THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 30, pp. 20206–20214, July 24, 2009

GAREM, a Novel Adaptor Protein for Growth Factor Receptor-bound Protein 2, Contributes to Cellular Transformation through the Activation of Extracellular Signal-regulated Kinase Signaling*

Received for publication, May 13, 2009, and in revised form, June 2, 2009 Published, JBC Papers in Press, June 9, 2009, DOI 10.1074/jbc.M109.021139

Kyoko Tashiro[‡], Takumi Tsunematsu[§], Hiroko Okubo[§], Takeshi Ohta[§], Etsuko Sano[‡], Emiko Yamauchi[‡], Hisaaki Taniguchi[‡], and Hiroaki Konishi[‡§1]

From the [§]Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023 and the [‡]Division of Disease Proteomics, Institute for Enzyme Research, the University of Tokushima, 3-15-18 Kuramotocho, Tokushima 770-8503, Japan

Adaptor proteins for the various growth factor receptors play a crucial role in signal transduction through tyrosine phosphorylation. Several candidates for adaptor proteins with potential effects on the epidermal growth factor (EGF) receptor-mediated signaling pathway have been identified by recent phosphoproteomic studies. Here, we focus on a novel protein, GAREM (Grb2-associated and regulator of Erk/MAPK) as a downstream molecule of the EGF receptor. GAREM is phosphorylated at tyrosine 105 and 453 after EGF stimulation. Grb2 was identified as its binding partner, and the proline-rich motifs of GAREM are recognized by the N- and C-terminal SH3 domains of Grb2. In addition, the tyrosine phosphorylations of GAREM are necessary for its binding to Grb2. Because the amino acid sequence surrounding tyrosine 453 is similar to the immunoreceptor tyrosine-based inhibitory motif, Shp2, a positive regulator of Erk, binds to GAREM in this phosphorylation-dependent manner. Consequently, Erk activation in response to EGF stimulation is regulated by the expression of GAREM in COS-7 and HeLa cells, which occurs independent of the presence of other binding proteins, such as Gab1 and SOS, to the activated EGF receptor. Furthermore, the expression of GAREM has an effect on the transformation activity of cultured cells. Together, these findings suggest that GAREM plays a key role in the ligand-mediated signaling pathway of the EGF receptor and the tumorigenesis of cells.

The interactions between receptor tyrosine kinases and adaptor proteins are crucial for the transduction of intracellular growth signals from the plasma membrane to the nucleus: these signals are propagated by the tyrosine phosphorylation of each molecule (1, 2). Among the numerous adaptor proteins, the complex of Grb2 and the Grb2-associated binder (Gab)[2] family protein can directly bind to several growth factor receptors. This complex can also regulate the activity of downstream protein kinases such as Erk and Akt, which are known regulators of various cellular functions (3–5). These adaptor proteins contain functional domains such as the proline-rich, Src-homology (SH) 2, SH3, phosphotyrosine-binding, or pleckstrin homology (PH) domains (1, 6–8) required for interaction with their partner proteins. In addition, Gab or insulin receptor substrate family proteins have multiple tyrosine phosphorylation sites and are recognized as substrates by tyrosine kinases. Therefore, Gab or insulin receptor substrate family proteins are targets for interaction with other proteins possessing SH2 domains (9).

A great deal of excellent work on the epidermal growth factor (EGF) receptor has established the EGF signaling pathway as a paradigm for growth factor-mediated signal transduction (10). The EGF receptor is known for being involved not only in normal cell proliferation but also in the origin or development of various human cancers (11). Many research groups have applied proteomic techniques, such as mass spectrometry, to identify novel molecules and the post-translational modifications involved in the EGF signaling pathway (12–17).

The functions in the growth factor receptor-mediated signaling pathway of any molecule identified by phosphoproteomic studies must be deciphered by performing the appropriate biochemical and cell biological experiments. To identify the proteins acting downstream of the EGF receptor, we isolated all the proteins by column chromatography. The column was packed with three different anti-phosphotyrosine antibodies from the lysate of EGF-stimulated A431 cells. Over 150 proteins were detected by mass spectrometric analysis, including well studied proteins and several previously unidentified ones.

* This work was supported in part by Grants-in-Aid for Scientific Research and by the Knowledge Cluster Initiative from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by basic research grants from the Prefectural University of Hiroshima.

[1] To whom correspondence should be addressed: Faculty of Life and Environmental Science, Prefectural University of Hiroshima, 562 Nanatsuka, Shobara 727-0023, Japan. Tel.: 81-82-474-1776; Fax: 81-82-474-1776; E-mail: hkonishi@pu-hiroshima.ac.jp.

[2] The abbreviations used are: Gab, Grb2-associated binder; CBB, Coomassie Brilliant Blue; EGF, epidermal growth factor; Grb2, growth factor receptor-bound protein 2; GST, glutathione *S*-transferase; HEK, human embryonic kidney; LC, liquid chromatography; MS, mass spectrometry; PH, pleckstrin homology; Shp2, SH2 domain-containing phosphatase 2; siRNA, small interference RNA; SOS, Son of sevenless; Erk, extracellular signal-regulated kinase; nt, nucleotide(s); GAREM, Grb2-associated and regulator of Erk/MAPK; GAREM(S), internal deleted form of GAREM; CMV, cytomegalovirus; MAPK, mitogen-activated protein kinase.

Recently, we reported the functions of three unique adaptor proteins that were identified by this proteome analysis (18–20). In this study, we focus on and analyze the protein encoded by the cDNA clone of FLJ21610. FLJ21610 has been identified as a tyrosine-phosphorylated protein in our phosphoproteomic study. This protein and one of its phosphorylation sites (tyrosine 453) have also been studied by phosphoproteomic experiments performed by several research groups (12, 15, 16). Although FLJ21610 has been hypothesized to function in the EGF signaling pathway, there has been no biological evidence of its role thus far.

In this study, we found that Grb2 is one of the binding partners of FLJ21610, and that it has a regulatory effect on the Erk activity associated with SH2 domain-containing phosphatase 2 (Shp2) (21) in response to EGF stimulation. Therefore, this protein has been named Grb2-associated and regulator of Erk/MAPK (GAREM). A functional analysis demonstrates the crucial role of GAREM as an adaptor protein in the activated EGF receptor complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7, A431, 293T, and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. For maintaining the NIH3T3 cells, fetal bovine serum was substituted with 10% calf serum. Plasmid transfection into COS-7 cells was carried out by electroporation by using Gene-Pulser (Bio-Rad). Prior to EGF stimulation, the cells were serum-starved for 16 h, and 100 ng/ml EGF (Sigma) dissolved in a serum-free medium was added.

cDNA Cloning and Vector Construction—GAREM (FLJ21610) cDNA, provided by the National Institute of Technology and Evaluation, Japan, was subcloned into pFLAG-CMV6a to be expressed as an N-terminal FLAG-tagged protein. Point mutations or internal deletions were introduced by using the QuikChange kit (Stratagene) according to the manufacturer's protocol. Grb2 and Shp2 cDNAs were cloned from a HeLa cDNA library by PCR and inserted into a pCMV-3Tag-2 vector (Stratagene) and expressed as 3× Myc-tagged proteins at the N terminus. The dominant negative construct of the Shp2 fragment containing residues 1–220 (22) was amplified by PCR and inserted into a pFLAG-CMV6 vector. All the nucleotide sequences were determined and verified using an ABI prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI Prism 3100-Avant genetic analyzer.

Antibodies—An anti-GAREM rabbit polyclonal antibody was raised against a GAREM fragment containing residues 391–488, which was bacterially produced as a glutathione *S*-transferase (GST) fusion protein by using the pGEX4T vector (Amersham Biosciences). This antibody was purified by using HiTrap *N*-hydroxysuccinimide-activated Sepharose columns (Amersham Biosciences) coupled with an immunizing antigen. An anti-EGF receptor rabbit polyclonal antibody was raised against a 15-amino acid peptide corresponding to the C terminus of the protein. The following other antibodies were obtained commercially from various companies: anti-FLAG M2, anti-β-actin (Sigma); anti-phosphotyrosine (4G10, Upstate Biotech Inc.); anti-Grb2, anti-Gab1, anti-SHP2, and anti-SOS1 (Santa Cruz Biotechnology); anti-Erk1/2 and anti-phospho-Erk1/2 (Cell signaling Technology); anti-GST (Nacalai Tesque); anti-Akt and anti-phospho-Akt (BD Transduction); and anti-myc (9E10, Roche Applied Science).

Semi-quantitative Reverse transcription-PCR—To assess the relative expression levels of the GAREM transcripts, reverse transcription-PCR was performed on each panel of eight different human culture cell and tissue cDNAs cultures (human tissue and cell line MTC panel, Clontech) by using the primers 5′-GTTTAAGCTTCAGGACTCTGGAGATAGT-3′ and 5′-GAAATCTAGATTACGTCTTTTGTCTAGGGTA-3′.

Small Interference RNA—The mammalian expression vector pSUPER-Retro-puro (Oligoengine) was used for expressing siRNA in HeLa cells. The targeted sequence of human GAREM was 5′-AAGAGAAACTGCCCAGCACCTTT-3′ (from nt 1981 to nt 2003) and 5′-AAGTCACTACGGTTCATTGGTTT-3′ (from nt 2449 to nt 2471). Each resulting plasmid for the knockdown of GAREM was named as pSuper-GAREM 1 or 2, respectively. The empty pSuper vector was used as the control.

Immunoprecipitation and Immunoblot Analysis—The following procedures were carried out at 0–4 °C. The transfected cells were lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and a complete protease inhibitor mixture (Roche Applied Science) to produce a total cell lysate (TCL). For the immunoprecipitation experiments, the total cell lysate was centrifuged, and the supernatant was incubated for 2 h with either the primary antibody or an anti-FLAG affinity gel (Sigma). Protein G-Sepharose (Amersham Biosciences) was added, and the resulting mixture was rotated at 4 °C for 1 h. The beads were subsequently washed three times with the lysis buffer. The processed samples were performed as described previously (18).

Purification of FLAG-GAREM Derivatives for GST Pulldown Assay—COS-7 cells or 293T cells (1×10^8 cells) transiently expressing FLAG-tagged GAREM or ΔP-rich were lysed with the lysis buffer. The lysate was centrifuged, and the supernatant was incubated with a FLAG-affinity gel (bed volume: 50 μl) for 2 h. The gel was applied to an empty mini column (Bio-Rad) and washed several times with the lysis buffer. FLAG-GAREM was eluted with the FLAG-peptide. GST pulldown assays were performed as described previously (18).

GST Overlay Assay—Samples were fractionated by SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking in 1% skim milk and TBST (20 mM Tris-HCl buffer, (pH 8.0), 100 mM NaCl, 0.1% Tween 20), the membrane was incubated with 500 ng/ml GST or GST-Grb2 in TBST for 30 min. Then the membrane was washed, incubated with anti-GST antibody for 2 h at room temperature. The blots were then washed and incubated for 1 h with horseradish peroxidase-conjugated anti-goat antibody (Santa Cruz Biotechnology) and were developed by ECL (Amersham Biosciences).

Focus Formation and Anchorage-independent Growth Assay—Primary focus-formation assays were performed in NIH3T3 cells exactly as described (23). NIH3T3 cell lines that stably transfected with pcDNA3, pcDNA3-GAREM, or pcDNA3-

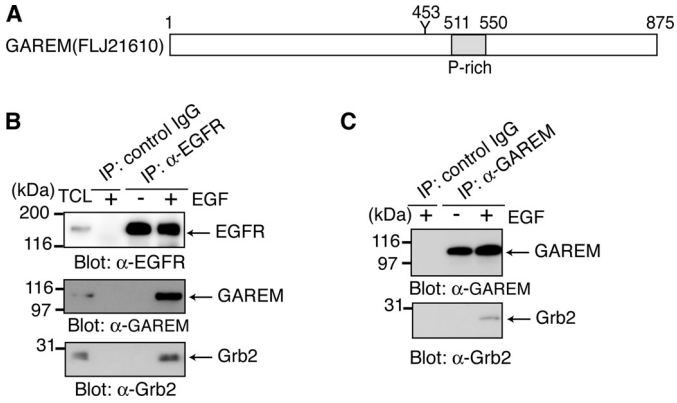

FIGURE 1. **GAREM involvement in the activated EGF receptor complex.** *A*, schematic representation of the primary structure of GAREM. The location of the relevant amino acid residues and a representative tyrosine residue at one end of the sequence are indicated. The location of the proline-rich region is indicated as a *shaded box*. *B*, presence of endogenous GAREM in the activated EGF receptor complex. Co-immunoprecipitation experiments were carried out with control IgG and anti-EGF receptor antibody by using HeLa cell lysates with (+) or without (−) EGF stimulation for 10 min. Immunoblot analysis was performed using anti-EGF receptor (*upper panel*), anti-GAREM (*middle panel*), and anti-Grb2 antibodies (*lower panel*). *C*, EGF stimulation-dependent association of endogenous GAREM and Grb2. Co-immunoprecipitation and immunoblot experiments were carried out using the indicated antibodies and HeLa cell lysates with (+) or without (−) EGF stimulation for 10 min.

GAREMΔP-rich were generated. Following transfection, cells were selected after 14 days in a growth medium supplemented with G418. Cells were then fixed in 10% acetic acid and 10% methanol and stained with crystal violet. These NIH cell lines or HeLa cells stably transfected with pSUPER vector or pSUPER-GAREM (2.0×10^5 cells) were suspended in 3 ml of 0.3% agarose Dulbecco's modified Eagle's medium onto a semisolid 0.6% agarose Dulbecco's modified Eagle's medium layer (Dulbecco's modified Eagle's medium containing 5% fetal bovine serum) in a ~60-mm dish and incubated in a CO_2 incubator for 2 weeks to form colonies. Colonies of 50 cells or more were counted from at least four randomly selected fields per triplicate well. In proliferation assays, NIH3T3 cell lines were seeded onto 6-well plates, and cell numbers were directly counted.

RESULTS

Involvement of GAREM in the Activated EGF Receptor Complex—The GAREM protein was originally identified as a tyrosine-phosphorylated protein by phosphoproteomic studies performed by three independent research groups. The researchers found that Tyr-453 is one of the phosphorylation sites of GAREM (12, 15, 16). By using a similar strategy, we independently identified GAREM from the lysate of EGF-stimulated A431 cells. The GAREM protein comprises 875 amino acid residues and contains typical proline-rich motifs for interaction with SH3 domains (Fig. 1*A*). GAREM cDNA has been cloned in full-length human cDNA-sequencing projects in Japan and has been stored as FLJ21610 or FAM59A (gi 12232415). However, the function of GAREM in the EGF-signaling pathway remains to be elucidated. At first, we focused on the physical association between GAREM and the EGF receptor. Endogenous GAREM and Grb2 were found to co-immunoprecipitate with the activated EGF receptor in the lysate from the EGF-stimulated HeLa cells (Fig. 1*B*). Furthermore, endogenous GAREM and Grb2 formed a complex in EGF-stimulated 293 cells (Fig. 1*C*). These results suggest that GAREM might regulate the EGF receptor by binding to Grb2.

A Proline-rich Motif in GAREM Is the Tyrosine Phosphorylation-dependent Binding Site to Grb2—According to Scan-site, the proline-rich motif in GAREM was speculated to be a suitable binding site for the SH3 domains of various signaling molecules (intersectin, Crk, phospholipase Cγ, Src, Grb2, the p85 subunit of phosphatidylinositol 3-kinase, and cortactin). Specifically, we focused on the APPVPPRSAKPLS and PSIPPR (amino acid residues: 532–544 and 547–552, respectively) sequences in GAREM, which matched well with the *XX*P*XXX*R*XX*KP*XX* and the P*XX*P*X*R (where *X* is any amino acid residue) sequences that have been identified as the targets of the C-terminal and N-terminal SH3 domains of Grb2, respectively (Fig. 2*A*) (24). To analyze the binding mechanisms of GAREM and Grb2 in detail, several constructs were expressed in COS-7 cells and their interaction was assessed by immunoprecipitation (Fig. 2*B*). Endogenous Grb2 was found in the immune complex of anti-FLAG antibody obtained from the lysate of EGF-stimulated COS-7 cells expressing wild-type FLAG-GAREM. The substitution of the proline 547 and 550 residues by alanine and deletion of amino acid residues from proline 533 to arginine 538 did not abolish the binding ability to Grb2 in mutants. Furthermore, the combination mutant in this region (P547/550A+ΔP-rich) also bound to small amounts of Grb2. The obtained results revealed that the proline-rich region of over 50 amino acid residues (amino acid residues: 498–550) is required for the binding of GAREM to Grb2. Furthermore, binding to the activated EGF receptor was not observed in this mutant (Fig. 2*C*). Therefore, the Grb2 protein may be necessary for indirect association of GAREM and the EGF receptor.

Next, we analyzed the effect of the tyrosine phosphorylation of GAREM on its binding to Grb2. Three research groups have reported that Tyr-453 in GAREM is phosphorylated by growth factor stimulation (12, 15, 16). However, precise analysis for detecting the phosphorylation sites of GAREM by using point mutants revealed that the Y453F mutant of GAREM replaced the tyrosine with phenylalanine continued to be phosphorylated in COS-7 cells upon EGF stimulation (Fig. 2*D*). From further analysis using various truncated mutants of GAREM, we found that other tyrosine phosphorylation sites might exist in the N-terminal domain of GAREM (data not shown). In addition to the Tyr-453 mutant, a single mutant of Y105F led to a slight reduction in the phosphorylation level as compared with the level detected in the wild-type GAREM. Furthermore, in a double mutant in which both Tyr-105 and Tyr-453 were replaced with Phe, EGF-dependent tyrosine phosphorylation was abolished (Fig. 2*D*). Therefore, in addition to Tyr-453, the Tyr-105 residue of GAREM is also phosphorylated after EGF stimulation. Interestingly, the ΔP-rich mutant, which contained both Tyr-105 and Tyr-453, was not phosphorylated. Grb2 binding to the Y105F mutant was lower than that to wild-type GAREM. Furthermore, the Y105F/Y453F mutant of GAREM did not bind to Grb2 (Fig. 2*E*).

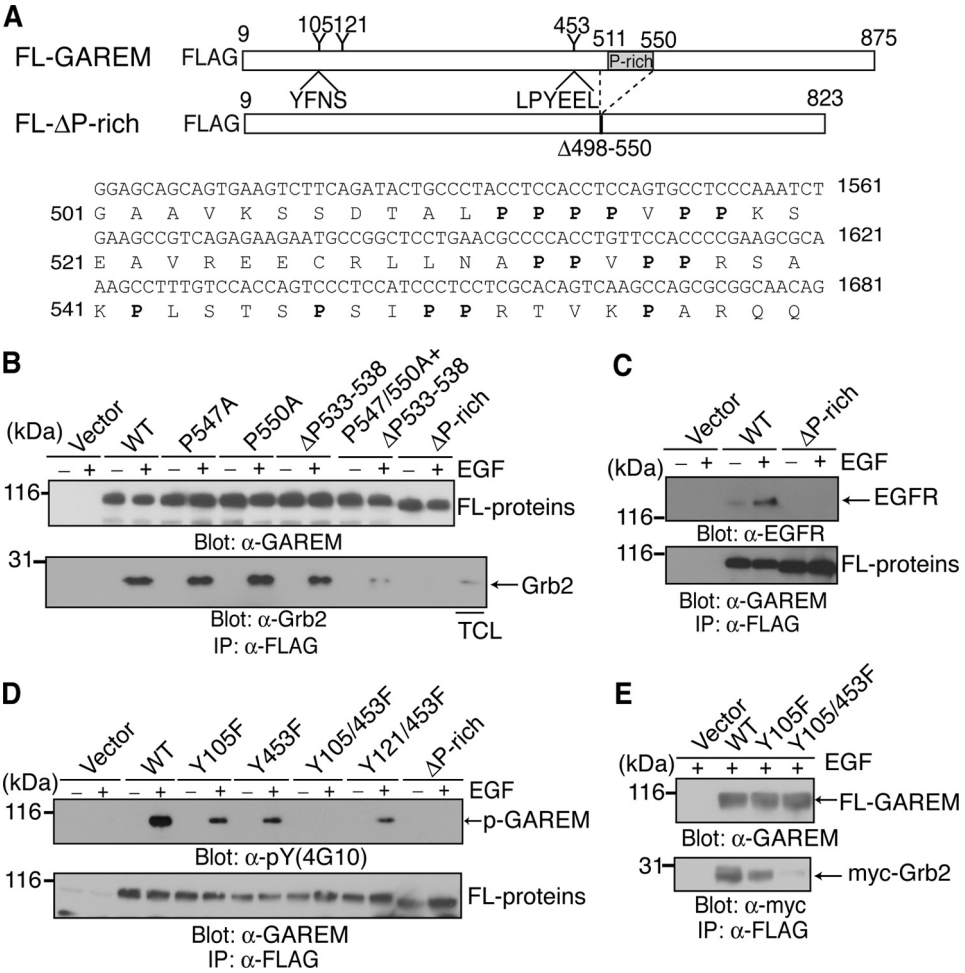

FIGURE 2. **The proline-rich motifs in GAREM enable its binding to Grb2; this binding is dependent on the tyrosine phosphorylations of GAREM upon EGF stimulation.** *A*, schematic representation of the constructs of FLAG-tagged wild-type and ΔP-rich GAREM mutants. The positions of the representative tyrosine residues in GAREM and the surrounding amino acid sequence in GAREM are indicated. *Numbers* indicate amino acid residues (*upper*). Nucleotide and deduced amino acid sequences of the proline-rich region of GAREM (*lower*). *B*, the binding of FLAG-tagged GAREM to endogenous Grb2. Co-immunoprecipitation studies were carried out using the lysates from COS-7 cells transfected with the empty vector or an expression plasmid encoding FLAG-GAREM in which proline (*P*) residues had been substituted with alanine (*A*) or deleted (Δ); the number of substituted or deleted amino acid residues substituted or deleted is indicated. Cells were treated with (+) or without (−) EGF stimulation for 10 min, and each FLAG-tagged molecule was immunoprecipitated with the respective anti-FLAG antibody. Immunoblot analysis was performed using anti-GAREM (*upper panel*) and anti-Grb2 antibodies (*lower panel*). *C*, Grb2-dependent association of the EGF receptor and GAREM. COS-7 cells were transfected with the indicated plasmid, and EGF treatment and immunoprecipitation were performed as described above. Immunoblot analysis was carried out using the indicated antibodies (*lower panel*). *D*, Tyr-105 and Tyr-453 are tyrosine phosphorylation sites of GAREM that are phosphorylated in an EGF-stimulation dependent manner. COS-7 cells were transfected with each indicated plasmid encoding the FLAG-tagged construct of GAREM derivatives in which tyrosine residues had been substituted with phenylalanine. Immunoprecipitation of the expressed molecule and EGF stimulation of the cells were performed as described above. Immunoblot analysis was carried out using anti-FLAG (*lower*), and anti-phosphotyrosine (*upper*) antibodies. *E*, effects of the tyrosine phosphorylation of GAREM induced by EGF stimulation of the binding of GAREM to Grb2. Co-immunoprecipitation studies were carried out with the anti-FLAG antibody by using the lysates of COS-7 cells transfected with Myc-tagged Grb2 or an expression plasmid of FLAG-GAREM derivatives and EGF treatment. Immunoblot analysis was carried out using the anti-FLAG (*upper panel*) or anti-Myc antibodies (*lower panel*).

The SH3 Domains of Grb2 Are Important Binding Sites for the Proline-rich Region of GAREM—To clarify whether the SH3 domains of Grb2 directly bind to GAREM, GST overlay (Fig. 3*B*), and pulldown (Fig. 3*C*) assays were carried out for each isolated functional domain of Grb2 indicated in Fig. 3*A*. Although each fusion protein containing the SH3 domain of Grb2 was able to bind directly to the purified FLAG-GAREM protein but not in the ΔP-rich mutant, the binding affinity of the C-terminal SH3 domain to GAREM was higher than that of the N-terminal SH3 domain. Next, to determine the function of Tyr-105 phosphorylation in GAREM, we performed a GST pulldown assay with the total cell lysate of COS-7 cells expressing GAREM protein derivatives from EGF-stimulated cells. The amino acid sequence surrounding Tyr-105 in GAREM is YFNS. This sequence is a good match to the SH2 domain binding site of Grb2: Y*X*N*X* (where *X* is any amino acid residue) (25). However, small amounts of wild-type and Y453F but not Y105F mutants of GAREM associated with the SH2 domain of Grb2. In contrast, the binding efficiency of the EGF receptor to the SH2 domain of Grb2 was apparently higher than that of GAREM under the same conditions (Fig. 3*D*). These results suggest that the interaction between GAREM and Grb2 depends on the activation of the EGF receptor and may be mainly mediated by the proline-rich domains of GAREM and the SH3 domain of Grb2. Although the Tyr-105 and Tyr-453 phosphorylation sites of GAREM may not be direct binding sites for Grb2, they may be necessary for the association of GAREM and Grb2 in the EGF-stimulated cells.

Regulation of Erk Activity in Response to EGF Stimulation by GAREM Expression—To understand the function of GAREM in the activated EGF receptor complex, we investigated the effect of the GAREM expression on Erk and Akt activation, which are known to be downstream protein kinases in the signaling pathway of the EGF receptor (3–5). For this purpose, we established a GAREM-knockdown HeLa cell line by using a retrovirus gene transfer system. Then, the activation of each protein kinase in response to EGF stimulation was measured using the phospho-specific antibody of each kinase. GAREM knockdown inhibited Erk activation but not Akt activation after EGF stimulation (Fig. 4, *A* and *B*). If GAREM knockdown suppresses Erk activation, overexpression of the GAREM protein should reverse this effect. We found that Erk activation is enhanced after EGF stimulation in cells overexpressing FLAG-GAREM but not in cells transfected with

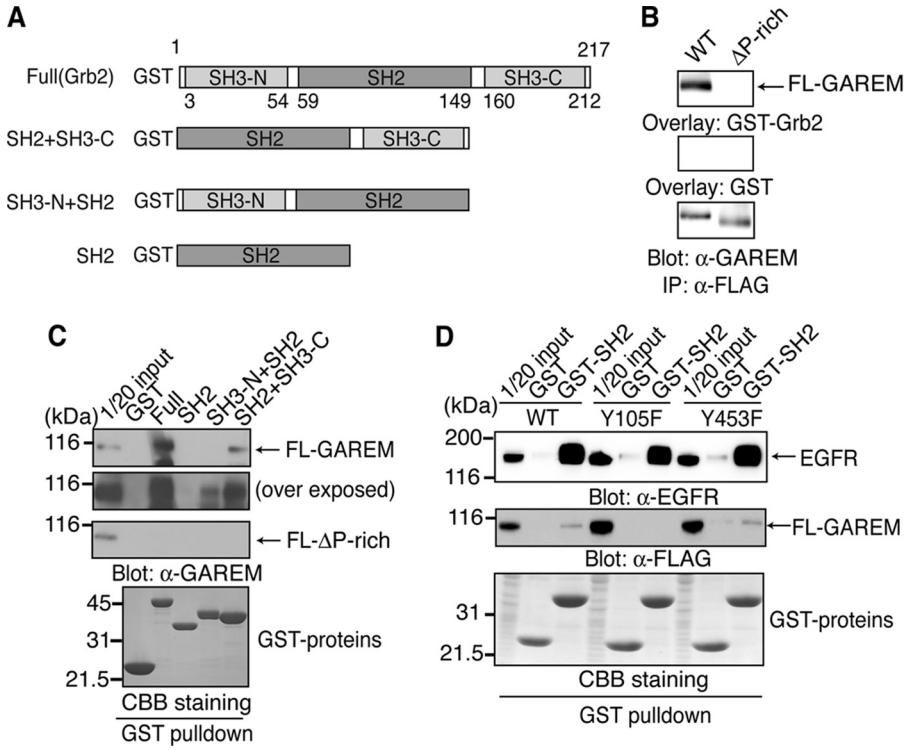

FIGURE 3. ***In vitro* interaction between the SH3 and SH2 domains of Grb2 and GAREM was confirmed by GST overlay and pulldown assays.** *A*, schematic representation of the constructs of GST-fused Grb2. *Numbers* indicate the amino acid residues. *B*, COS-7 cells were transfected with the expression vectors of FLAG-GAREM or the FLAG-ΔP-rich mutant. Each FLAG-tagged molecule was immunoprecipitated with anti-FLAG antibody. Overlay assays were carried out using GST-Grb2 (*upper panel*) and GST (*middle panel*). Immunoblot analysis was performed using anti-FLAG antibody (*lower panel*). *C*, GST fusion proteins used in this assay were visualized by Coomassie Brilliant Blue (*CBB*) staining (*bottom panel*). Immunoblotting was used to determine the amount of FLAG-GAREM (*top panel*) and FLAG-ΔP-rich (*lower middle panel*) bound to GST fusion proteins by using anti-GAREM antibody. The *upper middle panel* shows the result of subjecting FLAG-GAREM to long exposure times for immunoblotting. The purified protein (1/20th the amount used in this assay) from COS-7 cells expressing FLAG-proteins is present in the *left lane*. *D*, *in vitro* interaction between tyrosine-phosphorylated GAREM and the SH2 domain of Grb2. GST fusion proteins used in this assay were visualized by CBB staining (*bottom panel*). Immunoblotting analysis of the amounts of FLAG-GAREM and mutant FLAG-GAREM (Y105F and Y453F) bound to the GST fusion proteins was performed using anti-EGFR (*top panel*) and anti-GAREM (*middle panel*) antibodies. The total lysate (1/20th the amount used in this assay) from EGF-stimulated COS-7 cells expressing FLAG-proteins is shown in the *left lane*.

mock or expression plasmid for ΔP-rich mutant (Fig. 4*C*). Furthermore, the Y105F/Y453F mutant could not enhance Erk activation (Fig. 4*D*). These data suggest that GAREM has a positive effect on Erk activation.

Erk Is Activated by GAREM Expression Due to the Tyr-453 Phosphorylation-dependent Binding of Shp2 to GAREM—To understand the mechanism of the regulation of Erk by GAREM, we focused on the amino acid sequence of the region surrounding the phosphorylation site Tyr-453:LP$_{phospho}$YEEL region. This motif is a good match to the consensus sequence of the binding site of Shp1 and Shp2, (I/V/L)$X_{phospho}$Y*XX*(I/V/L) (where *X* is any amino acid residue). The Shp family protein is known to be required for the full activation of Erk (21). Immunoprecipitation experiments revealed that the endogenous GAREM and Shp2 proteins associate in an EGF stimulation-dependent manner (Fig. 5*A*), and that this association depends on the phosphorylation of the Tyr-453 amino acid of GAREM (Fig. 5*B*). Furthermore, the enhancement of Erk activation by the expression of recombinant GAREM was abolished in cells expressing the dominant negative construct of Shp2-(1–220) (22) (Fig. 5, *C* and *D*). Therefore, GAREM may up-regulate Erk activity by binding to Shp2 downstream of the EGF receptor. On the other hand, Gab1 is known as a Grb2-binding protein (3–5), and the binding properties of Gab1 to Grb2 are similar to those of GAREM. Therefore, GAREM might have a competitive effect on the interaction between Gab1 and Grb2. However, we found that the expression of FLAG-GAREM had no effect on the association of the EGF receptor and Gab1. The amounts of Gab1 in the immunoprecipitated EGF receptor did not change in the EGF-stimulated COS-7 cells expressing FLAG-GAREM (Fig. 5*E*). Moreover, the suppression of GAREM expression had no effect on the Grb2-Son of sevenless (SOS) complex (Fig. 5*F*), suggesting that the enhancement of Erk activation might be dependent on the binding of Shp to GAREM.

Effects of GAREM Expression on the Focus and Colony Formation of the Cultured Cells—Our observations suggest that GAREM might have the potential to regulate the growth and transformation of the cells through the pathways between receptor tyrosine kinases and Erk. Transformed HeLa cells grow and form colonies in soft agar, which is one of the hallmarks of transformation (26). To further examine the involvement of GAREM in cellular transformation, HeLa cell lines infected with an siRNA vector were assayed for anchorage-independent growth when suspended in soft agar. Although the number of colonies formed in soft agar was similar for each cell line, the cells in which the expression of GAREM was suppressed by siRNA formed smaller colonies than the vector-infected cells (Fig. 6, *A* and *B*). Furthermore, we performed conventional focus- and colony-formation assays in NIH3T3 cells. Focus and colony formation was observed for the stable cell line expressing wild-type GAREM but not for cells transfected with an empty vector or an expression plasmid for GAREMΔP-rich (Fig. 6, *C* and *D*). Furthermore, GAREM expression positively regulated cell proliferation (Fig. 6*E*). Therefore, the expression level of GAREM may be one of the critical factors for transformation or malignancy of the cells.

In addition, we have noticed that the internal deleted form of GAREM (GAREM(S)), which was recorded in the NCBI data base during this study, lacks the proline-rich region (gi 111307706) (Fig. 7*A*). To assess the expression of two GAREM mRNA species, we performed semi-quantitative reverse transcription-PCR by using a wide variety of cDNA

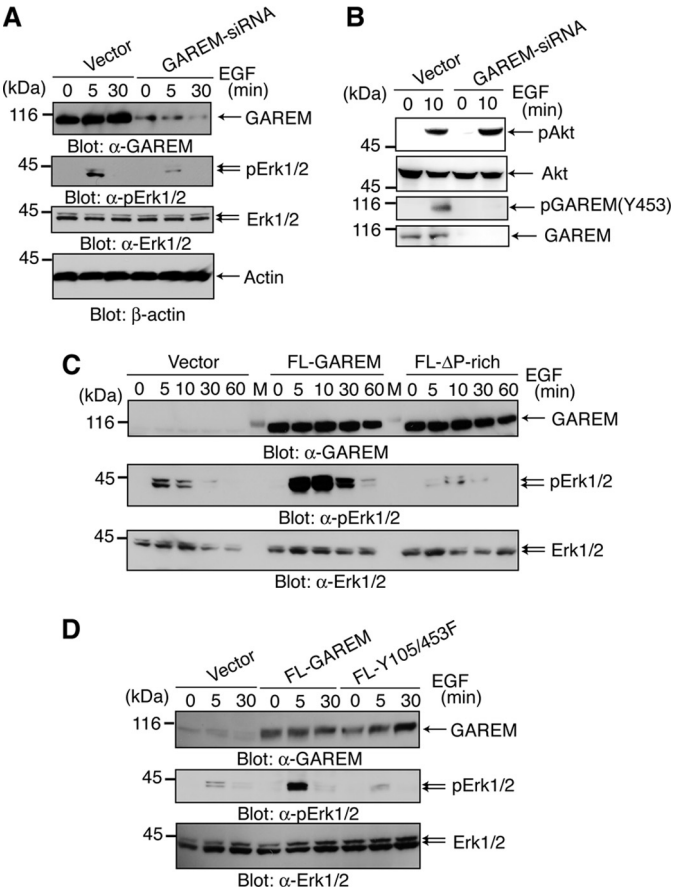

FIGURE 4. **Effect of GAREM expression on Erk activation in response to EGF stimulation.** *A*, effect of siRNA knockdown of GAREM on Erk activation in response to EGF stimulation. The amounts of GAREM (*upper*), phospho-Erk1/2 (*upper middle*), Erk1/2 (*lower middle*), and β-actin (*lower*) were compared by immunoblotting with each specific antibody. Total cell lysates were prepared using vector-infected (*left three lanes*) and siRNA construct-infected (*right three lanes*) HeLa cells stimulated with EGF for the indicated times; 20 μg of each lysate was applied in each lane. *B*, effects of knockdown of GAREM on phosphorylation, and the activation of Akt in response to EGF stimulation. Total cell lysates were prepared using the vector-infected (*left two lanes*) and siRNA construct-infected (*right two lanes*) HeLa cells stimulated with EGF for the indicated times; 20 μg of each lysate was applied in each *lane*, and immunoblotting analyses were performed using the indicated antibodies. *C* and *D*, the amount of each protein was compared by immunoblotting with anti-GAREM (*upper panel*), anti-phospho-Erk1/2 (*middle panel*), and anti-Erk1/2 antibodies. Total cell lysates were prepared from COS-7 cells transfected with the empty vector (*left lanes* in *C* and *D*), the expression plasmid for FLAG-GAREM (*center lanes* in *C* and *D*), the ΔP-rich mutant (*right lanes* in *C*), or the Y105F/Y453F mutant (*right lane* in *D*) and stimulated with EGF for the indicated times; 20 μg of each lysate was applied in each *lane*.

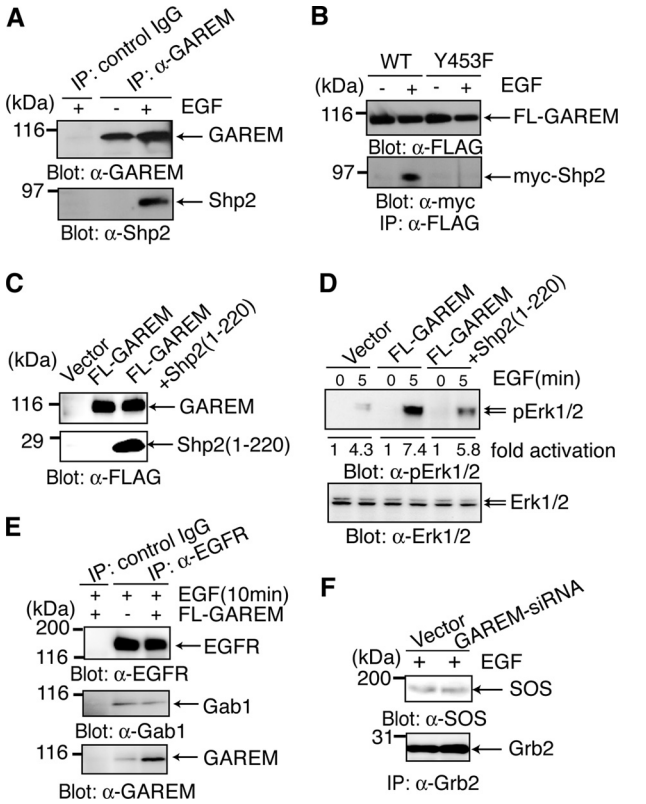

FIGURE 5. **Binding of Shp2 to GAREM upon EGF stimulation.** *A*, EGF stimulation-dependent association of endogenous GAREM and Shp2. Co-immunoprecipitation experiments were carried out with IgG (control) and anti-GAREM antibody by using the HeLa cell lysates with (+) or without (−) EGF treatment. Immunoblot analysis was performed using anti-GAREM receptor (*upper panel*) and anti-Shp2 (*lower panel*). *B*, Tyr-453 phosphorylation is required for the binding of GAREM to Shp2. COS-7 cells were transfected with a plasmid expressing Myc-Shp2, and each derivative molecule of FLAG-GAREM. Co-immunoprecipitation experiments and EGF stimulation were carried out as described in Fig. 2. Immunoblot analysis was performed using anti-FLAG (*upper panel*) and anti-Myc (*lower panel*) antibodies. *C* and *D*, effect of co-expression of the dominant negative construct of Shp2 and GAREM on Erk activation in response to EGF stimulation. *C*, expression of FLAG-GAREM (*upper panel*) and FLAG-Shp2-(1–220) (*lower panel*) in COS-7 cells. The amount of each protein was compared by immunoblotting that was performed as described above. *D*, the amount of each protein was monitored by immunoblotting with anti-phospho-Erk1/2 (*upper panel*) and anti-Erk1/2 (*lower panel*) antibodies. Total cell lysates were prepared from COS-7 cells transfected with the indicated expression vectors and stimulated with EGF for the indicated times; 20 μg of each lysate was applied. The levels of Erk phosphorylation were quantified by densitometry and normalized with total Erk. The ratio of Erk phosphorylation to the total Erk present in the cells with the indicated plasmid at time 0 was set as 1. *E*, GAREM forms a ternary complex with the EGF receptor and Gab1, and the effects of expression of GAREM on the EGF receptor-Gab1 complex are shown. The complex was monitored by immunoprecipitation performed using HeLa cells. EGF-stimulated cells with (+) or without (−) expressing FLAG-GAREM were lysed, and the immunoprecipitation was performed using anti-EGF receptor antibody. Immunoprecipitated EGF receptor (*upper panel*), co-precipitated Gab1 (*middle panel*), and GAREM (*lower panel*) are indicated, respectively. *F*, effects of knockdown of GAREM on the interaction between SOS and Grb2 in response to EGF stimulation. Immunoprecipitated Grb2 complex prepared from vector-infected (*left*) and siRNA construct-infected (*right*) HeLa cells stimulated with EGF was applied in each *lane*. Immunoblot analysis was carried out using anti-SOS1 (*upper panel*) and anti-Grb2 (*lower panel*) antibodies.

libraries derived from human tissues and cultured cells. The mRNA of wild-type GAREM was expressed ubiquitously. However, only a small population of cells expressed the small transcript of GAREM as compared with wild-type GAREM (Fig. 7*B*). We have obtained the cDNA of GAREM(S) and expressed it as a FLAG-tagged recombinant protein in COS-7 cells. Similar in behavior to the ΔP-rich mutant of GAREM, the small form did not associate with Grb2 (Fig. 7*C*). Thus, the expression level of GAREM and GAREM(S) in tumor cells may have a role in malignancy.

DISCUSSION

Members of the Gab family are known Grb2-binding proteins and regulators of downstream protein kinases such as Erk and Akt (3–5). Unlike Gab1, GAREM does not have multiple phosphorylation sites or other functional domains such as PH domains; instead, it has proline-rich sequences that enable its binding to N- and C-terminal SH3 domains of Grb2 in response to EGF stimulation (Figs. 2 and 3). Several phosphoproteomics studies have identified Tyr-453 to be one of the phosphoryla-

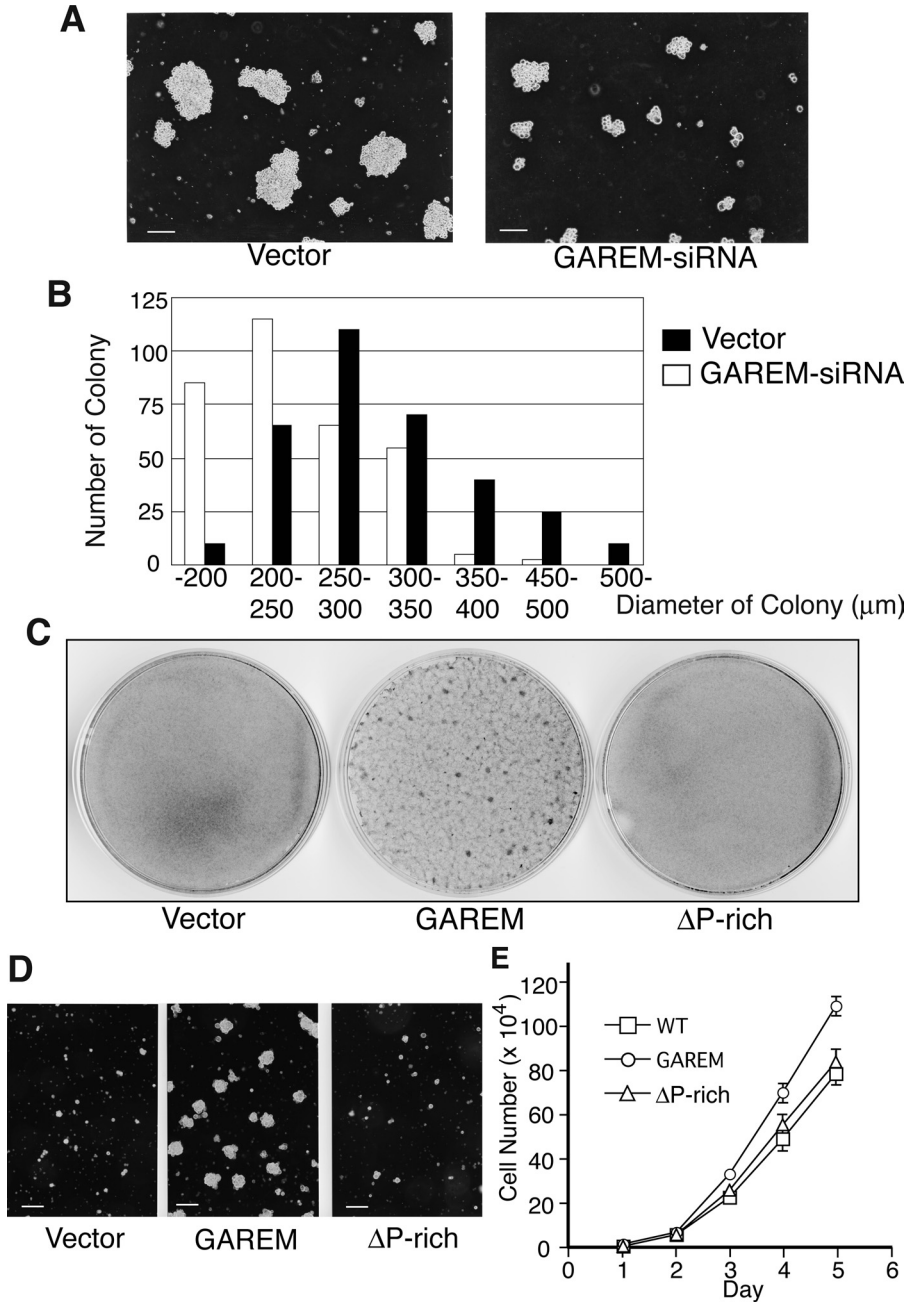

FIGURE 6. **Effects of GAREM expression on growth and transformation of the cultured cells.** *A*, colony formation in soft agar of HeLa cells infected with vector (*left panel*) and siRNA construct (*right panel*) was assayed as described under "Experimental Procedures." *B*, the *graph* shows the histogram distribution of the diameter of the colonies of each cell line. The *open* and *solid bars* indicate GAREM-siRNA and vector-infected cells, respectively. Data were obtained from 200 randomly selected colonies in one experiment, and three independent sets of experiments were performed. A representative result is shown. *C* and *D*, effects of the expression of GAREM on the focus and colony formation of NIH3T3 cells. *C*, the cell lines of NIH3T3 cells transfected with a vector (*left*), wild-type GAREM (*center*), and ΔP-rich mutant (*right*) were passaged in a growth medium supplemented with 10% calf serum. After 21 days, the cells were fixed and stained with crystal violet. Three independent sets of experiments were carried out, and representative pictures of the focus are shown. *D*, NIH3T3 cells transfected with vector (*left*), wild-type GAREM (*center*), and ΔP-rich mutant (*right*) were cultured in soft agar for 14 days. Three independent sets of experiments were carried out, and representative pictures of the colonies are shown. The *scale bars* represent 200 μm. *E*, NIH3T3 cells transfected with vector (□), wild-type GAREM (○), and ΔP-rich mutant (△) were seeded onto 6-well plates, and cells were counted at the time points shown. Data are shown as mean ± S.D. of the three experiments.

tion sites of GAREM (12, 15, 16). In addition to this site, we identified that the Tyr-105 phosphorylation occurs upon EGF stimulation. Although both tyrosine phosphorylation sites of GAREM were crucial for its binding to Grb2, neither Tyr-105 nor Tyr-453 is an effective direct binding site for the SH2 domain of Grb2. From these results, we speculate that the tyrosine phosphorylations might contribute to the conformational alteration of GAREM into its active form to facilitate interaction between the proline-rich motif of GAREM and SH3 domain of Grb2 in the EGF-stimulated cells.

On the other hand, the phosphorylation of Tyr-453 in GAREM plays an important role in the biological function of protein. The amino acid sequence surrounding the phosphorylation site Tyr-453 is $LP_{phospho}YEEL$: this site is a good match to the consensus sequence of the Shp binding site present in the Gab or the insulin receptor substrate-1 family (27, 28), which is also called an immunoreceptor tyrosine-based inhibitory motif (29, 30). In this study, we found that the phosphorylation of Tyr-453 and the immunoreceptor tyrosine-based inhibitory-like sequence surrounding GAREM may be necessary for the binding of GAREM to Shp2. Therefore, this binding might be directly caused by the enhanced activation of Erk in response to EGF stimulation. Immunoreceptor tyrosine-based inhibitory motifs normally exist in some receptor proteins. GAREM may provide an extra binding site for Shp2 on the plasma membrane. It may be involved in the formation of the activated EGF receptor complex, or it may indirectly associate with the EGF receptor via Grb2. In general, Gab1 was translocated to the plasma membrane in a manner dependent on the PH domain and the phospholipids generated by the stimulation of several growth factors (31). In contrast to Gab1, GAREM localized at the plasma membrane with or without EGF stimulation (data not shown). GAREM cDNA has been cloned as a gene coding for the SAM domain-containing protein at its extreme C terminus. Recently, SAM domains have been shown to play a role in homotypic or heterotypic protein-protein interactions (32, 33). Currently, we do not have any

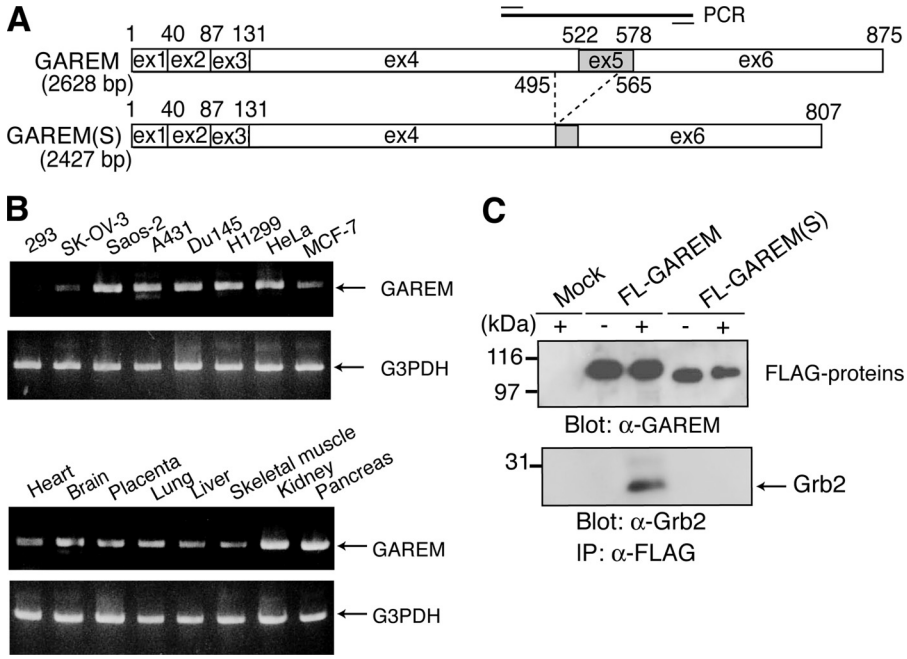

FIGURE 7. **Expression of the variants of GAREM in various human culture cells and tissues.** *A*, schematic representation of the primary structure of GAREM isoforms. Wild-type GAREM is composed of six exons (gi 12232415), and the truncated forms (GAREM(S)) lack the region from isoleucine 495 to proline 565 (gi 111307706). *Numbers* indicate amino acid residues of the boundaries. *B*, expression of GAREM mRNA in various human tissues and cultured cells. Quantitative PCR was carried out using specific internal oligonucleotide primers as indicated in *A*. The results of studies on various human culture cells (*upper panel* of *top panel set*) and tissues (*upper panel* of *bottom panel set*) are shown. The amplified DNA fragments of expected size derived from GAREM cDNA are indicated by an *arrow* in each *panel*. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was used as loading control. The PCR cycle number of all samples was 30. *C*, the binding property of FLAG-tagged GAREM(S) to Grb2. Co-immunoprecipitation studies were carried out using the cell lysates from COS-7 cells transfected with the empty vector or an expression plasmid encoding FLAG-GAREM or FLAG-GAREM(S). Each FLAG-tagged molecule was immunoprecipitated using anti-FLAG antibody. Immunoblot analysis was carried out using anti-GAREM (*upper panel*) and anti-Grb2 antibodies (*lower panel*).

experimental evidence to show that a portion of the C-terminal region of GAREM could function as a SAM domain. Further study will be required to determine the mechanism of cellular localization of GAREM.

An interesting aspect of this complex is the question of whether or not the Gab family is included. However, GAREM may be able to bind the complex of Grb2 and Shp2 downstream of the EGF receptor independent of Gab1 and SOS. Consequently, a new independent pathway, Grb2-GAREM-Shp2, may be functionally generated for Erk activation in addition to the previously known pathways such as Grb2-SOS, Shc-Grb2-SOS, and Grb2-Gab-Shp2 at the plasma membrane. The precise relationship between GAREM and other binding proteins to the EGF receptor remains to be elucidated.

In addition to the EGF receptor, the signaling pathways of the receptor tyrosine kinases are closely linked to the transformation of mammalian cells (11). We have shown the relationship between the expression level of GAREM and the transformation of the cells may be important. Although NIH3T3 cells have been known to be devoid of endogenous EGF receptor (34), the effect of GAREM on tumorigenesis could be observed in NIH3T3 cells and HeLa cells without adding EGF. Thus, GAREM may function as a downstream molecule for not only the EGF receptor but also other receptor tyrosine kinases.

Interestingly, a GAREM mutant lacking the proline-rich domain has been identified, and this mutant was not able to bind to Grb2 (Fig. 7). Because this mutant is not a splice variant of the wild-type GAREM, it may be difficult to generate it by simple gene mutation. The expression levels of this mutant protein in cultured cells or animal tissues are not yet clear. In our study, only a single protein band of GAREM was detected in the lysate of certain types of cultured cells such as COS-7, HeLa, 293, and A431 cells (data not shown). The expression of the GAREM gene (FLJ21610) has been identified by microarray analysis of multiple myeloma cells (35). Further, this gene is hypomethylated in various human colorectal carcinoma cell lines (36). The function of GAREM at physiological levels in normal cells or at abnormal levels in various tumor cells remains to be elucidated. We are currently analyzing the amount of GAREM protein and the level of Tyr-453 phosphorylation in various carcinoma cells to help understand the relationship between GAREM expression and tumorigenesis. Furthermore, screening of other interacting molecules in various cell lines, and the analysis of the function of SAM-like domains in the C-terminal of GAREM will help achieve a detailed understanding of its physiological functions.

In conclusion, we have identified GAREM, a novel adaptor protein in the growth factor-mediated signaling pathway. This protein may serve a crucial role in the regulation of the downstream molecules of the growth factor receptor as well as the Gab or insulin receptor substrate-1 family proteins.

Acknowledgments—We thank Dr. T. Horiuchi and T. Morinaga for the microscopic analysis. We also thank Dr. Ryan K. Louie for the helpful discussions.

REFERENCES

1. Pawson, T., and Nash, P. (2003) *Science* **300,** 445–452
2. Seet, B. T., Dikic, I., Zhou, M. M., and Pawson, T. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 473–483
3. Gu, H., and Neel, B. G. (2003) *Trends Cell Biol.* **13,** 122–130
4. Liu, Y., and Rohrschneider, L. R. (2002) *FEBS Letters* **515,** 1–7
5. Nishida, K., and Hirano, T. (2003) *Cancer Sci.* **27,** 4–14
6. DiNitto, J. P., and Lambright, D. G. (2006) *Biochim. Biophys. Acta* **1761,** 850–867
7. Mayer, B. J. (2001) *J. Cell Sci.* **114,** 1253–1263
8. Pawson, T. (2004) *Cell* **116,** 191–203
9. White, M. F. (2002) *Endocrinol. Metab.* **283,** E413–422

10. Schlessinger, J. (2002) *Cell* **110,** 669–672
11. Hynes, N. E., and Lane, H. A. (2005) *Nat. Rev. Cancer* **5,** 341–354
12. Blagoev, B., Ong, S. E., Kratchmarova, I., and Mann, M. (2004) *Nat. Biotechnol.* **22,** 1139–1145
13. Kratchmarova, I., Blagoev, B., Haack-Sorensen, M., Kassem, M., and Mann, M. (2005) *Science* **308,** 1472–1477
14. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) *Cell* **127,** 635–648
15. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) *Nat. Biotechnol.* **23,** 94–101
16. Thelemann, A., Petti, F., Griffin, G., Iwata, K., Hunt, T., Settinari, T., Fenyo, D., Gibson, N., and Haley, J. D. (2005) *Mol. Cell Proteomics* **4,** 356–376
17. Zhang, Y., Wolf-Yadlin, A., Ross, P. L., Pappin, D. J., Rush, J., Lauffenburger, D. A., and White, F. M. (2005) *Mol. Cell Proteomics.* **4,** 1240–1250
18. Konishi, H., Tashiro, K., Murata, Y., Nabeshi, H., Yamauchi, E., and Taniguchi, H. (2006) *J. Biol. Chem.* **281,** 28919–28931
19. Sano, E., Shono, S., Tashiro, K., Konishi, H., Yamauchi, E., and Taniguchi, H. (2008) *Biochim. Biophys. Acta* **1783,** 1036–1047
20. Tashiro, K., Konishi, H., Sano, E., Nabeshi, H., Yamauchi, E., and Taniguchi, H. (2006) *J. Biol. Chem.* **281,** 24612–24622
21. Neel, B. G., Gu, H., and Pao, L. (2003) *Trends Biochem. Sci.* **28,** 284–293
22. Chang, Y., Ceacareanu, B., Dixit, M., Sreejayan, N., and Hassid, A. (2002) *Circ. Res.* **91,** 390–397
23. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) *Methods Enzymol.* **255,** 395–412
24. Lewitzky, M., Kardinal, C., Gehring, N. H., Schmidt, E. K., Konkol, B., Eulitz, M., Birchmeier, W., Schaeper, U., and Feller, S. M. (2001) *Oncogene* **20,** 1052–1062
25. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., Cooper, J. A., and Schlessinger, J. (1994) *Mol. Cell Biol.* **14,** 509–517
26. Freedman, V. H., and Shin, S. I. (1974) *Cell* **3,** 355–359
27. Case, R. D., Piccione, E., Wolf, G., Benett, A. M., Lechleider, R. J., Neel, B. G., and Shoelson, S. E. (1994) *J. Biol. Chem.* **269,** 10467–10474
28. Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993) *Mol. Cell Biol.* **13,** 7418–7428
29. Pinheiro da Silva, F., Aloulou, M., Benhamou, M., and Monteiro, R. C. (2008) *Trends Immunol.* **29,** 366–373
30. Ravetch, J. V., and Lanier, L. L. (2000) *Science* **290,** 84–89
31. Maroun, C. R., Moscatello, D. K., Naujokas, M. A., Holgado-Madruga, M., Wong, A. J., and Park, M. (1999) *J. Biol. Chem.* **274,** 31719–31726
32. Qiao, F., and Bowie, J. U. (2005) *Sci. STKE 2005,* RE7
33. Zhuang, G., Hunter, S., Hwang, Y., and Chen, J. (2007) *J. Biol. Chem.* **282,** 2683–2694
34. Honegger, A., Dull, T. J., Bellot, F., Van Obberghen, E., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1988) *EMBO J.* **7,** 3045–3052
35. Claudio, J. O., Masih-Khan, E., Tang, H., Gonçalves, J., Voralia, M., Li, Z. H., Nadeem, V., Cukerman, E., Francisco-Pabalan, O., Liew, C. C., Woodgett, J. R., and Stewart, A. K. (2002) *Blood* **100,** 2175–2186
36. Frigola, J., Ribas, M., Risques, R. A., and Peinado, M. A. (2002) *Nucleic Acids Res.* **30,** e28