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Research Article

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A Novel Variant of Human Grb7 Is Associated with Invasive Esophageal Carcinoma

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Abstract

The cDNAs of a putative growth factor-bound (Grb) 7 signal transduction molecule and Grb7V novel splice variant were isolated from an invasive human esophageal carcinoma. Although both Grb7 isoforms share homology with the Mig-10 cell migration gene, the Grb7V isoform lacks 88 base pairs in the C terminus; the resultant frame shift leads to substitution of an SH2 domain with a short hydrophobic sequence. The wild-type Grb7 protein, but not the Grb7V isoform, is rapidly tyrosyl phosphorylated in response to EGF stimulation in esophageal carcinoma cells. Analysis of human esophageal tumor tissues and regional lymph nodes with metastases revealed that Grb7V was expressed in 40% of Grb7-positive esophageal carcinomas. More importantly, Grb7V expression was enhanced after metastatic spread to lymph nodes as compared to the original tumor tissues. Finally, transfection of an antisense Grb7 RNA expression construct lowered endogenous Grb7 protein levels and suppressed the invasive phenotype exhibited by esophageal carcinoma cells. These findings suggest that Grb7 isoforms are involved in cell invasion and metastatic progression of human esophageal carcinomas. (*J. Clin. Invest.* 1998. 102:821–827.) Key words: Grb7 • Mig-10 • splice variant • esophageal carcinoma • metastasis

Introduction

Metastatic spread of tumor cells is one of the major risk factors affecting clinical prognosis. There are several sequential steps in this process including cell migration into the surrounding host tissue, infiltration, and penetration into blood and/or lymphatics for dissemination, attachment to capillary beds of distant organs, cell extravasation, and subsequent growth in tissues (1). Some of the mechanisms involving normal cell migration observed during development are believed to be closely associated with the properties of tumor cell invasion. Thus, the so-called “cell migration genes” may be frequently used by cancer cells to allow for metastatic spread of disease.

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One of the most interesting approaches to define molecular components involved in the control of cell movement is the genetic analysis of mutations that disrupt specific cell migration patterns and the subsequent characterization of the corresponding genes involved in these processes. *Caenorhabditis elegans* is an excellent animal model to perform a genetic analysis of cell migration (3). Positional cloning of the corresponding genes have defined various key molecules required for cell migration during embryogenesis. For example, several mutations affecting the long-range anterior migration of the canal-associated neurons during embryogenesis have been defined (4). Characterization of the Mig-10 gene has revealed important information regarding such cell migration associated events (5). The Mig-10 gene encodes for two isoforms that contain proline-rich sequences in both of the NH₂ and COOH termini and a distinct central region composed of ~300 amino acids with a pleckstrin homology (PH)¹ domain. Interestingly, the central region of Mig-10 has been found to be shared by a recently described family of mammalian Src-homology-2 (SH2) domain-containing proteins designated as growth factor-bound (Grb) 7, Grb10/IRb, and Grb14 (6, 7, 8). These molecules appear to function as essential components in signal transduction pathways after receptor tyrosine kinase (RTK) activation by EGF, insulin, or PDGF. The structural homology between the Grb7 family and Mig-10 proteins in the central part of the molecule has been designated as the GM region (5). The Grb7 family of proteins also contains a proline-rich sequence in the NH₂ terminus and an SH2 domain in the COOH terminus, whereas the Mig-10 protein lacks such a domain.

Because of these structural similarities that may imply a biologic function with respect to cell migration, we searched for the expression of Grb7 and potential isoforms in esophageal carcinomas with metastatic spread to adjacent tissues (9). In this study, a novel splice variant designated as Grb7V was isolated and characterized. We found that both Grb7 and Grb7V contain the GM domain. However, the Grb7V isoform lacks an SH2 domain in the COOH terminus. Analysis of the human esophageal tumor tissues revealed that enhanced expression of Grb7 and Grb7V was associated with metastatic progression of the disease. Our findings suggest a role for these proteins in cell migration and invasion by human esophageal carcinomas.

Methods

Cloning of human Grb7 cDNA. The NH₂-terminal sequence and GM region of the human Grb7 cDNA has been previously reported (9). To clone the full-length cDNA of human Grb7, we used RACE and degenerate PCR primer sequences reported for murine Grb7 as de-

1. Abbreviations used in this paper: Grb, growth factor-bound; PH, pleckstrin homology; PTP, protein tyrosine phosphatase; RT, reverse transcriptase; RTK, receptor tyrosine kinase; SH, Src-homology.

scribed (6). Total RNA was extracted from an esophageal carcinoma tissue or from the KYSE410 esophageal carcinoma cell line followed by reverse transcriptase (RT) PCR amplification (9). The PCR products were cloned using the TA cloning kit (Invitrogen Inc., Carlsbad, CA) and DNA sequences determined by a cycle sequencing procedure (Applied Biosystems Inc., Foster City, CA). To certify the presence of transcripts in esophageal carcinoma cells, the full coding region of human Grb7 was amplified by RT-PCR using the two primers (5'-GGACAAGGGCACACAACCTGGTTC-3' and 5'-CGCCCCAGATCCACTGGATGG-3').

Analysis of Grb7 protein expression in human esophageal carcinoma cells. Grb7 protein expression was detected by immunoblot analysis using three different antibodies directed against the NH₂-terminal 1–205 aa residues of the Grb7 proline-rich and GM regions (Transduction Laboratories, Lexington, KY), NH₂-terminal 2–21 aa residues (N-20; Santa Cruz Biochemistry, Santa Cruz, CA) and COOH-terminal 516–535 aa residues (C-20; Santa Cruz Biochemistry) as described (10). Cell lysates were prepared in cold Triton-lysis buffer (50 mM Tris-HCl, pH 7.5 containing 1% Triton, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM Na₄P₂O₇, 2 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml aprotinin, 3.5 mg/ml pepstatin A and 25 mg/ml leupeptin) after cells were treated with 6-h serum starvation followed by the addition of 100 ng/ml EGF for 10 min. Next, 100 µg of total protein was electrophoresed through SDS-polyacrylamide gels and proteins transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). Immunodetection of proteins was performed with Grb7 antibodies using the enhanced chemiluminescence system (Amersham Corp., Arlington, IL). To determine tyrosyl phosphorylation of Grb7, cell lysates containing 500 µg of protein were incubated with 10 µg of the Grb7 antibody and immunoprecipitated with protein A-agarose. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis followed by immunodetection with the specific antibody (PY20) directed against phosphotyrosine residues (Transduction Laboratories).

Analysis of Grb7 expression in human esophageal carcinoma tissue and tumor metastatic to regional lymph nodes. To analyze the pattern of Grb7 expression in clinical samples, we studied 43 primary squamous cell esophageal tumors and eight metastatic lymph nodes obtained at the time of surgical resection. Amplification of transcripts by 25 cycle RT-PCR was performed using the following primers as previously described (9); Grb7: 5'-GCCTGGAGGAAGAAGACA-AACCAC-3' and 5'-CTCCTCATCCCGTCCCCTGTGG-3' (product size = 505 bp or 417 bp). As a control, we performed RT-PCR analysis of glyceraldehyde-3-phosphatase dehydrogenase mRNA (GAPDH) using 5'-GTCAACGGATTGGTCTGTATT-3' and 5'-AGTCTTCTGGGTGGCAGTGAT-3' sequences as primers (product size = 560 bp). All PCR primers were selected to span introns in order to detect specific mRNA sequences. To detect Grb7 protein expression, tissue lysates were prepared in cold Triton-lysis buffer and 100 µg of total protein was electrophoresed through SDS-polyacrylamide gels and proteins transferred onto Immobilon-P membranes (Millipore Corp.). Immunodetection of proteins was performed with Grb7 antibodies using the enhanced chemiluminescence system (Amersham Corp.). For histological examination, tissues were fixed with periodate-lysine-paraformaldehyde at 4°C at the time of surgical resection, embedded in OCT compound (Miles, Elkhart, IN), and stored at -80°C. In addition, specimens (*n* = 20) were stained with hematoxylin-eosin and cellular identification of the Grb7 protein was performed with an antibody specific for the NH₂ terminus followed by immunodetection using the avidin-biotin-peroxidase complex method.

Effects of antisense Grb7 mRNA on invasion of human esophageal carcinoma cells. The 5'-region of Grb7 was amplified by PCR and subcloned in the reverse orientation into the pcDNA3 vector and subsequently transfected into KYSE410 esophageal carcinoma cells followed by G418 selection as described (11). After selection in the presence of 600 µg/ml of G418, the levels of Grb7 protein were examined in each clone by immunoblot analysis using an anti-Grb7 anti-

body directed against the NH₂ terminus. Grb2 protein expression was also analyzed as a control using an anti-Grb2 rabbit polyclonal antibody (Santa Cruz Biotechnology). Cell growth in culture was assessed as described (11). Assays of cell invasion properties were performed according to Albini et al. (14). This technique uses a modified Boyden chamber with polyethylene terephthalate filter inserts coated with a Matrigel matrix in 24-well plates containing 8-mm pores. In brief, 10⁵ esophageal tumor cells were suspended in serum-free medium with 0.5% BSA, and plated onto the upper chamber followed by filling the lower chamber with the same medium in the presence or absence of 100 ng/ml EGF. Cells were then cultured for 18 h, fixed with 5% glutaraldehyde in PBS, and stained with 0.5% trypan blue in 2% Na₂CO₃. Cells on the upper side of the filter were carefully removed and those cells invading the lower side were counted by microscopic examination. The mean migration rate of three independent experiments was determined for each clone with reduced Grb7 protein expression as the result of antisense effects.

Results

Two isoforms of human Grb7 are found in esophageal carcinomas. Previous studies have established that coexpression of Grb7 and RTK were associated with invasive esophageal carcinomas. However, 30% of Grb7-positive esophageal tumors did not express RTK (9). To analyze the pattern of Grb7 expression in these invasive carcinomas, a full-length human Grb7 cDNA was isolated from a cDNA library prepared from an esophageal carcinoma using a partial NH₂-terminal sequence of Grb7 (9). Two individual cDNA clones (2.2 and 2.1 kb) were obtained and designated as human Grb7 and Grb7V spliced variant. These two isoforms were also detected in the human esophageal carcinoma cell line KYSE410. The human Grb7 cDNA encodes for 532 aa (Fig. 1) and this molecule shares 90% identity with the murine Grb7 protein as reported by Margolis et al. (6). The predicted Grb7 protein contains a proline-rich sequence in the NH₂ terminus and a SH2 domain in the COOH terminus. The Grb7 central region includes a PH domain and is homologous to the Mig-10 cell migration molecule identified in *C. elegans* as shown in Fig. 2, A and B.

In contrast, the Grb7V isoform lacks an 88-bp region encoding for the SH2 domain found in human Grb7. In the Grb7V cDNA, a frame shift has led to a coding region for 447 amino acids and the deleted SH2 domain has been substituted with a short hydrophobic sequence in the COOH terminus (Fig. 2 B). It is of interest that this sequence shares 50% identity with the COOH-terminal tail of the human homeobox protein HoxB4 (SGGAAGSAGGPPGRPPNGGPRAL; underlined are the identical sequences between the two proteins) (12).

Expression and tyrosyl phosphorylation of Grb7 isoforms in esophageal carcinoma cells. We searched for in vitro expression of Grb7 isoforms in the KYSE410 human esophageal carcinoma cell line by RT-PCR. As shown in Fig. 3 A, two cDNA fragments of 1731 and 1643 bp in length were recognized and certified as human Grb7 and Grb7V by direct sequencing. Immunoblot analysis using an antibody directed against the NH₂-terminal region of Grb7 (Transduction Laboratories) revealed two distinct proteins of 59 and 50 kD that are the predicted sizes of the Grb7 and Grb7V proteins, respectively (Fig. 3 B, Grb7-N). In contrast, a different Grb7 antibody directed against the COOH terminus (C-20; Santa Cruz Biochemistry) identified the 59-kD protein of Grb7 but not the 50-kD protein of Grb7V, as it is truncated at the COOH

Grb7

atggagctggatctgtctccaactcatttagcaagctctccggaagaccttggccagcc 60
M E L D L S P P H L S S S P E D L W P A 20
cctgggaccctcctgggactccccgccctgataccctctgctgaggaggtaaag 120
P G T P P G T P R P P D T P L P E E V K 40
aggtccagcctctcctcatcccaaccaccggcaggaaacttcgagaggaggagggcgt 180
R S Q P L L I P T T G R K L R E E E R R 60
gccacctccctccctctatcccaacccttccctgagctctgcaagctccctccacag 240
A T S L P S I P N P P P E L C S P P S Q 80
agcccaattctcggggccctccagtgcaaggggctctcccccgcgatgccagccgc 300
S P I L G G P S S A R G L L P R D A S R 100
ccccatgtagttaaagtgtagcagtgaggatggggcctgcaaggtctgtaggtggcaca 360
P H V V K V Y S E D G A C R S V E V A A 120
ggtgccacagctcggccagctgtggaatgctggtgcaagcagctcagccttgagcgc 420
G A T A R H V C E M L V Q R A H A L S D 140
gagacctggggctggtggagtgcacccccctcagcactggagcgggtttggaggac 480
E T W G L V E C H P H L A L E R G L E D 160
cagcagctcctggtggaagtgcagctgctggccctggggggagatagccgctctgctc 540
H E S V V E V Q A A W P V G G D S R F V 180
ttccggaaaaactcggcaagtgcacactgttcaagagctcccccactccctgttccca 600
F R K N P A K Y E L F K S S P H S L L F P 200
gaaaaaatggtctccagctgtctcagatgcacacctgglatcccatgaagacctcctc 660
E K M V S S C L D A H T G I S H E D L I 220
cagaacttctgaatgctggcagcttccctgagatccagggctttctcagctcgggggt 720
Q N F L N A G S F P E T Q G F L Q L R G 240
tcaggacggaaacttggaaagcctttctgtttcttgcgcagatcggcctctattac 780
S G R K L W K R F F C F L R R S G L Y Y 260
tccaccaaggccactcagaagtcaggcagcctgcagtagctggcagatgtgaacgag 840
S T K G T S K D P R H L Q Y V A D V N E 280
tccaaactgtacgtggtgacgagggcggcagctcagggatgccactgacttcgggt 900
S N V Y V V T Q G R K L Y G M P T D F G 300
ttctgtcgaagcccaacaagctcgaatggacacaggggcttcggatctctcagct 960
F C V K P N K L R N G H K G L R I F C S 320
gaagtagagcagagccgacctgctggctggctgctccgctctcagtagcgggggtg 1020
E D E Q S R T C W L A A F R L F K Y G V 340
cagctgtacaagaattaccagcaggcagctcgcactcgcactcctctgtttgggc 1080
Q L Y K N Y Q Q A Q S R H L H P S C L G 360
tccccaccttgagaagtgcctcagataataccctggtggccatggactctctggccat 1140
S P P L R S A S D N T L V A M D F S G H 380
gctgggctgtcattgagaaccocgggaggtctgagvtggtgcccctggaggagccag 1200
A G R V I E N P R E A L S V A L E E A Q 400
gcctggaggaagaagacaaaccaccgctcagcctgccatgccagcctccggcagcagc 1260
A W R K K T N H R L S L P M P A S G T S 420
ctcagtgcaagcctccacgaccccaactcgttccacgggcccattccctgaggag 1320
L S A A I H R T Q L W F H G R I S R E E 440
agccagcggcttattggacagcgggcttggtagcagcctgttccctggtccggagag 1380
S Q R L I G Q Q G L V D G L F L V R E S 460
cagcggaaaccocgggcttctcctctcttggccacctgcagaaagtgaagcattat 1440
Q R N P Q G F V L S L C H L Q K V K H Y 480
ctcactcgcagagcaggagggtgctgtaactcagcagatgatggcagacc 1500
L I L P S E E B G R L Y F S M D D G Q T 500
cgcttactgacctgctgagcctggtggattccaccagctgaaccggcagcctccg 1560
R F T D L L Q L V E F H Q L N R G I L P 520
tgcttctgctgcccattgctgcaagcgggtggccctctga 1596
C L L R H C C T R V A L * 532

Grb7V

atggagctggatctgtctccaactcatttagcaagctctccggaagaccttggccagcc 60
M E L D L S P P H L S S S P E D L W P A 20
cctgggaccctcctgggactccccgccctgataccctctgctgaggaggtaaag 120
P G T P P G T P R P P D T P L P E E V K 40
aggtccagcctctcctcatcccaaccaccggcaggaaacttcgagaggaggagggcgt 180
R S Q P L L I P T T G R K L R E E E R R 60
gccacctccctccctctatcccaacccttccctgagctctgcaagctccctccacag 240
A T S L P S I P N P P P E L C S P P S Q 80
agcccaattctcggggccctccagtgcaaggggctctcccccgcgatgccagccgc 300
S P I L G G P S S A R G L L P R D A S R 100
ccccatgtagttaaagtgtagcagtgaggatggggcctgcaaggtctgtaggtggcaca 360
P H V V K V Y S E D G A C R S V E V A A 120
ggtgccacagctcggccagctgtggaatgctggtgcaagcagctcagccttgagcgc 420
G A T A R H V C E M L V Q R A H A L S D 140
gagacctggggctggtggagtgcacccccctcagcactggagcgggtttggaggac 480
E T W G L V E C H P H L A L E R G L E D 160
cagcagctcctggtggaagtgcagctgctggccctggggggagatagccgctctgctc 540
H E S V V E V Q A A W P V G G D S R F V 180
ttccggaaaaactcggcaagtgcacactgttcaagagctcccccactccctgttccca 600
F R K N P A K Y E L F K S S P H S L L F P 200
gaaaaaatggtctccagctgtctcagatgcacacctgglatcccatgaagacctcctc 660
E K M V S S C L D A H T G I S H E D L I 220
cagaacttctgaatgctggcagcttccctgagatccagggctttctcagctcgggggt 720
Q N F L N A G S F P E T Q G F L Q L R G 240
tcaggacggaaacttggaaagcctttctgtttcttgcgcagatcggcctctattac 780
S G R K L W K R F F C F L R R S G L Y Y 260
tccaccaaggccactcagaagtcaggcagcctgcagtagctggcagatgtgaacgag 840
S T K G T S K D P R H L Q Y V A D V N E 280
tccaaactgtacgtggtgacgagggcggcagctcagggatgccactgacttcgggt 900
S N V Y V V T Q G R K L Y G M P T D F G 300
ttctgtcgaagcccaacaagctcgaatggacacaggggcttcggatctctcagct 960
F C V K P N K L R N G H K G L R I F C S 320
gaagtagagcagagccgacctgctggctggctgctccgctctcagtagcgggggtg 1020
E D E Q S R T C W L A A F R L F K Y G V 340
cagctgtacaagaattaccagcaggcagctcgcactcgcactcctctgtttgggc 1080
Q L Y K N Y Q Q A Q S R H L H P S C L G 360
tccccaccttgagaagtgcctcagataataccctggtggccatggactctctggccat 1140
S P P L R S A S D N T L V A M D F S G H 380
gctgggctgtcattgagaaccocgggaggtctgagvtggtgcccctggaggagccag 1200
A G R V I E N P R E A L S V A L E E A Q 400
gcctggaggaagaagacaaaccaccgctcagcctgccatgccagcctccggcagcagc 1260
A W R K K T N H R L S L P M P A S G T S 420
ctcagtgcaagcctgttccgctcgggagagctcagcggaaaccocgggctttgtcctct 1320
L S A A I H R T Q L W F H G R V S G T P R A L S S 440
ctttgtgccacctgcagaaagtga 1351
L C A T C R K * 447

Figure 1. Coding region and predicted amino acid sequences of Grb7 and Grb7V isoforms isolated from human esophageal carcinoma tissue (EC3). The underlined amino acid sequence indicates the GM region and SH2 domain. The bold sequences indicate the proline-rich sequences in the NH₂-terminal region of Grb7 proteins. The arrows represent potential splicing sites. These Grb7 and Grb7V sequences are available from GenBank/EMBL/DDBJ under accession numbers AB008789 and AB008790, respectively.

terminus (Fig. 3 B, Grb7-C). Murine Grb7 has been reported to be tyrosyl phosphorylated after EGF stimulation in breast carcinoma cell lines (13). The tyrosyl phosphorylation profile of Grb7 and Grb7V proteins was then analyzed using the KYSE410 esophageal carcinoma cell line with or without EGF additions as shown in Fig. 3 B. We found that the 59-kD Grb7 protein became tyrosyl phosphorylated in EGF-stimulated as compared to quiescent serum-starved cells. In contrast, EGF additions were unable to increase the tyrosyl phosphorylation of the 50-kD Grb7V protein.

Analysis of Grb7 expression in human esophageal carcinoma. We have previously reported overexpression of the Grb7/Grb7V gene in invasive esophageal carcinoma tissues (9). In this study, expression of Grb7/Grb7V proteins in invasive esophageal carcinomas was determined by immunohistochemistry using an NH₂-terminal Grb7 specific antibody (N-20; Santa Cruz Biochemistry). As demonstrated in Fig. 4 A, the invading esophageal carcinoma cells overexpress the proteins, whereas other cells and surrounding tissue elements have little if any expression. To examine the potential clinical

A

Grb7 VKVYSEDGACRSVEVAAGATARHVCEMIVQRA--H-ATSDETWGLVECHEHTALERGLE
Mig-10 VKFFVEDGEALQLLIDERWT--VADTLKQLAEKNHIALM-EDHCTVEEYELYIKRVYE

Grb7 DHSSVVEVQAAWFVGGDSRFV--FRKNFAKYELFKSSPHS-IFPEK---MVSSCIDAHT
Mig-10 DHKVVFENIQMW-WO-DSPNKLYFMRRPDKYA-FISRELYILTRTSDHEIPSGDOWT

Grb7 GTSHEDLICNFLNAGSF-----PEICGFLCIRGSGRKLWKRFFCFLRSGLYY---ST
Mig-10 -I---DVKKQKFVSEY-FHREPVVPEFMEGELYLKSDCRKSWKKHYFVLPSGLYYAPKSK

Grb7 RGTSKDPRHLOYVADVNESN-VYVVTOGRKLYGMPTDFGFCVKPNKL---RNGH-KGLRT
Mig-10 KPTTKDLTCLMNL---HSNQVYTGIGWEKKYKSPTPWCISIKLTALOMKRSOFIKY--I

Grb7 FCSEDEQSRTQWLAAFRLFKYGVQIYKNYQCA-CSRHLHPSCLGSPPLRSA-SDNTL---
Mig-10 -CAEDEMTFKKWLVAIRIAKNGAELLENYERACQIRR---ETLGPASSMSAASSTAISE

Grb7 VAMDFSGH
Mig-10 VPHSISHH

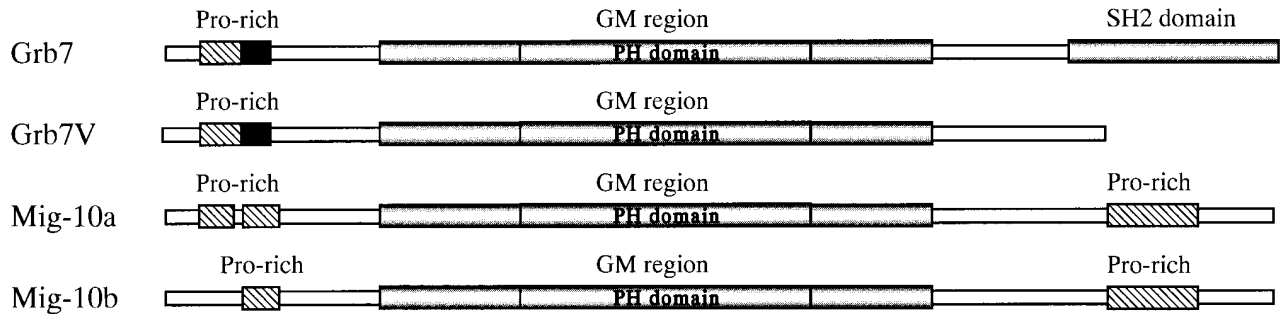
B

Figure 2. This is a sequence comparison of the GM domains of Grb7 and Mig-10 proteins. (A) Regions of identity are shaded. The underlined amino acid sequences indicate the PH domains. (B) Scheme demonstrating the structural and functional domains of human Grb7 isoforms and *C. elegans* Mig-10. The Mig-10 gene encodes two isoforms (Mig-10a and 10b) as described (6). Black boxes indicate proline-rich sequences that bind to the SH3 domain of c-Abl.

significance of Grb7 and Grb7V expression, a specific RT-PCR was performed to distinguish the parent molecule from its isoform in human esophageal tumor tissues as well as in regional lymph nodes containing metastatic disease, as shown in Fig. 4, B and C. The primers selected spanned the spliced region to detect Grb7 as a 505-bp band and Grb7V as 417-bp band, respectively. The Grb7 mRNA expression was detected in 20 of 43 tumors (47%). In addition, expression of the Grb7V isoform was present in eight of Grb7-positive (40%) but not in Grb7-negative tumors. More importantly, Grb7V expression was found only in the carcinomas that had invaded the adventitia and, therefore, demonstrated a metastatic phenotype. In this regard, we have previously shown that increased expression of Grb7 was associated with invasive esophageal carcinomas that also overexpress RTK, as exemplified by tumors EC4 and EC5 presented in Fig. 4, B and C. In contrast, Grb7V expression was not only recognized in RTK-overexpressing carcinomas (EC4) but also in invasive carcinomas, without such overexpression as exemplified by EC2 and EC3. Additionally, we searched for Grb7 and Grb7V expression in regional lymph nodes derived from eight metastatic carcinomas of the esopha-

cus. Five metastatic lymph nodes contained detectable Grb7 expression that matched the primary tumors. It is noteworthy that Grb7-positive lymph nodes containing metastatic disease had enhanced expression of Grb7V compared to the original tumor tissues as demonstrated by the representative examples EC6 in Fig. 4, B and C. These findings suggest a relationship of Grb7V expression to invasion and metastatic spread of human esophageal carcinoma tumor cells.

Effects of reduced Grb7 protein levels on the migration properties of human esophageal carcinoma cells. Finally, the effect of antisense mRNA expression on Grb7 protein levels with respect to alterations of the phenotype of KYSE410 esophageal carcinoma cells was investigated. Stable cloned cell lines were isolated and grown for further analysis after transfection of mock DNA or antisense Grb7-expression vectors containing the neomycin resistance gene (11). As shown by the immunoblots demonstrated in Fig. 5 A, mock DNA-transfected cells express both the 59- and 50-kD proteins to a similar degree as observed in the parental KYSE410 esophageal carcinoma cell line. We obtained three independent stable clones designated as A-1, A-2, and A-3, that express variable

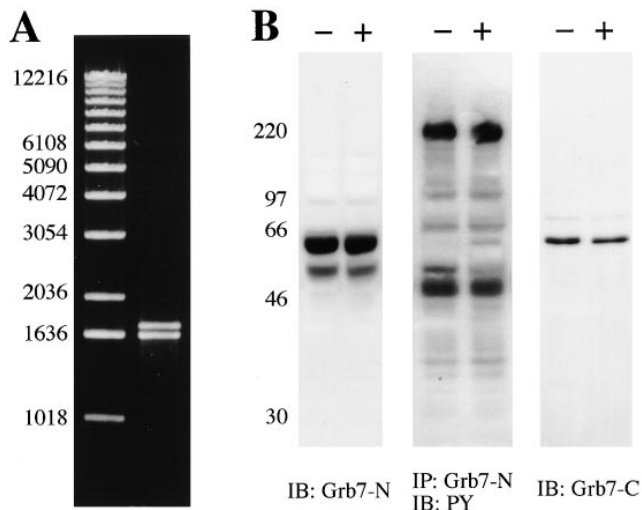


Figure 3. Transcription and protein expression of two Grb7 isoforms in a human esophageal cell line. (A) A RT-PCR of the full coding regions of human Grb7 revealed two mRNA fragments (1731 bp and 1643 bp) in the KYSE410 cells. (B) Left lane shows immunoblot analysis using an antibody directed against the NH₂-terminal of the human Grb7 protein. Two distinct proteins of 59 and 50 kD are detected in KYSE410 cells with or without EGF stimulation. Middle lane shows tyrosyl phosphorylation of the proteins after immunoprecipitation with the anti-NH₂-terminal Grb7 antibody. Phosphorylation of the 59-kD Grb7 protein was detected only in EGF-stimulated cells, whereas the 50-kD Grb7V isoform does not respond to EGF stimulation. The other bands have the predicted size of known proteins that bind to phosphorylated Grb7, such as RTK, SHPTP2, and Shc protein as previously described (13, 16). Right lane shows that the antibody directed against the COOH terminus of Grb7 reacts with the 59 kD (Grb7) protein but not with the 50 kD protein (Grb7V), as it is truncated at the COOH terminus.

but low amounts of both Grb7 and Grb7V proteins after stable transfection of the antisense Grb7 expressing construct into KYSE410 cells. The expression of a control protein such as Grb2 was not effected in each cell line. There was no difference with respect to the doubling times of the transfectants; 30.5 ± 1.8 h for A-1, 29.9 ± 2.6 h for A-2, and 30.7 ± 1.1 h for A-3, compared to 31.2 ± 1.1 h for parental KYSE410 cells and 29.5 ± 1.6 h for mock DNA-transfected cells, indicating that the downregulation of Grb7 expression by antisense RNA had no growth inhibitory effects on these esophageal carcinoma cell lines. However, the potential for cell invasion was altered as demonstrated by the Matrigel assay (14). Fig. 5 B depicts the number of cells invading the Matrigel complex either as a basal rate or after stimulation with EGF. The results demonstrated that esophageal carcinoma cell invasion was strikingly suppressed when Grb7 and Grb7V protein levels were reduced by expression of the antisense RNA.

Discussion

Transmission of intracellular signals requires the sequential interaction of signaling molecules (15). Specific amino acid sequences present in certain proteins have been shown to be essential with respect to forming these molecular interactions. Proteins containing phosphotyrosine motifs are well known targets for binding of signaling molecules that have SH2 do-

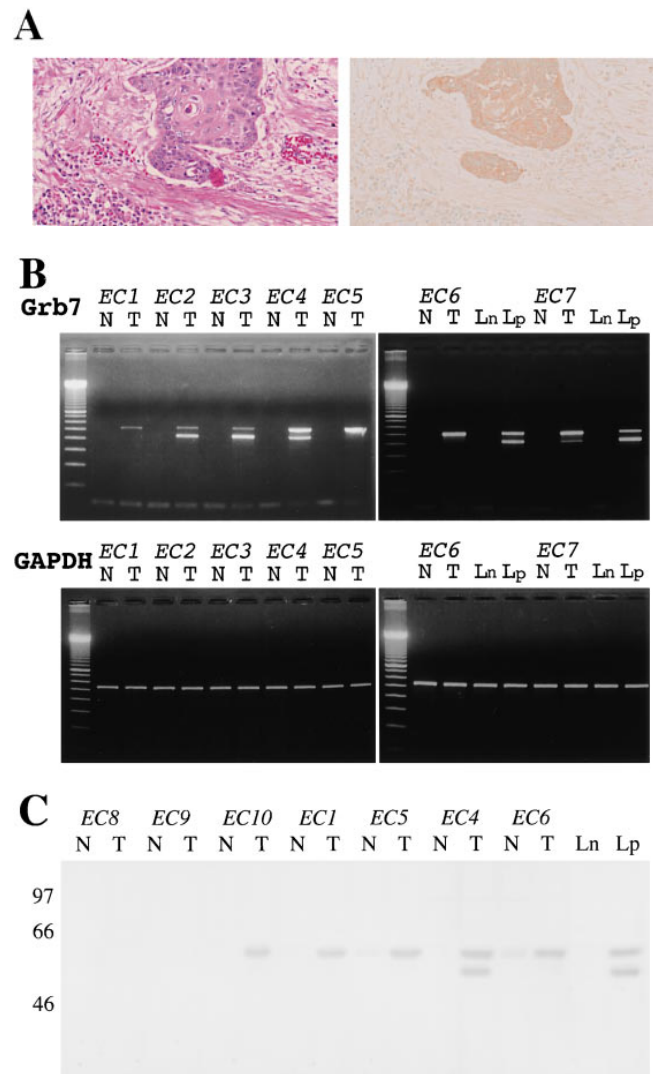


Figure 4. Protein and RNA expression of Grb7 in clinical tumor samples obtained at the time of surgical resection. (A) Representative example of an invasive esophageal carcinoma (EC4). Left lane shows hematoxylin and eosin staining ($\times 100$). Right lane shows immunohistochemical staining reaction using an antibody specific for the NH₂ terminus of the Grb7 protein. Positive staining for Grb7 protein expression is present in invasive nests of esophageal carcinoma cells ($\times 100$). (B) Expression of Grb7 isoforms and GAPDH in human esophageal tissues and lymph nodes as measured by RT-PCR. Esophageal carcinoma tumors (T), normal adjacent mucosa (N), regional lymph nodes without metastasis (Ln), and lymph nodes with metastasis (Lp) were studied. The case numbers are represented in italics. All of the tumors presented are derived from invasive or metastatic esophageal carcinomas, except for EC1. Molecular markers: 100 bp ladder. (C) Immunoblot analysis using an antibody for the NH₂ terminus of Grb7 protein demonstrates different expression of 59-kD Grb7 and 50-kD Grb7V proteins in tumors, and are similar to the results obtained by RT-PCR analysis.

mains, such as Grb, Shc, insulin receptor substrate-1, and recently reported c-Met binding domain of the Gab1 protein. For example, the murine Grb7 cDNA was originally isolated and characterized using the CORT (cloning of receptor targets) method by taking advantage of the interaction between the SH2 domain and the autophosphorylated kinase region of

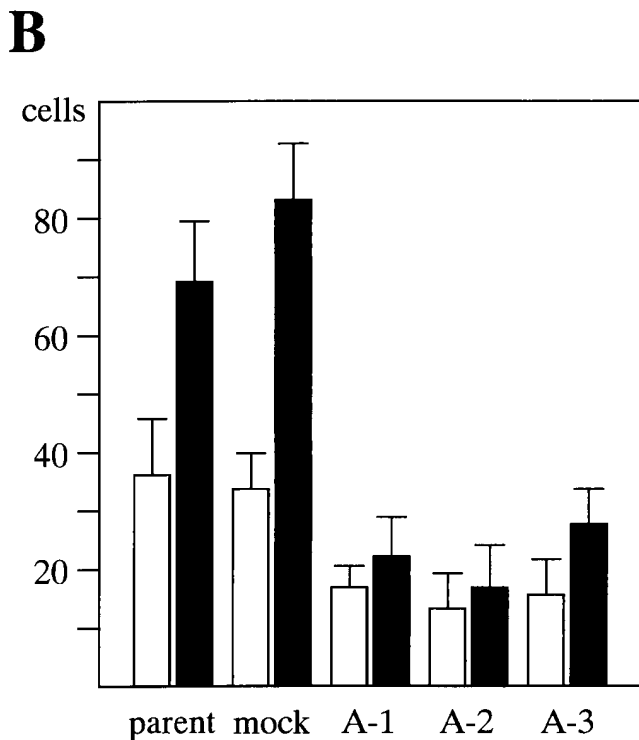
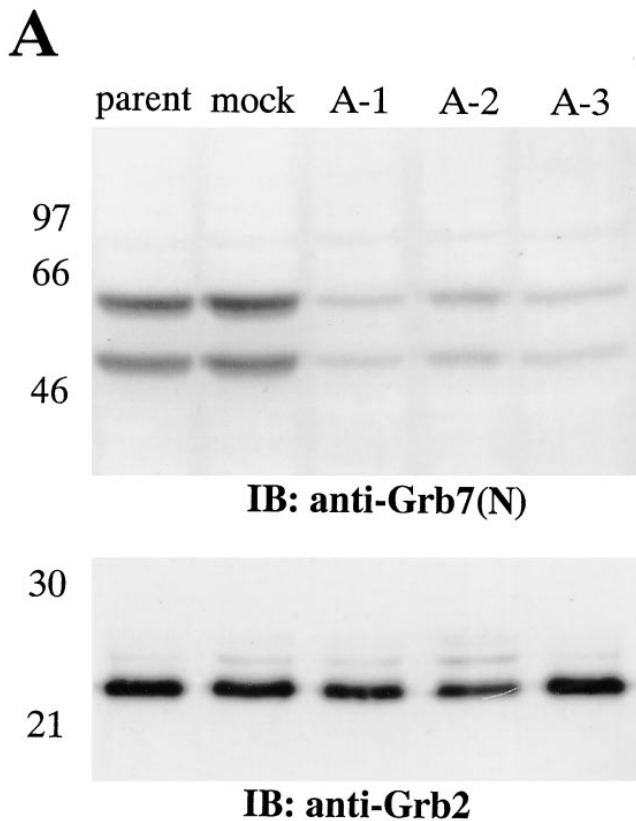


Figure 5. Effect of antisense Grb7 RNA expression on the phenotype of esophageal carcinoma cells. (A) Analysis of Grb7 expression in KYSE410 esophageal carcinoma cell clones transfected with antisense Grb7 (A-1, A-2, and A-3) compared to parental (*parent*) and mock DNA-transfected KYSE410 cells. Expression of a Grb2 control protein was not altered in these cell lines. (B) Results of the Matrigel invasion assay. The number of cells invading into the Matrigel

the EGF receptor (6). In the present study, a Grb7 protein containing a SH2 domain in the COOH terminus was shown to be tyrosyl phosphorylated by EGF stimulation in esophageal carcinoma cells. Thus, the activated EGF receptor will bind the Grb7 SH2 domain and phosphorylate the protein. On the other hand, tyrosyl-phosphorylated Grb7 was not detectable in cell serum-starved quiescent cells. The mechanism of Grb7 dephosphorylation has not been identified. One possibility is that protein tyrosine phosphatases (PTP) may be involved in the dephosphorylation of Grb7. Recently, a member of this PTP family named SHPTP2 or PTP1D was found to be associated with the SH2 domain of Grb7 (16). Therefore, wild-type Grb7 is presumably dephosphorylated by the PTP activity after a Grb7 interaction with SHPTP2 via its SH2 domain. In contrast, dephosphorylation of Grb7V that clearly lacks this SH2 domain in the COOH terminus may not be possible even in serum-starved quiescent cells. Because Grb7V cannot be targeted by such PTP binding, the tyrosyl phosphorylation of the protein found in esophageal carcinoma cells is of interest with respect to metastatic potential in the context of a constitutive activated signal transduction pathway.

The Grb7V is composed of a proline-rich sequence and a GM domain. The proline-rich sequence is generally thought to interact with SH3 WW domains (15, 17). Recent studies of human Grb10, a member of the Grb7 family (18), revealed that the NH₂-terminal proline-rich region SLPAIPNPFPEL may bind to the SH3 domain of the c-Abl oncoprotein. The c-Abl protein also contains a tyrosine kinase domain. More recently, the SH3 domain of c-Abl was found to bind to a similar proline-rich sequence of the ataxia telangiectasia mutated protein (19) and c-Abl will tyrosyl phosphorylate the ataxia telangiectasia mutated protein directly (20). A similar proline-rich SLPSIPNPFPEL sequence is also present at aa 63–74 of the Grb7 and Grb7V proteins. In this regard, preliminary *in vitro* binding data demonstrates a direct interaction of the Grb7V isoform to the c-Abl SH3 domain (our unpublished observation). Although a SH3-containing tyrosine kinase such as c-Abl protein might phosphorylate both Grb7 and Grb7V proteins, the Grb7V isoform lacking the SH2 domain may not be sensitive to PTP activity and this could contribute to phosphorylation of the molecule in esophageal carcinoma cells.

We have previously reported that tyrosyl phosphorylation of the insulin receptor substrate-1 is followed by binding to SH2-containing proteins, such as Grb2, and this interaction induces the mitogen-activated protein kinase signaling pathways associated with transformation of cells to the malignant phenotype (10). Because many tyrosine residues are present in the GM region of both Grb7 and Grb7V as shown in Fig. 2, this domain may be essential for Grb7 function. It is of interest that the GM region includes a PH domain. Recent studies suggest that the PH domain interacts with Gαβ subunits (21) and phospholipids (22) and may be involved in signal transduction pathways related to cell growth. Our previous investigations also indicate that the PH domain was required for effective interaction of signaling molecules related to malignant transforma-

tion. The number of cells invading into the Matrigel matrix were determined for each clone. White bars indicate the basal invasion rate and black bars indicate the rate of invasion after stimulation of chemotaxis by EGF. Results are derived from three individual experiments and error bars indicate the standard deviation from the mean.

tion of hepatocytes (11). However, the role of the GM domain in Grb7 and its isoforms has not been clarified. A possible clue to its function is derived from the genetic analysis of *C. elegans* Mig-10 mutant phenotypes. An amber Mig-10 (ct41) mutant results in a protein with a truncation upstream to the GM region. Such a mutation induces an incomplete long range anterioposterior migration of neurons. Thus, the function of Mig-10 is closely associated with directional cell migration (4). This finding is consistent with our observations that Grb7 and Grb7V expression is related to metastatic spread of esophageal carcinoma. Indeed, in vitro analysis using antisense constructs revealed that lowering the intracellular levels of Grb7 and Grb7V proteins was associated with reduced invasion of esophageal carcinoma cells into Matrigel.

Human esophageal carcinomas are known to be highly invasive, frequently metastasize, and, therefore, have a poor prognosis (23). The present investigation has revealed a relationship between expression of Grb7 and the novel Grb7V isoform and the biologic properties of invasion and metastatic spread of these cells. Further studies will be needed to identify signal transduction pathways and molecules that interact with the GM domain of Grb7 (20) to further clarify its molecular role in this disease.

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