terns are shown in Fig. 2 for two cells, one recorded on E18, the second on E20. The discharge patterns of most units were irregular. Some units, however, showed periodic patterns of discharge when monitored for 10 min (7).

We report that mammalian retinal ganglion cells are spontaneously active in prenatal life. In this time period the axons of ganglion cells reach the superior colliculus and the lateral geniculate nucleus (4) and functional synapses are formed (8); during the same time period projections are refined and many erroneous connections are eliminated (4, 9). Impulse activity in ganglion cells may play a key role in these events.

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10. We thank M. Fitzgerald and P. W. Land for advice; A. Fiorentini and M. P. Stryker for helpful discussions on the manuscript; and B. Margheritti, M. Antoni, V. Alpigiani, M. Benvenuti, D. Moriconi, A. Bertini, and P. Taccini for technical assistance.

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Localization of an Arg-Gly-Asp Recognition Site Within an Integrin Adhesion Receptor

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Many adhesive interactions are mediated by Arg-Gly-Asp (RGD) sequences within adhesive proteins. Such RGD sequences are frequently recognized by structurally related heterodimers that are members of the integrin family of adhesion receptors. A region was found in the platelet RGD receptor, gpIIb/IIIa, to which an RGD peptide becomes chemically cross-linked. This region corresponds to residues 109 to 171 of gpIIIa. This segment is conserved among the β subunits of the integrins (76 percent identity of sequence), indicating that it may play a role in the adhesive functions of this family of receptors.

TWO RECENT DEVELOPMENTS HAVE aided our understanding of the molecular mechanisms of cell adhesion. First, a family of structurally and functionally related adhesion receptors has been identified (1). This family of receptors, the integrins, can be separated into three distinct subfamilies in which members share a common β subunit that combines with unique α subunits to create functionally distinct receptors. Second, many of the adhesive proteins that serve as ligands for the integrin receptors contain the tripeptide sequence, Arg-Gly-Asp (RGD) (2). This sequence has been directly implicated in the recognition of the fibronectin receptor of the VLA (very late antigens) subfamily, Mac-1 of the leukocyte adhesion proteins, and gpIIb/IIIa and the vitronectin receptor of the cytoadhesin subfamily (3, 4).

GpIIb/IIIa, a platelet integrin, is involved in the platelet adhesive response through its interaction with fibrinogen, fibronectin, and von Willebrand factor (5). An RGD recognition site mediates these interactions, because RGD-containing peptides inhibit the binding of these ligands to either intact platelets or purified gpIIb/IIIa, and RGD-containing peptides interact directly with gpIIb/IIIa (3, 6). Activation of platelets with an agonist, an event necessary for binding of adhesive proteins to gpIIb/IIIa, markedly and selectively enhances the chemical cross-linking of small RGD peptides to gpIIIa (7, 8). Here we have defined a discrete site within gpIIIa to which an RGD peptide becomes cross-linked. This region is conserved in the primary sequence of other members of the integrin family, suggesting that it could have a role in receptor function.

The RGD peptide used, Fn-7, has the structure Lys-Tyr-Gly-Arg-Gly-Asp-Ser (KYGRGDS). When ^{125}I-labeled Fn-7 was bound to stimulated platelets and then bifunctional reagents, such as bis(sulfosuccinimidyl)suberate (BS^3), were added (8), Fn-7 was cross-linked predominantly to gpIIIa (Fig. 1, lane 1). When the gpIIIa:Fn-7 complex was digested with either V8 protease or chymotrypsin, the radioactivity of the Fn-7 peptide migrated as two major bands. In the V8 protease digest, the major derivatives were 8 and 10 kD (Fig. 1, lane 3). A fainter doublet was variably noted at 18 and 20 kD. Fn-7 does bind and becomes cross-linked to gpIIIa on resting platelets, albeit less efficiently than on stimulated platelets (8). The radiolabeled fragments obtained by V8 protease digestion of gpIIIa from non-stimulated and stimulated platelets had the same electrophoretic mobility (Fig. 1, lane 5). With chymotrypsin, gpIIIa:Fn-7 from stimulated platelets yielded a major band at 23 kD and a less prominent band at 14 kD (Fig. 1, lane 7). Both proteases generated numerous proteolytic fragments; the V8 protease digest, for example, contained 37 peaks on a C 18 high-performance liquid chromatography (HPLC) column monitored at 212 nm. Thus, Fn-7 becomes cross-linked to gpIIIa in a systematic orientation such that only a limited number of radiolabeled fragments are obtained. Therefore, we undertook to localize the cross-linked Fn-7 within gpIIIa.

We cross-linked ^{125}I-labeled Fn-7 to thrombin stimulated platelets and isolated the gpIIIa:Fn-7 complex by SDS–polyacrylamide gel electrophoresis (PAGE). The eluted product was digested with chymotrypsin or V8 protease and applied to a C18 column. Radioactive peaks were pooled, separated on a 15% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to NH_2-terminal sequence analysis. The 23-kD chymotryptic fragment yielded a single predominant sequence (Fig. 2, step 3). At all of the determined positions, the amino acids were identical to the NH_2-terminal sequence of gpIIIa (9). In the 14-kD chymotryptic fragment, a major sequence predominated for 22 cycles and coincided to residues 91–112 of gpIIIa (Fig. 2, step 3). A preferred chymotrypsin cleavage site, Leu, is in position 90 of gpIIIa. The 14-kD gpIIIa fragment should extend to residues 200–220 of the glycopro-

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Table 1. Alignment of the RGD cross-linked site with other integrins. Deduced amino acid sequences (*9*, *18*) are aligned by the GAP and PRETTY computer programs (*19*). The consensus sequence requires exact matches of at least four of the five sequences compared. Residues that match the consensus sequence are in uppercase.

Cytoadhesin (gpIIIa):	DYPvDiYY LM	DLSYSMKDDL	w s i qnLGTkL	a tqM rklTSn	lRIGFGaFVd	KpV s PymYIS	p pe
Leukocyte adhesion proteins:	g YPIDLYYLM	DLSYSM l DDL	r NVKkLGgdL	l ra l nelTes	gRIGFGSFVd	KTV I Pfvn. .	ThP
VLA:	DYPIDLYYLM	DLSYSMKDDL	e NV K s LGTdL	mneM rrITSd	fRIGFGSFVe	KTVmP. . YIS	TtP
Integrin (avian):	DYPIDLYYLM	DLSYSMKDDL	e NV K s LGTaL	mreMekITSd	fRIGFGSFVe	KTVmP. . YIS	TtP
Integrin (*Xenopus*):	DYPIDLYYLM	DLS fSMKDDL	e NV K s LGTaL	mteMekITSd	fRIGFGSFVe	KTVmP. . YIS	TtP
Consensus:	DYPIDLYYLM	DLSYSMKDDL	- NVK - LGT - L	- - - M - - ITS -	- RIGFGSFV -	KTV - P - - YIS	T-P

tein, and the NH_2-terminal 23-kD fragment should also terminate within the same region. Thus, the Fn-7 cross-linking sites within these chymotryptic fragments are consistent with one another.

The 10-kD fragment produced by V8 protease yielded a single NH_2-terminal sequence that extended for 16 residues (Fig. 2, step 4) and corresponded precisely to residues 109–124 of gpIIIa. A preferred cleavage site of V8 protease, Glu, is in position 108 of gpIIIa. Although there was a strong signal for Met^{124}, there was no signal for Lys^{125}, the next predicted residue. Thus, Lys^{125} is a candidate for direct cross-linking of Fn-7. The 10-kD fragment extending from Asp^{109} is predicted to terminate in the vicinity of residue 200. The sequence signal of the 8-kD V8 fragment was not as strong as that of the 10-kD derivative, but the first four positions were clear and identical to those of the 10-kD fragment (Fig. 2, step 4). Thus the 8-kD fragment should terminate in the 170–180 region; two glutamic acids occur within this 10–amino acid stretch. As the NH_2-termini of the 8- and 10-kD V8 fragments reside within the 14-kD chymotryptic fragment, these independent results are internally consistent.

The above amino acid sequences indicate that the RGD cross-linking site may be confined to a region as small as 63 amino acids, extending from residue 109 to Glu^{171}. The primary structures of this region among the beta subunits of the human, avian and *Xenopus* integrins were aligned (Table 1), and found to be conserved in all species. In the consensus sequence 48 of the 63 residues (76%) are specified, which exceeds the overall identity among the integrins. Such conservation suggests that this region contributes to the common functions of the integrins. The localization of the RGD cross-linking site is the first direct implication of this region in integrin function. A nonconserved region of gpIIIa, residues 129–149, is flanked by two very highly conserved regions. This nonconserved region could impart functions to gpIIb/IIIa, such as its high affinity for multiple RGD ligands, that distinguish it from the other integrins.

To correlate the cross-linking reaction of the NH_2-terminal region to the function of gpIIb/IIIa, two additional sets of experiments were performed. First, ^{125}I-labeled Fn-7 was cross-linked to thrombin-stimulated platelets, and the intact cells were digested with chymotrypsin. Chymotrypsin releases the NH_2-terminal portion of gpIIIa, and a COOH-terminal fragment of 66 kD is retained by the cell (*10*). In four experiments, chymotrypsin released 50 to 70% of the cell-associated radioactivity. Moreover, the retained radioactivity was associated exclusively with undigested gpIIIa; none was associated with the 66-kD fragment (Fig. 2, step 2). Nevertheless, this fragment was the predominant gpIIIa species identified in immunoblotting analysis with both polyclonal and monoclonal antibodies to gpIIIa. In addition, platelets were digested with chy-

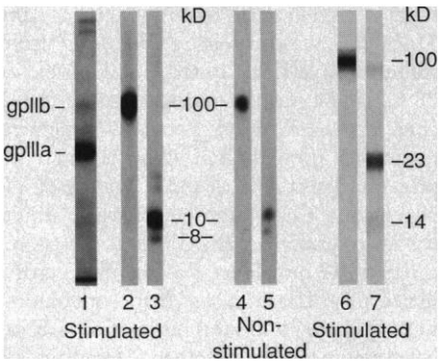

Fig. 1. Cross-linking of an RGD peptide to discrete sites in the β subunit (gpIIIa) of an integrin adhesion receptor. The ^{125}I-labeled RGD peptide Fn-7 (20 μ*M*) was bound to platelets (6×10^8 per milliliter) for 45 min at 22°C and cross-linked with BS^3 (0.2 m*M*). The fragmentation patterns after proteolysis were analyzed by SDS-PAGE (*14*) and autoradiography. (Lane 1) SDS-PAGE (7.5% gel, nonreducing conditions) showing the predominance of ^{125}I-labeled Fn-7 cross-linked to the β subunit (gpIIIa), with less cross-linked to the α subunit (gpIIb) on thrombin-stimulated platelets. (Lanes 2 to 7) gpIIIa:Fn-7 was excised from gels, subjected to proteolysis, and analyzed by SDS-PAGE (15% gels, reducing conditions). Intact gpIIIa:Fn-7 complexes (lane 2) and V-8 protease digests (2 mg/ml) performed within the gel slice (*15*) (lane 3) from thrombin stimulated platelets. Intact gpIIIa:Fn-7 (lane 4) and V8 protease digests (lane 5) from nonstimulated platelets. (Lanes 6 and 7) GpIIIa:Fn-7 (~18 μg) was extracted and digested with 20 μg of chymotrypsin for 1 hour at 22°C (lane 7) or nondigested (lane 6). No bonds within Fn-7 are known to be susceptible to V8 protease. If the tyrosylglycyl peptide bond within ^{125}I-labeled Fn-7 were cleaved by chymotrypsin, the radioactivity would remain cross-linked to gpIIIa.

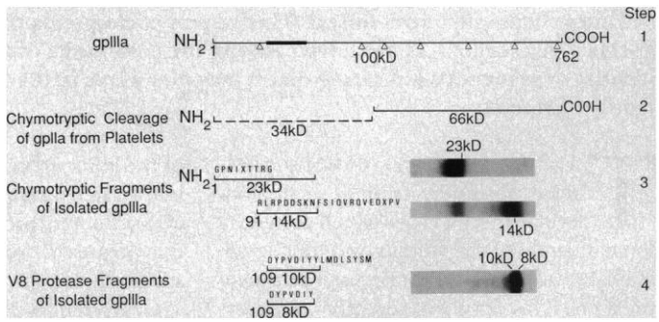

Fig. 2. Localization of the RGD cross-linking site within the β subunit (gpIIIa). The diagram illustrates the isolated proteolytic fragments of the gpIIIa:Fn-7 complexes, their NH_2-terminal amino acid sequences, and their location within gpIIIa. In step 2, the NH_2-terminal 34-kD localization is based upon the chymotryptic cleavage of intact platelets (see text). GpIIIa:Fn-7 was extracted from gels in 1% SDS, phosphate-buffered saline, *p*H 7.3, and then digested with chymotrypsin at a 1:1 (w/w) substrate/enzyme ratio for 24 hours at 22°C. Samples were boiled and applied to a C 18 HPLC reverse phase column, equilibrated in 0.1% trifluoroacetic acid (TFA) and 10 μ*M* dithiothreitol (DTT). The peptides were eluted with an acetonitrile gradient containing 0.08% TFA and 10 μ*M* DTT. Radioactive peaks were pooled, concentrated, and separated on SDS-PAGE by a 15% gel under reducing conditions. The gels were transferred to PVDF membranes (*16*) and then autoradiographed. Radioactive bands, at 23 and 14 kD from the chymotryptic digest (step 3) and at 10 and 8 kD from the V8 protease digest (step 4), were excised and subjected to NH_2-terminal sequence analysis in an Applied Biosystem Model 475A gas-phase sequenator. The sizes of the fragments and the position of the NH_2-terminal residues of each fragment are drawn to scale relative to gpIIIa. The open triangles represent potential glycosylation sites, "X" in amino acid sequences indicates undetermined residues, and the heavy bar indicates the location of the RGD cross-linking region (*17*).

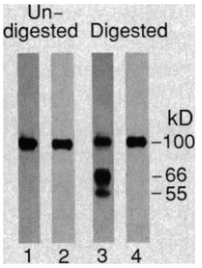

Fig. 3. RGD–affinity chromatography of platelets incubated in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of chymotrypsin. After incubation, the platelets were solubilized in octylglucoside, and applied to an RGD–affinity column (*3*). The flow-through (lanes 1 and 3) and the bound (lanes 2 and 4) materials were analyzed on SDS-PAGE (7% gels, nonreducing conditions), transferred to PVDF membranes, and then probed with a monoclonal antibody to gpIIIa.

motrypsin such that approximately 50% of the gpIIIa was degraded to the 66-kD derivative. The digested and control (undigested) platelets were then solubilized and passed over RGD affinity columns (*3*). After washing to obtain a flow-through fraction, the columns were eluted with free RGD peptide. The flow-through and the eluate were subjected to SDS-PAGE, transferred, and immunoblotted with a monoclonal antibody (MAb) to gpIIIa. Intact gpIIIa was observed in the flow-through from the undigested platelets, and a mixture of intact gpIIIa and the 66-kD gpIIIa fragment was present in the flow-through from the chymotrypsin-treated platelets (Fig. 3). In the RGD eluates from both columns, only intact gpIIIa was detected. Thus, the 66-kD fragment was not retained on the RGD affinity column. This result is compatible with interpretation that the site to which RGD peptides bind and to which they crosslink coincide and reside in the NH_2-terminal aspects of gpIIIa.

In sum, our results indicate that the lysine residues within gpIIIa 109–170, particularly Lys^{125}, reside in close proximity to the lysine in Fn-7 when it interacts with the RGD binding site in gpIIb/IIIa. In addition, the NH_2-terminal region of gpIIIa may be involved in the function of this adhesion receptor; and, on the basis of homology, the related sequences in the β subunits of the other integrins may also be involved in their adhesive functions. This is also consistent with the inhibition of platelet aggregation by a MAb to the NH_2-terminal region of gpIIIa (*11*). It must be emphasized, however, that the identified region resides in close proximity but may not be the RGD binding site. In fact, the RGD cross-linking site on gpIIIa probably does not constitute the complete recognition site or sites involved in the binding of adhesive proteins to gpIIb/IIIa or other integrins. Clearly, the α subunits play an important role in regulating the specificity of the integrins (*12*), and it is likely that the recognition site contains elements contributed by both subunits. The localization of the gpIIb region to which RGD and fibrinogen γ chain peptides crosslink (*7, 8*) may provide a means of identifying the sequences within an α subunit that may also contribute to the function of this family of adhesion receptors.

Note added in proof: With a different peptide and cross-linker, Smith and Cheresh (*13*) have identified a similar region (61–203 of the β subunit) in a vitronectin receptor from placenta involved in cross-linking.

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$tRNA_i^{met}$ Functions in Directing the Scanning Ribosome to the Start Site of Translation

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The mechanism by which the scanning ribosome recognizes the first AUG codon nearest the 5′ end of eukaryotic messenger RNA has not been established. To investigate this an anticodon change (3′-UCC-5′) was introduced into one of the four methionine initiator ($tRNA_i^{met}$) genes of *Saccharomyces cerevisiae*. The ability of the mutant transfer RNA to restore growth properties to *his4* initiator codon mutant yeast strains in the absence of histidine was then assayed. Only the complementary codon, AGG, at the *his4* initiator region supported His^+ growth. The mutant transfer RNA also directed the ribosome to initiate at an AGG placed in the upstream region of the *his4* message. Initiation at this upstream AGG precluded initiation at a downstream AGG in accordance with the "scanning" model. Therefore, an anticodon:codon interaction between $tRNA_i^{met}$ as part of the scanning ribosome and the first AUG must function in directing the ribosome to the eukaryotic initiator region.

A FUNDAMENTAL DIFFERENCE between prokaryotic and eukaryotic organisms is the mechanism of initiation of protein synthesis. In prokaryotes, the 30*S* ribosomal subunit binds the start site of translation in mRNA (*1*). This recognition process is predominantly mediated by the 3′ end of 16*S* ribosomal RNA, a component of the 30*S* subunit, which base pairs with a complementary ribosomal binding site (Shine/Dalgarno sequence) that is located 5′ to a start codon. In eukaryotes, ribosomal binding sites of the prokaryotic type are not present in mRNA (*2*). Instead, the ribosomal scanning model (*3*) proposes that the 40*S* ribosomal subunit in association with methionine initiator tRNA ($tRNA_i^{met}$)

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