The region of fibronectin encompassing type III repeats 4–6 contains a low affinity heparin binding domain, but its physiological significance is not clear. We have studied whether this domain is able to interact with cells as already shown for other heparin binding domains of fibronectin. A computer search based on homologies with known active sites in fibronectin revealed the sequence KLDAPT located in FN-III5. A synthetic peptide containing this sequence induced lymphoid cell adhesion upon treatment with the activating anti-β1 monoclonal antibody (mAb) TS2/16 or with Mn2⁺, indicating that KLDAPT was binding to an integrin. A recombinant fragment containing repeat III5 (FN-III5) also mediated adhesion of TS2/16/Mn2⁺-treated cells while the FN-III6 fragment did not. Soluble KLDAPT peptide inhibited cell adhesion to FN-III5 as well as to a 38-kDa fibronectin fragment and VCAM-1, two previously known ligands for α4β1 integrin. KLDAPT also competed with the binding of soluble alkaline phosphatase-coupled VCAM-Ig to Mn2⁺-treated α4β1. Furthermore, mAbs anti-α4 and anti-αβ7, but not mAbs to other integrins, inhibited cell adhesion to FN-III5 and KLDAPT. These results therefore establish a cell adhesion function for the FN-III5 repeat and show that KLDAPT is a novel fibronectin ligand for activated α4 integrins.

Fibronectin (Fn) is an extracellular matrix and plasma protein composed of structural domains that contain binding sites for other macromolecules (fibrin, heparin/proteoglycans, collagen), as well as for cells (reviewed in Hynes (1)). The NH2-terminal heparin binding domain or Hep I (see Fig. 1) also interacts with cell surfaces by binding to uncharacterized molecules and is mainly involved in formation of Fn matrices (1, 2). The Hep II domain displays the highest avidity for heparin and contains specific sequences which bind proteoglycans and/or the α4β1 integrin at the cell surface and induce cell adhesion (3–5). One of these sites is H1, which is a ligand for α4β1 in melanoma (6) and lymphoid cells (7). Two other sequences, CS-1 and CS-5, located within the IIICS segment (outside the Hep II domain) are also ligands for α4β1 (8–11) and bind with different affinities (12). These previous studies have established that α4β1 may bind several sequences in the COOH-terminal region of Fn and that these interactions are particularly important in lymphoid cells, where α4β1 is highly expressed.

The central Hep III domain is less well studied because it binds heparin only at low salt concentration, and the physiological significance of this interaction is unclear. Using DNA affinity chromatography, we and others previously isolated proteolytic fragments from this region of Fn, which displayed low affinity binding to heparin. These include a 14-kDa fragment corresponding to FN-III1 repeat (13) and two fragments of 18–20 kDa (FN-III4–5 repeats) (14) and 30 kDa (FN-III4–6 repeats) (15) from human and bovine plasma Fn, respectively. We also previously reported that an 80-kDa tryptic fragment (FN-III4–12/FN-III11) binds heparin with low avidity (16). It is not known whether this heparin binding domain also interacts with cells.

The present study was undertaken to further characterize the properties of the Hep III domain of Fn. To this effect, we have prepared recombinant fragments encompassing type III homology repeats from this region and have studied their cell binding activities. We show that a fragment containing the FN-III5 repeat mediates adhesion of T and B lymphoid cells efficiently. Furthermore, we have identified a novel 6-amino acid-sequence as the active site in FN-III5, and we show that activated α4β1 and α4β7 integrins are receptors for this sequence and the FN-III5 fragment. These results therefore establish a novel function for the Hep III Fn domain.

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Fibronectin Type III5 Repeat Contains a Novel Cell Adhesion Sequence, KLDAPT, Which Binds Activated α4β1 and α4β7 Integrins*

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1 The abbreviations used are: Fn, fibronectin; mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin.
**EXPERIMENTAL PROCEDURES**

**Fibronectin Fragments, Synthetic Peptides, and VCAM-1**—To prepare recombinant Fn fragments extending type III repeats 1-2-11 (without the ED-B repeat) (see Fig. 1B), we made a construct using Fn cDNA from clones pFNHI14 (17) and AF10. The AF10 clone was obtained by screening a human melanoma cDNA library in Agt11 (CLONTECH, Palo Alto, CA) using IST-3 mAb (see below). The cDNA fragments, which contained nucleotides 2229–4787 (17), were inserted into pQE-12 or pQE-3/5 vector using the QAExpress kit (Qiagen, Chatsworth, CA). Recombinant FN-III1/2-11 was isolated by immunoaffinity chromatography using mAb 3E3 (18) conjugated to Sepharose 4B (Pharmacia Biotec, Uppsala, Sweden). Further purification of this fragment was achieved by gel filtration chromatography on AcA44 (Pharmacia) columns equilibrated with 50 mM sodium phosphate buffer, pH 7.1, 1 mM EDTA, 0.5% SDS.

Recombinant fragments containing type III homology repeats 5 (FN-III5), and 6 (FN-III6) were produced by polymerase chain reaction amplification using UTmA DNA-polymerase (Perkin-Elmer), cDNA from FN-III1/2-11 clones, and appropriate primers. Polymerase chain reaction products were cloned into pQE-12 or pQE-3/5 vector using the QAExpress kit and expressed in Escherichia coli. All cloned cDNAs were sequenced using a Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp.). FN-III5 fragment, without the 6 x His tag, was purified by immunoaffinity chromatography using IST-4 mAb (see below). FN-III6 fragment contains the 6 x His tag and was purified using nickel-chelating nitritolactic acid-agarose chromatography. Purity of recombinant fragments was confirmed by SDS-polyacrylamide gel electrophoresis gradient gels (4–18%, see Fig. 1).

The proteolytic native fragments 80 kDa, containing the RGD sequence, and 38 kDa, containing the HEP II domain and the CS-1 site, have been previously described (16, 19). The synthetic peptides KLDAPT (H2), and KDLAPT (scramble H2 or H2.sc) were synthesized on an automated multiple peptide synthesizer (AMS 422, ABIMED, Laingenfeld, Germany) using standard solid phase procedures. Peptides were purified by reverse phase high performance liquid chromatography. Peptide CDELFLQVLTPHIPLGHEIDVPST (CS1) was purchased from AMS biotechnologies (Madrid, Spain). Peptides were covalently conjugated to keyhole limpet hemocyanin (KLH, Calbiochem-Novabiochem Int., La Jolla, CA) with glutaraldehyde as described previously (7). Recombinant VCAM-1 was kindly provided by Dr. Roy Lobb (Biogenc Inc., Cambridge, MA). Amino acid sequence comparisons among Fn cell binding sites were done using software from The Phylip package (Phylic manual version 3.0. J. A. Felsenstein, University of Washington, distributed by the author).

**Antibodies and Enzymes**—IST-3 and IST-4 mAbs reactive with FN-III4 and FN-III5 repeats, respectively, were produced as reported previously (20). The following mAbs were obtained as gifts: activating anti-VLA2 monoclonal antibody TS2/16 (17) and function-blocking anti-α4 HP2/1, both obtained from culture supernatant (Dr. Francisco Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain); anti-αVβ3 mAb LM609, ascitic fluid (Dr. David Cheresh, Scripps Research Institute, La Jolla, CA); and anti-α4β7 mAb Act-1, purified Ab (Dr. Douglas J. Ringler, Leuksite, Cambridge, MA). Anti-α5 mAb P1D6 (ascitic fluid) was purchased from Calbiochem.

**Cells and Cell Cultures**—The human B cell line Ramos was obtained from the American Type Culture Collection (Rockville, MD); RPMI 8866 (B lymphoblastic) and K562 (erythrocytic) cells were obtained from Dr. Bernabeu (Centro de Investigaciones Biológicas), respectively. Cells were maintained in RPMI 1640, 10% fetal bovine serum (ICN Pharmaceuticals, Costa Mesa, CA), and 24 μg/ml gentamycin (Life Technologies, Inc., Middlesex, UK).

**Immunofluorescence Analyses**—5 × 10⁵ cells were incubated for 30 min at 4 °C with 100 μl of culture supernatants (1:2 dilution) or ascittis (1:100 dilution) containing the appropriate mAb. Cells were washed twice with cold phosphate-buffered saline, 1% bovine serum albumin and resuspended in 100 μl of a 1:30 dilution of fluorescein-conjugated Fab’ fragments of rabbit antibodies to mouse IgG (Dakopatts, Glostrup, Denmark). After 30 min at 4 °C, cells were washed twice, resuspended in 200 μl of phosphate-buffered saline, and analyzed by flow cytometry on an EPICS-CS (Coulter Científica, Móstoles, Spain).

**Cell Adhesion Assays**—These assays were performed as described elsewhere (7, 21) using flat bottom high binding 96-well microtiter plates (Nunc, Nalge, Cambridge, MA) for synthetic peptides, native fragments, and VCAM-1, or flat bottom 8-well strips with N-oxysuccinimide amine binding surface (Costar) for recombinant fragments (diluted in 0.1 M sodium borate, pH 9.0). Assays were carried out for 1–3 h at 37 °C, and attached cells were stained with toluidine blue and quantitated by reading the absorbance at 620 nm on a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland) and visually on an inverted microscope (Nikon Diaphot, Japan) (21). Integrin activation with TS2/16 mAb or Mn²⁺ was performed by incubating the cells with 1:10 dilution of hybridoma supernatant or 10 μM Tris, 150 μM NaCl, 1% bovine serum albumin, 1 mM Mn²⁺, pH 7.2, for 15 min at 37 °C prior to the attachment assay. For inhibition experiments, cells were incubated in a total volume of 400 μl, with appropriate supernatant (1:5 dilution), ascitic fluid (1:100 dilution), or peptides (various concentrations) for 30 min at room temperature. The cell suspension was then diluted to 5 × 10⁵ cells/ml, and 100 μl were added to each substrate-coated well. Inhibition of binding of alkaline phosphatase-coupled VCAM-Ig fusion protein to α4β1 was performed exactly as described previously (22).

**RESULTS**

**Identification of KLDAPT in FN-III5 Repeat as a Novel Cell Adhesion Sequence in Fn**—To test the possibility that the central region of Fn comprising the HEP III domain contains cell binding sites, we performed a computer search using the Phylip package program to look for structural homologies with known cell binding sequences in the COOH-terminal region of Fn, namely EILDVPST (CS-1) and IDAPS (H1). The sequence KLDAPT located at the NH2 terminus of repeat III-5 (Fig. 1) showed the highest homology to CS-1 and H1 (Fig. 2). We have named this sequence H2 because of its similarity with H1 both in primary structure and location in a heparin-binding region of Fn (Fig. 1).

To determine whether H2 mediated cell adhesion, the synthetic peptide KLDAPT was coupled to KLH and used as adhesion substrate. Peptides KLDAPT-KLH (scramble H2), and CS-1-KLH, were employed as negative and positive controls, respectively. The various cell types used for these experiments and their pattern of surface expression of some integrin receptors are listed in Table I. As shown in Fig. 3, none of the cell lines tested adhered to the H2 peptide under resting conditions. However, incubation with 1 mM Mn²⁺ (RPMI 8866) or with anti-β1 mAb TS2/16 (Ramos, Jurkat) induced a very efficient dose response to this peptide, similar to that attained for

![Fig. 1](https://example.com/f1.png)

**FIG. 1.** A, schematic drawing of the human Fn molecule showing the three types of internal homology units with type III repeats numbered. Shaded regions indicate the alternatively spliced segments (ED-B, ED-D, HICIS) present in only certain Fn subunits. The location of the main sequences that promote cell adhesion via integrin receptors, including the novel site reported here (H2) is indicated. The heparin binding domains (Hep I, II, III) of Fn are also shown. B, drawing and SDS-polyacrylamide gel electrophoresis analysis (4–18% gradient) of the recombinant fragments prepared and used in the present study. Numbers indicate the molecular masses (kDa) of known standards.
adhesion to CS-1 (Fig. 3). These results suggested that the KLDAPT motif was binding to an integrin and required an activated form of the receptor. TS2/16- or Mn2+-treated cells did not bind to the scramble peptide KDLAPT at any concentration tested (Fig. 3).

The Recombinant Fragment FN-III5 Mediates Cell Adhesion, Inhibition by Soluble H2 and CS-1 Peptides—As mentioned above, the KLDAPT sequence is located in FN-III5 repeat. We therefore studied whether a fragment containing this repeat above, the KLDAPT sequence is located in FN-III5 repeat. We did not bind to the scramble peptide KDLAPT at any concentration tested (Fig. 3).

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These results suggested that KLDAPT was responsible for the adhesion activity observed in FN-III5. To establish whether this sequence could also bind to the cell surface in soluble form and inhibit cell adhesion, TS2/16 treated-Ramos cells were preincubated with various concentrations of H2 or scramble H2 peptides prior to the adhesion assay. As shown in Fig. 5A, soluble H2 peptide inhibited adhesion of Ramos cells to FN-III5 in a dose-dependent manner, while the control scramble peptide had no effect. Moreover, soluble CS-1 peptide also inhibited Ramos cell adhesion to FN-III5 (Fig. 5A), suggesting that α4β1 integrin was the receptor for FN-III5. The calculated IC50 values in this assay were 5 and 51 μM for CS-1 and H2 peptides, respectively (Fig. 5A and Table II).

We also examined whether soluble H2 could affect binding of α4β1 to its known ligands. TS2/16-treated Ramos cells were preincubated with 0.5 mg/ml of H2 or the control peptides CS-1 and scramble H2, and tested for adhesion to two Fn fragments of 38 and 80 kDa, which we previously showed to be ligands for resting or activated α4β1, respectively (9, 22), or to the endothelial ligand VCAM-1 (23). The synthetic peptide H2-KLH was included as control substrate in this assay. As shown in Fig. 5B, preincubation with soluble H2 or CS-1 produced 100% inhibition of adhesion to immobilized H2-KLH, confirming the specificity of the α4β1 recognition of this sequence. Moreover, H2 completely abolished adhesion to the 80- and 38-kDa fragments, and to VCAM-1, while the control peptide KDLAPT did not affect cell adhesion to any of these substrata (Fig. 5B). To obtain some insight on the affinity of the interaction of KLDAPT with α4β1 integrin we determined the inhibitory effect of various concentrations of H2, CS-1, and scramble H2 peptides on two sets of experiments: 1) on cell adhesion to the 38-kDa fragment (Fig. 5A) or to VCAM-1 (not shown); 2) on binding of recombinant alkaline phosphatase-coupled
VCAM-Ig fusion protein to Mn$^{2+}$-activated α4β1 (22). The IC$_{50}$ values calculated from these experiments are shown in Table II.

### α4β1 and α4β7 Integrins Are Receptors for KLDAPT and FN-III5—

The preceding results indicated that adhesion to KLDAPT and FN-III5 was mediated by α4β1. This was further confirmed by using specific mAbs to various integrins. As shown in Fig. 6, preincubation of TS2/16-treated Ramos cells with HP2/1 (anti-α4) mAb completely inhibited adhesion to both substrata, while Act-1 (anti-α4β7) or LM609 (anti-αVβ3) mAbs had no effect. These results are in agreement with the lack of expression of the last two integrins on Ramos cells (24) (Table I) and clearly establish that α4β1 is the receptor for H2 and FN-III5 on these cells.

However, RPMI 8866 cells, which bound well to H2 and FN-III5 (Figs. 5 and 6), express α4β7 and very low levels of β1 on their surface (Table I) (24, 25), suggesting that α4β7 could be involved in this adhesion. As shown in Fig. 6, both HP2/1 and Act-1 mAbs completely inhibited adhesion of Mn$^{2+}$-treated RPMI 8866 cells to H2-KLH or FN-III5, therefore indicating that α4β7 is also a functional receptor for the H2 sequence. LM609 mAb had a minor effect (15% inhibition) on adhesion of RPMI 8866 cells to FN-III5, perhaps indicating a small contribution of αVβ3 to the attachment of these cells to this fragment. These results together with the fact that K562 cells, which lack α4 integrins (Table I), do not bind to FN-III5 (or H2-KLH, not shown), clearly establish that KLDAPT is a novel adhesion sequence specifically recognized by activated α4 integrins.

### DISCUSSION

In this report we have characterized the function of FN-III5 homology repeat located in the Hep III domain of Fn. The major conclusions are: 1) recombinant fragments containing the FN-III5 repeat mediate adhesion of lymphoid cells, 2) the sequence KLDAPT is the active site in FN-III5 and is sufficient to induce cell adhesion, 3) the synthetic peptide KLDAPT in soluble form efficiently inhibits cell adhesion to different recombinant or proteolytic Fn fragments and to VCAM-1, and 4) the α4β1 and α4β7 integrins are functional receptors for KLDAPT and FN-III5 and require activation for binding.

The function of the region of Fn encompassing repeats III4–6 has been largely unknown. Early studies have indicated that this segment contains a weak heparin and DNA binding site(s) (named Hep III domain), but its physiological relevance is unclear (13–16). A recent study has proposed that the region comprising Fn repeats III1–7 may play a regulatory role in the process of matrix formation (26). We show in the present report that a recombinant fragment containing the FN-III5 repeat promotes cell adhesion efficiently and therefore constitutes a new cell binding domain in Fn. We have identified the sequence KLDAPT, located at the amino terminus of FN-III5, as the active site. A synthetic peptide containing this sequence was an effective inhibitor of cell adhesion to FN-III5 and also mediated cell attachment when coupled to KLH. These results clearly demonstrate that interaction with KLDAPT is sufficient for cell binding to the FN-III5 fragment.

The present report also establishes that KLDAPT (called H2 throughout this study) is a novel ligand for α4β1 and α4β7 integrins and requires previous activation of both integrins for binding. As we show here, H2 was approximately 5-fold less efficient than the previously identified α4β1 ligand CS-1 in inhibiting cell adhesion to the Fn 38-kDa fragment and VCAM-1, and 10-fold less efficient in inhibiting adhesion to FN-III5 fragment (see Table II). H2 was also 10-fold less efficient than CS-1 in inhibiting binding of soluble VCAM-Ig-alkaline phosphatase, but it was a specific competitor in this assay when compared with the control scramble peptide KDAPT, which did not inhibit. While these apparent IC$_{50}$ values certainly provide information on the affinity of the interaction, it should be considered that the different length of H2 and CS-1 peptides (6 versus 25 amino acid residues) may also account for the higher efficiency observed for CS-1 inhibition. On the other hand, the fact that recognition of H2 requires an activated integrin for binding suggests a low affinity interaction. This is also supported by the fact that CS-1 is a more efficient inhibitor of adhesion to FN-III5 than to the 38-kDa fragment or VCAM-1 (see Table II). We have recently shown that the low affinity α4β1 ligands H1 and RGD induce different intracellular responses than CS-1 (7). Therefore low affinity interactions such as the newly described here are probably phsiologically important in regulating such responses.

We and others have previously shown that α4β1, the main Fn receptor in lymphocytes, recognizes several sites located in different regions of Fn. These include CS-1 and CS-5 in IICS (9–12), H1 in repeat III14 (6), and RGD in repeat III10 which binds only to activated α4β1 (22). These ligands have a common recognition motif which can be LDV (CS-1), EDV/GDV (CS-5), IDA (H1), and GDS (RGD). The KLDAPT sequence reported here fits well into this pattern and shows that LDA is a novel recognition motif for activated α4β1 and α4β7, present in the FN-III5 repeat. In agreement with this, a recent report (27) has shown that mutants of IICS in which the wild-type sequence IDLDVP was changed to IDLDAP, supported adhesion of CHO-α4 transfected cells almost as effectively as the native sequence. This group also reported that increasing the time of the assay could overcome the impaired adhesion observed for certain mutants. In this regard, we have observed that, while cell adhesion to the synthetic peptide KLDAPT-KLH reached saturation within 30 min, adhesion to FN-III5 required longer incubation times. Although we do not have an explanation for this, it is possible that the higher ligand density obtained when

### Table II

| α4β1 ligand | H2 | CS-1 | H2.sc |
|-------------|----|-----|------|
| FN-III5     | 51 | 5   | 5.9  |
| 38 kDa      | 145| 30.5|      |
| VCAM-1      | 133| 25.5|      |
| VCAM-Ig-AP  | 81.4| 7.9 | >718 |

**DISCUSSION**

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The present report also establishes that KLDAPT (called H2 throughout this study) is a novel ligand for α4β1 and α4β7 integrins and requires previous activation of both integrins for binding. As we show here, H2 was approximately 5-fold less efficient than the previously identified α4β1 ligand CS-1 in inhibiting cell adhesion to the Fn 38-kDa fragment and VCAM-1, and 10-fold less efficient in inhibiting adhesion to FN-III5 fragment (see Table II). H2 was also 10-fold less efficient than CS-1 in inhibiting binding of soluble VCAM-Ig-alkaline phosphatase, but it was a specific competitor in this assay when compared with the control scramble peptide KDAPT, which did not inhibit. While these apparent IC$_{50}$ values certainly provide information on the affinity of the interaction, it should be considered that the different length of H2 and CS-1 peptides (6 versus 25 amino acid residues) may also account for the higher efficiency observed for CS-1 inhibition. On the other hand, the fact that recognition of H2 requires an activated integrin for binding suggests a low affinity interaction. This is also supported by the fact that CS-1 is a more efficient inhibitor of adhesion to FN-III5 than to the 38-kDa fragment or VCAM-1 (see Table II). We have recently shown that the low affinity α4β1 ligands H1 and RGD induce different intracellular responses than CS-1 (7). Therefore low affinity interactions such as the newly described here are probably phsiologically important in regulating such responses.

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using the peptide as a substrate overcomes the low affinity/avidity of the interaction.

The novel active sequence KLDAPT found in FN-III5 repeat is highly homologous to AIDAPS or H1 site previously identified in FN-III4 repeat (6). It is interesting that both sequences lie in exposed loops outside the β strand structures present in type III homology repeats (28). This observation and the fact that both repeats are in heparin binding regions of Fn suggest that there is a structural and functional homology between FN-III5 and FN-III4 modules. However, the ability of both repeats to interact with α4β1 is different because FN-III5 induces adhesion only through an activated integrin. The results reported here therefore extend our previous findings showing that there is a structural and functional homology between FN-III5 and FN-III4 modules. However, the ability of both repeats to interact with α4β1 is different because FN-III5

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