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RHODOSTOMIN, AN RGD-CONTAINING PEPTIDE EXPRESSED FROM A SYNTHETIC GENE IN *Escherichia coli*, FACILITATES THE ATTACHMENT OF HUMAN HEPATOMA CELLS

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SUMMARY: Rhodostomin (Rho) from snake venom, a potent inhibitor of platelet aggregation, contains 68 amino acids having an RGD sequence and 12 cysteine residues. A chemically synthesized *Rho* gene was cloned and expressed in *Escherichia coli*. The expression of *Rho* gene fused with the *glutathione S-transferase* (GST) gene was about 10-30% of total cell proteins. The Rho-fusion protein could be recognized by antibodies raised against either a native Rho peptide or a synthetic peptide. The purified GST-Rho coated on culture plates facilitated the attachment of human hepatoma cells, which was inhibitable by co-incubation with a synthetic hexapeptide GRGDSP but not with a related peptide of GRGESP, suggesting that the *E. coli*-expressed Rho-fusion protein was properly folded and biologically functional.

At least 10 small peptides, ranging from 48 to 84 amino acids and containing the tripeptide Arg-Gly-Asp (RGD) and 8 to 14 cysteine residues, have been isolated from snake venoms and reported to have high potency for inhibition of platelet aggregation (for a review, see 1). Reduction of disulfides of RGD-containing snake venom polypeptides dramatically reduces their ability to inhibit platelet aggregation, suggesting that a correct disulfide bond-mediated folding is essential for their biological activity (2,3). The RGD sequence is recognized by an adhesion receptor family containing

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Abbreviations: bp, base pair(s); EDTA, ethylene diaminetetraacetic acid; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RGD, Arg-Gly-Asp; Rho, rhodostomin; SDS, sodium dodecyl sulfate.

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heterodimeric polypeptides termed integrin (4,5), including the GPIIb-IIIa complex of platelets. The binding of fibrinogen to activated platelets is mediated by its RGD sequence with fibrinogen receptor associated with GPIIb-IIIa complex (6,7). Thus the RGD of snake venoms prevents fibrinogen from binding to GPIIb-IIIa on platelets and inhibits platelet aggregation (8,9). Recent studies further demonstrated that RGD-containing snake venom peptides also prevent melanoma cells from binding to extracellular matrix, i.e., fibronectin, vitronectin, and collagen, through the competition of RGD binding to integrin (3,10). These RGD-containing venom peptides, therefore, are recently termed "disintegrin" (1, 11).

Rhodostomin (Rho) from *Calloselasma rhodostoma*'s venom, consisting of 68 amino acids including 12 residues of cysteine and an RGD sequence at positions of 49-51 (see Fig 1A, 1, 12), is one of the most potent inhibitors of platelet aggregation among the known disintegrins (1,8). It is of a great interest to elucidate the structure of Rho and the mode of interaction of Rho with integrin for possible anti-thrombosis or anti-cancer usages. However, there is a major problem for studies of Rho, namely, only a small amount of peptide is available *via* purification of crude venom. To circumvent this difficulty, we attempted to overexpress a chemically synthesized *Rho* gene in *E. coli*. In this communication, we report such a protein constituted about 10-30% of total cell proteins and is biologically functional as manifest from the facilitation of attachment of human hepatoma cells to culture plates through the interaction of RGD and integrin. With this system, it will be possible to produce large amounts of Rho mutants (substitutions or deletions) for further studies on Rho-integrin interaction.

MATERIALS AND METHODS

Reagents: All restriction enzymes and DNA size markers were purchased from BRL (Gaithersburg, MD). The kit of polymerase chain reaction (PCR) was from Perkin Elmer Cetus (Norwalk, CT) and the kit of DNA sequencing from Promega (Madison, WI). Glutathione-conjugated Sepharose beads were from Pharmacia (Uppsala, Sweden) and synthetic hexapeptides of GRGDSP and GRGESP from Peninsula Lab. Inc. (Belmont, CA).

Bacterial strains: *Escherichia coli* strains DH5α, JM109(DE3), RR1, and K38/pGP1-2 were used for cloning or expression.

Oligonucleotide synthesis: For the synthesis of the artificial *Rho* gene, we divided the coding strand into 5 continuous oligonucleotides and the complementary strand into 6 continuous oligonucleotides as shown in Fig. 1A. It is to be noted that these oligonucleotides from the two strands can overlap among themselves to form, after ligation, the full length synthetic *Rho* gene. These oligonucleotides ranging from 24- to 45-mers in length were synthesized on a DNA synthesizer (Applied Biosystems 380B) and purified by denaturing PAGE on a 6% acrylamide/8 M urea gel. After elution from the gel by Maxam and Gilberts' buffer (0.1 M Tris-HCl, 5 mM EDTA and 0.5 N NaCl, pH 8.0), the oligonucleotides were desalted by passage over a Millipore C_{18} Sep-Pak cartridge (13). Each of oligonucleotides was phosphorylated in the presence of 1 mM ATP and T4 polynucleotide kinase.

Assembly and cloning of the synthetic *Rho* gene: A mixture of eleven oligonucleotides (~1 nM of each fragment) coding for two strands of Rho was heated to 95°C and then slowly cooled at room temperature for annealing. Ligation was carried

out at 16°C for 15 h in a total volume of 30 µl containing 66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 30 units of T4 DNA ligase. After ligation, one µl ligation mixture was used as a template for PCR by adding of two 5'-overhanging oligonucleotides (1.25 pM) as primers and 1.5 units of Taq enzyme. The reaction was performed following the supplier's protocol. The *Rho* gene amplified by PCR was checked by 2% agarose gel and then ligated into the *Sma*I site of pGEM7 (Promega) to yield the pGEM7-RHO recombinant.

Construction of Rho-expression plasmids: Two expression vectors, pGEMX-1 (14) and pGEX-2KS (15), were used to express Rho in *E. coli*. The constructions of expressed plasmids, pGEMX-RHO and pGEX-2KSRHO, are partially illustrated in Fig. 1B.

Protein analysis and purification: The production of Rho containing proteins in different strains of *E. coli* carrying different plasmids was analyzed by SDS-PAGE (16) with Coomassie blue staining or Western blotting (17). The GST-Rho fusion protein produced by the pGEX-2KSRHO harboring cells was purified by a glutathione column and its purity was analyzed by SDS-PAGE as described by Smith et al. (15).

Cell attachment assay: The method is described by Yamada and Kennedy (18). Briefly, the purified GST-Rho was coated on 24 or 96 well microtiter plates for 3 h, and then washed with PBS twice. After blocking with bovine serum albumin overnight, a proper number of human hepatoma cell, HuH-7 (19), was seeded. After 15 min, the unattached cells were washed off by PBS and the attached cells were fixed and photographed. To quantify cell attachment, the HuH-7 cells were first metabolically labeled with [^{35}S]-methionine. Attached cells were freed from plates by a treatment of trypsin and EDTA and the amount of isotope-labeled cells was then determined by a beta-scintillation counter.

RESULTS AND DISCUSSION

Design and construction of the synthetic *Rho* gene: In the beginning of this work, although the amino acid sequence of Rho has already been determined, there was no *Rho* gene available (1,12). We decided to express Rho by using a chemically synthesized gene in *E. coli*. As indicated in Fig. 1A, the synthetic *Rho* gene is composed of 11 oligonucleotides covering two complementary strands of gene. Five of them are continuous and represent the coding strand of *Rho* gene. The complementary strand is covered by six continuous segments. These segments are the opposing strands overlapping with each other to allow the generation of a double-stranded *Rho* gene after ligation.

Several features of the synthetic *Rho* gene should be noted: (i) It is 225-bp long, including 68 codons for Rho and 7 extra codons for cloning and expression purposes (see below). (ii) Codons highly utilized in *E. coli* gene expression (20) were selected for the *Rho* gene except for the codon of CGT (encoding Arg) located at the 3'-end of gene, creating a *Hin*cII site. (iii) Two extra codons, coding for Thr and Met, were placed at the 5'-end of gene to create an *Nco*I site. This together with the *Hin*cII or *Eco*RI site at the 3'-end allows the intact *Rho* gene to be excised out for recloning if desired. Furthermore, this synthetic gene generates a 5' sequence ACCATGG, optimal for initiation of translation in most eukaryotic cells. This would ensure high level expression of *Rho* gene in eukaryotic cells (21). (iv) Two residues of Arg and restriction sites of *Hin*cII and *Eco*RI were introduced at the 3'-terminus of the gene.

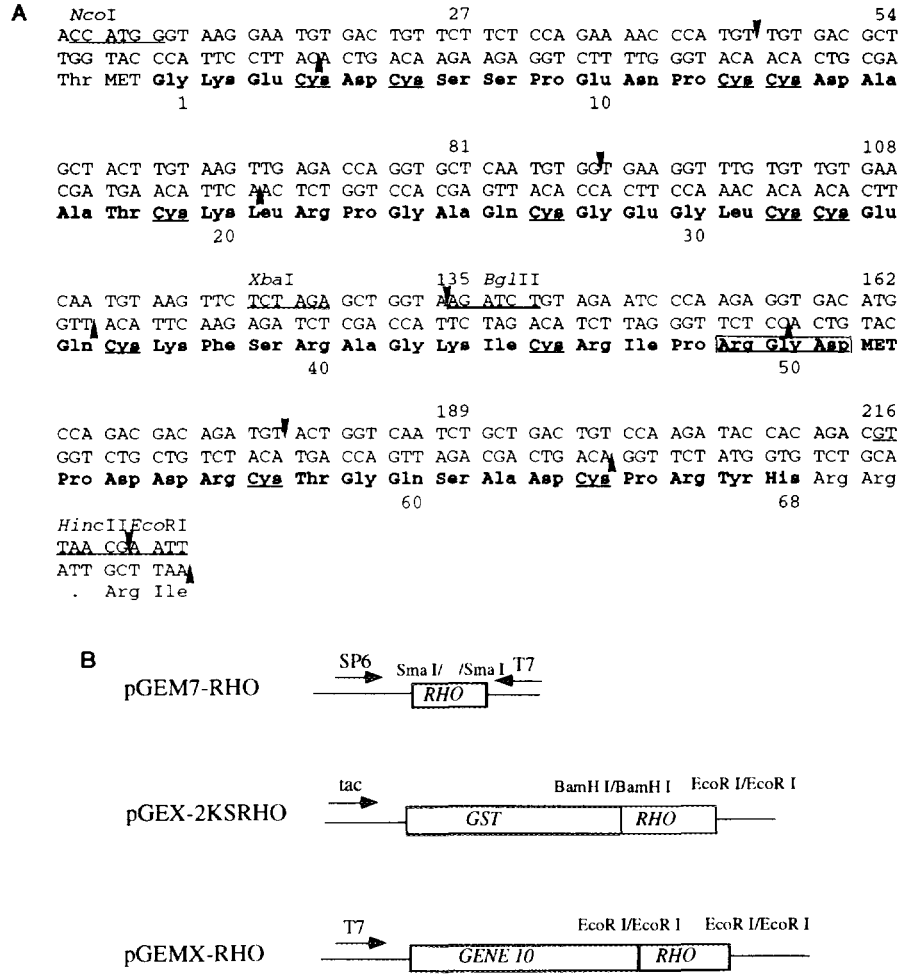

Fig.1. Nucleotide sequence of the synthetic *Rho* gene and the Rho-containing plasmids. (A) The gene was designed from the known amino acid sequence of Rho (1) and the codons selected for each amino acid were adapted from those found in highly expressed *E. coli* gene (20). The 11 oligonucleotides used to assemble the *Rho* gene are delineated by small arrowheads either above the coding strand or below the non-coding strand. The amino acid sequence is represented in a three letter abbreviation and the 68 amino acids of Rho are indicated by boldface letters. The residues of cysteine are underlined and the Arg-Gly-Asp sequence is boxed. Specific restriction sites are shown above the coding sequence. The nucleotide number of synthetic gene is given above the sequence and the amino acid number of Rho is indicated below the sequence. (B) Partial restriction maps of three Rho-containing plasmids, pGEM7-RHO, pGEX-2KSRHO, and pGEMX-RHO. Only the *Rho* gene and its relevant upstream regions are shown.

The *Eco*RI site was originally designed for directional cloning the synthetic *Rho* gene into the pGEM7 vector (see below). The *Hinc*II site supplies a blunt cutting site to facilitate fusing with any other gene downstream. The two Arg residues at the end of Rho provides a cutting site by protease when Rho is expressed as a fusion protein.

Attempts to directly clone the ligated 11 oligonucleotides fragment into the *Sma*I and *Eco*RI sites of pGEM7 were failed, which might due to the low abundance of

ligated fragment. The ligated fragment was then amplified by adding of two 5'-overhanging oligonucleotides for PCR and the amplified fragment was inserted into the *Sma*I site of pGEM7. The resultant pGEM7-RHO was initially checked by the presence of *Xba*I and *Bgl*II sites in the middle portion of *Rho* gene (see Fig. 1A) and then confirmed by the method of dideoxy-chain-termination sequencing (22).

Expression of Rho-fusion proteins: In order to obtain a large quantity of Rho in *E. coli*, the *Bam*HI-*Eco*RI fragment containing the synthetic *Rho* gene was excised out from the pGEM7-RHO plasmid and cloned into two different expression vectors, pGEX-2KS and pGEMX-1, to yield the pGEX-2KSRHO and pGEMX-RHO recombinants, respectively (see Fig. 1B). In the pGEX-2KSRHO, the *Rho* gene is fused downstream to the *glutathione S-transferase* (GST) gene and a fusion protein of 35 kDa was expected. The expression of GST-Rho in this construct is under the control of *tac* promoter. In the pGEMX-RHO, the *Rho* gene is fused downstream to a 780-bp fragment of phage T7 gene 10. In this construct, the expression of Rho-fusion protein is under the T7 RNA polymerase regulation and the expected molecular weight of Rho-fusion protein is about 41 kDa.

As shown in Fig. 2A, a dominant band of 35 kDa protein was observed in the pGEX-2KSRHO harboring cells under an IPTG induction, while no such band was detected in non-induction cells. Judged from its relative intensity of Coomassie blue stain, the amount of GST-Rho fusion protein was accounting for about 10-30% of total cell proteins. Western blot analysis using the anti-Rho antibodies revealed that the 35-kDa protein was indeed immunoreacted with the anti-Rho antibodies (Fig. 2B). These results, therefore, strongly suggest that the fusion protein of 35 kDa contains a Rho

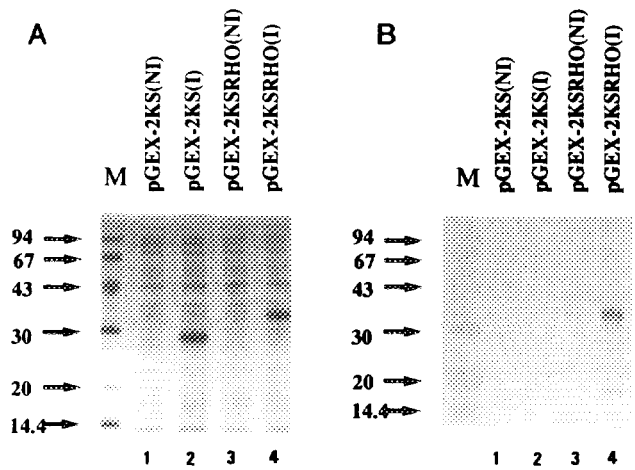

Fig. 2. Analysis of the expression of GST-Rho in *E. coli.* by Coomassie blue stain (A) and immunoblot (B). Lanes 1 and 2 are cells harboring the vector plasmid pGEX-2KS; lanes 3 and 4 are cells with the plasmid pGEX-2KSRHO. Lanes 1 and 3 are without IPTG induction and lanes 2 and 4 are with induction. The protein standard markers are indicated at left. Immunoblot was with a rabbit polyclonal antibody against a synthetic peptide (amino acid positions 45-59) of Rho. A similar result was obtained by using a rabbit polyclonal antibody against the native Rho peptide.

peptide. A similar amount of T7 gene 10-Rho was also obtained from the pGEMX-RHO containing cells under an IPTG induction (data not shown).

Cell attachment assay: To investigate the biological function of *E. coli*-expressed GST-Rho, cell attachment assay was adapted because it has been widely used in analyzing the function of RGD-containing proteins (23). Fig. 3 shows the attachment of human hepatoma cells on culture plates coated with or without proteins. These results clearly indicated that at least 10 to 20 folds higher number of cells attached to the GST-Rho-coated plates (micrograph 3a) than to the GST-coated plates (micrograph 3b). It was also noticed in micrograph 3c that there was a fewer number of cells on the non-protein-coated area (lower left region) than on the GST-Rho-coated area (upper right region). These results show that the Rho-containing protein is able to facilitate cell attachment on plates.

To further test whether the GST-Rho mediated cell attachment is due specific with Rho and involves the RGD sequence, quantitative analyses of attached cells on plates coated with various amounts of GST-Rho or in the presence of a synthetic hexapeptide of GRGDSP or of GRGESP were performed. Fig. 4A shows that cell attachment was gradually increased when the plates were coated with GST-Rho ($\geq$ 6 μg/ml) but not when coated with GST (up to 4 mg/ml). As compared with the number of attached cells on GST-coated plates, the GST-Rho-coated plates promoted about 10 to 25 folds of cell attachment. These results are in accordance with above mentioned microscopic observations.

We also observed that cell attachment on the GST-Rho-coated plates was decreased by the presence of increasing amounts of the hexapeptide GRGDSP but not the hexapeptide GRGESP (Fig. 4B). These results fully support the notion that the facilitation of HuH-7 cell attachment on plates is in part, if not all, through the

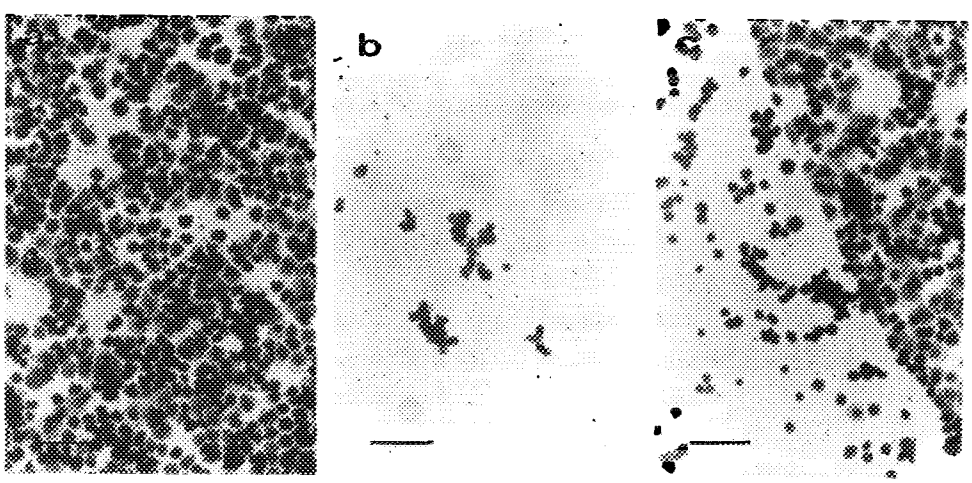

Fig. 3. Light microscopic analysis of cell attachment. (a) and (c) show cells attached on GST-Rho coated plates while (b) shows cells on GST-coated plates. (a) and (b) show the center of protein-coating region while (c) shows the boundary of protein-coating region. The bar indicates 20 μm.

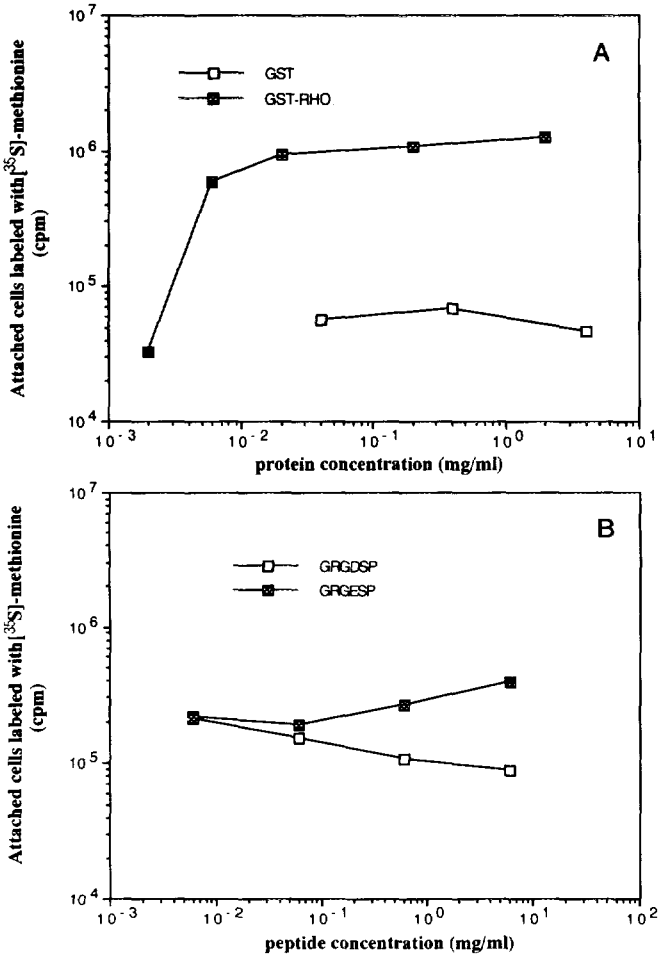

Fig. 4. Quantitative analysis of cell attachment. (A) Cell attachment on GST- or GST-Rho-coated plates. The amount of isotope from human hepatoma cells attached on various amount of GST-coated plates or on GST-Rho-coated plates are represented by open and closed squares, respectively. (B) Effect of synthetic hexapeptides on cell attachment. The amount of cells attached on the GST-Rho-coated plates in the presence of synthetic hexapeptides GRGDSP and GRGESP are represented by open and closed squares, respectively.

interaction of the RGD sequence of Rho and the integrin of cells. They also demonstrated that the GST-Rho fusion protein produced from a chemically synthesized gene in *E. coil* is biologically functional.

According to the data of computer search by Titani et al. (24), there are more than 100 different known proteins containing the RGD sequence. However, not all RGD-containing proteins can serve as cell adhesion molecules, e.g., IgG, tubulin, and various intracellular enzymes (24), indicating that proper conformation and exposition of RGD sequence of protein are essential. In this study, we successfully demonstrate that the GST-Rho fusion protein is able to serve as cell adhesion molecules. This suggests that GST-Rho is properly folded as in the native Rho to maintain proper configuration and exposition of the RGD sequence, allowing its binding to the integrin

of human hepatoma cells. The newly created synthetic gene of Rho may, therefore, facilitate the study of interaction of Rho and integrin *via* generating more mutated Rho peptides.

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