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IMMUNOSELECTION OF GRP94/ENDOPLASMIN FROM A KNRK CELL-SPECIFIC λgt11 LIBRARY USING ANTIBODIES DIRECTED AGAINST A PUTATIVE HEPARANASE AMINO-TERMINAL PEPTIDE

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Induction of an invasive phenotype by metastatic tumour cells results in part from inappropriate expression of extracellular matrix-degrading enzymes normally involved in embryonic morphogenesis, tissue remodelling, angiogenesis and wound healing. Such enzymes include endoglycosidases that degrade heparan sulfate (HS) in endothelial basement membrane, as well as better characterized proteases. Heparanase, an endo-β-D-glucuronidase initially detected in B16 melanoma cells, has been described as a M_r 96 000 glycoprotein with pI of 5.2, and has been immunolocalized to the cell surface and cytoplasm. We have utilized a polyacrylamide-gel-based HS degradation assay to demonstrate that KNRK, a rat kidney fibroblast cell line transformed by v-K-*ras*, exhibits HS-degrading activity similar to that of B16F10 mouse melanoma cells. To immunoselect heparanase-expressing clones from a KNRK-cell-specific λgt11 cDNA library, we have also prepared a rabbit anti-serum directed against a putative amino-terminal peptide of B16F10 cellular heparanase. Lysogens from one clone expressed a β-galactosidase fusion protein whose staining with peptide anti-serum was inhibited by competition with excess peptide. Dideoxy-mediated sequencing of the insert termini of this recombinant revealed that it represents a rat homologue of M_r 94,000 glucose-regulated protein (GRP94/endoplasmin), a molecular chaperone that contains the exact amino-terminal sequence previously attributed to heparanase. Our results call into question the specificity of this peptide sequence, as well as previous immunolocalization studies of heparanase carried out using such anti-sera.
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The metastatic cascade is a complex series of processes whereby invasive sub-populations of cells within a primary tumour detach from the tumour mass and degrade the surrounding extracellular matrix (Liotta *et al.,* 1991). Penetration of vascular sub-endothelium results in the blood-borne transmission of these cells to other sites, where they extravasate into organ parenchyma by local degradation of basement membrane and proliferate as secondary masses. Because the likelihood of cancer mortality is greatly increased by the development of metastases within the host, an understanding of the role of degradative enzymes in mechanisms of metastatic progression is of potential benefit to the development of new cancer therapies.

It is currently understood that induction of an invasive phenotype by tumour cells results from insufficient modulation of extracellular-matrix-degrading enzymes whose normal roles include embryonic morphogenesis and tissue remodelling, as well as angiogenesis. The most extensively characterized of these enzymes are proteases, of which several have been implicated in tumour metastasis. Elevated expression of urokinase-type plasminogen activator (uPA), thiol proteases (cathepsins B and L) and matrix metalloproteases of the collagenase and stromelysin sub-families strongly correlates with oncogenic transformation and enhancement of a metastatic phenotype in many murine and human tumour systems (Moscatelli and Rifkin, 1988; Liotta *et al.,* 1991).

Less extensively characterized, but also strongly associated with metastatic progression, are endoglycosidases involved in proteoglycan cleavage, of which heparan sulfate (HS) is the major species resident in endothelial basement membrane. Enhanced release of HS fragments from $Na_2[^{35}SO_4]$-labeled extracellular matrix was initially correlated with metastatic potential in cellular extracts, but not in conditioned media, of B16 murine melanoma clones (Nakajima *et al.,* 1983). Analyses of substrate specificity, and examination of reducing termini of HS fragments led to the characterization of heparanase as an endo-β-D-glucuronidase that is inhibited by heparin (Nakajima *et al.,* 1984). In addition to expression in numerous human tumour cell lines, heparanases are also expressed in circulatory cells whose functional roles require penetration of endothelial basement membranes, *i.e.,* neutrophils, mast cells, macrophages and activated T lymphocytes (Matzner *et al.,* 1985; Bashkin *et al.,* 1990; Nakajima *et al.,* 1988). Platelets also produce a distinct HS- and heparin-degrading enzyme, heparitinase (Oosta *et al.,* 1982).

Although its biochemical activity has been well documented, the molecular biology of heparanase-gene expression is little known. Purified heparanase from B16 melanoma cells has been characterized as a glycoprotein with M_r of approximately 96 000 and a pI of 5.2, and has been localized immunochemically to the cell surface and cytoplasm using a polyclonal anti-serum directed against a putative amino-terminal sequence from purified B16 melanoma cell heparanase (Jin *et al.,* 1990). In the present study, we demonstrate that v-K-*ras*-transformed normal rat kidney (KNRK) cells also exhibit HS-degrading activity. Using the putative heparanase amino-terminal peptide (Jin *et al.,* 1990), we prepared a similar anti-serum with the aim of immunoselecting heparanase-expressing clones from a KNRK mRNA-derived λgt11 cDNA library. We now report that we have immunoselected a clone representing a previously characterized molecular chaperone, GRP94/endoplasmin, a 94-kDa protein that contains the exact amino-terminal sequence previously attributed to heparanase.

MATERIAL AND METHODS

Cells and viruses

Kirsten murine-sarcoma-virus-transformed normal rat kidney cells (KNRK) were obtained from the ATCC (Rockville, MD). B16F10 murine melanoma cells were obtained from Dr. R. Liteplo. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

The construction of a KNRK-specific λgt11 cDNA library, used for screening of recombinants reactive with putative heparanase peptide anti-serum, has been described (De Vouge

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and Mukherjee, 1992). Cultures of *Escherichia coli* strains Y1089, used to generate lysogens and induce synthesis of β-galactosidase fusion proteins, and Y1090, used to screen the library, were routinely grown in LB medium, or plated on 1.5% agar in LB exactly as described by Huynh *et al.* (1985).

Preparation of mammalian and bacterial extracts

Tissue-culture plates of confluent cells (150 mm, containing 7.5×10^6 cells) were washed with Ca^{2+}, Mg^{2+}-free PBS, and detached from the substrate with 15 ml PBS containing 2 mM EDTA for 5 min at 37°C. After centrifuging at 900*g* for 10 min at 4°C, the cells were washed with 10 ml of PBS, spun again, re-suspended in 1 ml of PBS and microfuged for 1 min. Cell pellets were re-suspended in 100 μl of HEB buffer [50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.5, 5 mM N-ethylmaleimide, 0.05% sodium azide, 0.1 mM *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA), 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF, added freshly from a 20-mM stock solution in methanol), and 50 μM chymostatin (added from a 10-mM stock in DMSO after dilution to 1 mM with H_2O)]. After sonicating the suspensions on ice, using 2 bursts of 5 sec each at power setting 1 from a 50-watt Microson automatic cell disruptor, glycerol was added to 30% (v/v) and concentrations of cellular protein were measured using a fluorescamine assay (below). Extracts were assayed for heparanase activity immediately, or were stored at −20°C for several weeks without significant loss of activity.

Lysogens of λgt11 clones in *E. coli* Y1089 were isolated by infection at a multiplicity of infection of 5 (Huynh *et al.*, 1985). True lysogens were isolated by replica-plating colonies grown at 32°C on LB agar plates, and incubating either at 32°C or at 42°C. Colonies exhibiting growth only at 32°C were selected. Bacterial extracts expressing β-galactosidase fusion protein were prepared from uninduced or IPTG-induced cultures of lysogenic *E. coli* Y1089 grown as described (Huynh *et al.*, 1985). Cells were pelleted at 1000*g* for 5 min at room temperature, and re-suspended in 0.02 of the original culture volume of re-suspension buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM CDTA). The cell suspensions were quick-frozen in liquid N_2, and thawed immediately or stored at −100°C until required. PMSF was added to 2 mM and cells were lysed by treatment with lysozyme (0.25 mg/ml) for at least 1 hr on ice. The lysates were sonicated at setting 5 for 2 × 15 sec bursts.

Quantitation of protein was carried out using fluorescamine (Sigma, St. Louis, MO). A standard curve was prepared using BSA (0.88 to 35 μg) in 0.5 ml dH_2O. After adding 1 ml of a solution containing 0.3 M sodium borate, pH 9.2, 0.1% SDS and 5 mM CDTA, 1 ml of fluorescamine solution (0.015% in acetone) was added with rapid mixing. The fluorescence of samples was measured in a Perkin-Elmer (Norwalk, CT) model LS-5 fluorescence spectrophotometer (λ_{ex} = 390 nm; λ_{em} = 475 nm).

Heparanase assay

HS from bovine kidney (Sigma) was size-fractionated on Sephadex G-75 (Pharmacia, Uppsala, Sweden) equilibrated with CT (10 mM Tris-HCl, pH 8.0, 0.1 mM CDTA). Fractions corresponding to the void volume were pooled, and this high-molecular-weight HS was adjusted to 1 mg/ml and used for heparanase assays. Ten microlitre reactions contained cellular extracts and 1 to 2 μg of HS in 20 mM cacodylate buffer, pH 6.1. Incubation was carried out at 37°C, typically for 16 hr. At the end of incubation, 0.25 volumes of micrococcal nuclease (9 U/ml in 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 9.5, 30% v/v glycerol) was added, and the samples were incubated for 1 hr at 37°C. Proteinase K and SDS were added to 0.5 μg/μl and 0.1% respectively, and the samples were incubated at 60°C for ≥1 hr. Controls included (i) HS without cell extract, (ii) cell extract without HS and (iii) HS plus cell extract incubated for 0 hr instead of 16 hr. After adding 0.25 volumes of 5X glycerol loading buffer (80% glycerol, 5 mM CDTA), the entire sample volumes were loaded onto 7.5% polyacrylamide mini-gels in TAC buffer (40 mM Tris, 20 mM sodium acetate, 1 mM CDTA, pH 7.8) and electrophoresed at 100 V for 30 min. Gels were soaked in H_2O for 10 min to remove SDS, stained with 0.1% methylene blue in 50% ethanol for 5 to 10 min and de-stained under running water.

Preparation of anti-sera, immunoblotting

An anti-serum directed against a portion of the putative amino-terminal sequence of heparanase (EEDLGKSREG-SRTDDC-amide) (Jin *et al.*, 1990) was prepared in New Zealand white rabbits. This peptide, coupled to keyhole limpet hemocyanin (KLH) by *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, was prepared by Multiple Peptide Systems, San Diego, CA. Four rabbits were immunized with 400 μg of antigen emulsified 1:1 with complete Freund's adjuvant, administered as 5 s.c. injections of 0.1 ml per site. Boosts of 480 μg antigen emulsified with incomplete adjuvant were administered as 4 or 5 s.c. injections (0.6 ml total) 3 weeks later, and at 2-week intervals thereafter. Test bleeds and serum preparation were carried out at the time of boosts. Rabbits were exsanguinated 3 weeks after the fourth boost. Titres of anti-peptide antibody were measured by an ELISA test.

Immunoblotting of proteins from KNRK or B16F10 cell extracts was used to select optimal anti-serum titres for immunoselection of heparanase from the KNRK-specific λgt11 library. Cell lysates containing 200 to 300 μg of protein were separated by SDS-PAGE, in 7.5% preparative mini-gels (Bio-Rad, Richmond, CA). The proteins were transferred to nitrocellulose by electroblotting for 2 hr at 150 V in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), and blocked with 1% BSA in TBST for 1 hr at room temperature (or overnight at 4°C). Various dilutions of pre-immune or immune serum in TBST were applied to membranes immobilized in a Bio-Rad Mini-Protean II Multiscreen manifold. After 1-hr incubation at room temperature, 3 washes of 5 min each with TBST were carried out, and conjugate anti-serum (goat anti-rabbit IgG coupled to alkaline phosphatase; Sigma) was applied at 1:1000 in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 hr at room temperature. After washing with TBST, membranes were stained for 5 to 15 min with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 66 μl nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 66 μl 5-bromo-4-chloro-3-indolylphosphate (25 mg/ml in 50% dimethylformamide) per 10 ml buffer.

Immunoscreening of λgt11 library

Initial high-density screening of the KNRK-specific λgt11 library was carried out essentially as described (Huynh *et al.*, 1985). Aliquots of library containing 3×10^4 pfu/plate (67% recombinant) were grown with *E. coli* Y1090 for 3.5 hr at 42°C, and overlaid with dry nitrocellulose filters which had previously been impregnated with 10 mM IPTG. Duplicate filters were sequentially applied to plates and incubated at 37°C for 2.5 and 2 hr respectively. The filters were rinsed, blocked and stained as described for immunoblotting, except that primary anti-serum was applied at a dilution of 1:40. Positive plaques were cored out with a Pasteur pipette into 1 ml SM [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2% $MgSO_4$, 0.01% gelatin (added from a stock of 2% autoclaved gelatin)], and sterilized with 10 μl of chloroform.

Plaque purification was carried out in 35-mm Petri dishes containing 2 ml of 1.5% agar base in LB. Aliquots of phage suspension were diluted 100-fold in SM and 4 μl were combined with 30 μl of *E. coli* Y1090. After 15 min of

incubation at 37°C, 0.8 ml of 0.7% top agarose in LB was added and the suspensions were poured onto the agar plates. Plates were incubated as described above, then overlaid with IPTG-impregnated nitrocellulose filters (Schleicher and Schuell, Dassel, Germany, BA83, 0.22 μm) for 2 hr at 37°C. Phage populations were subjected to several rounds of purification until all plaques on a filter stained positively. One additional round of purification was then carried out.

Because initial screening attempts resulted in selection of rare recombinants which reacted strongly with antibodies present in pre-immune serum, the anti-serum was pre-absorbed with lysate derived from an IPTG-induced culture of Y1089 bacteria expressing one such clone (clone F) as a lysogen. A 200-ml culture of this lysogen was grown and IPTG-induced as described above. Bacterial lysate was then prepared, and vacuum-filtered through Immobilon-P membrane (Millipore, Bedford, MA; wetted briefly in methanol, followed by TBS). Three such filters were required to process the entire volume of bacterial extract. Anti-serum (1 ml) was diluted 5× in TBST and sequentially incubated with each filter overnight at 4°C. The adequacy of pre-absorption was monitored by diluting aliquots of anti-serum to 1:40 and staining a nitrocellulose filter containing 10^4 pfu of clone F recombinant.

Phage DNA isolation and sub-cloning

Rapid phage DNA isolation was carried out using established protocols (Sambrook *et al.*, 1989). Each of 3 100-mm Petri dishes containing a base layer of 1.5% agarose in LB was plated with 10^5 pfu of purified recombinant phage and *E. coli* Y1090, and grown for 5 to 6 hr at 42°C. Phages were eluted from the plates with 5 ml of gelatin-free SM overnight at 4°C. A second 1-hr elution was carried out, and the lysates were pooled. After pelleting bacterial debris at 1500*g* for 10 min at 4°C, the phage suspension was treated with deoxyribonuclease I and ribonuclease A at 1 μg/ml each for 15 min at 37°C. An equal volume of 20% (w/v) PEG-8000 and 2 M NaCl in gelatin-free SM was added and phages were precipitated overnight at 4°C. After pelleting at 10,000*g* for 10 min, the phage pellet was re-suspended in 400 μl of gelatin-free SM. After extraction with chloroform, CDTA, proteinase K, and SDS were added to 20 mM, 50 μg/ml, and 0.5%, respectively. After 1-hr incubation at 56°C, the cooled suspension was extracted once with phenol/chloroform and once with chloroform, and the nucleic acids in the aqueous phase were precipitated with 2 volumes of cold ethanol. The pelleted nucleic acids were re-suspended in 100 μl of MOPS-acetate (50 mM MOPS, 0.1 M sodium acetate, pH 8.0, 1 mM CDTA), precipitated with ethanol, and re-suspended in 50 μl of CT containing 20 μg/ml boiled ribonuclease A. Digestion of 20 μl of DNA with 10 U of EcoRI was carried out for 2 hr at 37°C. The fragments were then labeled with 1 U of *E. coli* DNA polymerase I Klenow fragment and 0.1 μCi of [α-^{35}S]dATP for 30 min at 30°C. Wells of a 1.5% agarose gel contained 20 μl of the 30 μl final reaction volume.

Sub-cloning of intact recombinant inserts was carried out by excision of the intact insert with NotI, followed by ligation of the unpurified insert into NotI-cleaved and dephosphorylated pBluescript II KS(+) (Stratagene, LaJolla, CA). The constructs were transformed into the XL1-Blue strain of *E. coli* (Stratagene) previously rendered transformation-competent by repeated washing and concentration of log-phase cells with ice-cold 0.1 M $CaCl_2$ (Sambrook *et al.*, 1989).

Sequencing and identification of recombinant clones

Sub-cloned pBluescript II KS(+) plasmid harbouring recombinant insert selected from the λgt11 screening was extracted from overnight cultures of transformant grown in LB, using a modified alkaline lysis protocol (Birnboim, 1983). Plasmid DNA purification was carried out by binding to and eluting from Sephaglas (Pharmacia). Dideoxy-mediated sequencing of 5′ insert termini was carried out on alkali-denatured, double-stranded plasmid using a Sequenase 2.0 kit (U.S. Biochemical, Cleveland, OH), and T7 and T3 promoter-derived sequencing primers. An additional anti-sense oligonucleotide (GTCGTC-GGTCCTGCTGCCCTCCCTRCTYTTNCCCAG), deduced from a portion of the putative heparanase amino-terminal polypeptide (LGKSREGSRTDD), was also used as a sequencing primer. Separation of [α-^{35}S]dATP-labeled DNAs were in 6% polyacrylamide gels in 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), containing 7 M urea. Unfixed gels were dried and exposed to X-ray film.

RESULTS

Heparanase assay of B16F10 and KNRK cell extracts

Heparanase activity is commonly assayed using $Na_2[^{35}SO_4]$labeled extracellular matrix as a substrate for enzyme present either in conditioned medium or in cell extracts. Chromatographic fractionation and scintillation counting of fractions enable assessment of HS fragment size. Because such assays are relatively impractical for the analysis of large numbers of samples, we have developed a semi-quantitative adaptation of an earlier heparanase assay, based on the digestion of unlabeled HS and assessment of activity by fragment separation in polyacrylamide gels (Nakajima *et al.*, 1983). Cellular extracts are incubated with size-fractionated, high-molecular-weight HS from bovine kidney. After incubation at 37°C, the samples are digested with micrococcal nuclease and proteinase K to prevent cellular protein or nucleic acid from interfering with the separation or staining of HS fragments in the gel. The fragments are then specifically stained with methylene blue. Using this approach, we have demonstrated that B16F10 and KNRK cell lysates exhibit HS degradative activity (Fig. 1). Densitometric traces of cell lysate incubated with HS for 16 hr illustrate HS peaks that migrate slightly more rapidly than control reactions placed on ice immediately after addition of components (0 hr samples), or HS incubated in the absence of cell extracts. Reactions proceeding for 16 hr also show broader smears and higher levels of partially digested HS migrating towards the bottom of the gel, although in comparison with controls containing HS only, 0 hr samples also appear to exhibit low levels of heparanase activity. In general, B16F10 extracts tended to be less active than KNRK extracts using bovine kidney HS as a substrate. Control samples containing cell extracts but no HS yielded only background, or a faint, rapidly migrating smear, due to small amounts of residual undigested protein or nucleic acid. KNRK extracts were confirmed to be active for heparanase using a column chromatographic assay and Na_2 [$^{35}SO_4$]labeled ECM as substrate (I. Vlodavsky, personal communication).

Characterization of putative anti-heparanase peptide antibodies

Rabbit antibodies were prepared against an oligopeptide reported to represent an amino-terminal sequence of heparanase (Jin *et al.*, 1990), in order to immunoselect and characterize heparanase-expressing clones from a KNRK-specific λgt11 library. Serum antibody titres were initially monitored by enzyme-linked immunosorbent assay (ELISA), using 96-well dishes coated with 1 μg of heparanase peptide in 50 μl of 10 mM $NaHCO_3$, pH 9.0. Although the results of these studies indicated that 3 of the 4 rabbits immunized (designated R5–R8) expressed reactive antibody at titres of 1:10,000 or less (data not shown), suitability of the anti-sera for immunodetection of λgt11 clones was more stringently assayed by immunoblotting of KNRK cell extracts. As shown in Figure 2, immune anti-serum R8 (IS R8) strongly stained a band with an average apparent M_r of 98,000 (based on 3 determinations) at dilutions of 1:10 to 1:640. Occasional weak staining of other bands was noticed in individual trials, usually proteins with approximate

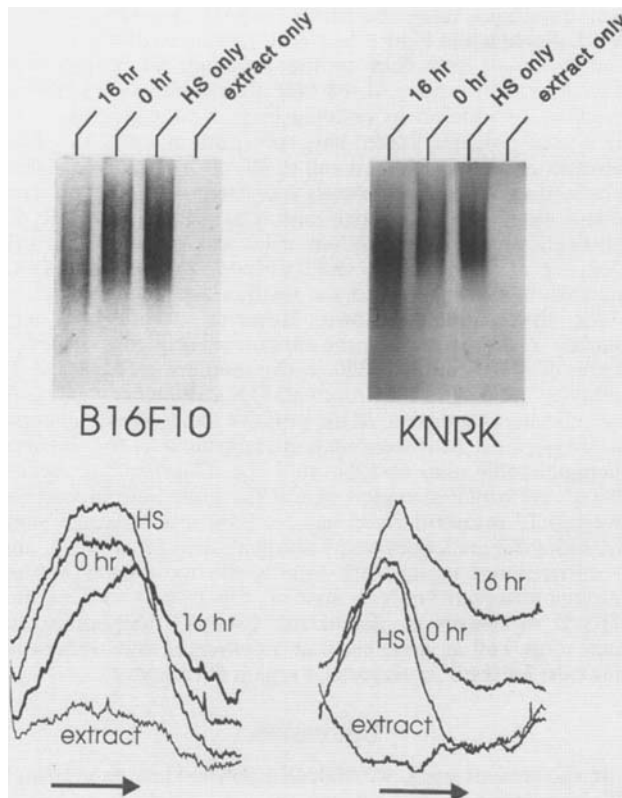

FIGURE 1 – Heparanase assay of cellular extracts from B16F10 murine melanoma and KNRK fibroblast cells. Aliquots of cellular extracts (12 μg) were combined with 1-2 μg of bovine kidney HS and incubated at 37°C for 0 or 16 hr. After micrococcal nuclease and proteinase K digestion, and addition of glycerol loading buffer concentrate, the total sample volumes were run in 7.5% polyacrylamide gels in TAC buffer as described in "Material and Methods". Densitometric traces under each gel are of photographic negatives used in reproducing the gels in this figure. Lanes designated HS only and extract only denote reactions carried out in the absence of cellular extracts or HS respectively.

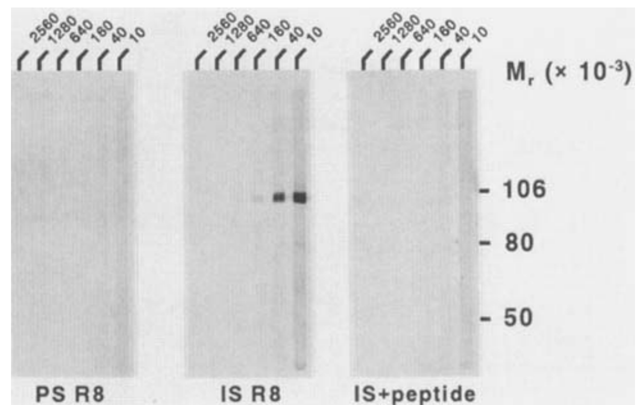

FIGURE 2 – Immunoblotting of KNRK lysate using various dilutions of pre-immune serum (PS R8), immune serum directed against KLH-conjugated, putative heparanase peptide (IS R8), or immune serum pre-incubated with peptide at 0.5 μg/μl for 2 hr at 37°C (IS + peptide). KNRK lysate (300 μg) was run in a preparative 7.5% SDS-PAGE gel and transferred to nitrocellulose as described in "Material and Methods". Individual lanes were incubated with sera at the dilutions indicated at the top of the figure. Washing, incubation with alkaline-phosphatase-conjugated anti-rabbit IgG, and staining are also as described in "Material and Methods". Positions of pre-stained M_r standards run in the same gel are indicated at right.

M_r of 150,000 and 40,000. However, the M_r-98,000 protein remained the strongest and most consistently stained in KNRK cell extracts. Pre-incubation of IS R8 with putative heparanase peptide greatly inhibited staining of the KNRK M_r-98,000 band, even at 1:10 dilution (Figure 2), demonstrating that IS R8 expresses antibodies specific to the peptide, and that the M_r-98,000 protein represents a specific substrate for this antibody. Pre-immune serum from rabbit 8 (PS R8) failed to stain KNRK polypeptides at comparable dilutions (Fig. 2). Immune sera from rabbits R5–R7 were judged to be unsuitable for immunoselection, as numerous KNRK polypeptides were non-specifically stained with these sera (data not shown).

Immunodetection, characterization and sub-cloning of λgt11 recombinants reactive with R8 anti-serum

Because IS R8 was observed to strongly stain the M_r-98,000 protein at a dilution of 1:40, and because staining of this polypeptide was successfully abolished when the anti-serum was pre-incubated with the putative heparanase peptide, these conditions were also used for immunoscreening and plaque purification of the KNRK-specific λgt11 library. An estimated total of 240,000 recombinant plaques were screened in duplicate with anti-serum thoroughly pre-absorbed with bacterial extract. Forty-six positive plaques were obtained from the initial screening, and these were subjected to plaque purification. Duplicate filters of each plaque were stained in the absence or presence of peptide at 20 μg/ml. The results shown in Figure 3*a* illustrate filters containing impure populations of positively staining plaques against a background of weakly staining contaminants. A representative clone (1–2) is shown as an example of a positive recombinant whose staining was not inhibited by pre-incubation of IS R8 with peptide.

Of the 46 positives examined, a single plaque whose staining was inhibited by competition with putative heparanase peptide was detected (Fig. 3*a*). This recombinant was designated clone 10-4, and was purified to homogeneity. Phage DNA from clone 10-4 was isolated and characterized by cleavage with EcoRI to determine insert size and assess whether internal EcoRI sites were present. As shown in Figure 3*b*, EcoRI-cleaved clone 10-4 DNA yielded 2 bands of approximately 2.2 and 0.75 kb. Because the KNRK-specific λgt11 library was constructed using EcoRI/NotI adaptors to ligate cDNA inserts into λgt11 vector arms, full-length inserts may be excised using NotI, a rare-cutting restriction endonuclease. This enzyme was used to sub-clone phage clone 10-4 by ligation of the excised insert into a plasmid vector suitable for sequencing, pBluescript II KS(+). Cleavage of the recombinant plasmid obtained (pHEP2) with NotI confirmed an intact insert size of approximately 3 kb (data not shown).

Characterization and heparanase assay of β-galactosidase-clone 10-4 fusion proteins induced in bacterial lysogens

To determine whether lysogens expressing β-galactosidase-clone 10-4 fusion proteins actually exhibit heparanase activity, extracts were prepared from IPTG-induced cultures of clone 10-4 lysogens and compared with lysates from uninduced or IPTG-induced lysogens of non-recombinant λgt11, as well as KNRK cell extracts. In Figure 4*a*, immunoblots of protein from bacterial or mammalian cell extracts were stained with IS R8 in the absence or presence of 20 μg/ml of putative heparanase peptide. Because large amounts of protein were applied to the lanes of these gels, and because anti-serum not pre-absorbed with bacterial lysate was used for these experiments, substantial amounts of non-specific staining are evident in these blots. In particular, β-galactosidase (M_r 114,000) is visible as a prominent band in lanes containing lysates of

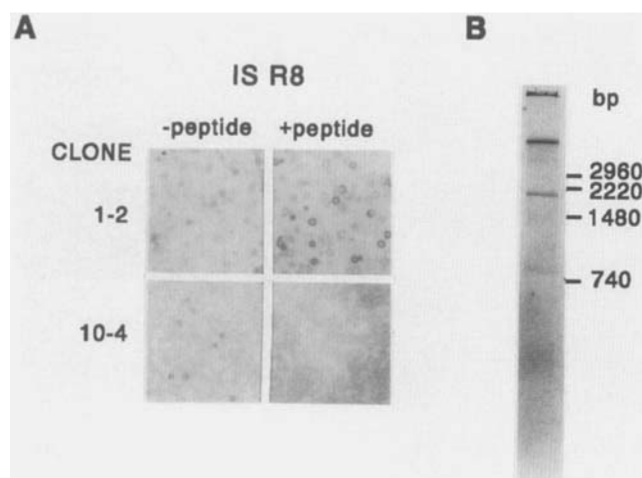

FIGURE 3 – *(a)* Peptide competition assay of positively staining recombinants immunoselected using putative heparanase peptide anti-serum IS R8. Duplicate filters containing impure populations of clones 1-2, 10-4 and 44 other positives were incubated with IS R8 at 1:40 in the absence (−peptide), or presence (+peptide) of putative heparanase peptide at 20 μg/ml. Washing of filters, incubation with alkaline-phosphatase-conjugated anti-rabbit IgG, and staining are as described in "Material and Methods". *(b)* Assessment of insert size by EcoRI cleavage of extracted clone 10-4 DNA. An aliquot of DNA extracted from clone 10-4 phage was digested with EcoRI and radiolabeled with *E. coli* DNA polymerase I Klenow fragment and [α-^{35}S]dATP as described in "Material and Methods". Fragments were separated in a 1.5% agarose gel which was stained with 0.5 μg/ml ethidium bromide, photographed, dried and autoradiographed. Marker fragments, run in the same gel, are partial digest fragments of pCB10, a derivative of pCB6 (ATCC).

IPTG-induced, non-recombinant λgt11 lysogen. However, this staining was not inhibited by peptide, nor was any other non-specific staining of bacterial or cell lysate protein. In contrast, clone 10-4 lysate exhibits a diffuse, but intensely stained smear of high-M_r proteins, whose staining is greatly reduced in the presence of peptide. Although some leaky expression of these proteins is evident in the absence of IPTG, their expression is greatly increased by IPTG, indicating that they represent a specific product of the fused β-galactosidase-clone 10-4 gene. The heterogeneity of migration of the IPTG-induced proteins is most likely due to degradation of the protein within the bacterial cell. It is also apparent from Figure 4*a* that B16F10 and KNRK lysates express proteins of M_r 98 000 and approximately 200 000, whose staining is also reduced in the presence of peptide. Figure 4*b* shows the heparanase activities of lysates from clone 10-4 or non-recombinant λgt11 lysogens. These lysates were observed to be uniformly negative for heparanase activity in comparison with KNRK cell lysate, irrespective of whether uninduced or IPTG-induced bacterial lysates were assayed. These results indicate that either the heparanase sequences encoded by clone 10-4 are non-functional, or clone 10-4 does not code for heparanase.

Sequencing of the insert termini of pHEP2 and identification of pHEP2 as GRP94/endoplasmin

To determine whether the pHEP2 insert bears significant homology to known cellular proteins, its termini were sequenced using 2 promoter-derived sequencing primers (T3, T7) found in pBluescript II KS(+). A third sequencing primer was a 16-fold divergent anti-sense oligonucleotide that binds to sense sequences encoding the last 12 amino acids in the putative amino-terminal heparanase peptide. Sequences obtained were compared with sequences present in various gene bank databases, using the BLAST algorithm (Altschul *et al.*, 1990). As shown in Figure 5*a,b* both termini of pHEP2 exhibit high levels of homology to the sequence of a previously characterized protein, M_r-94 000 glucose-regulated protein (GRP94, also known as endoplasmin). At the 5′ end of the cDNA sense strand, 9 third-base substitutions, and 3 first-base substitutions were observed, out of 49 codons surveyed. These substitutions are not necessarily consistent with differences in codon usage between mice and rats, since 6 of the 12 substitutions generated codons used less commonly in rats (Aota *et al.*, 1988), and GAT-to-GAC and GAC-to-GAT conversions were observed for distinct aspartic-acid codons within the sequences shown. However, despite the large number of altered codons, the amino-acid sequences of the 5′ region of pHEP2 and GRP94/endoplasmin were identical. In addition, the 5′ end also revealed DNA sequences encoding the amino-acid sequence of the putative amino-terminal heparanase peptide. Anti-sense sequences primed by the deduced oligonucleotide also corroborated the T7-primed sequence. The 3′ untranslated region of GRP94/endoplasmin and the inverted T3 primer-derived sequence are also strikingly similar, with 94% nucleotide sequence similarity. Since the 5′ and 3′ untranslated regions are usually the most divergent sequences observed between species, this finding implies that pHEP2 represents a cloned rat GRP94/endoplasmin sequence, as well as implying that a conserved functional role may exist for the 3′ untranslated region of this gene.

DISCUSSION

In the present study, we utilized a polyacrylamide-gel-based HS degradation assay to demonstrate that KNRK (a rat kidney fibroblast cell line transformed by the v-K-*ras*-expressing Kirsten murine sarcoma virus) exhibits heparan-sulfate-degrading activity similar to that exhibited by B16F10 mouse melanoma cells (Nakajima *et al.*, 1983). Using a rabbit anti-serum directed against a KLH-conjugated peptide purported to represent an amino-terminal sequence of purified B16F10 heparanase (Jin *et al.*, 1990), we attempted to immunoselect heparanase-expressing clones from a KNRK cell-specific λgt11 cDNA library. Of 46 positively staining clones obtained from our initial screen of 240 000 recombinants, we identified one lysogen that expressed a β-galactosidase fusion protein whose staining with heparanase peptide anti-serum was inhibited by competition with excess peptide. Sequencing of approximately 200 nucleotides from each end of the 3-kb insert revealed that it very likely represents a rat homologue of M_r-94 000 glucose-regulated protein (GRP94/endoplasmin).

GRP94/endoplasmin was initially detected as one of several proteins whose expression is induced in response to glucose deprivation (Shiu *et al.*, 1977). Tunicamycin and other inhibitors of protein glycosylation, as well as the Ca^{2+} ionophore A23187, are also capable of enhancing expression of GRPs 78 and 94 (Olden *et al.*, 1979; Welch *et al.*, 1983). Protein and cDNA sequence analyses have revealed that GRP94, endoplasmin, tumour-rejection antigen and ERp99 are identical gene products (Koch *et al.*, 1986; Lee *et al.*, 1984; Srivastava *et al.*, 1987; Mazzarella and Green, 1987). The complete cDNA of GRP94/endoplasmin contains an open reading frame of 3 kb encoding a peptide of M_r 92 475, with high mannose oligosaccharides attached to at least 1 of 6 N-linked glycosylation sites (Mazzarella and Green, 1987).

GRPs have been localized to the lumen of rough endoplasmic reticulum (Koch *et al.*, 1986), where they have been implicated in regulating the folding of nascent polypeptides. Induction of both GRPs 78 and 94 are observed when mutant forms of influenza virus haemagglutinin are over-expressed, resulting in malfolding and blockage of transport through the ER (Kozutsumi *et al.*, 1988). The extrapolated amino-acid sequence of GRP94/endoplasmin features a hydrophobic

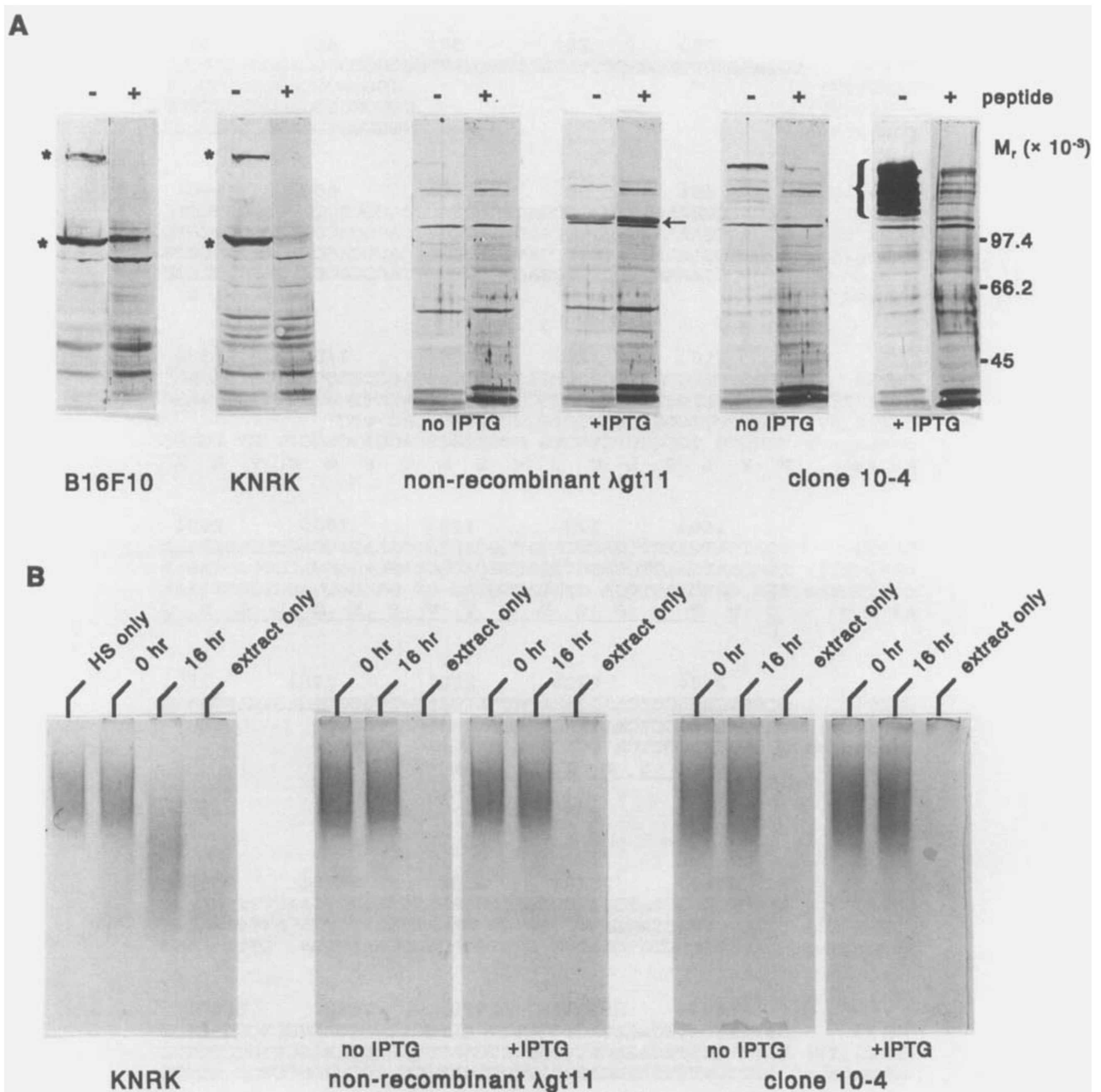

FIGURE 4 – *(a)* Immunoblots of proteins from lysates of B16F10 and KNRK cells, and *E. coli* Y1089 lysogenic for either non-recombinant λgt11 or recombinant clone 10-4. B16F10 and KNRK cell extracts, and extracts from uninduced (no IPTG) or IPTG-induced (+IPTG) bacterial cultures were prepared as described in "Material and Methods". Aliquots of 100 μg of lysate protein were separated in 7.5% SDS-PAGE and transferred to nitrocellulose by electroblotting for 3 hr at 70 V. Gels run in parallel were also stained with Coomassie blue R-250 to ensure equal loading of protein. Blots were blocked and treated with anti-serum IS R8 in the absence (−) or presence (+) of putative heparanase peptide at 20 μg/ml. Positions of M_r standards run in parallel are indicated at right. The M_r 98 000 and approximately 200 000 proteins from KNRK and B16F10 lysates are indicated by *, whereas β-galactosidase (M_r 114 000) and β-galactosidase-clone 10-4 fusion protein are indicated by the arrow and bracket respectively. Positions of M_r standards run in parallel with the samples are shown at right. *(b)* Heparanase assays of lysates from KNRK cells and *E. coli* Y1089 lysogenic for either non-recombinant λgt11 or recombinant clone 10-4. Reactions of 30 μl contained 1.5–3 μg of bovine kidney HS and 60 μg of extracts from uninduced (no IPTG) or IPTG-induced (+IPTG) bacterial cultures, or KNRK cells. After overnight incubation, reactions were treated with micrococcal nuclease, proteinase K and SDS as described in "Material and Methods", and ⅓ of the total reaction volumes were loaded onto 7.5% polyacrylamide gels in TAC buffer. Lanes designated HS only or extract only denote reactions carried out in the absence of cellular extract or HS respectively.

region characteristic of membrane-spanning domains, and a cleaved amino-terminal signal implicated in ER translocation (Mazzarella and Green, 1987). No discrete enzymatic function has yet been associated with GRP94, although it binds Ca^{2+} with high capacity and relatively low affinity (Macer and Koch, 1988). The induction of GRPs in response to numerous types

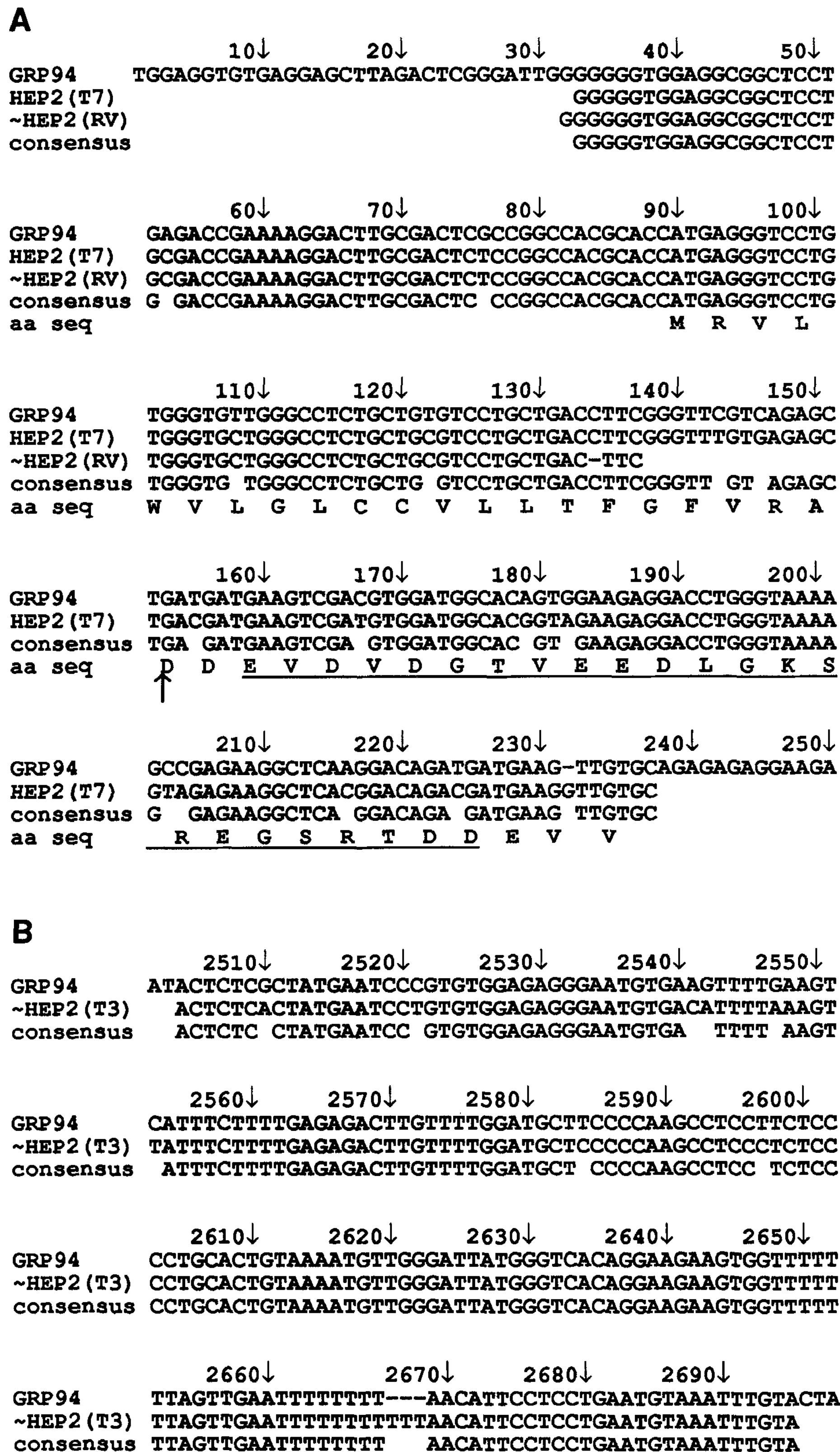

FIGURE 5 – Sequence comparison of 5′ *(a)* and 3′ *(b)* insert termini of the sense strand of pHEP2 (clone 10-4 insert sub-cloned in pBluescript II KS(+)), with GRP94/endoplasmin. Sequencing primers used (T7, T3, RV-anti-sense oligo) are indicated. Inverted sequences are prefixed by ~. Nucleotide sequences in common and deduced peptide sequences are shown in lines marked consensus and aa seq respectively. Amino-acid sequences matching the putative heparanase amino terminus are underlined. The cleavage site of the GRP94/endoplasmin amino-terminal signal is indicated by an upward arrow. Nucleotides are numbered according to the cDNA sequence of GRP94/endoplasmin (Mazzarella and Green, 1987); using this numbering system, the termination codon is located at nucleotides 2496-8, and the polyadenylation signal is at nucleotides 2740-5.

of diverse stimuli, as well as significant homologies to heat-shock proteins hsp70 and hsp90, indicate that GRP94/endoplasmin belongs to a superfamily of stress-related proteins (Gething and Sambrook, 1992).

Evidence obtained from our study leaves little doubt that anti-serum against a putative amino-terminal sequence from B16F10-derived heparanase has specifically selected a clone representing GRP94/endoplasmin. The insert size of clone 10-4 is approximately 3 kb and contains a single EcoRI site approximately 750 bp from the 5′ terminus of the sense strand, consistent with the known size and sequence of full-length GRP94/endoplasmin mRNA (Mazzarella and Green, 1987). Terminal sequences from clone 10-4 insert exhibit homology of 90 to 95% to both the 5′ coding, and 3′ untranslated sequences of murine GRP94/endoplasmin, with 100% conservation of amino-acid sequence over the 49 amino-terminal residues. Immune serum R8, directed against the putative amino-terminal heparanase peptide, stains proteins of M_r 98 000 and approximately 200 000 in immunoblots of B16F10 and KNRK cell lysates. The staining of both proteins is inhibited upon competition by heparanase peptide. The appearance of the 200-kDa protein evident in these lysates is also consistent with GRP94/endoplasmin, since it has been observed to form a homodimer (Mazzarella and Green, 1987). Immunochemical staining of β-galactosidase-clone 10-4 fusion protein with IS R8 is also inhibited by competition with putative heparanase peptide, both in immobilized plaques and in Western blots. However, this clone is inactive when assayed for heparanase activity in lysates of lysogens expressing the fusion protein. Although we cannot rule out the possibility that heparanase contains an identical peptide sequence, or that clone 10-4 insert sequences are inactive in the fusion protein, the results of this study call into question the specificity of this peptide sequence to heparanase, as well as previous immunolocalization studies of heparanase carried out using such an anti-serum (Jin *et al.,* 1990). Interestingly, the putative amino-terminal heparanase sequence is located close to the amino terminus of mature GRP94/endoplasmin, only 2 amino acids to the carboxyl side of the signal peptide cleavage site. We speculate that GRP94/endoplasmin may have been co-purified with heparanase in previous studies from which the peptide sequence originated (Jin *et al.,* 1990). This is especially likely given the role of GRP94/endoplasmin, a molecular chaperone with protein-binding properties. Co-purification may have occurred through incidental protein-protein interactions, or alternatively, GRP94/endoplasmin may be required for the proper folding of heparanase during its maturation and targeting as a membrane-associated or secreted molecule.

We have also demonstrated that KNRK cells, like B16F10 melanoma cells, express HS-degrading activity, although at present we cannot state whether these enzymes are identical or distinct. Potential roles and contexts for heparanase expression in KNRK cells may relate to normal physiological functions of heparanases in non-transformed cells. Although heparanases are associated with intravasation and extravasation in metastatic cells, they are also involved in neovascularization or tissue repair by releasing ECM-associated basic fibroblast growth factor (bFGF), a potent mitogen for mesenchymal cells that lacks signal sequences for secretion (Saksela *et al.,* 1988). Release of active bFGF from the ECM is thought to trigger neovascularization or wound healing in normally quiescent endothelium (Vlodavsky *et al.,* 1991). In such a context, it may be speculated that mesenchymally derived NRK cells would constitutively express heparanase only in sub-confluent culture, a condition that would mimic wound healing *in vivo.* Preliminary assays of NRK cellular heparanase activity have indicated that these cells may indeed be capable of degrading HS (data not shown), although we have not yet attempted to relate heparanase activity to culture density, or compared heparanase activity before and after transformation by v-K-*ras.* Expression of heparanase in actively growing NRK cells would also imply that heparanase expression by KNRK cells would result from unrestricted growth and absence of density-dependent growth inhibition, and not necessarily from oncogenic transformation *per se.* Previous studies have shown that 10T1/2 cells transfected with activated H-*ras* exhibited both elevated heparanase activity and a strongly metastatic phenotype (Schwarz *et al.,* 1990). However, NIH 3T3 cells transfected with activated H-*ras* did not exhibit increased heparanase activity, indicating that enhancement of heparanase activity upon transformation may actually depend on cell type and its capacity for heparanase expression during normal growth-related processes. Further characterization of the KNRK HS-degrading activity and its possible roles in metastasis and normal cellular processes await its cloning and transfection into non-transformed cells.

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