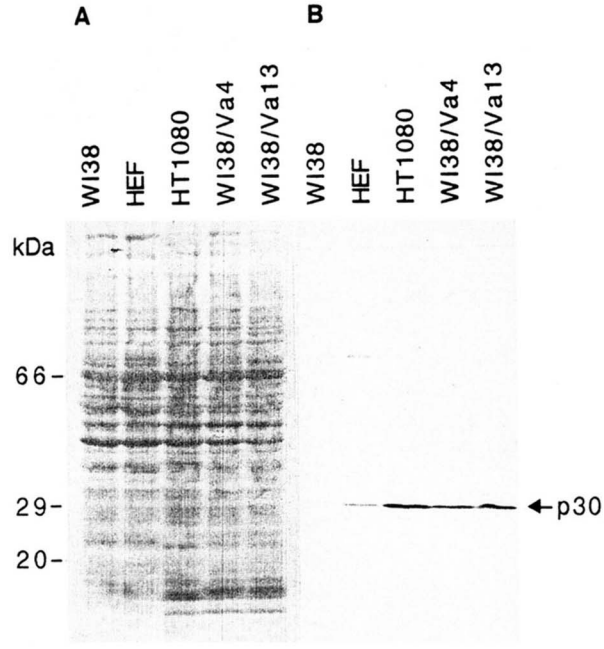

FIG. 5. **Amphoterin protein in different fibroblast cell lines.** Subconfluent cultures were solubilized in 1% SDS and samples corresponding to 20 μg of total cellular protein were separated in reduced SDS-PAGE and transferred to a nitrocellulose filter. The filter was stained with Ponceau S (*A*) and incubated with anti-peptide I at 100 ng/ml (*B*). The bound antibodies were visualized with alkaline phosphatase-conjugated anti-rabbit IgG.

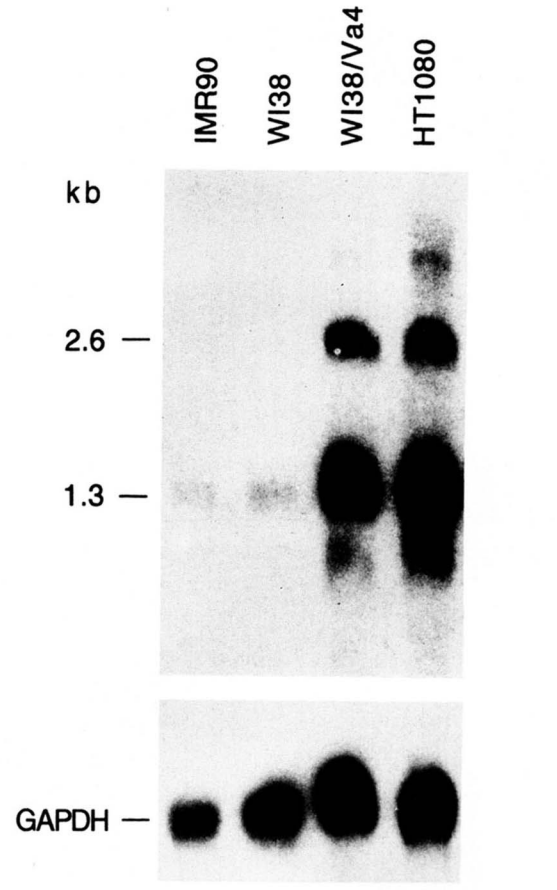

FIG. 6. **Amphoterin mRNA in different fibroblast cell lines.** RNA was purified from subconfluent cultures and samples corresponding to 20 μg of total cellular RNA were separated on a 1.2% agarose gel and transferred to a nylon filter. The filter was probed with ^{32}P-labeled rat amphoterin cDNA (*top*) and human glyceraldehyde-3-phosphate dehydrogenase cDNA (*GAPDH*).

cells. When the gel was loaded with a similar amount of total RNA from the different cell lines, the transformed cells gave 20–100-fold higher hybridization signals with ^{35}P-labeled amphoterin cDNA as compared to the normal fibroblast lines (Fig. 6). The transformed cells also gave a higher hybridization signal with human glyceraldehyde-3-phosphate dehydrogenase cDNA, but the difference was at least an order of magnitude smaller than that obtained with amphoterin cDNA.

To find out whether amphoterin expression varies with respect to cell proliferation, RNA was isolated from 3T3 cells growing at different cell densities and hybridized with amphoterin cDNA on a Northern blot. Hybridization signals of similar intensity were obtained with RNA originating from proliferating subconfluent and quiescent confluent cultures (not shown). This suggested that the proliferation rate does not have any notable influence on amphoterin expression.

Localization of Amphoterin in Cells and in Substratum-attached Material—To compare the localization of amphoterin in transformed cells of neuroectodermal and mesenchymal origin, N18 neuroblastoma and HT1080 fibrosarcoma cells spreading on laminin were fixed, permeabilized, and stained with affinity-purified anti-peptide II and anti-rec-Atn antibodies. These antibodies are specific for amphoterin within the HMG1-type protein family (Fig. 3) and specifically recognize the 30-kDa amphoterin band in immunoblotting of solubilized cells (Fig. 1). Both antibodies stained intensively filopodia in N18 cells (Fig. 7, *A* and *C*), whereas in HT1080 cells membrane ruffles at the leading edges were visualized (Fig. 7, *B* and *D*). The anti-rec-Atn antibodies gave in both cells a more intense cytoplasmic staining than anti-peptide II but no nuclear staining was observed. Nonimmune chicken IgG gave a diffuse cytoplasmic staining (Fig. 7, *F* and *G*) that was more intense than that obtained with nonimmune rabbit IgG. A similar detection of the leading edge structures was observed with anti-peptide III. Anti-peptide IV, which binds to all HMG1-type structures (see above), stained the cytoplasm, the nuclei, and leading edge structures (not shown). The peptide sequence detected by anti-peptide V does not appear to be exposed in the protein structure since these antibodies did not stain the cells.

When the cells (HT1080, N18, and B16) were cultured for longer periods (1–2 days) under ordinary culture conditions in the presence of serum, the immunostaining showed a diffuse cytoplasmic localization in a high proportion of cells (Fig. 8*A*), which is very similar to that shown by anti-HMG1 antibodies previously (Bustin and Neihart, 1979). However, when the cells were induced to grow processes on a laminin-coated surface, amphoterin became concentrated at the leading edges and extending processes (Figs. 7 and 8*B*). Similarly, in cells that were clearly motile under ordinary culture conditions, such as dividing cells (Fig. 7*E*), amphoterin became concentrated to the leading edges. Amphoterin was not found in focal contacts at the ventral surface of HT1080 cells that were visualized by staining with anti-vinculin and anti-u-PA antibodies (not shown).

Surface labeling of amphoterin was studied with anti-peptide II and anti-rec-Atn antibodies in living cells on ice bath and after mild fixation with paraformaldehyde. Immunostaining of living HT1080 cells on ice bath revealed a patchy staining of cell margins and cytoplasmic extensions (Fig. 9*A*). Similarly, in fixed, nonpermeabilized cells local areas in cell bodies and cytoplasmic extensions were visualized (Fig. 9*B*). The impermeability of the plasma membrane for the antibodies in these cells was supported by the lack of cytoplasmic

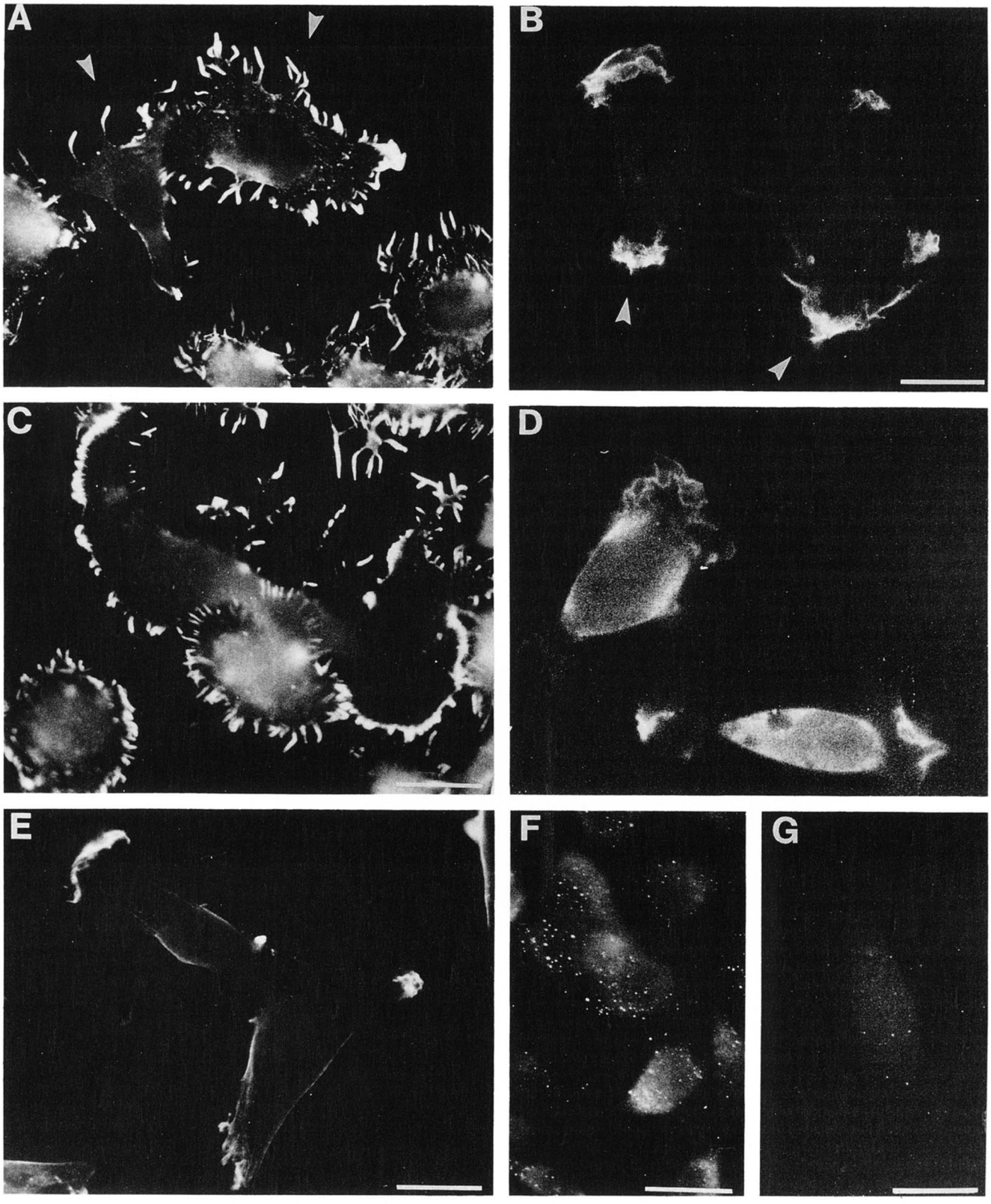

FIG. 7. **Localization of amphoterin in N18 and HT1080 cells spreading on laminin.** N18 (*A*, *C*, and *F*) and HT1080 (*B*, *D*, and *G*) cells were kept on laminin for 40 min, fixed with 4% paraformaldehyde, 0.05% glutaraldehyde, and permeabilized with methanol. The cells were incubated with antipeptide II at 2 μg/ml (*A* and *B*), anti-rec-Atn at 0.5 μg/ml (*C* and *D*) or nonimmune chicken IgG at 2 μg/ml (*F* and *G*) for 1 h, and the bound antibodies were visualized with FITC-conjugated anti-rabbit (*A* and *B*) or anti-chicken IgG (*C*, *D*, *F*, and *G*). *E* shows HT1080 cells cultured in 10% FCS for 20 h, fixed and permeabilized as above, and immunostained as in *A* and *B*. *Arrowheads* point to filopodia and membrane ruffles. *Bars* = 20 μm.

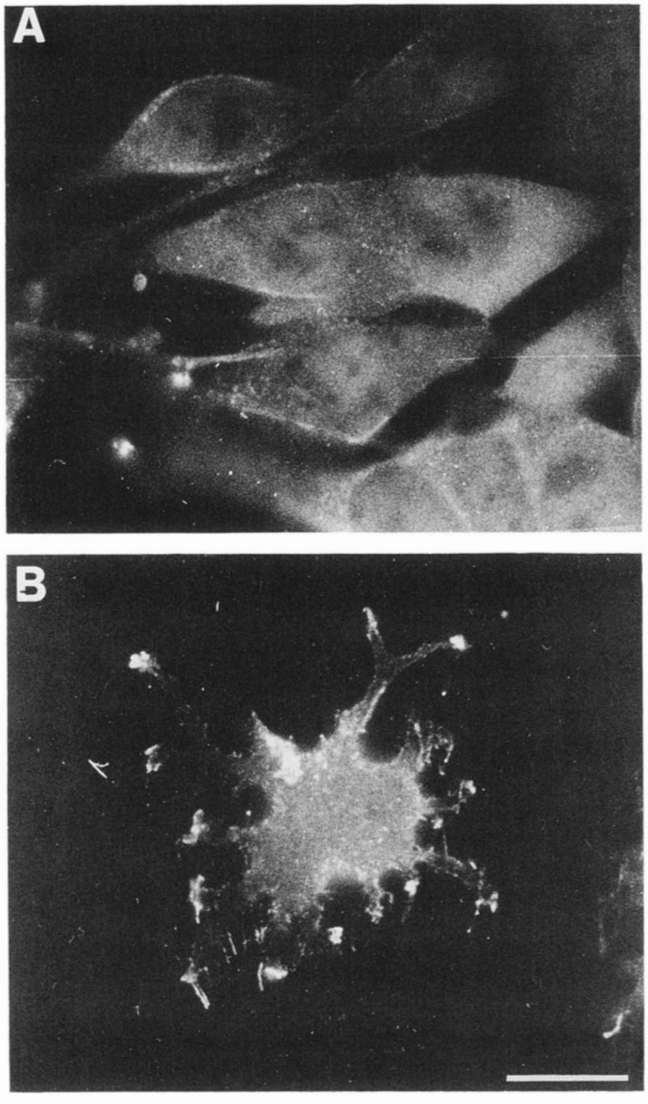

FIG. 8. **Comparison of amphoterin localization in stationary and spreading cells.** B16 cells were cultured under ordinary conditions in the presence of serum (*A*) or were induced to grow processes on laminin for 1 h (*B*). The cells were fixed and permeabilized as in Fig. 7 and incubated with anti-peptide II at 10 μg/ml for 1 h. The bound antibodies were visualized with FITC-conjugated anti-rabbit Ig. *Bar* = 20 μm.

staining as compared to permeabilized cells stained under similar conditions (Fig. 7*D*). No surface staining of cells was observed with nonimmune rabbit or chicken IgG (Fig. 9*C*).

Immunostaining of spreading embryonic fibroblasts with the amphoterin-specific antibodies also suggested a localization to the leading edge of the lamellipodia (not shown). The leading edge of normal fibroblasts showed less ruffling than that of HT1080 cells, and amphoterin was visualized mainly in microspike protrusions. The localization of amphoterin in microspikes was also verified by immunogold labeling of cells grown on electron microscopy grids (Fig. 9*D*). The immunogold labeling indicated that amphoterin mainly lines the microspikes, but is rarely found in the cental core of these structures.

Amphoterin was previously shown to be present in the substratum-attached material of N18 cells after removal of the cells under conditions that preserve the cell-substratum contact areas (Merenmies *et al.*, 1991). Amphoterin was also deposited to the substratum by HT1080 cells as indicated by immunofluorescence staining and immunoblotting with anti-rec-Atn antibodies (Fig. 10). The amount of amphoterin in the substratum-attached material was lower when the cells were grown in the presence of 10% serum as compared to cells grown in a plasminogen-depleted serum or without serum (Fig. 10). Addition of plasminogen to the culture medium of cells resulted in a dose-dependent disappearance of amphoterin from the substratum, whereas addition of aprotinin to the culture medium effectively increased it (Fig. 10).

Effects of Amphoterin on Plasminogen Activation—The above results indicate that the level of endogenous, substratum-attached amphoterin is regulated by extracellular plasminogen in the culture medium. Previous studies using amphoterin isolated from brain suggested that amphoterin binds both plasminogen and t-PA in a manner that enhances the generation of active plasmin and leads to degradation of amphoterin (Parkkinen and Rauvala, 1991). We therefore tested, whether rec-Atn is able to interact with the plasmin-generating system in a similar manner. In a homogeneous assay in solution, rec-Atn effectively accelerated plasminogen activation by t-PA and was even more effective than tissue amphoterin at a corresponding concentration (Fig. 11*A*). Determination of the kinetic parameters of t-PA-catalyzed plasminogen activation in the presence of 2 μM rec-Atn indicated 1–2 μM for K_m and about 0.2 s^{-1} for K_{cat}. The overall enzymatic efficiency (K_{cat}/K_m) was 4–10-fold lower than in the presence of CNBr-fragmented fibrinogen at a corresponding mass concentration (Fig. 11*B*) but about 200-fold higher than without any promoter. Rec-Atn had only a low enhancing effect on plasminogen activation by scu-PA in solution, the acceleration being about 3-fold at 8 μM rec-Atn concentration (not shown).

Denaturating treatments, such as incubation in concentrated urea, trifluoroacetic acid, or perchloric acid, that is commonly used for the purification of HMG1 protein (Walker *et al.*, 1980), decreased the plasminogen activation-enhancing effect of rec-Atn (5% perchloric acid for 15 min in an ice bath to about 50%). This suggests that promotion of plasminogen activation requires a native form of amphoterin. As a further control, we tested another heparin-binding protein, rec-HB-GAM, that contains similar lysine clusters as amphoterin and was produced by the same expression system (Raulo *et al.*, 1992). Rec-HB-GAM bound both t-PA and plasminogen with a comparable affinity as amphoterin and in a lysine-dependent manner (not shown). However, in the plasminogen-activation assay rec-HB-GAM alone had no effect and, when added together with rec-Atn, competitively inhibited the enhancing effect of rec-Atn (Fig. 11*A*).

As the physiological site of amphoterin function is proposed to be the cell-matrix interface, we studied the effect of solid phase-bound rec-Atn on the generation of bound plasmin. The effect of rec-Atn was compared with fibrinogen fragments and other proteins that have been reported to bind plasminogen, *i.e.* fibronectin, laminin, and gelatin (Salonen *et al.*, 1985; Stack *et al.*, 1990). When incubated with plasminogen and either t-PA or scu-PA, plasmin activity was generated to the wells coated with rec-Atn or fibrinogen fragments, whereas little if any plasmin was generated to wells coated with laminin, fibronectin, gelatin, or BSA only under the conditions used (Fig. 12). t-PA was severalfold more potent than scu-PA in generating plasmin to amphoterin and fibrinogen fragment surfaces. Generation of surface-bound plasmin also took place when the amphoterin surface was first incubated with the activator, washed, and then incubated with plasminogen, which indicated that both t-PA and scu-PA bind to amphoterin. Binding of the activator proteins to the amphoterin surface was also indicated by immunochemical

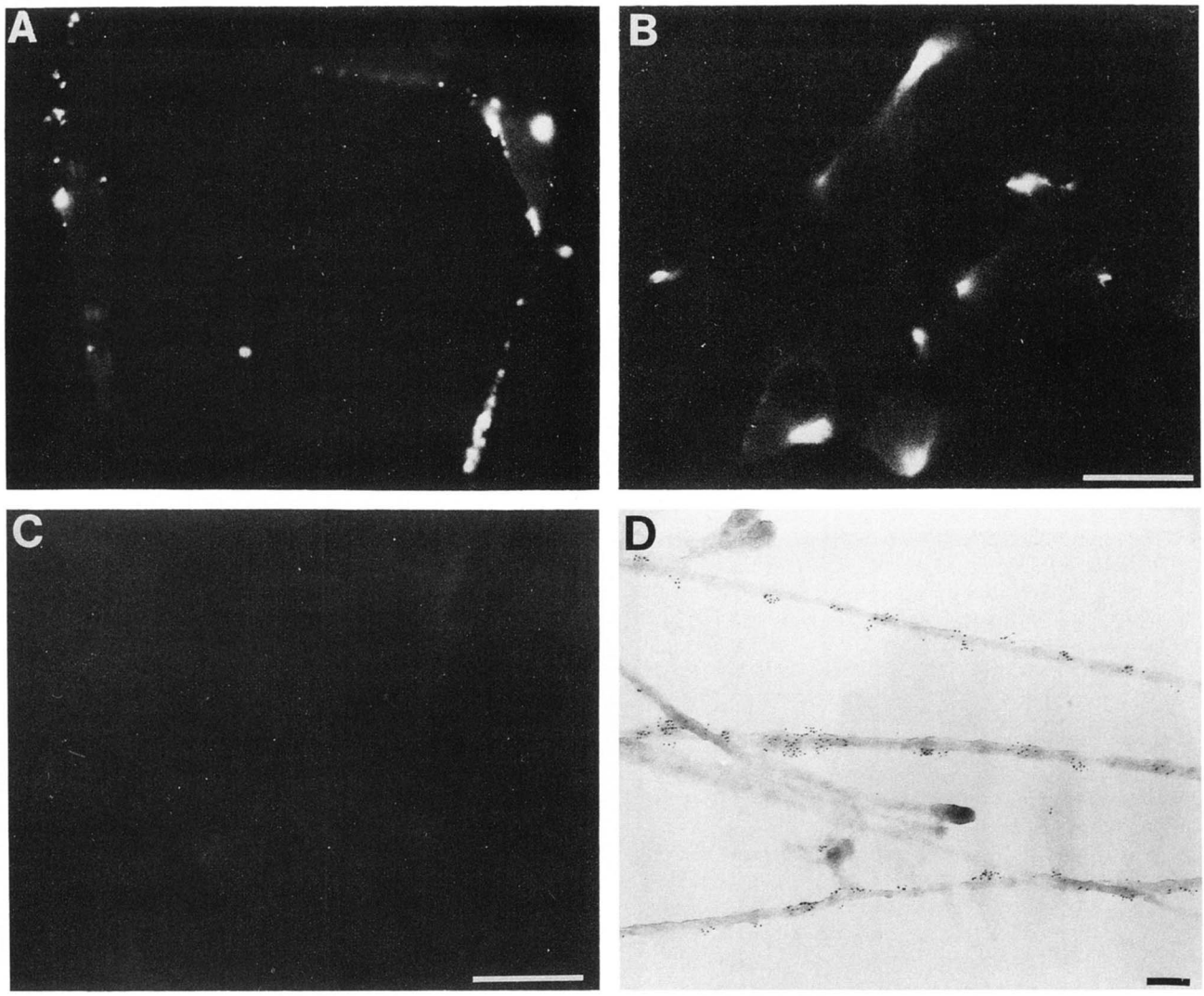

FIG. 9. **Immunostaining of amphoterin on the cell surface and in microspikes.** For surface labeling, HT1080 cells were kept on Matrigel for 3 h. In *A*, living cells were incubated with anti-peptide II at 4 μg/ml for 30 min on an ice bath and fixed with 4% paraformaldehyde. In *B* and *C*, the cells were first fixed with 4% paraformaldehyde for 30 min and then incubated with anti-rec-Atn (*B*) or nonimmune chicken IgG (*C*) at 4 μg/ml for 1 h. The bound antibodies were visualized with FITC-conjugated anti-rabbit (*A*) and anti-chicken IgG (*B* and *C*). For immunogold labeling of microspikes (*D*), embryonic fibroblasts were cultured overnight on gold grids, fixed and permeabilized as in Fig. 7, and incubated with anti-peptide II at 2 μg/ml for 1 h. The bound antibodies were visualized with anti-rabbit Ig-coated gold particles, 5 nm in size. *Bars*: *A–C*, 20 μm; *D*, 100 nm.

detection (Parkkinen and Rauvala, 1991), which suggested a low affinity interaction for both activators with amphoterin (not shown).

Generation of surface-bound plasmin resulted in effective degradation of immobilized amphoterin as indicated by release of radioactive amphoterin fragments into the solution. Degradation of immobilized amphoterin took also place in EDTA-plasma supplemented with either t-PA or scu-PA (Fig. 13). If plasminogen was depleted from plasma, no degradation of amphoterin was observed, whereas replenishing plasminogen restored degradation (Fig. 13). This indicated that amphoterin-associated plasminogen activation took place in a plasma milieu and that the amphoterin-bound plasmin was protected from its inhibitors in plasma.

DISCUSSION

Amphoterin and Related HMG1-type Proteins—Amphoterin has been previously defined as a cytoplasmic and extracellular protein that is involved in neurite outgrowth (Rauvala *et al.*, 1988; Merenmies *et al.*, 1991). The cytoplasmic and cell surface localization has been found for neuroblastoma cells and brain neurons (Rauvala *et al.*, 1988) and for epithelial cells (Salmivirta *et al.*, 1992). A similar localization has been found in peripheral neurons and in Schwann cells in studies that suggest a role for amphoterin in neuron-glia interactions (Daston and Ratner, 1991).

Unexpectedly, molecular cloning studies (Merenmies *et al.*, 1991) revealed that the coding sequence of amphoterin is identical to the sequence cloned in several studies (Paonessa *et al.*, 1987; Bianchi *et al.*, 1989; Kaplan and Duncan, 1988; Tsuda *et al.*, 1988; Wen *et al.*, 1989) on the basis of the peptide sequences of the isolated HMG1 protein (Walker *et al.*, 1980). The HMG1 protein has been defined on the basis of its electrophoretic mobility and binding to DNA (Johns, 1982). The HMG1 protein has been localized to the cell cytoplasm, from where it could be transported into the nuclei (Bustin and Neihart, 1979; Mosevitsky *et al.*, 1989). It has been also reported that in some tissues, like in liver and brain, the HMG1 protein does not enter the nuclei (Mosevitsky *et al.*, 1989). Binding of the HMG1 protein to DNA *in vitro* has

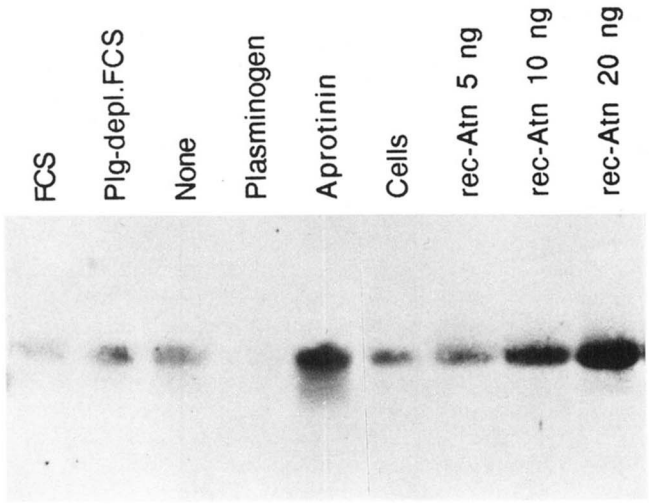

FIG. 10. **Immunoblotting of the substratum-attached material of HT1080 cells with anti-rec-Atn antibodies.** HT1080 cells were cultured in Matrigel-coated 34-mm tissue culture wells (about 5×10^5 cells/well) in the medium supplemented with 10% FCS, plasminogen-depleted FCS, 0.1% BSA alone (*None*), or with plasminogen (10 μg/ml) or aprotinin (10 μg/ml) as indicated, for 16 h. The cells were removed and the substratum-attached material was solubilized in SDS-PAGE sample buffer. 1/4 of the samples and 1/200 of solubilized cells from a corresponding well were run in 10–20% SDS-PAGE, transferred to nitrocellulose, and incubated with anti-rec-Atn at 10 ng/ml followed with horseradish peroxidase-conjugated anti-chicken IgG. The bound antibodies were visualized by the ECL reaction and autoradiography.

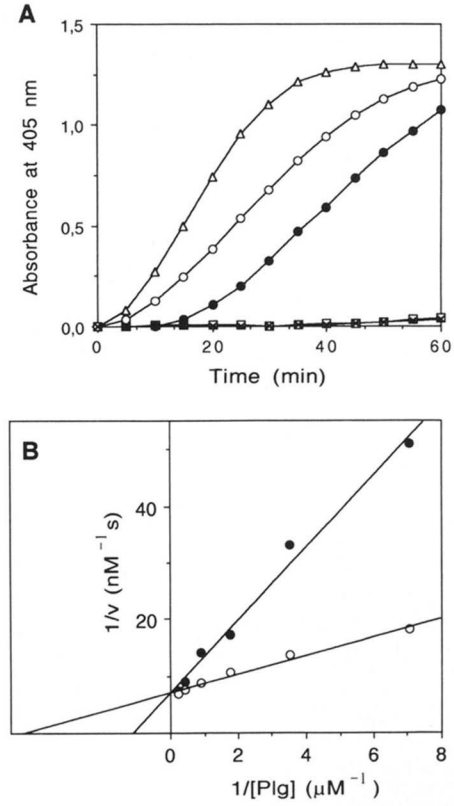

FIG. 11. **Effect of recombinant and tissue amphoterins on plasminogen activation in solution.** *A*, plasminogen (0.5 μM), t-PA (0.7 nM), and the chromogenic plasmin substrate S-2251 (0.6 mM) were incubated with rec-Atn at 2 μM (○) or 9 μM (△), tissue amphoterin at 2 μM (●), rec-HB-GAM at 2 μM (×), or without any compound added (□). *B*, Lineweaver-Burk plots for the initial activation rates of plasminogen (0.14–4.5 μM) by t-PA (0.7 nM) in the presence of rec-Atn, 2 μM (60 μg/ml) (●) or CNBr-fragmented fibrinogen, 60 μg/ml (○).

been extensively studied (for reviews, see Johns (1982) and Bustin *et al.* (1990)), but the cellular function of the HMG1 protein is still unknown (Bustin *et al.*, 1990).

To study the apparent discrepancies of the amphoterin and the HMG1 concepts, we have in the present study expressed the amphoterin cDNA with the aid of a baculovirus vector in the SF9 cells. These studies indicate that the amphoterin cDNA encodes an apparently homogeneous 30-kDa protein that has the same or a very similar structure as compared to the corresponding heparin-binding 30-kDa protein isolated from developing rat brain. Interestingly, the tissue-derived samples contain two major co-purifying proteins (p28 and p29) that are not produced in the expression of the amphoterin cDNA. p28 and p29 are difficult or impossible to separate from amphoterin under nondenaturating conditions but can be purified by reverse phase HPLC. Mapping of amphoterin, p28, and p29 by seven different antibodies indicates homologous but distinct structures that are also distinct from the HMG2 protein (Shirakawa *et al.*, 1990). Partial amino acid sequencing confirms that p28 and p29 have novel sequences that are homologous with the amphoterin sequence. Amphoterin can thus be regarded as one of the proteins having an HMG1-type sequence, electrophoretic mobility, and the ability to strongly bind to a polyanionic matrix, like heparin. The occurrence of several different but homologous polypeptides may have caused confusion in previous studies on HMG1/amphoterin-type proteins.

Multiple bands have been previously observed in Southern blotting using the cDNA of amphoterin[2] or of the corresponding HMG1 protein (Tsuda *et al.*, 1988). The reason for this heterogeneity is not known, but it could be due to long intron sequences or to the occurrence of pseudogenes or multiple functional genes (Tsuda *et al.*, 1988). The present results show the occurrence of multiple homologous proteins in tissues, whereas only one protein form (p30) is produced in the expression of the amphoterin cDNA. This suggests that p28 and p29 are not alternative protein variants produced co-translationally or post-translationally from the amphoterin cDNA, but they may be products of different gene(s). This inference is supported by the finding that the sequence differences found in p28, p29, amphoterin/HMG1, and the HMG2 protein are local, often single amino acid changes. These findings suggest the occurrence of a novel functional multigene family. However, further studies are required to establish the mechanism that creates the sequence variation in the amphoterin family of proteins and whether the variation is actually more extensive than that observed in the present study.

The occurrence of several homologous amphoterin-type sequences in the same animal species may be unexpected from the viewpoint that the amphoterin/HMG1 sequence is well conserved across different species. For example, the sequences of man (Wen *et al.*, 1989), pig (Tsuda *et al.*, 1988), cow (Kaplan and Duncan, 1988), and rat (Bianchi *et al.*, 1989; Merenmies *et al.*, 1991) are all composed of 214 amino acids, and the degree of homology is more than 98%. The variation between the sequences is due to two or three aspartate-glutamate changes in the polyanionic region. The 184-amino acid polycationic regions, within which the variation was found in this study, are identical in the sequences of man, pig, cow, and rat except for one histidine-glutamine change in the pig sequence (amino acid 141; Tsuda *et al.* (1988)). The strong conservation of the amphoterin/HMG1 sequence suggests that the se-

[2] J. Merenmies and H. Rauvala, unpublished results.

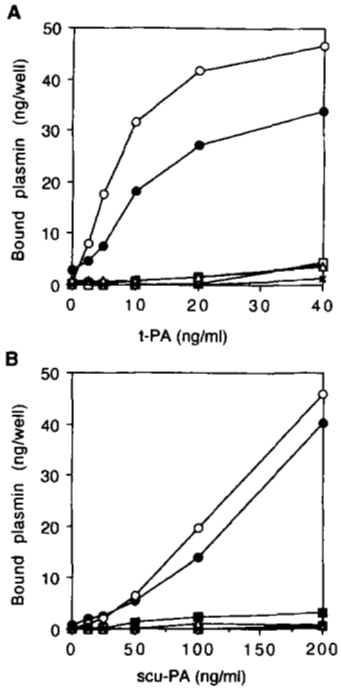

FIG. 12. **Plasminogen activation on protein surfaces.** Wells coated with rec-Atn (●), fibrinogen fragments (○), laminin (■), fibronectin (□), gelatin (△), or BSA (×) were incubated with t-PA (*A*) or scu-PA (*B*) and plasminogen, 10 μg/ml in 0.1% BSA-PBS, for 30 min at 37 °C. After washing the wells, the bound plasmin activity was determined by the thioesterase assay. Mean values of duplicate wells are shown.

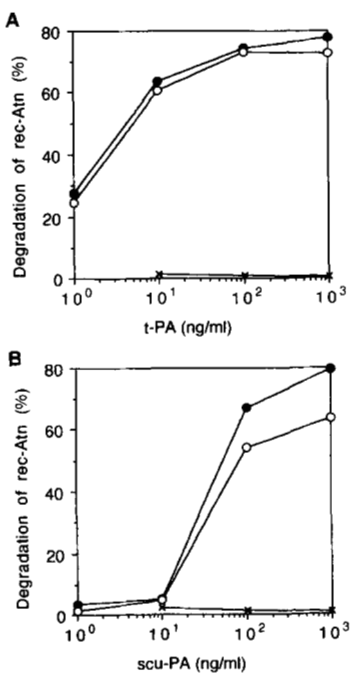

FIG. 13. **Degradation of amphoterin by plasmin generated in plasma.** Wells coated with ^{125}I-labeled rec-Atn were incubated with t-PA (*A*) or scu-PA (*B*) in 10% plasma (●), plasminogen-depleted plasma (×) or plasminogen-depleted plasma supplemented with plasminogen, 10 μg/ml (○) for 2 h at 37 °C. The proportion of radioactivity released into the solution during incubation was determined. Mean values of duplicate wells are shown.

quence variation observed in the present study is not meaningless but has been developed to change the specificity of the interactions in which the polycationic regions of the proteins participate. The local type of sequence variation in proteins of the same tissue of the same animal species is an unusual one and has some resemblance to the sequence variation found in immunoglobulins.

Since p28 and p29 are not detected or are only weakly detected by polyclonal antibodies against amphoterin, the protein structures of these components might be significantly different despite the apparent clear homology as compared to amphoterin. It is currently unclear whether p28 and/or p29 might correspond to chromosomal HMG1 and how these proteins are distributed in a cell. Elucidation of these questions awaits production of specific probes to detect p28 and p29. However, the present results make it possible to distinguish amphoterin from other HMG1-type proteins that have previously remained unidentified.

Functional Role of Amphoterin at the Leading Edges of Cells—A strong expression of amphoterin has been previously found in early brain and in neuroblastoma cells (Rauvala and Pihlaskari, 1987; Merenmies *et al.*, 1991). The present results show that amphoterin is clearly expressed in many cultured cell lines. Interestingly, in the fibroblast lines studied the expression was one to two orders of magnitude higher in transformed cells as compared to their normal counterparts. The expression pattern of amphoterin suggests a general functional role in embryonic and transformed cells. However, there is currently no evidence that amphoterin would be required for cell proliferation. For example, no enhancing or inhibitory effect has been observed in standard mitogenic assays.[3]

To gain insight into the functional role of amphoterin, we have in the present paper carried out localization studies using cell types that express abundant amounts of the protein and antibodies that discern it from the other homologous proteins. These studies confirm the localization of amphoterin to the leading edges and their filopodia in neuroblastoma cells, which was previously reported (Merenmies *et al.*, 1991). Interestingly, amphoterin does not only distribute to the growth cones and their filopodia in neural type cells, but an analogous leading edge localization is clearly observed in motile non-neuronal cells. In fibroblastoid cells, the amphoterin-specific antibodies labeled membrane ruffles and microspike protrusions that have been suggested to have an analogous pathfinding function as the filopodia of the neuronal growth cone (Singer and Kupfer, 1986). Previously, actin has been the only protein identified in both microspikes and filopodia (Singer and Kupfer, 1986). The finding that amphoterin is concentrated in both types of membrane extensions further supports their functional similarity.

The term HMG box is currently used to describe a homologous sequence of DNA-binding nuclear proteins (Jantzen *et al.*, 1990; Ner, 1992). Amphoterin also contains an HMG box, but its distribution in cells is clearly different from that expected for a nuclear protein. This calls for caution in the interpretation of functional roles of proteins on the basis of sequence motifs only. There are also other examples of the occurrence of common structural motifs in proteins that are targeted to different cellular compartments and have different functional roles (Baron *et al.*, 1991). An extreme example of different cellular localizations of the same or very homologous proteins has been shown for the heparin-binding fibroblast growth factors. Basic fibroblast growth factor is found in the cytoplasm, in the nucleus, and in the extracellular matrix (Vlodavsky *et al.*, 1991). Slight sequence differences have been suggested to be the reason for the targeting of basic fibroblast growth factor to different cellular compartments (Imamura *et*

[3] H. Rauvala and R. Nolo, unpublished results.

al., 1992). Since several proteins that are homologous with amphoterin exist in tissues, the possibility that different homologous protein forms could be targeted to different cellular compartments should be taken into consideration and is currently being studied. It seems clear that indirect evidence on the localization and function of amphoterin, such as the occurrence of the HMG box sequence or the consensus sequence of extracellular heparin-binding proteins (found in the amino-terminal region of amphoterin; see Merenmies *et al.* (1991)) or the binding of the protein to DNA or to heparin and heparan sulfate proteoglycans *in vitro*, lead to contradictory ideas and may be of little value for revealing a functional role in living cells.

Amphoterin lacks a classic-type secretion signal sequence and resembles in this respect several other proteins, such as the acidic and basic fibroblast growth factors, interleukin 1, and transglutaminase (for a review, see Muesch *et al.* (1990)) that are thought to have an extracellular function but lack a signal sequence. Ciliary neuronotrophic factor (Stöckli *et al.*, 1989; Lin *et al.*, 1989) and the lactose-binding lectin L-14 (Cooper and Barondes, 1990) are also examples of such proteins. Interestingly, basic fibroblast growth factor has been shown to be released from migrating, uninjured cells and mediate functions in an autocrine manner (Mignatti and Rifkin, 1991). Studies on the lectin L-14 have suggested a novel type of secretion via exocytosis that is independent of the classic endoplasmic reticulum-Golgi route. A diffuse cytoplasmic localization has been shown for L-14. At certain developmental stages, however, the lectin becomes concentrated at the plasma membrane as patches, from which it is pinched off and binds to the extracellular matrix (Cooper and Barondes, 1990). The cellular distribution of amphoterin appears very similar to that of L-14; although a diffuse cytoplasmic distribution can be observed, amphoterin also forms patches at the plasma membrane and is found in the extracellular matrix. This suggests that amphoterin is secreted in a similar manner as L-14. The finding that amphoterin is localized mainly in the leading edges of spreading cells and is found on the cell surface at this location suggests that its externalization may take place mainly at the leading membrane. As the above-mentioned proteins are involved in developmental processes, it appears that selective, Golgi-independent protein externalization is not an uncommon phenomenon during development and differentiation.

There are several clues as to the functional role of amphoterin at the leading membrane. Amphoterin has an exceptional dipolar structure with a highly basic 184-amino acid long amino-terminal region, in which on an average every fourth amino acid is a lysine residue, and a 30-amino acid long carboxyl terminus consisting of only aspartate and glutamate. This type of exceptionally charged structure suggests that amphoterin is sticky to many types of surfaces. As expected from the dipolar structure, amphoterin has a strong tendency to form polymeric structures, which was actually noted for the protein isolated from brain (Rauvala and Pihlaskari, 1987). As expected from the polylysine-type structure (Merenmies *et al.*, 1991), amphoterin is highly adhesive for cells *in vitro* (Rauvala and Pihlaskari, 1987). Amphoterin may therefore enhance the binding of the advancing plasma membrane to various surfaces and thereby serve as a multifunctional, local adhesion protein during migratory phenomena. In this manner, the cells could have a general ability to advance cytoplasmic processes along the multitude of structures that they encounter, whereas the matrix receptors could have a more specific role, for example in determining the direction of the movement. The localization of amphoterin to membrane ruffles and microspike protrusions in various cell types is in agreement with the proposed general function at the leading edges of cells.

Studies with rec-Atn confirmed the earlier findings of the strong promoting effect of amphoterin on plasminogen activation by t-PA (Parkkinen and Rauvala, 1991). Furthermore, surface amphoterin was found to promote the generation of surface-bound plasmin as opposed to extracellular matrix proteins, like fibronectin and laminin, and gelatin that have been shown to bind plasminogen (Salonen *et al.*, 1985; Stack *et al.*, 1990). Amphoterin was in this effect comparable with fibrinogen fragments. The finding that amphoterin-associated plasminogen activation and plasmin-mediated degradation of amphoterin also took place in a plasma milieu supports the biological significance of these interactions. This finding, together with the leading edge localization of amphoterin, suggests that amphoterin may contribute to the spatial and temporal regulation of plasminogen activation in invasive cells.

It is noteworthy that amphoterin surfaces promote plasminogen activation both by t-PA and scu-PA. Amphoterin might thus serve as a plasminogen binding site at the advancing membrane of various cells, irrespective of whether the cell utilizes t-PA or u-PA for plasminogen activation. Various types of cells use u-PA for tissue invasion, whereas t-PA is generally thought to function in fibrinolysis. However, there is evidence that at least cells of neuroectodermal origin utilize t-PA for generation of surface-bound plasmin (Krystosek and Seeds, 1986; Neuman *et al.*, 1989; Bizik *et al.*, 1990). Furthermore, the recent finding that t-PA is induced as an immediate early gene following neuronal activity suggests a role for t-PA in neuronal plasticity (Qian *et al.*, 1993). Typical for both plasminogen activators is that they are localized to the leading edge in motile cells. As amphoterin binds with a low affinity both activators, it might also participate in the localization of the activator proteins to the leading membrane, possibly as a complex with heparin-type structures that also bind plasminogen activators (Stein *et al.*, 1989; Stephens *et al.*, 1992).

Taken together, our results indicate a general role for amphoterin at the advancing membrane of various invasive cells. The adhesive properties and the ability to localize and enhance plasmin generation suggest that amphoterin may be a link between adhesive and proteolytic regulation at the leading membrane; adhesive interactions mediated by amphoterin are reversed upon complex formation with plasminogen and plasminogen activators, and plasmin generation is strongly enhanced upon the complex formation, which enhances penetration of the processes during migration. This type of function is in agreement with the strongly enhanced expression of amphoterin in transformed and malignant cells.

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