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Citation	Journal of Biological Chemistry. 1989, 264(26), p. 15165-15168
Version Type	VoR
URL	<a href="https://hdl.handle.net/11094/71443">https://hdl.handle.net/11094/71443</a>
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## A Novel Cell Adhesive Protein Engineered by Insertion of the Arg-Gly-Asp-Ser Tetrapeptide\*

(Received for publication, April 21, 1989)

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A tetrapeptide Arg-Gly-Asp-Ser (RGDS) has been shown to be a versatile cell recognition signal of extracellular matrix components for the interaction with cells. We introduced the RGDS tetrapeptide into a truncated form of protein A, a staphylococcal immunoglobulin-binding protein, by inserting an oligonucleotide cassette encoding the tetrapeptide into the coding region of the protein A expression vector pRIT2T. The mutagenized protein was capable of not only binding to immunoglobulin G but also mediating cell attachment and spreading onto an inert substrate. Cell adhesion mediated by the mutagenized protein was inhibitable by a synthetic peptide Gly-Arg-Gly-Asp-Ser but not by a related peptide Gly-Arg-Gly-Glu-Ser, confirming that the inserted RGDS tetrapeptide served as a recognition signal for cell adhesion. Furthermore, the RGDS-containing protein was capable of adhering cells onto an immunoglobulin-coated surface which could not by itself support cell adhesion. Thus, the cell adhesive and immunoglobulin binding activities of the mutagenized protein appear to function coordinately. The protocol described here is essentially applicable to any protein and, therefore, provides a general principle in tailoring novel multifunctional proteins having cell adhesive activity.

The interaction of cells with the extracellular matrix components plays an important role in many biological processes such as cell migration, differentiation, and possibly proliferation. Recently, a family of proteins capable of mediating cell adhesion onto biologic as well as artificial substrates has been isolated from the extracellular matrix and body fluids (for recent reviews, see Refs. 1-4). These cell adhesive proteins, characterized by the presence of a conserved tripeptide Arg-Gly-Asp (RGD), include fibronectin (5), vitronectin (6), laminin (7), von Willebrand factor (8), type I collagen (9), fibrinogen (10), thrombospondin (11), osteopontin (12), and tenascin (13). The RGD sequence serves as a recognition signal for the interaction with cell surface receptor molecules, col-

lectively termed integrins (3, 14).

In the present investigation, we attempted to genetically engineer an artificial cell adhesive protein by insertion of the Arg-Gly-Asp-Ser (RGDS) tetrapeptide, the minimal cell adhesive sequence of fibronectin (5), into a truncated form of the bacterial IgG-binding protein, protein A'.<sup>1</sup> We report here that the modified bacterial protein was capable of binding not only to IgG but also to the RGD-directed cell surface receptor(s), thereby serving as a mediator of cell adhesion onto the substrate coated with IgG.

### EXPERIMENTAL PROCEDURES

**Oligonucleotide Synthesis**—Oligonucleotides were chemically synthesized using an Applied Biosystems DNA synthesizer model 380A (Foster City, CA). The double-stranded DNA oligomer encoding a heptapeptide SLRGDSA ("RGDS cassette") was obtained by annealing 5'AGCTTACGTGGTGATAGCGCT3' and 5'AGCTAGCGCT-ATCACACGTA3' (Fig. 1). Another double-stranded DNA oligomer encoding SLRGESA ("RGES cassette") was prepared by annealing 5'AGCTTACGTGGTGAAAGCGCT3' and 5'AGCTAGCGCTTTC-ACCACGTA3'.

**Vector Constructions**—A plasmid expression vector pRIT2T (Pharmacia, Uppsala, Sweden) was partially digested with *Hind*III (Takara Shuzo, Kyoto, Japan), and the linearized plasmid was ligated with >1000-fold molar excess of the unphosphorylated oligonucleotide cassette and used to transform *Escherichia coli* HB101. The plasmid containing the cassette at the downstream *Hind*III site was selected upon double digestion with *Nhe*I and *Pst*I, by which the desired construct gave rise to a 385-base pair fragment. The pRIT2T derivative was used to transform *E. coli* N4830 to express the RGDS- or RGES-containing protein under the control of the temperature-sensitive  $\lambda$  repressor (15).

**Protein Purification**—The *E. coli* N4830 harboring pRIT2T or its derivative was grown in LB medium containing 50  $\mu$ g/ml ampicillin at 30 °C until OD<sub>600 nm</sub> became approximately 1.0. The temperature of the culture was then shifted to 42 °C by adding an equal volume of LB medium preheated to 54 °C. Bacterial cells were cultured at 42 °C for 90 min and then harvested by centrifugation at 5,000  $\times$  *g*. The cells were suspended in 0.1 volume of TS (50 mM Tris buffer, pH 7.6, containing 150 mM NaCl) and lysed by sonication. The lysate was centrifuged at 20,000  $\times$  *g* for 20 min at 4 °C, and the supernatant was applied to a IgG-Sepharose column (Pharmacia) after dilution with 5 volumes of TS. After extensive washing with TS, the bound protein was eluted with 0.5 M ammonium acetate, pH 3.4, and dialyzed against 2 mM CAPS<sup>2</sup> buffer, pH 10.5, containing 0.5 mM EDTA at 4 °C. The protein concentration was estimated from the absorbance at 280 nm assuming that the absorption coefficient of 0.1% protein A' is 0.38 cm<sup>-1</sup>. Purity of the modified proteins thus obtained was verified by SDS-PAGE using 10% acrylamide gels, followed by staining with Coomassie Blue.

**Protein Sequencing**—Protein A' mutagenized with the RGDS cassette was cleaved at room temperature with CNBr in 70% formic acid as described by Gross (16). The fragment containing the RGDS sequence was isolated by reversed-phase HPLC on a column of SynChropak RP8 (4.1  $\times$  250 mm, SynChrom, Lafayette, IN). Automated sequence analysis of the purified fragment was performed with an Applied Biosystems protein sequenator model 470A (Foster City, CA).

**Cell Adhesion Assay**—The cell adhesive activity of the mutagenized

\* This investigation was supported by a grant-in-aid from Fujita-Gakuen Health University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The truncated form of protein A encoded by the protein A expression vector pRIT2T is referred to as "protein A'" in this paper to distinguish it from the intact protein.

<sup>2</sup> The abbreviations used are: CAPS, 3-(cyclohexylamino)propane-sulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

protein A's was determined essentially as previously described (17, 18). Twenty-four-well bacteriological plates (Petray SH-T24FS, Terumo, Tokyo, Japan) were coated with the modified protein A' upon appropriate dilutions with 0.1 M NaHCO<sub>3</sub>, pH 9.5, overnight at room temperature and then blocked with 0.1% bovine serum albumin for 2 h. The baby hamster kidney fibroblasts were detached from the culture dish with 0.25% trypsin and 0.02% EDTA, washed once with adhesion medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Hepes-buffered saline (20 mM Hepes, 137 mM NaCl, 3 mM KCl, pH 7.1)) containing 0.01% soybean trypsin inhibitor and twice with adhesion medium without the inhibitor. The cells were incubated on the precoated polystyrene plates at  $4 \times 10^5$  cells/well for 60 min in adhesion medium at 37 °C and then gently washed three times with adhesion medium. The attached cells were fixed with 4% formaldehyde and photographed using phase contrast microscopy. The number of cells adopting well spread morphology was counted with four independent fields/well.

**Protein Adsorption Isotherms**—The amount of proteins adsorbed onto plastic substrate was determined using <sup>125</sup>I-labeled proteins. Protein iodination was performed by the chloramine-T method (34). The 24-well plastic plates were coated with serially diluted protein solutions as described for the cell adhesion assay, except that the protein solutions contained a trace amount of the iodinated protein. The plates were washed three times with phosphate-buffered saline (8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and then the radioactivity adsorbed on each well (2 cm<sup>2</sup>/well) was determined with a well-type  $\gamma$  counter (Aloka ASP-120, Aloka, Tokyo, Japan).

## RESULTS AND DISCUSSION

A plasmid vector pRIT2T encodes a truncated form of protein A (residues 23–269), hereafter referred to as protein A', which contains four consecutive IgG-binding domains (15, 19). There are two *Hind*III sites within the coding region of the plasmid, of which the downstream site was chosen for the insertion of the oligonucleotide cassette encoding either SLRGDSA or SLRGESA heptapeptide (Fig. 1). The amino acid residues flanking the RGDS or RGEA tetrapeptide are encoded by the *Hind*III linkers attached to the oligonucleotides encoding these tetrapeptides. The double-stranded oligonucleotides contain a unique *Nhe*I site at the 3' end which facilitates the identification of the construct containing the insert at the correct *Hind*III site with a right orientation. The expression of the mutagenized protein was induced by heat inactivation of the temperature-sensitive  $\lambda$  repressor of the host *E. coli*.

The mutagenized proteins retained the IgG binding activity,

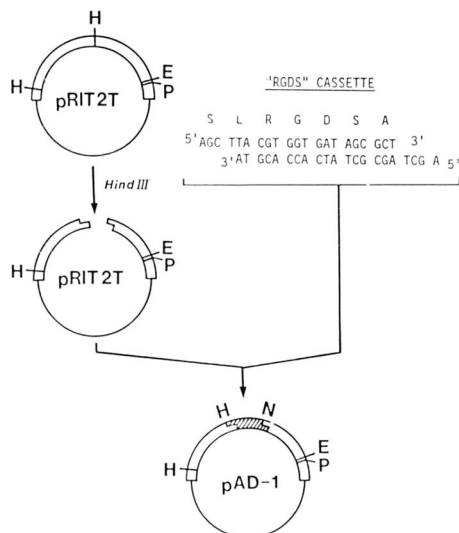


FIG. 1. Construction of the plasmid vector pAD-1 by insertion of an oligonucleotide cassette encoding SLRGDSA. Restriction sites indicated are: H, *Hind*III; P, *Pst*I; E, *Eco*RI; N, *Nhe*I.

since they could bind to an IgG-Sepharose column. The mutagenized proteins migrated slightly above the unmodified protein upon SDS-PAGE (Fig. 2A), in agreement with the insertion of the extra heptapeptide, either SLRGDSA or SLRGESA. This was further confirmed by direct protein sequencing of the CNBr-cleaved fragment containing the SLRGDSA heptapeptide (Fig. 2B).

Fig. 3 shows the cell adhesive activity of the mutagenized protein. The protein A' containing the RGDS sequence was capable of mediating cell attachment and subsequent spreading when coated on the inert plastic surface (*panel C*), although the unmodified protein A' was totally inactive (*panel B*). Substitution of aspartic acid with glutamic acid in the extra heptapeptide completely abolished the cell adhesive activity of the modified protein (*panel D*). Thus, acquisition of the cell adhesive activity by protein A' strictly requires the RGDS sequence and is not simply due to the insertion of the extra heptapeptide. The specificity of the cell adhesive activity of the modified protein was further confirmed by inhibition experiments with synthetic pentapeptides. Cell adhesion onto the substrate coated with the protein A' containing the RGDS sequence was completely inhibited by GRGDS (*panel E*) but not by GRGES (*panel F*).

Although the protein A' with the RGDS sequence displayed a significant cell adhesive activity, it was less active than intact fibronectin (Fig. 4). The maximal level of cell spreading achieved was only half as much as that achieved by fibronectin. The difference in the cell adhesive activity between these two proteins was not due to the difference in the amounts of the proteins adsorbed on plastic substrate, since more modified protein A' than fibronectin was adsorbed on the substrate at the protein concentrations where significant cell spreading was observed (Fig. 4, *inset*). It is likely that the affinity of the RGDS cell adhesion signal with the cell surface receptors was

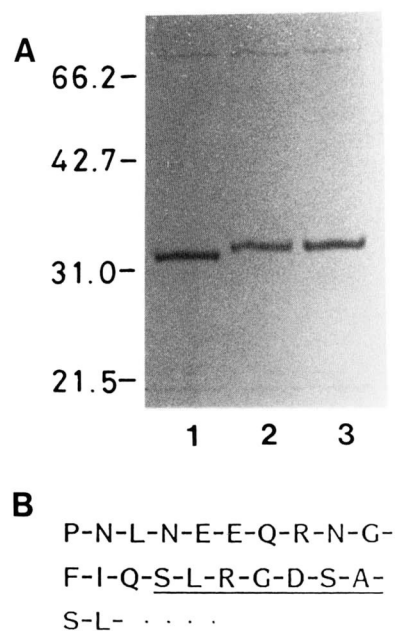
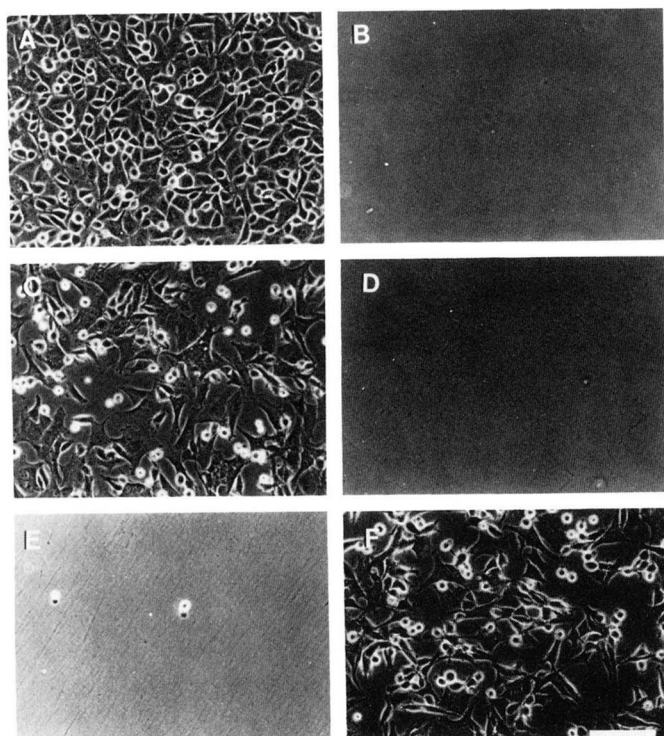


FIG. 2. SDS-PAGE and partial amino acid sequencing of protein A's modified by insertion of extra heptapeptides. A, SDS-PAGE of control protein A' (*lane 1*), protein A' mutagenized with the RGDS cassette (*lane 2*), and protein A' mutagenized with the RGEA cassette (*lane 3*). Positions of the molecular weight standards are shown in the left margin. B, partial amino acid sequence of the CNBr fragment of the protein A' mutagenized with the RGDS cassette. The CNBr fragment of the modified protein containing the extra heptapeptide was isolated by HPLC and sequenced with an automated protein sequenator.

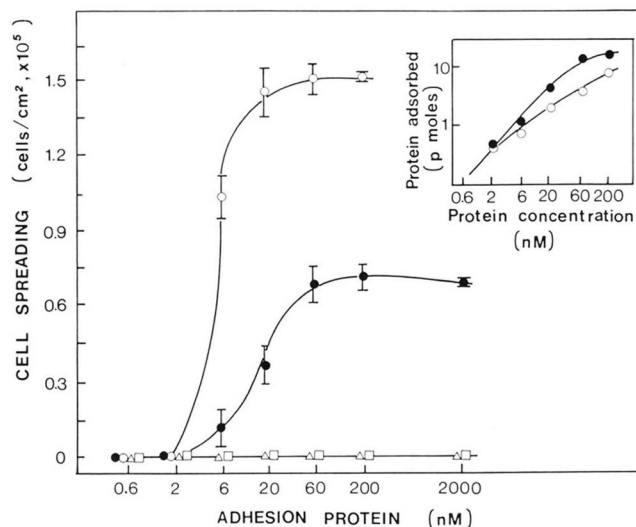


**FIG. 3. Cell adhesive activities of protein A's mutagenized by insertion of extra heptapeptides.** Trypsinized baby hamster kidney fibroblasts were incubated on the plastic substrates precoated with the following proteins at 5 µg/ml: A, fibronectin; B, control protein A'; C, E, and F, protein A' containing the extra SLRGDSA peptide; D, protein A' containing the extra SLRGESA peptide. The synthetic pentapeptide GRGDS (E) or GRGES (F) was included in the adhesion medium in order to examine its inhibitory effects on the cell adhesion onto the substrate coated with protein A' containing the SLRGDSA peptide. Bar = 100 µm.

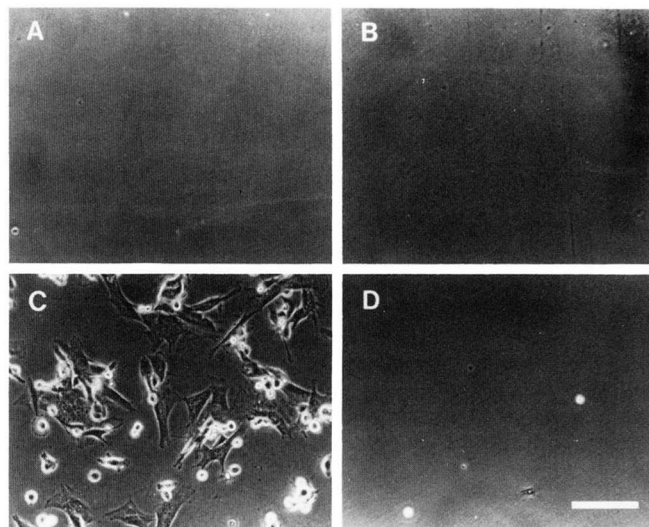
significantly lower when transplanted in protein A' than situated at the original locus in intact fibronectin.

Since the protein A' with the RGDS sequence was capable of not only binding to IgG but also of being recognized by the RGD-directed cell surface receptors, we examined whether the modified protein could serve as an adaptor for the interaction of the RGD-directed receptors with IgG. Although the cells could not adhere to the substrate coated with IgG (Fig. 5A), they could attach and spread on the same substrate after pretreatment with protein A' containing the RGDS sequence (Fig. 5C). Pretreatment with the unmodified protein (Fig. 5B) or the protein containing the RGES sequence (Fig. 5D) did not allow the cells to attach. These results clearly demonstrate that the cell adhesive and IgG binding activities of the RGDS-containing protein A' function coordinately. It should be noted, however, that the RGDS-containing protein A' adsorbed on the substrate via IgG was less active than that adsorbed directly (compare Fig. 5C with Fig. 3C). This could be due to either partial blockade or conformational change of the RGDS signal upon binding to the substrate-adsorbed IgG.

A computer search of the protein sequence data base has identified more than 100 different proteins containing the RGD tripeptide, many of which are unlikely to possess the cell adhesive activity (3, 20). Indeed, no significant affinity to the RGD-directed cell surface receptors has been detected with IgG (Ref. 21; also the present study) or a peptide derived from epidermal growth factor receptor (22), both of which contain the RGD sequence. Thus, the presence of the RGDS sequence *per se* does not guarantee the coincidence of the cell adhesive activity. It is likely that, besides being exposed to



**FIG. 4. Quantitative cell spreading assay on the substrates coated with the mutagenized protein A's.** The plastic substrates were coated with different concentrations of fibronectin (○), control protein A' (△), protein A' containing the RGDS sequence (●), and protein A' containing the RGES sequence (□). Cells were incubated on the substrates for 60 min in a CO<sub>2</sub> incubator. The extent of cell spreading was expressed as the number of cells adhered to unit surface area (*i.e.* cm<sup>2</sup>). The concentration of fibronectin is expressed as that of fibronectin monomer in order to compare the cell adhesive activity of fibronectin and the mutagenized protein A' on a "per RGDS site" basis. *Inset* shows the adsorption isotherm of fibronectin and the RGDS-containing protein A'.



**FIG. 5. Cell adhesion onto the substrates precoated with IgG.** The plastic substrates were first coated with human IgG (200 µg/ml) and then blocked with 0.1% bovine serum albumin. The substrates were incubated with phosphate-buffered saline (A), control protein A', 5 µg/ml (B), protein A' containing the SLRGDSA peptide, 5 µg/ml (C), or protein A' containing the SLRGESA peptide (D) before incubation with trypsinized cells. Bar = 100 µm.

the surface of the molecule, the RGD(S) sequence must assume a certain conformation to be recognized by its cell surface receptors with an affinity sufficient for stable cell adhesion (3, 23).

In the present investigation, we have shown that the insertion of the RGDS sequence into one of the four IgG-binding domains of protein A' confers the cell adhesive activity to this bacterial protein. Based on the x-ray crystallographic analysis of the IgG-binding domain of protein A complexed

with the Fc fragment of IgG (24), it is predicted that the RGDS sequence is inserted to one of the two anti-parallel  $\alpha$ -helices composing the framework of the IgG-binding domain and is exposed at the surface of the molecule. Furthermore, prediction of the secondary structure by the algorithm of Chou and Fasman (25) suggests that the RGDS sequence within protein A' is likely to assume a  $\beta$ -turn conformation, as considered to be the case within intact fibronectin. Thus, it is not irrational to speculate that the inserted RGDS tetrapeptide assumes a conformation recognizable by the cell surface receptors with an affinity enough to support stable cell adhesion.

It should be noted, however, that the affinity of the modified protein A' to the RGD-directed receptors was significantly lower than that of intact fibronectin. This is most likely due to the incomplete fitness of the RGDS signal with its receptors when situated as a "transplant" in the foreign protein. The reduced activity of the RGDS signal of the modified protein could be also partly due to the lack of the putative second signal required for the full cell adhesive activity (26). The cell adhesive protein A' described in this paper will provide a suitable experimental system for identification and localization of the putative second signal by simply inserting the candidate sequence alone or together with the RGDS signal into protein A'.

The RGD cell recognition signal of many cell adhesive proteins has been shown to be recognized by a family of cell surface receptor molecules, termed integrins (3, 14). It is of real interest, therefore, which integrin(s) is involved in the cell adhesion mediated by the RGDS-containing protein A'. Recently, Singer *et al.* (35) reported that both fibronectin and vitronectin receptors became concentrated at focal contacts when cells spread out on the substrate coated with the synthetic RGDS peptide-albumin conjugate, suggesting that both receptors can recognize the synthetic peptide. Although these observations cannot be simply extended to the cell adhesion mediated by the mutagenized protein A', both receptors may also be involved in the cell adhesion mediated by the mutagenized protein.

Recent advances in genetic engineering make it possible to introduce essentially any desired mutation to a protein. To date, most of the interest in protein engineering has been focused on the modification of the specificity and/or the affinity of enzymes to their substrates (27-29) as well as their stability under nonphysiological conditions (30-32). However, the strict requirements of the three-dimensional structure of enzymes for their catalytic activities make it extremely difficult to transplant the catalytic site of an enzyme to other proteins without losing its activity. In contrast, the cell adhesive activity associated with the RGD-containing proteins can be partly, although not fully, reproduced by a short synthetic peptide or a small cell-binding fragment (5, 33). This unique feature of these cell adhesive proteins offers an excellent model for "active site transplantation" by insertional mutation. The technology described here is essentially applicable to any protein and allows us to design a wide variety of artificial cell adhesive proteins possibly without destroying their intrinsic biological activities. These man-made multifunctional proteins may be useful in modulating cell-to-cell and cell-to-substrate interactions during tissue

remodeling, platelet aggregation, and possibly tumor metastasis.

*Acknowledgment*—We are grateful to Masami Suzuki for excellent technical assistance in protein sequencing and peptide synthesis.

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