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cDNA and amino acid sequences of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptors

(molecular cloning/cytoplasmic domain/proteolytic processing/lymphocyte function-associated antigen 1/macrophage antigen 1 homology)

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ABSTRACT **Cells adhere to vitronectin substrates through a cell surface receptor that recognizes an Arg-Gly-Asp sequence in vitronectin. The receptor is a glycoprotein composed of a 150-kDa α and a 115-kDa β subunit. The α subunit consists of two disulfide-bonded chains of 125 kDa and 25 kDa. cDNA clones were isolated for the α subunit of the vitronectin receptor from a phage λgt11 expression library made with RNA from a human fibroblast cell line, IMR-90. The identity of the clones that had been selected from the library based on immunological criteria was verified by comparison of DNA and protein sequences. NH_2-terminal sequences were obtained for each of the α-subunit chains. The sequence of the 25-kDa chain of the α subunit was found in a cDNA clone, and the amino acid sequence deduced from the cDNA establishes the complete amino acid sequence of the 25-kDa chain. This chain contains a membrane-spanning domain as well as a putative intracytoplasmic region that is 32 amino acids long and consists mostly of polar amino acids. Comparison of the cDNA and protein sequences shows that the 25-kDa chain is generated by proteolytic cleavage of an α-subunit precursor, the partial sequence of which is contained in the cDNA clones. These clones contain 1910 base pairs of open reading frame and a 3′ untranslated sequence. RNA blot hybridization detected one transcript of about 7 kilobases in RNA from fibroblastic and epithelial cells. Together, the cDNA clones cover 4442 bases of this RNA. The α-subunit sequence showed strong homology with the sequence of the α subunit of fibronectin receptor. Moreover, the NH_2-terminal protein sequence of the 125-kDa chain was homologous with the NH_2-terminal sequences of two other cell surface proteins, lymphocyte function-associated antigen 1 (LFA-1) and macrophage antigen 1 (Mac-1), which have been implicated as receptors for adhesion proteins of leukocytes. These results establish several of the structural features in the vitronectin receptor and suggest the existence of a superfamily of receptors for cell adhesion proteins.**

Adhesive interactions between cells and their environment are of fundamental importance in processes such as embryonic development, organ function, and tumor-cell invasion as well as in the function of cells comprising the immune system. It has become clear that the major mediators of such interactions are cell surface glycoproteins that specifically bind extracellular protein ligands. Many of these receptors belong to a group of receptors that recognize an Arg-Gly-Asp (RGD in the single-letter code for amino acids) sequence in their ligand protein (1, 2). This receptor group includes the fibronectin receptor (FNR) (3), the vitronectin receptor (VNR) (4), and the gpIIb/IIIa glycoprotein of platelets (5), which recognizes the Arg-Gly-Asp sequence of fibrinogen, fibronectin, and vitronectin (6, 7) as well as that of von Willebrand factor (8). In addition, a chicken receptor appears to recognize fibronectin and laminin in an Arg-Gly-Asp-dependent manner (9, 10).

The VNR is present in various types of cells and can be isolated in chemical amounts by affinity chromatography (4, 7). VNR consists of two major subunits, one of about 150 kDa and another of about 115 kDa. We refer to these subunits as the α and β subunit, respectively. Upon reduction, the larger (α) subunit loses about 25 kDa of its apparent molecular mass and a small polypeptide can be seen on gels, suggesting the existence of a small chain disulfide bonded to the rest of the subunit. VNR is thought to be an integral membrane protein because it can be incorporated into liposomes (4). The structures of FNR and gpIIb/IIIa are similar to those of VNR. VNR and gpIIb/IIIa are especially closely related to each other with regard to the molecular sizes of the subunits (7). All of these structural and functional similarities suggest that the receptors are homologous membrane proteins with a shared evolutionary origin. However, since no amino acid sequence data have been available for these receptors, such relationships remain unproven.

We report here protein and cDNA sequencing results that reveal a typical transmembrane domain in VNR, establish sequence homologies within the Arg-Gly-Asp receptor family, and suggest that a group of leukocyte proteins is related to the Arg-Gly-Asp receptor family.

MATERIALS AND METHODS

Isolation of cDNA Clones. A phage λgt11 expression cDNA library (11) was made from the poly(A)-containing mRNA of IMR-90 human fibroblasts as described (12) and was screened by using antibodies affinity-purified from a rabbit anti-VNR antiserum. The antiserum was prepared by immunizing with VNR isolated from human placenta (13). Anti-VNR antibodies were purified from the antiserum by chromatography on VNR-Sepharose, and antibodies specific to the α and β subunits of VNR were affinity-purified by using each of the subunits immobilized on nitrocellulose filters (14). Positive clones from the antibody screening were plaque-purified and subjected to further immunological analysis in which the hybrid proteins produced by the positive clones were used to purify antibodies. About 1×10^5 plaque-forming units were

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Abbreviations: kbp, kilobase pair(s); FNR, fibronectin receptor; LFA-1, lymphocyte function-associated antigen 1; Mac-1, macrophage antigen 1; VNR, vitronectin receptor.

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plated on a 7.5-cm Petri dish and incubated for 2.5 hr at 42°C until small plaques became visible. A nitrocellulose filter paper soaked with isopropyl β-D-thiogalactopyranoside was put on top of the agarose and incubated for 6 hr at 37°C. The resultant filter was removed and washed four times with 50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween-20 and then incubated with antiserum. The bound antibody was eluted with 5 mM glycine·HCl, pH 2.3/0.5 M NaCl/0.5% Tween 20/0.01% bovine serum albumin (14). The reactivity of the resultant antibodies was studied by immunoblotting (15). VNR (0.5 μg) was separated by $NaDodSO_4$/PAGE using 7% gels. The separated polypeptides were blotted on nitrocellulose filters and treated with the appropriate antibodies. The bands were visualized with horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad).

DNA Sequencing. Phage DNAs were purified by the plate lysate method (16). After *Eco*RI digestion, the inserts were separated on agarose gels, extracted, and subcloned into phage M13mp19. The DNA sequences were determined by the dideoxynucleotide chain-termination method of Sanger *et al.* (17) using adenosine 5′-[α-thio]triphosphate labeled at the α-thio position with ^{35}S. To facilitate the sequencing, a series of overlapping deletion clones of both strands were made as described by Dale *et al.* (18). In both strands, several undetermined parts remained, which were then sequenced by using an oligonucleotide priming method as described (19).

NH_2-Terminal Amino Acid Sequencing. The NH_2-terminal amino acid sequences of the VNR subunits were determined as follows. Approximately 300 μg of the isolated VNR, containing ^{125}I-labeled VNR as a radioactive tracer, was separated by $NaDodSO_4$/PAGE under reducing conditions. The subunits were localized on the dried gel by autoradiography, excised, and electrophoretically eluted from the rehydrated gel. After extensive dialysis against 0.01 M NH_4HCO_3/0.02% $NaDodSO_4$ and lyophilization, the protein was subjected to gas-phase microsequencing using an Applied Biosystems (Foster City, CA) model 470A sequencer and the trifluoroacetic acid-based chemistry provided by the manufacturer.

Other Procedures. mRNA was isolated from cultured cells as described by Rowe *et al.* (20). RNA and DNA blot analyses were performed as described by Thomas (21). The probes were labeled by nick-translation or by random oligonucleotide primer extension (22).

RESULTS

Identification of a cDNA Coding for the Vitronectin Receptor. Screening of 10^6 plaques from a λgt11 library constructed from the human fibroblast cell line IMR-90 revealed 14 clones that reacted with anti-VNR antibodies. These clones were classified into four different groups based on DNA cross-hybridization and reactivity with antibodies against the subunits of VNR.

One of the groups consisted of two clones (λVNR10 and λVNR11) that appeared to code for the α subunit. Each of the clones reacted with the affinity-purified anti-VNR antibody in blotting experiments; when subunit-specific antibodies were used, the antibody against the α subunit of VNR recognized the clones, whereas the anti-β subunit antibody did not. Moreover, antibodies affinity-purified on the hybrid proteins produced by these clones reacted with the α subunit of VNR but not with the β subunit. The result for the λVNR10 fusion protein is shown in Fig. 1. These results indicate that the α subunit of VNR and the two hybrid proteins share one or more epitopes.

DNA Sequence of the VNR α-Subunit Clones and the Deduced Amino Acid Sequence. The combined DNA sequence of the λVNR10 and λVNR11 inserts and the deduced amino acid sequence are shown in Fig. 2. The size of the

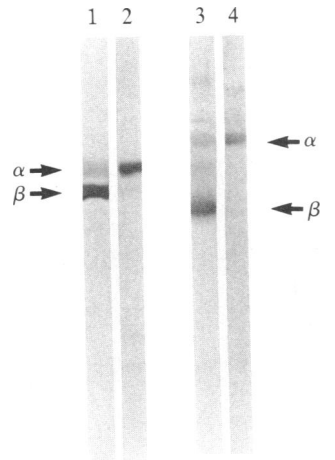

FIG. 1. Staining of electrophoretically separated VNR polypeptides by antibodies purified with a hybrid protein encoded by cloned cDNA. Antibody bound from anti-VNR antiserum to the hybrid protein encoded by λVNR10 was eluted and used for immunoblotting analysis of VNR separated under reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. The antibody preparation obtained with the hybrid protein stains the upper band of the VNR receptor (lanes 2 and 4), whereas the original antibody preparation stains both bands (lanes 1 and 3). The 25-kDa subunit was not reproducibly detected.

λVNR10 insert is about 1.3 kilobase pairs (kbp) and that of λVNR11 about 3.9 kbp. The two inserts share 773 bp of overlapping sequence. One open reading frame of 1910 bp extends from the 5′ end of the λVNR10 insert to the middle of the λVNR11 insert. The 3′ half of the λVNR11 insert corresponds to an untranslated region, but since no poly(A) tail is present, it may not be complete.

A possible membrane-spanning domain consisting of hydrophobic amino acid residues is seen near the COOH terminus of the open reading frame. At the COOH terminus is a putative intracytoplasmic region that is 32 amino acids long and has a highly hydrophilic character. Ten possible N-linked glycosylation sites are present throughout the open reading frame; they are all located in the region believed to be extracellular.

A protein homology search carried out by using the Bionet system showed that the VNR sequences established from the cDNA clones are unique, with no significant homologies with other proteins in the database. Protein sequencing performed to confirm the identification of the clones and to correlate the cDNA sequences with the two chains of the α subunit did reveal homologies with known sequences (see below).

NH_2-Terminal Amino Acid Sequences of the α-Subunit Chains. NH_2-terminal amino acid sequences determined for the 25-kDa and 125-kDa chains of the α subunit are shown in Fig. 3. The NH_2-terminal sequence of the 25-kDa chain was found in the cDNA-derived sequence (bases 1437–1469 in Fig. 2). This confirms the identity of our cDNA clones as VNR α-subunit clones and shows that the complete sequence of the light chain is contained within the sequences derived from these clones. The NH_2 terminus of the 25-kDa chain is preceded in the cDNA sequence by a sequence coding for two basic amino acids, which presumably serve as a recognition signal for the cleavage, as is the case with many other proteins. Also shown in Fig. 3 is the homology of the NH_2-terminal sequence of the 125-kDa chain with the NH_2-terminal sequences of the leukocyte adhesion proteins Mac-1 (macrophage antigen 1) and LFA-1 (lymphocyte function-associated antigen 1) reported earlier (23).

mRNA Analysis. RNA blotting with the VNR clones revealed one strongly hybridizing transcript of about 7 kilobases in the IMR-90 fibroblasts (Fig. 4). The same

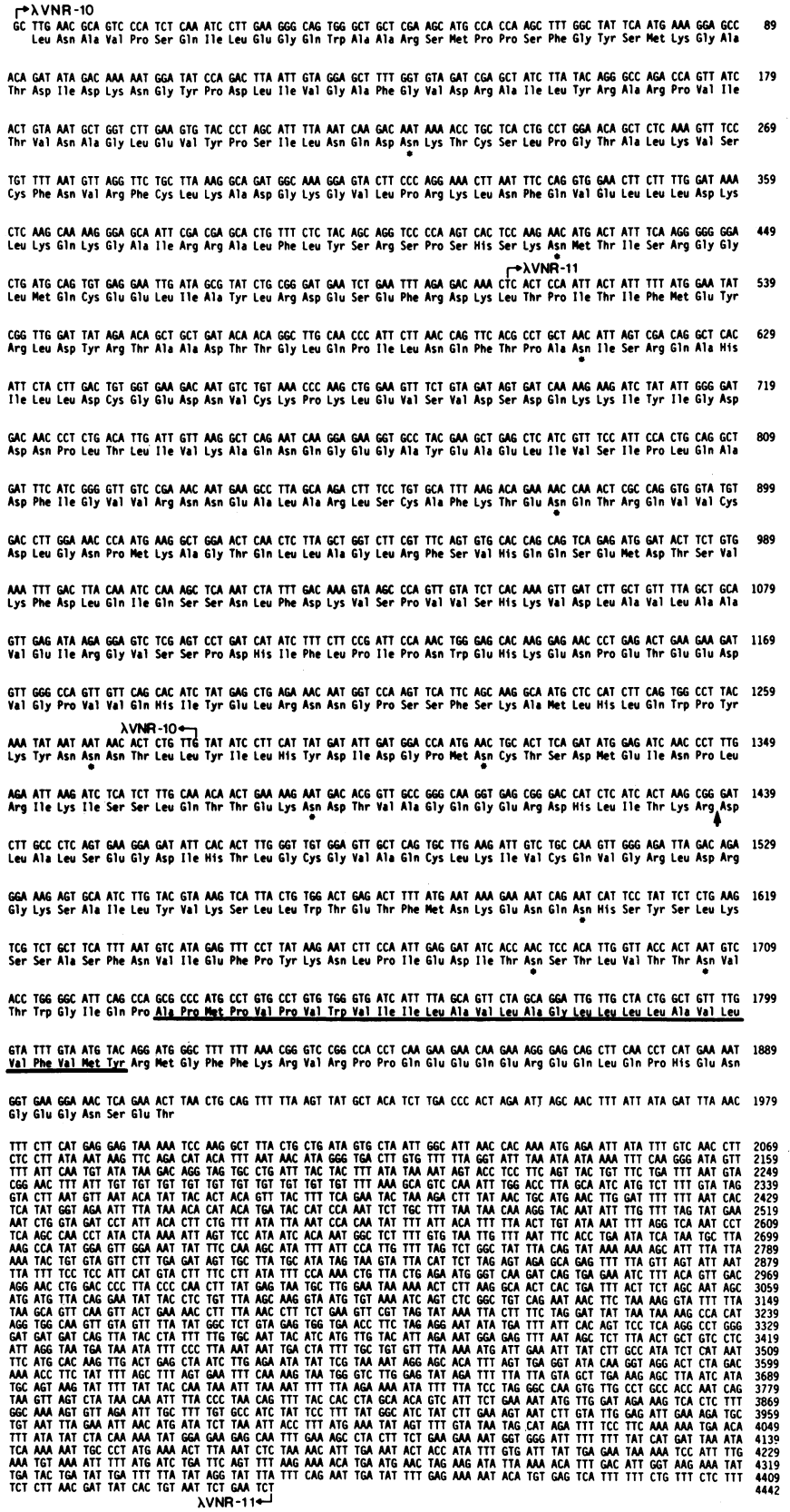

FIG. 2. DNA sequence and deduced amino acid sequence of cDNA clones λVNR10 and λVNR11. Both strands of λVNR10 and λVNR11 were sequenced by the dideoxynucleotide chain-termination method (17). The ends of the λVNR10 and λVNR11 clones are indicated. The cleavage site at the NH_2 terminus of the 25-kDa chain is marked by the arrow. The possible N-linked glycosylation sites are indicated by asterisks, and the hydrophobic stretch of amino acids contained in the putative membrane-spanning domain is underlined.

7-kilobase transcript was found in smaller amounts in MG-63 osteogenic sarcoma cells and in A431 carcinoma cells.

DISCUSSION

We have isolated two overlapping VNR cDNA clones and have derived the complete amino acid sequence of the α-subunit 25-kDa chain and a portion of the α-subunit 125-kDa chain sequence from them. That our cDNA clones belong to the α subunit of VNR is based on two pieces of evidence. First, the hybrid proteins produced by the clones crossreact immunologically with the α subunit of VNR. Secondly, the cDNA-derived sequence contains the NH_2-terminal protein sequence of the α-subunit 25-kDa chain.

	1	2	3	4	5	6	7	8	9	10	11
VNR α 25 kDa	X	Leu	Ala	Leu	Ser	Glu	Gly	Asp	Ile	X	Thr
VNR α 125 kDa	Phe	Asn	Leu	Asp	Val	X	Ser	Pro	Ala	Glu	Tyr
Mac-1 α	Phe	Asn	Leu	Asp	Thr	Glu	His	Pro	Met	Thr	Phe
LFA-1 α	Tyr	Asn	Leu	Asp	Thr	Arg	Pro	Thr	Gln	Ser	Phe

FIG. 3. NH_2-terminal amino acid sequences of the 25-kDa and 125-kDa chains of VNR α subunit and homology of the 125-kDa chain with the α subunits of Mac-1 and LFA-1. VNR polypeptides (1150 pmol) were separated on $NaDodSO_4$/PAGE under reducing conditions, and the α-subunit polypeptides were cut out, eluted from the gels electrophoretically, and subjected to gas-phase amino acid sequencing. The yields for the 25-kDa and 125-kDa sequences were 300 and 580 pmol as determined from the recoveries for the second and first residues, respectively. Residues 1 and 10 of the 25-kDa polypeptide and residue 6 of the 125-kDa polypeptide could not be identified. The Mac-1 and LFA-1 sequences are from ref. 23. The boxes indicate identical residues and the conservative replacements, Tyr/Phe, Ser/Thr, or Thr/Val. The Thr/Val substitution is considered conservative because the side chains of Thr and Val are similar in size and have been found to be interchangeable in functionally active synthetic peptides (24).

Comparison of this 10-amino acid sequence with the corresponding sequence in the FNR (Fig. 5; ref. 25) shows that, although the two receptors show considerable overall homology, this region is not strongly homologous in the VNR and FNR. Since the 25-kDa chain NH_2-terminal sequence we have used in the identification of the cDNA clones is poorly conserved in the VNR and FNR, it is unlikely that some related as-yet-unknown receptor would contain the same sequence as the VNR, supporting the assumption that the cDNA clones are VNR clones.

The sequence corresponding to the 25-kDa chain is near the 3′ end of the open reading frame, suggesting that the 25-kDa chain is formed by proteolytic processing of a precursor. The remaining part of the cDNA corresponds to the 125-kDa chain of the α subunit. Although the cDNA sequence does not extend far enough to include the NH_2-terminal protein sequence of the 125-kDa chain, our immunological results show that the 125-kDa chain of the α subunit and the hybrid proteins coded for by these clones are immunologically crossreactive. Thus, we can conclude that the cDNA sequences contain the complete amino acid sequence of the 25-kDa chain and a large part of the 125-kDa chain sequence. The open reading frame contained in the cDNA clones codes for ≈70 kDa of polypeptide sequence. The molecular mass of the VNR α subunit is ≈150,000 (4, 7). Depending on the carbohydrate content of this subunit, which is not known, the sequence presented here includes about half or more of the polypeptide. $NaDodSO_4$/PAGE

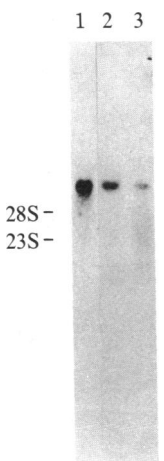

FIG. 4. RNA hybridization analysis. RNA from IMR-90, MG-63, and A431 cells (lanes 1–3, respectively) was electrophoresed on agarose gels, blotted onto nitrocellulose, and probed with ^{32}P-labeled λVNR10 insert. The amount of RNA was 10 μg in each case, and the RNAs in all three lanes were electrophoresed and probed together.

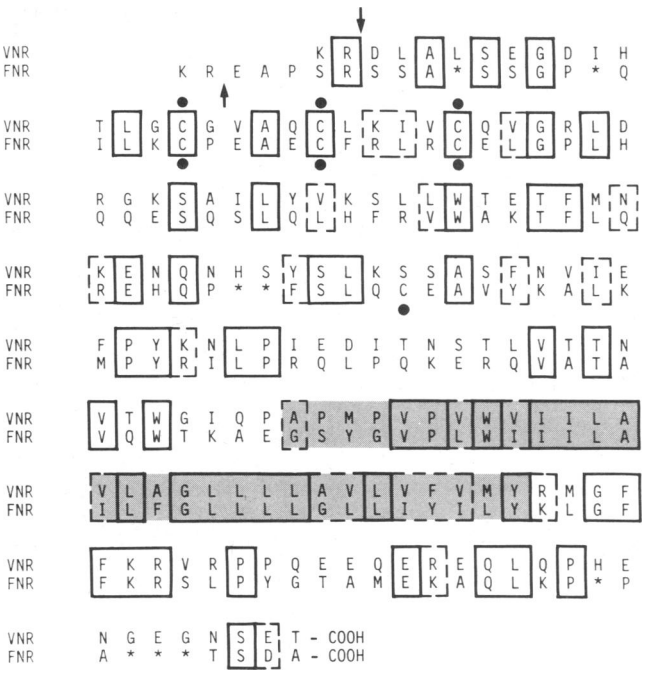

FIG. 5. Comparison of the deduced amino acid sequence of the 25-kDa chain of the VNR α subunit with the corresponding portion of the FNR α subunit (standard single-letter amino acid code). The boxes with solid lines indicate identical residues. Conservative replacements are boxed with dashed lines. The hydrophobic stretches believed to be transmembrane domains have been shaded. The boundaries of these domains were chosen by making them coincide with the first charged residue in either sequence. The FNR sequence is from ref. 25. A cleavage site analogous to the one in VNR (arrow pointing down) may generate a small chain from the α subunit of the FNR (arrow pointing up) at the KRD and KRE sequences, respectively.

analysis of VNR presented here as well as earlier results (4, 7) indicates that the two chains that make up the α subunit of VNR are linked by disulfide bonding. The extracellular domain of the 25-kDa chain contains three cysteine residues; at least one of these apparently forms a disulfide bond to the 125-kDa chain.

The 25-kDa chain contains a domain consisting of hydrophobic amino acid residues. This domain has features shared by transmembrane domains of other proteins, such as the uninterrupted row of hydrophobic amino acids and the basic residues commonly present on the cytoplasmic side of transmembrane domains (26–29). These structural features, added to the fact that the cell surface localization, the receptor function, and the capacity to be incorporated into liposomes suggest that VNR is an integral membrane protein, make it highly likely that this domain spans the cell membrane. Membrane insertion of the putative transmembrane domain could leave the 32 amino acids at the COOH terminus of the 25-kDa domain excluded from the membrane, if the transmembrane domain ended at the first arginine residue that follows the hydrophobic stretch. The high content of hydrophilic amino acids among this 32-amino acid domain is characteristic of cytoplasmic domains of membrane proteins, some of which are short like ours (27–29). This domain is likely to be on the cytoplasmic side of the membrane. Therefore, the structural arrangement of the α subunit appears to be that the 25-kDa chain anchors the α subunit of the VNR to the cell membrane and the 125-kDa chain is attached to the 25-kDa chain through a disulfide bond.

The α subunits of the VNR and FNR are clearly related to one another and both appear to be related to the gpIIb subunit of the platelet adhesive protein receptor gpIIb/IIIa. The

relatedness of the VNR and FNR is apparent from the amino acid sequences of the VNR 25-kDa chain and the corresponding portion of the FNR sequence, which we have also elucidated from cDNA (25). The sequence comparison in Fig. 5 shows that the sizes of their putative membrane-spanning and cytoplasmic domains are nearly identical and there is an overall amino acid sequence homology of 34% when the two sequences are analyzed on the basis of similarity (30). If conservative replacements are considered, the overall homology approaches 48%. The gpIIb subunit is composed of two chains, and the molecular sizes of these chains are closely similar to those of the VNR α subunit (31), and there is some evidence to suggest that gpIIb is made from a single mRNA and that its light chain has a transmembrane domain (32). Moreover, the VNR and gpIIb/IIIa are immunologically crossreactive (our unpublished results with M. Ginsberg and E. Plow). The structural similarities of the vitronectin, fibronectin, and platelet receptors as well as the similarity of their functions as Arg-Gly-Asp receptors (6) clearly define them as members of a family of proteins. Our results also suggest that this family may have more than these three members.

Examination of the NH_2-terminal sequence of the 125-kDa chain of the VNR α subunit (Fig. 3) reveals a high degree of homology with the recently published NH_2-terminal sequences of Mac-1 and LFA-1 (23). This homology is especially striking with Mac-1, which shares the first four residues with VNR (Fig. 3). The homology is less pronounced for residues 6–11, but when conservative replacements are allowed, the overall degree of homology between VNR and Mac-1 is 63%. The same extent of homology exists between Mac-1 and LFA-1.

These results provide a link connecting two previously described families of adhesion proteins, the VNR, FNR, and gpIIb/IIIa family and the one consisting of LFA-1 (33), Mac-1 (34), and gp150/95 (35), which shares a subunit with the other two (36). The suggestion that these two protein families are homologous is based not only on their related NH_2-terminal sequences which we report here, but also on their similar subunit structure and size and their related functions in adhesion interactions at the cell surface. Two members of this superfamily (VNR and FNR) are found in various cell types, including fibroblasts and lymphocytes (37). The other four (gpIIb/IIIa, Mac-1, LFA-1, and gp150/95) appear to occur primarily in blood cells. The existence of a connection between the Mac-1 and LFA-1 proteins and the Arg-Gly-Asp-directed receptors VNR, FNR, and gpIIb/IIIa has also been suggested recently by Cosgrove *et al.* (38), who found that a single genomic clone could lead to the expression of a protein or proteins that reacted with antibodies to Mac-1, LFA-1, and gpIIb/IIIa. It remains to be determined to what extent the ligand specificity of LFA-1, Mac-1, and gp150/95 might resemble that of the Arg-Gly-Asp-directed receptors.

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