Antiserum to a Synthetic Peptide Recognizes the HTLV-III Envelope Glycoprotein

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In a study performed to determine which regions of the human T-cell lymphotrophic virus type III (HTLV-III) may represent vaccine candidates to prevent the acquired immune deficiency syndrome (AIDS), a synthetic peptide corresponding to amino acid sequence 735 to 752 of the precursor envelope glycoprotein of HTLV-III was used to immunize rabbits. The resulting rabbit antiserum to the synthetic peptide specifically recognized the precursor envelope glycoprotein (gp160) of HTLV-III. Human sera positive for antibody to HTLV-III reacted with this peptide. These findings indicate that synthetic peptides can be used to induce an immune response directed against a native envelope glycoprotein epitope of HTLV-III. The data are discussed in terms of using synthetic peptides to identify antigenic determinants involved in the induction of protective immunity and possibly as vaccine candidates against the etiologic agent of AIDS.

ANTIBODIES TO SYNTHETIC PEPtides that recognize the full-length parent protein are useful for studying macromolecular processes (*1, 2*). Synthetic peptides can be either selected from amino acid sequences determined for a given protein or chosen from an amino acid sequence that has been predicted from the DNA sequence. In the latter instance, isolation and amino acid sequencing of the protein in question is not required. Synthetic peptides offer advantages in the production of specific antibodies in that they eliminate the necessity to use the full-length protein as the antigen, and essentially unlimited quantities of the synthetic peptide antigen can be chemically produced.

The acquired immune deficiency syndrome (AIDS) was first described in 1981 (*3–5*). The etiologic viral agent associated with AIDS has been isolated, cloned, and the nucleotide sequence determined (*6–13*). Most, if not all, AIDS patients or patients with AIDS-related complex (ARC) have specific antibodies directed against the virus (*13, 14*). Antibodies from AIDS and ARC patients contain viral neutralizing activity (*15, 16*); however, infection presumably had occurred in these patients before they developed neutralizing antibody.

With retroviruses, as with other enveloped viruses, it is generally assumed that the antigenic determinants or epitopes associated with the induction of neutralizing antibodies are associated with the surface glycoprotein (*17, 18*). This has not been established for the AIDS-associated viruses; however, it has been demonstrated that envelope glycoproteins (gp120 and gp160) of HTLV-III are the most immunogenic in virus-exposed individuals (*14, 19*). On the basis of these observations, it is not unreasonable to speculate that the critical epitopes involved in the induction of protective virus-neutralizing antibody are associated with the two viral envelope subunits gp120 and gp41 (*19, 20*).

To develop an effective vaccine for AIDS, it will be essential to define the epitopes that elicit protective antibody. Synthetic peptide analogs that contain viral polypeptide amino acid sequences are useful for defining these epitopes (*21*). We have recently used a modified computer program (*22*) based on the hydrophilicity index described by Hopp and Woods (*23*) to predict the hydrophilic regions associated with gp160, the HTLV-III envelope gene product. In addition, we used the secondary structure prediction of Chou and Fasman (*24*) to analyze the envelope protein component of HTLV-III. Our computer analysis revealed a highly hydrophilic area associated with predicted β turns with residues 735–752 of HTLV-III. This region also had a relatively invariant stretch of amino acids when we compared the predicted amino acid sequences of HTLV-III, LAV, and ARV. In this study, we characterized a rabbit antibody response to a synthetic peptide containing HTLV-III amino acid residues 735–752. From previous observations of the primary nucleotide sequence (*9, 10*), we infer that this synthetic peptide is expressed on gp41.

Tyr/Asp-Arg-Pro-Glu-Gly-Ile-Glu-Glu-Glu-Gly
735
-Gly-Glu-Arg-Asp-Arg-Asp-Arg-Ser/Gly-Cys
752

The peptide was synthesized by solid-phase methodology (*25*) on a Biosearch Sam II peptide synthesizer. *N-t*-butyloxycarbonyl-*S*-4-methylbenzyl-L-cysteine coupled to polystyrene was used as the solid-phase support for the synthesis. The α amino groups were protected with *t*-butyloxycarbonyl (*t*-BOC), and the side-chain protecting groups were as follows: benzyl ether for the hydroxyl of serine, dichlorobenzyl ether for the phenolic hydroxyl of tyrosine, and γ and β benzyl esters for the carboxyl groups on glutamic acid and aspartic acid, respectively. Trifluoroacetic acid (40 percent in CH_2Cl_2) was used to remove *t*-BOC, and the resulting salt was neutralized with *N,N*-diisopropylethylamine (10 percent in CH_2Cl_2). Diisopropylcarbodiimide was used to couple the *t*-BOC amino acids. The specific steps of the synthesis have been described (*26*).

The protecting groups were removed, and the peptide was cleaved from the resin at 0°C with anhydrous HF containing 10 percent anisole and 1 percent ethanedithiol as scavengers. The HF reagent was removed under vacuum at 0°C, and the peptide was then precipitated and washed with anhydrous ether. After extraction of the peptide from the resin with trifluoroacetic acid, the solvent was evaporated at 15°C, and the peptide was again precipitated with ether. The ether was decanted after centrifugation, and the pellet was dissolved in 5 percent acetic acid with 6*M* guanidine hydrochloride.

This solution was desalted on a BioGel P-2 column equilibrated in 5 percent acetic acid, and the peptide-containing fractions were pooled and lyophilized. A cysteine residue was added to the carboxyl-terminus of the peptide to provide a functional–SH group for the coupling of the peptide to

Table 1. Amino acid composition of synthetic peptide. Analyses were performed on a Durram D500 amino acid analyzer equipped with a Digital PDP8A computer after the amino acid was hydrolyzed in a vacuum for 24 hours at 110°C in 6*M* HCl. The theoretical values are given in parentheses.

Amino acid	Peptide 735–752
Cysteine*	(1)
Serine†	0.26 (1)
Arginine	4.14 (4)
Aspartic acid	3.36 (3)
Glutamic acid	5.12 (5)
Glycine	4.01 (4)
Isoleucine	0.97 (1)
Proline	1.18 (1)
Tyrosine	1.07 (1)

*Half cysteine was present in the amino acid analysis but was not integrated. †Values are not corrected for destruction during hydrolysis.

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carrier proteins. Glycine was included after the cysteine to provide a spacer amino acid between the cysteine residue to be used in coupling and the amino acid sequences analogous to gp160. A tyrosine residue was added to the amino-terminus of the peptide for radioactive labeling with ^{125}I to determine peptide-to-carrier protein coupling efficiency and to identify the peptide during purification by absorbance at 275 nm. After desalting and lyophilization, the peptide had the expected amino acid analysis (Table 1) and eluted as a single peak (92 percent) upon reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} column in a linear gradient of 0.05 percent trifluoroacetic acid and 2-propanol.

The peptide was conjugated through the –SH group on the cysteine to amino groups on keyhole limpet hemocyanin (KLH) (for immunization of rabbits) and bovine serum albumin (BSA) (for assaying antibody activity) by means of the heterobifunctional cross linkers *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (*27, 28*) and *N*-succinimidyl 3-(2-pyridylthio)propionate (SPDP), respectively. Briefly, 1 mg of KLH in 10 m*M* sodium phosphate (*p*H 7.2) was incubated with 4 mg of MBS in dimethylformamide for 30 minutes at 25°C. Unreacted MBS and solvent was removed on a Sephadex PD-10 column equilibrated in 50 m*M* sodium phosphate buffer (*p*H 6.0). Alternatively, 1 mg of BSA in 0.01*M* sodium acetate buffer (*p*H 4.6) was incubated with a 200-molar excess of SPDP in absolute ethanol for 30 minutes at 25°C. Unreacted SPDP was removed on a Sephadex G-50 column equilibrated in acetate buffer (*p*H 4.6). Activation of the cross-linker required treatment with a reducing agent prior to coupling. A 100-molar excess of the peptide relative to KLH or BSA, along with approximately 500,000 count/min of ^{125}I-labeled peptide, was added to the reaction mixture and incubated for an additional 3 hours at 25°C. Uncoupled peptide was removed by repeated dialysis. The coupling efficiency was determined by the amount of ^{125}I-labeled peptide associated with KLH and BSA and was approximately 62 and 68 percent, respectively.

Two rabbits were each immunized with peptide-KLH (100 μg per dose) emulsified in Freunds complete adjuvant. The rabbits received one intramuscular injection every 2 weeks for a total of three injections, and serum was obtained after each injection. The two rabbits produced a detectable antibody response (as measured by peptide-BSA) after a single injection of the peptide-KLH (Table 2). Serum obtained from each rabbit before immunization did not appreciably bind the peptide. Antibody levels increased after each injection of the peptide. The specificity of the antibody response was shown by the inability of the antiserum to peptide-KLH to bind a control peptide conjugated to BSA. The control peptide contained amino acid sequences analogous to one of the four subunits from the *Torpedo californica* acetylcholine receptor (γ subunit). This peptide was 23 amino acids in length and contained carboxyl terminal sequences of cysteine and glycine and an amino terminal tyrosine residue, similar to the synthetic HTLV-III 735–752 peptide. In addition, the synthetic HTLV-III peptide completely inhibited (100 percent) the binding of the rabbit antibody to peptide-BSA. These data indicate that both rabbits produced an antibody response specific for HTLV-III amino acid sequence 735 to 752. The two rabbits also produced high antibody titers to KLH; however, rabbit antiserum to KLH did not bind peptide-BSA.

We next examined the ability of the rabbit antibodies to the peptide to recognize native proteins associated with HTLV-III. Molt-3, an HTLV-III–infected T-cell line, was labeled with [^{35}S]cystine and used for immunoprecipitation to determine whether the antisera to the peptide would bind any radioactivity labeled HTLV-III native proteins. The procedures for radioimmunoprecipitation (RIP) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) have been described (*14, 19*). Rabbit antibodies to the peptide specifically precipitated a single band with a molecular

Table 2. Antibody responses in rabbits immunized with HTLV-III synthetic peptide 735–752. An ELISA was used to titrate the rabbit antibodies to the peptide. Briefly, 200 ng of either HTLV-III peptide coupled to BSA or *Torpedo* receptor peptide coupled to BSA (NH_2-Tyr-Ile-Asp-Pro-Glu-Asp-Phe-Thr-Glu-Asn-Gly-Tre-Thr-Ile-Arg-His-Arg-Pro-Ala-Lys-Gly-Cys-COOH) was adsorbed overnight at 4°C to the wells of polyvinyl microtiter plates. After the addition of 10 percent normal goat serum to block nonspecific sites, the rabbit antibodies to the peptide diluted in 10 percent goat serum were added and incubated for 2 hours at 37°C. The microtiter wells were washed with Tween-20 phosphate-buffered saline (T-PBS) and biotin-goat antibody to rabbit IgG (Vector Laboratories). After 1 hour at 37°C, the wells were washed, and Avidin conjugated with horseradish peroxidase (Av-HRP) was added. Unbound Av-HRP was removed by washing with T-PBS, and peroxidase activity was determined with a 1 m*M* solution of 2,2′-azino-di(3-ethyl-benzthiazoline-sulfonic acid) (Sigma) and 0.03 percent H_2O_2 as substrate. The reaction was stopped with 5 percent (weight by volume) SDS in water before spectrophotometric measurement at 410 nm with a plate reader (Dynatech). Each value represents the mean of triplicate determinations. N.D., not determined.

Immunization*	Reciprocal dilution of antiserum	A_{410} (mean ± SD)	
		735–752	*Torpedo*
	Rabbit 21		
None	10	0.17 ± 0.02	0.14 ± 0.02
	50	0.12 ± 0.03	0.11 ± 0.04
	250	0.02 ± 0.01	0.04 ± 0.02
Primary	50	1.4 ± 0.21	0.08 ± 0.03
	250	0.46 ± 0.02	0.06 ± 0.02
	1250	0.16 ± 0.01	0.05 ± 0.03
Secondary	50	1.4 ± 0.15	0.05 ± 0.03
	250	1.3 ± 0.25	0.04 ± 0.02
	1250	0.96 ± 0.04	N.D.
	6250	0.21 ± 0.04	N.D.
Tertiary	50	1.6 ± 0.04	0.08 ± 0.02
	250	1.4 ± 0.20	0.06 ± 0.03
	1250	1.4 ± 0.12	N.D.
	6250	0.95 ± 0.08	N.D.
	31250	0.23 ± 0.05	N.D.
	Rabbit 22		
None	10	0.17 ± 0.06	0.13 ± 0.03
	50	0.13 ± 0.02	0.10 ± 0.04
	250	0.02 ± 0.01	0.03 ± 0.02
Primary	50	1.4 ± 0.28	0.10 ± 0.02
	250	0.67 ± 0.06	0.06 ± 0.03
	1250	0.27 ± 0.05	0.04 ± 0.02
Secondary	50	1.6 ± 0.22	0.10 ± 0.04
	250	1.4 ± 0.18	0.06 ± 0.02
	1250	0.98 ± 0.11	N.D.
	6250	0.24 ± 0.02	N.D.
Tertiary	50	1.4 ± 0.10	0.08 ± 0.02
	250	1.4 ± 0.10	0.06 ± 0.02
	1250	1.4 ± 0.12	N.D.
	6250	1.1 ± 0.12	N.D.
	31250	0.86 ± 0.07	N.D.
	156250	0.28 ± 0.06	N.D.

*Antiserum was obtained 14 days after each immunization.

weight of approximately 160,000 (160K) as shown by autoradiographs of SDS-PAGE gels (Fig. 1). This band represents the precursor envelope glycoprotein gp160 of HTLV-III. No reactivity to HTLV-III proteins was demonstrated when preimmune rabbit sera were used in the immunoprecipitation experiments. The rabbit antibodies to the peptide did not recognize the gp120 envelope subunit that is detected with [^{35}S]cystine-labeled Molt-3 cells when human antiserum from AIDS patients is used in RIP (*18*). Gp41, the other envelope subunit produced by cleavage of gp160, is only weakly reactive by RIP compared to the strong activity to gp120 (*14*). Thus, these studies do not show whether the rabbit antibodies to the peptide also recognize gp41. However, the peptide consists of amino acid residues 735–752 that are associated with the gp41 subunit, and the antibodies to the peptide would be expected to recognize both gp160 and gp41. Data obtained with RIP using lectin-purified HTLV-III glycoproteins indicate that the antibodies do indeed recognize gp41 (*29*). In addition, serum from an AIDS patient specifically bound the peptide conjugated to BSA (Fig. 2). Rabbit antibodies to the peptide were much more efficient at binding the HTLV-III peptide when compared to the serum from an AIDS patient (Fig. 2). This was not surprising since the human response to an HTLV-III infection would be expected to contain many different antibody specificities, whereas the rabbit antibody induced by immunization with a given peptide should recognize epitopes associated with that peptide.

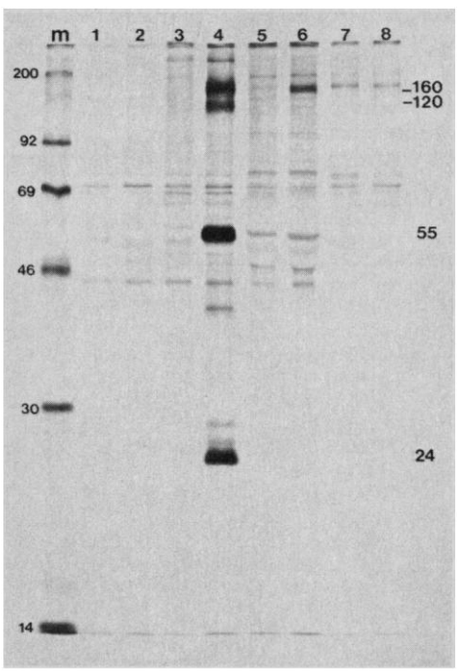

Fig. 1. Reactivity of synthetic peptide antisera to HTLV-III/LAV proteins. Soluble cell lysates labeled with [^{35}S]methionine from infected and uninfected Molt-3 cells were reacted with antibodies from the following sera: 10 μl of HTLV-III/LAV–negative human serum with uninfected (lane 1) and infected (lane 2) cells; 10 μl of HTLV-III/LAV–positive human serum with uninfected (lane 3) and infected cells (lane 4); 10 μl of rabbit antiserum to the synthetic peptide with uninfected (lane 5) and infected (lane 6) cells; and 10 μl of rabbit serum before immunization (preimmune) with uninfected (lane 7) and infected (lane 8) cells. Techniques for radiolabeling, immunoprecipitation, and RIP–SDS-PAGE have been described (*14, 19*). Molecular weight markers are included (m).

Table 3. Binding of human sera from AIDS patients, positive for antibodies to gp41, to HTLV-III peptide 735–752. The ELISA was performed as described in Fig. 3.

Serum	A_{410} (mean ± SD)*	Ratio†
Control 1	0.041 ± 0.009	1.0
Control 2	0.047 ± 0.009	1.1
AIDS 1	0.11 ± 0.01	2.7
AIDS 2	0.26 ± 0.007	6.3
AIDS 3	0.18 ± 0.02	4.4
AIDS 4	0.12 ± 0.01	2.9

*Represents the mean of triplicate values. †The ratio of the serum absorbance to that of control 1.

Antiserum from the AIDS patient specifically bound the HTLV-III peptide relative to the control *Torpedo* peptide (an absorbance at 410 nm of 0.30 for HTLV-III compared with 0.10 for *Torpedo* at a dilution of 1:50). This antiserum also bound the HTLV-III peptide more efficiently than two other control human sera from non-AIDS patients. The ratios of the absorbance of the AIDS serum to the absorbance of the two control sera at a dilution of 1:500 were 3.6 and 4.1. This represented an absorbance of 0.17 ± 0.01 (mean ± SD) for the AIDS serum compared to 0.047 ± 0.009 and 0.041 ± 0.009 for the two control sera. Four other human sera that were positive for gp41 by Western blot analysis also bound the HTLV-III peptide (Table 3). These results indicate that antibodies to the peptide recognize native epitopes associated with the envelope glycoprotein of HTLV-III.

Recent studies using immunofluorescence assays have indicated that the rabbit antibodies to the peptide will also bind to HTLV-III–infected cells (*30*). In addition, the rabbit antisera bind HTLV-III antigens in a commercially available immunoassay (*31*). This particular peptide, along with reagents against it, may be useful in the serodiagnosis of AIDS. Because only five human sera have been tested, further studies are required to determine the extent of antibodies to this particular peptide in AIDS patients. However, these data indicate that there is more than one native epitope associated with the gp41 transmembrane portion of HTLV-III envelope (*32*).

Synthetic peptides analogous to amino acid sequences associated with native proteins can be used to define the antigenic determinants involved in the immune response to that protein. In addition, synthetic peptides can be used to elucidate those epitopes associated with the production of a protective immune response against an infectious agent. Numerous studies have implicated the possible role of synthetic peptides as putative vaccine candidates against viral, bacterial, and protozoal agents that cause human diseases (*33, 34*). Whether or not the synthetic peptide 735–752 is associ-

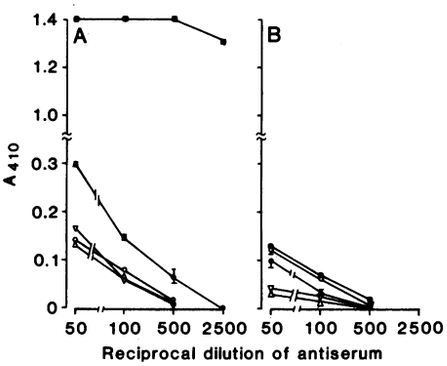

Fig. 2. The binding curves of human AIDS serum, a single rabbit antiserum to the peptide, two human sera from non-AIDS patients, and preimmune rabbit serum are shown reacted to peptide sequence 735–752 conjugated to BSA (A) and to *Torpedo* peptide-BSA (B) as determined by an enzyme-linked immunosorbent assay (ELISA). Five micrograms of each respective peptide conjugated to BSA was adsorbed to the solid phase of microtiter wells (Dynatech). Nonspecific sites were blocked with 10 percent normal goat serum and washed with T-PBS as described in Table 2. Human and rabbit sera were diluted in 10 percent normal goat serum, incubated for 1 hour at 37°C, and then washed with T-PBS. Biotin-goat antiserum to human IgG or biotin-goat antiserum to rabbit IgG (Vector Laboratories) was added to detect human and rabbit antibody binding to the peptides, respectively. After 1 hour at 37°C, the wells were washed and Av-HRP was added. Unbound Av-HRP was removed by washing with T-PBS, and peroxidase activity was determined with a 1 m*M* solution of 2,2′-azino-di(3-ethyl-benzthiazoline-sulfonic acid) (Sigma) and 0.03 percent H_2O_2 as substrate. The reaction was stopped with 5 percent (weight by volume) SDS in water before spectrophotometric measurement at 410 nm with a plate reader (Dynatech) similar to that described in Table 2. Optimal dilutions of each reagent were selected by titration. All reagents for determining specific binding except the substrate were diluted in 10 percent NGtS. Symbols: (■) rabbit antiserum to the peptide; (○) preimmune rabbit serum; (●) human AIDS serum; (▽) control human serum 1; and (△) control human serum 2. All tests were performed in triplicate; the brackets refer to a representative range of values obtained in each experiment for human serum.

ated with an epitope that induces protective immunity against HTLV-III is not known. These studies suggest that peptides of conserved epitopes of the envelope glycoprotein should be evaluated for diagnostic or immunoprotective purposes in the control of the HTLV-III infection.

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Platelet Membrane Glycoprotein IIb/IIIa: Member of a Family of Arg-Gly-Asp–Specific Adhesion Receptors

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Adhesive interactions of the platelet surface with plasma proteins such as fibrinogen and fibronectin play an important role in thrombosis and hemostasis. The binding of both of these proteins to platelets is inhibited by synthetic peptides containing the sequence Arg-Gly-Asp, which corresponds to the cell adhesion site in fibronectin and is also present in the α chain of fibrinogen. An affinity matrix made of an insolubilized heptapeptide containing the Arg-Gly-Asp sequence selectively binds the platelet membrane glycoprotein IIb/IIIa from detergent extracts of platelets. When incorporated into liposome membranes, the isolated protein confers to the liposomes the ability to bind to surfaces coated with fibrinogen, fibronectin, and vitronectin but not to surfaces coated with thrombospondin or albumin. This platelet receptor is related to the previously identified fibronectin and vitronectin receptors in that it recognizes an Arg-Gly-Asp sequence but differs from the other receptors in its wider specificity toward various adhesive proteins. These results establish the existence of a family of adhesion receptors that recognize the sequence Arg-Gly-Asp.

IN RECENT YEARS CONSIDERABLE PROgress has been made in identifying components that mediate the interaction between cells and extracellular matrix proteins. It has been shown that a tripeptide sequence, Arg-Gly-Asp, which is present in both fibronectin (*1*) and vitronectin (*2*), mediates the attachment of cultured fibroblasts to these two adhesive proteins (*1–3*). Distinct cell surface receptors for the Arg-Gly-Asp sequence in fibronectin (*4*) and vitronectin (*5*) have been identified in both fibroblastic and osteosarcoma cells. The Arg-Gly-Asp sequence is important in adhesive interactions at the platelet surface as well, since platelets also attach to fibronectin (*6*) and synthetic peptides containing Arg-Gly-Asp inhibit such attachment (*7*). Moreover, these peptides inhibit the binding of fibronectin and fibrinogen to platelets from solution and prevent platelet aggregation (*8*). This suggests that the Arg-Gly-Asp sequence, which occurs twice in fibrinogen (*9*), may serve as a platelet recognition site on this protein. Fibrinogen, however, does not promote the adhesion of fibroblastic cells (*1*). These findings suggest that platelets have a receptor capable of recognizing the Arg-Gly-Asp sequence in fibrinogen while fibroblasts do not. We report here that platelets have a receptor that recognizes the Arg-Gly-Asp sequence in several adhesive proteins, including fibrinogen, fibronectin, and vitronectin. We also report that this receptor is indistinguishable from the platelet membrane glycoprotein gpIIb/IIIa, but differs from the previously identified Arg-Gly-Asp receptors of fibroblastic cells that recognize only fibronectin or vitronectin.

The platelet receptor was identified by affinity chromatography on the heptapeptide Gly-Arg-Gly-Asp-Ser-Pro-(Lys) coupled to Sepharose (GRGDSP-Sepharose); the lysine residue was added to optimize coupling to the Sepharose. This peptide corresponds in sequence to the cell attachment site in fibronectin. A detergent extract of washed human platelets was applied to this affinity matrix, and specifically bound components were eluted with a solution of the GRGDSP peptide. As shown in Fig. 1, this resulted in the binding and selective release of two polypeptides, which, when reduced, migrated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with mobilities corresponding to molecular weights of 124,000 (124K) and 108K. These polypeptides were not released by a control elution with an inactive variant of the peptide in which the aspartic acid is substituted by a glutamic acid residue.

The sizes of the polypeptides of the putative Arg-Gly-Asp receptor from platelets are compatible with this protein being gpIIb/IIIa. The latter exists in platelets as a noncovalent heterodimer composed of gpIIb with a disulfide-linked heavy and light chain at about 120K and 20K and gpIIIa of about

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