Utilization of a Soluble Integrin-Alkaline Phosphatase Chimera To Characterize Integrin α8β1 Receptor Interactions with Tenascin: Murine α8β1 Binds to the RGD Site in Tenascin-C Fragments, but Not to Native Tenascin-C†

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ABSTRACT: The integrin α8β1 has been reported to bind to fibronectin, vitronectin, and tenascin-C in cell adhesion or neurite outgrowth assays. Here, we describe cDNA cloning of the murine α8 subunit, purification of a recombinant soluble heterodimer consisting of the extracellular domains of the murine α8 and β1 subunits, and development of a sensitive binding assay using a modified form of this heterodimer fused to alkaline phosphatase (AP). In binding assays, the purified α8β1-AP chimera exhibited the same divalent ion requirements for activation and binding specificity as cell surface α8β1: in the presence of Mn^{2+} it bound to fibronectin and vitronectin in an RGDS-peptide inhibitable manner. Contrary to previous reports, we found no evidence that α8β1, expressed on K562 cells or as an AP chimera, interacts strongly with native tenascin-C. In binding, adhesion, and spreading assays, significant interactions were observed only to short fragments of tenascin-C containing the third fibronectin type III repeat which contains an RGD sequence. Full length tenascin-C and longer fragments containing this repeat did not appear to serve as ligands, implying that the RGD site in native tenascin-C is a cryptic binding site for this integrin, exposed by removal of adjacent domains. Soluble integrin-AP chimeras should be generally useful for identifying and characterizing integrin interactions with ligands.

Integrins are a family of noncovalently linked αβ heterodimers which serve as cellular receptors for a wide variety of ligands including extracellular matrix (ECM)[1] glycoproteins, immunoglobulin, and cadherin-class cell adhesion molecules, and disintegrins (*1*−*3*). Integrins have been shown to play important roles in development, hemostasis, wound healing, immune responses, and malignant transformation (*1*). The integrin α8 subunit associates with the integrin β1, but not other integrin β subunits, to form a receptor for several ECM constituents including fibronectin (FN), vitronectin (VN), and osteopontin (*4*−*7*, *44*). Evidence has also been presented indicating that tenascin-C (TN-C) is a ligand for this integrin using cell adhesion and neurite outgrowth assays (*6*, *8*). Recently, we have shown that this integrin mediates epithelial-mesenchymal interactions essential for normal development of the metanephric kidney, although none of the known ligands for this integrin appear to have appropriate expression patterns to account for the requirement for this integrin during kidney morphogenesis (*9*). Motivated in part by these observations, we constructed a soluble truncated mouse α8β1 integrin heterodimer as a fusion protein with alkaline phosphatase (α8^{t}β1-AP) to use as a probe to characterize known receptor−ligand interactions and to help identify novel ligands. While AP chimeras have been used to identify the ligands for receptor tyrosine kinases (*10*, *11*), they have not been previously constructed with heterodimeric receptor molecules such as integrins. In previous work, detection of integrin ligands using purified integrins has been difficult because integrin interactions with ligands are often of comparatively low affinity. Interpretation of cell adhesion assays has often been difficult because

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‡ The sequence data are available from Genbank under accession number AF041409.

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[1] Abbreviations: AP, alkaline phosphatase; RGDS, arginine, glycine, aspartic acid, serine; ECM, extracellular matrix; TN-C, tenascin-C; FN, fibronectin; VN, vitronectin; GST, glutathione-S-transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNfn3, third fibronectin-type III repeat of tenascin-C; FN120, 120 kDa fragment of FN; TSP, thrombospondin-1; Col I, Collagen type I; Col IV, collagen type IV; LN-1, laminin-1; EGF, epidermal growth factor.

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many cells express several integrins capable of binding to the same ligand. Confidence in interpretation of results using function-blocking antibodies has been reduced by possibilities of indirect effects mediated by steric hindrance or perturbations of cellular signaling pathways. In the present work, we report successful expression, secretion, and purification of an $\alpha8^t\beta1$-AP chimera retaining ligand-binding activity and AP activity. Using the purified recombinant $\alpha8^t\beta1$-AP chimera in solid phase binding assays, we show that it provides a sensitive reagent for characterizing receptor−ligand interactions. We have reexamined ligand interactions of this integrin and present evidence that this integrin's interactions with human and chicken TN-C are mediated through a binding site containing an RGD sequence which appears to be cryptic in native TN-C, but is exposed in certain TN-C fragments.

EXPERIMENTAL PROCEDURES

Materials. Human plasma FN, the 120 kDa and 40 kDa FN fragments (FN120, FN40), bovine plasma VN, human TN-C, human thrombospondin-1, and the peptides GRGDSP and GRGESP were purchased from Gibco BRL (Gaithersburg, MD). Chicken TN-C was purified as described (*8*). Rat collagen types I and IV were purchased from Collaborative Research Inc. (Bedford, MA). Laminin-1/EHS laminin was purified as described (*12*). Recombinant chicken TN-C fragment fusion proteins were prepared according to published procedures (*13*). Recombinant human TN-C fragment proteins (*14*) and native human TN-C (*15*) were prepared as described. Recombinant mouse entactin/nidogen was a gift from Dr. R. Timple, Max-Planck Institute für Biochemie, Martinsried, Germany. Glutathione-S-transferase (GST)-entactin fragment fusion proteins (*16*) were a gift from Dr. A. E. Chung, University of Pittsburgh. A synthetic peptide containing the C-terminal residues of the human integrin α8 cytoplasmic domain was synthesized by Dr. C. W. Turck, Howard Hughes Medical Institute, University of California, San Francisco. The mouse integrin $\beta1$ cDNA clone (*17*) was a gift from Dr. S. Tominaga, Jichi Medical School, Tochigi, Japan. The human placental AP fusion vector (*10*) was a gift from Dr. J. G. Flanagan, Harvard Medical School.

Antibodies. Anti-GST-mouse α8 antibody was prepared by immunizing rabbits with powdered lyophilized gel slice containing a 130 kDa GST-mouse α8 fusion protein purified by SDS−PAGE from *Escherichia coli* inclusion bodies. This fusion protein was prepared by expressing the plasmid pGEX-4T3 (Pharmacia LKB Biotechnology Inc. Piscataway, NJ) containing the *Bam*HI−*Bsp*120I fragment of a PCR-amplified clone (see below) encoding amino acids 1−917 of the mature mouse integrin α8. This clone has a mutation (F to L) at the amino acid number 481. Anti-α8 cytoplasmic domain antibody was prepared as described (*4*), using the synthetic peptide (28 residues) of the cytoplasmic domain of human integrin α8 (*7*). These antibodies recognized specifically the human and mouse integrin α8 subunit in Western blots and in immunoprecipitations. The other antibodies were as follows: anti-human integrin $\alpha v\beta5$ mAb P1F6 (Gibco BRL); anti-human integrin α5 mAb B1E5 (*18*); anti-human integrin $\beta1$ mAb AIIB2 (*18*); anti-mouse integrin $\beta1$ mAb 9EG7 (*19*); anti-rat integrin $\beta1$ mAb HA2/11 (*20*); anti-human placental AP mAb MIA1801 (Medix Biotech, San Carlos, CA); anti c-Myc mAb 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA). IgG fractions were prepared from antisera or ascites using protein A or protein G-Sepharose (Pharmacia LKB Biotechnology).

cDNA Cloning and Plasmid Construction. The Tri-clone system (Invitrogen, San Diego, CA) was used to clone mouse integrin α8 cDNA: mRNA was purified from NIH 3T3 cells; cDNA was synthesized by reverse-transcriptase using random primers; several amplification reactions were performed using different sets of degenerate or gene-specific primers; the amplified fragments were gel-purified using Qiaex (Qiagen, Chatsworth, CA) and cloned into pCRII (TAcloning vector, Invitrogen). The degenerate primers used were sense primer A (nucleotide position 1−20, where position 1 is the 5′ end of coding sequence for mature α8 protein), 5′-TT(CT)AA-(CT)(CT)T(ACGT)GA(CT)G(AT)(ACGT)GA(AG)AA-3′; antisense primer H (position 3065−3046), 5′-GTCTTGT(CT)-ATTTGTCAG(CT)TG-3′; and antisense primer I (position 3078−3060), 5′-G(CT)(CT)A(ACGT)GC(ACGT)TCAG(G-T)(ACGT)GTCTTG-3′. The gene-specific primers used were primer B (position 1−35), C (position 919−938), D (position 1730−1746), E (position 2858−2877), F (position 962−946), and G (position 1821−1797). The plasmid inserts were sequenced using Sequenase 2.0 (Amersham, Arlington Heights, IL). The cDNA clone (pCRII-33) encoding the consensus sequence of mouse α8 (amino acids 1−1021) was generated by ligating the PCR-amplified clones. To express a secreted protein, a synthetic *Bam*HI-*Ava*I DNA fragment encoding the modified chicken α8 signal peptide fused to the N-terminal portion of the mature mouse α8 subunit (MARRQPPRPLLLLSALLCAPASA-FNLDVEKLTVYSG), generated by annealing two complimentary oligonucleotides, was ligated into *Bam*HI-*Ava*I sites of pCRII-33, resulting in the clone named pCRII-35. A clone encoding a truncated mouse α8 ($\alpha8^t$) was generated by PCR using pCRII-35 as a template, with a gene-specific 5′-primer containing a 5′ *Bam*HI site and a 3′-primer encoding the C-terminus of the deduced extracellular domain of mouse α8 plus a $(His)^6$-myc tag and a stop codon (VIWATPN-VSHHHHHHGEQKLISEEDL-stop). This amplified fragment was subcloned into the *Bam*H1-*Eco*RV site of the eucaryotic expression vector pCR3 (Invitrogen). The truncated mouse $\beta1$ ($\beta1^t$) was generated by introducing a stop codon after the end of extracellular domain (amino acid sequence number 728) by PCR using the mouse $\beta1$ cDNA clone ST1 (*17*) as a template with a gene specific 5′-primer containing a *Bam*HI site and a 3′-primer containing an EcoRI site. This was subcloned into the *Bam*HI and *Eco*RI sites within the multiple cloning site of pCR3. The mouse $\beta1$ extracellular domain-AP chimera was generated by isolating a modified pCR3-$\beta1^t$, in which the stop codon at the end of extracellular domain of $\beta1^t$ was eliminated by PCR. The *Sna*BI (filled in with Klenow)-*Xho*I fragment containing the AP from APtag-1 (*10*) was ligated into the *Eco*RI (filled in with Klenow) and *Xho*I sites of the pCR3-$\beta1^t$ plasmid modified to eliminate the stop codon. All of the PCR-generated clones were sequenced to confirm that they do not contain mutations caused by Taq polymerase.

Purification of Soluble Truncated Integrin Heterodimers. COS-7 cells were grown in 15 cm diameter dishes and transiently cotransfected using LipofectAmine Reagent (Gibco BRL) with the two plasmids, encoding extracellular domains of the α and β subunits plus indicated C-terminal

tags ($\alpha8^t$ and $\beta1^t$ to express $\alpha8^t\beta1^t$; $\alpha8^t$ and β1-AP to express $\alpha8^t\beta$1-AP). After treatment with the lipofection mixture for 5 h, the medium was changed to DMEM supplemented with Nutridoma HU (Boehringer, Indianapolis, IN) and antibiotics. The conditioned medium was collected every 2−4 days for 1 week. After addition of $MgCl_2$ (at final concentration of 1 mM), phenylmethanesulfonyl fluoride (PMSF, 1 mM), and sodium azide (0.02%), the conditioned medium was filtered (paper number 1, Whatman, Maidstone, UK) and concentrated 10−20-fold using a YM100 membrane (Amicon, Beverly, MA). The concentrated medium was supplemented with Tris-Cl, pH 8.0 (at final concentration of 20 mM), imidazole (10 mM), and PMSF (0.5 mM), and was then incubated at 4 °C for 2 h in batch with Ni-NTA beads (Qiagen). The beads were transferred into an empty column and washed with buffer A (20 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM $MgCl_2$, 0.02% sodium azide, 20 mM imidazole, 0.5 mM PMSF). After washing, bound proteins were eluted with elution buffer (20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.02% sodium azide, 100 mM imidazole, 0.5 mM PMSF). To remove imidazole, $\alpha8^t$ monomer, and nonspecifically bound contaminants of low molecular weight, the buffer of the eluate was exchanged by repeating concentration and dilution with the elution buffer without imidazole, using a Centricon 100 or Centriplus 100 filter apparatus (Amicon, Beverly, MA). Protein concentration of the purified integrins was determined by both Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and silver staining of proteins after fractionation by SDS−PAGE. The purified heterodimers retained activity for at least 5 months when stored at either 4 °C or −80 °C.

Radiolabeling and Immunoprecipitation. COS-7 cells were transfected with the plasmids as described above. After 1 day, the cells were metabolically labeled in methionine-and-cysteine-free DMEM supplemented with Nutridoma HU for 2 h with 500 μCi/mL Tran^{35}S-label (ICN, Irvine, CA). After labeling, the medium was replaced with fresh radioisotope-free medium, and the cells were further incubated for 17 h. The conditioned medium was harvested, precleared by incubation with Sepharose CL-4B (Pharmacia LKB Biotechnology), and immunoprecipitated with indicated antibodies and protein A or protein G-Sepharose beads (Pharmacia LKB Biotechnology). After collection, the beads were washed with buffer B (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.1% NP-40). To collect the secreted $\alpha8^t$ with Ni-NTA beads, the precleared medium was incubated with Ni-NTA beads, and beads were washed with buffer A. For immunoprecipitations or Ni-NTA binding of metabolically labeled integrins, 500 μL of medium was used directly for each sample without prior fractionation.

Solid-Phase Binding Assays. 96 Well plates (Maxisorp, Nunc, Rochester, NY) were coated with indicated concentrations of substrate proteins in TBS (25 mM Tris-Cl, pH 7.5, and 100 mM NaCl) at 4 °C overnight, blocked with 1% BSA (RIA grade, Sigma, St. Louis, MO) in TBS, and washed with TBS containing 1 mM $MnCl_2$ (TBS-Mn). Thrombospondin-1 was coated in the presence of 1 mM $CaCl_2$. As negative and positive controls, wells were coated with 1% BSA or 10 μg/mL FN120, respectively. A total of 100 μL of $\alpha8^t\beta$1-AP (5 μg/mL in TBS-Mn) was then added to each well, which was then incubated for 2 h at room temperature. After washing wells 5 times with TBS-Mn, 100 μL of AP substrate (12 mM *p*-nitrophenyl phosphate, 0.5 mM $MgCl_2$, and 1 M diethanolamine, pH 9.8) was added and incubated at room temperature for an appropriate time. Integrin binding was quantified by measuring absorbance at 405 nm. $\alpha8^t\beta1^t$ biotinylated with NHC-LC-biotin (Pierce, Rockford, IL) was also used in the binding assay, and binding was detected as described (*21*). Where indicated, the plates were coated first with nitrocellulose and then with proteins as described (*8*).

Cell Adhesion and Spreading Assays. The human erythroleukemia cell line K562, KA8 cells (chicken α8 subunit-transfected K562 cells), cell adhesion, and cell spreading assays have been described (*5*).

RESULTS

Cloning of the Coding Sequence of the Mouse α8 Integrin by RT-PCR. To obtain clones encoding the murine homologue of the integrin α subunit, degenerate primers were designed using the published sequences of cDNAs encoding the chicken and human α8 proteins (*4*, *7*). Using different sets of primers in RT-PCR reactions with the mRNA from NIH 3T3 cells, several overlapping cDNA clones were obtained. Multiple independently derived PCR clones were sequenced to determine the consensus sequence of the integrin coding region (Figure 1). The deduced amino acid sequence of the integrin coding region was 90% identical to the human and 76% identical to the chicken α8 subunit. In comparisons with other integrin α subunits, the α5 and αv subunits have been shown to be most closely related to α8 (e.g., refs *4* and *7*). Comparisons of the murine α subunit sequence described above to those of the published human and murine αv and α5 subunits revealed only 45−48% identity to each. Together, these results indicate that the subunit characterized above represents that of the murine integrin α8 subunit. As previously described for the avian and human homologues (*4*, *7*), the murine α8 subunit contains three potential metal-binding domains and a putative post-translational cleavage site. It also includes 16 potential N-glycosylation sites.

To provide independent evidence that our clones encode the murine integrin α8 subunit, we prepared antibodies to a bacterially expressed GST fusion protein containing the extracellular domain of the murine integrin α subunit described above. This antiserum was used in western blots using extracts of tissues of wild-type mice or mice lacking α8 protein due to a targeted mutation in the α8 gene (*9*). As predicted, a protein with the expected size of the α8 subunit was detected in extracts from the wild-type, but not mutant animals (ref *9* and data not shown). A second antibody was prepared to a peptide with the same sequence as the deduced cytoplasmic domain of the human α8 subunit (*7*). We expected that the antibody against the human cytoplasmic domain would recognize its murine counterpart due to the conservation of cytoplasmic domain sequences of integrin α subunits across species. This antibody was also shown to recognize a protein with the expected size of the α8 subunit in a variety of murine tissues from wild-type, but not mutant mice (*9*). The results using both antibodies provide further, independent evidence that the sequence presented here encodes the murine homologue of the integrin α8 subunit.

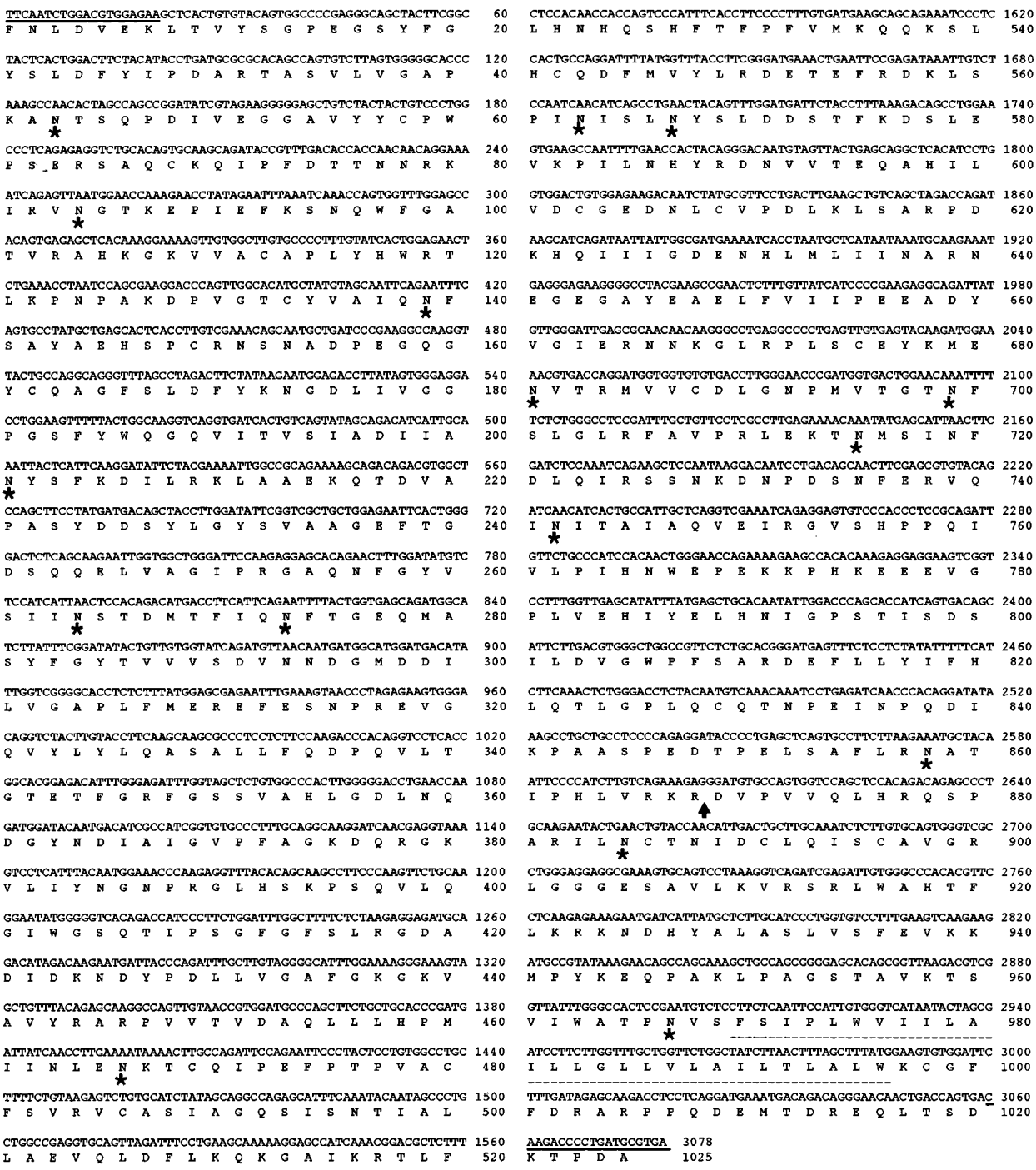

FIGURE 1: Mouse integrin α8 cDNA nucleotide sequence and deduced amino acid sequence. The consensus sequence of the clones obtained from different RT-PCR reactions is shown, except that the nucleotide sequence number 3046−3059 was derived from the clones obtained by a single RT-PCR. The amino acid sequence number 1 corresponds to the N-terminus of the mature protein determined by comparison to the deduced amino acid sequences of the previously cloned chicken and human α8 subunits. Underlined sequences at the 5′ and 3′ ends are amplified sequences derived from the degenerate primers (primer A and I, see Experimental Procedures) which may therefore contain mismatches to the true sequence. Broken underline, the putative transmembrane domain. Arrow, the putative posttranslational cleavage site. Asterisks, potential N-glycosylation sites (N-X-T/S). The nucleotide sequence of bases 21−3059 has been deposited in Genbank under the accession number AF041409.

Truncated Integrin α8 and β1 Subunits Are Secreted as a Heterodimer. Transient transfection experiments followed by immunoprecipitations confirmed that the expressed mature full-length murine α8 subunit formed cell surface heterodimers with the endogenous β1 subunit in human 293 cells and indicated that this α subunit is proteolytically processed into large and small disulfide-linked fragments during maturation (not shown), similar to maturation of this subunit in other species (*4*, *7*). To generate a secreted, soluble heterodimer containing the extracellular domains of the murine α8 and β1 subunits, we prepared constructs designed to express the extracellular domains of the mouse α8 and mouse β1 subunits by elimination of the transmembrane and cytoplasmic domains (Figure 2, panels A and B). Similar constructs have been previously used to express soluble extracellular domains of other integrin heterodimers (*22*−*27*). For purposes of affinity purification and detection, respectively, (His)^6 and c-myc epitope tags were added to

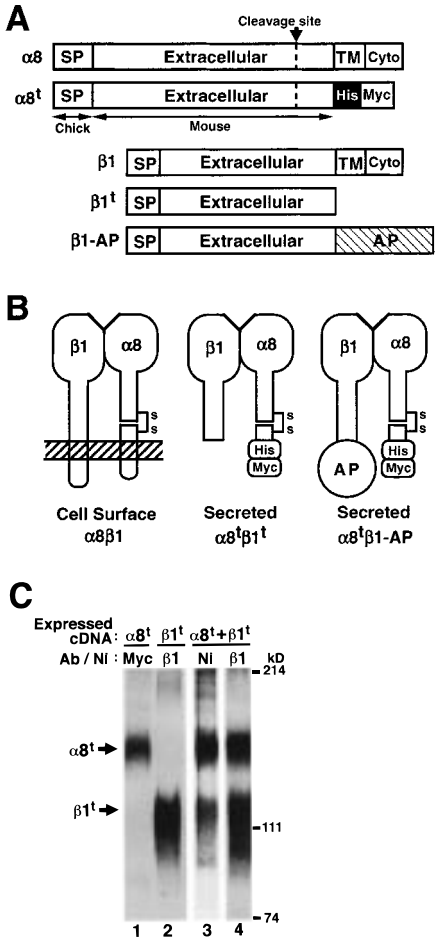

FIGURE 2: Expression of truncated forms of the mouse integrin α8 and β1 subunits. (A) Schematic representation of the expression constructs. Domain structures of the translated proteins are shown. After cleavage of the signal peptides (SP), the mature integrin α8 and β1 subunits consist of extracellular, transmembrane (TM), and cytoplasmic (Cyto) domains. To express the mouse α8 integrin subunit, the oligonucleotides encoding the sequence of the chicken α8 signal peptide were connected in frame to a clone encoding the mature mouse α8 protein. The truncated α8 ($\alpha8^t$) protein was expressed using an expression vector containing an insert consisting of a cDNA containing the chicken α8 signal peptide, the entire extracellular domain of mouse α8 (969 amino acids), and C-terminal $(His)^6$ and c-myc epitope tags. The truncated β1 ($\beta1^t$) consists of sequences from a cDNA encoding the signal sequence and entire extracellular domain (708 amino acids) of mouse β1 followed by a stop codon. The β1-AP chimera consists of the signal sequence and entire extracellular domain of β1 fused in frame to human placental AP. The putative posttranslational cleavage site of the α8 subunit, deduced by comparison to cleavage sites in other integrin α subunits, is marked with a dashed line. (B) Schematic drawings of the full length α8β1 heterodimer, the secreted soluble $\alpha8^t\beta1^t$ heterodimer obtained by expressing the $\alpha8^t$ and $\beta1^t$ constructs, and the soluble $\alpha8^t\beta1$-AP heterodimer obtained by expressing the $\alpha8^t$ and β1-AP constructs. (C) Detection of both monomers and heterodimers of truncated α8 and β1 integrins in the conditioned media. COS cells were transfected with vectors expressing either $\alpha8^t$ (lane 1), $\beta1^t$ (lane 2), or both $\alpha8^t$ and $\beta1^t$ (lanes 3 and 4). After metabolic labeling of the transfected cells with [^{35}S]methionine, media were either immunoprecipitated with antibodies (lane 1, anti-myc mAb; lanes 2 and 4, anti-β1 mAb HA2/11) or were incubated with Ni-NTA beads (lane 3). The precipitated/bound samples were fractionated by SDS−PAGE in 6% polyacrylamide gels in nonreducing conditions and analyzed by fluorography. Note that either truncated α8 or truncated β1 is secreted when expressed alone (lanes 1 and 2); when cells were cotransfected $\alpha8^t\beta1^t$ heterodimers were also secreted (lanes 3 and 4).

the C-terminal coding sequence of the α8 extracellular domain to form the truncated α8 construct named $\alpha8^t$. The construct $\beta1^t$ encoded the entire extracellular domain of β1 truncated with an artificial stop codon following amino acid 728. Human placental alkaline phosphatase (AP) was chosen for addition to the C-terminal coding sequence of the truncated β1 subunit to form the chimera named β1-AP because AP has well-characterized and sensitive detection properties (*10*, *11*).

To examine the abilities of the truncated proteins encoded by these constructs to be assembled into heterodimers and secreted, COS-7 cells were transfected with constructs encoding $\alpha8^t$ or $\beta1^t$ alone or in combination and grown in [^{35}S]methionine-containing media. Following immunoprecipitation or Ni-affinity chromatography, radiolabeled proteins were separated by SDS−PAGE and visualized by fluorography (Figure 2C). No secreted proteins corresponding in size to the $\alpha8^t$ and $\beta1^t$ subunits were seen in media conditioned by mock-transfected cells (not shown). When constructs encoding $\alpha8^t$ or $\beta1^t$ were transfected alone, each truncated subunit was secreted into the media (Figure 2C, lanes 1 and 2). When $\alpha8^t$ and $\beta1^t$ were cotransfected, both subunits were also secreted into the medium and apparently formed heterodimers as demonstrated by coelution of both subunits from Ni-affinity columns and coimmunoprecipitates with an anti-β1 mAb (Figure 2C, lanes 3 and 4). The differences in the relative intensities of the $\alpha8^t$ and $\beta1^t$ bands in lanes 3 and 4 suggest that subunit monomers as well as heterodimers were secreted from the COS-7 cells cotransfected with both $\alpha8^t$ and $\beta1^t$, and this will be more convincingly demonstrated below.

Purification of Soluble Truncated α8β1 Heterodimers. Two combinations of cDNA constructs encoding α8 and β1 subunits ($\alpha8^t$ with $\beta1^t$; $\alpha8^t$ with β1-AP) were coexpressed in COS-7 cells, and conditioned media from these cells were used to establish purification schemes for both the $\alpha8^t\beta1^t$ and the $\alpha8^t\beta1$-AP heterodimers (see Experimental Procedures and Figure 3). As a control, secreted AP was also expressed alone, not fused to the integrin β1 subunit. Purified proteins were fractionated by SDS−PAGE in reducing or nonreducing conditions to establish purity and to determine whether the secreted $\alpha8^t$ subunit was proteolytically cleaved into two disulfide linked polypeptides, similar to the full-length α8 subunit. Molecules were visualized either by silver staining or by antigen-blot employing subunit-specific antibodies or antibodies to AP (Figure 3). In silver-stained gels, only proteins with the expected sizes of $\alpha8^t$, $\beta1^t$, and β1-AP were visible, suggesting that the purification schemes yielded pure $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP heterodimers (Figure 3, lanes 1−4). Antigen blots with anti-α8, anti-β1, or anti-AP confirmed that the highly purified proteins did indeed correspond to $\alpha8^t$, $\beta1^t$, and β1-AP (Figure 3, lanes 6−13). Antigen blots revealed that the apparent molecular weight of the $\alpha8^t$ subunit was shifted from 152 to 137 kDa by addition of a reducing agent (Figure 3, lanes 6−9), strongly suggesting that the secreted α8 subunit had been cleaved into two disulfide linked polypeptides, similar to the normal α8 subunit.

Comparisons of antigen blots using conditioned media, eluates of Ni-affinity chromatography, or highly purified $\alpha8^t\beta1$-AP revealed that the COS-7 cell-conditioned medium contained 1.0−1.5 μg/mL of $\alpha8^t$. However, only 30−50% of the $\alpha8^t$ was purified as secreted $\alpha8^t\beta1$-AP heterodimer

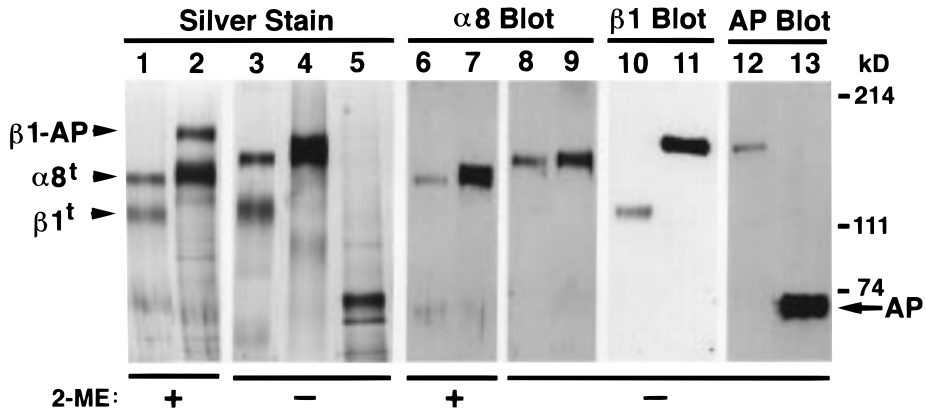

FIGURE 3: Purification of soluble truncated integrin heterodimers $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP. The purified $\alpha8^t\beta1^t$ heterodimer (lanes 1, 3, 6, 8 and 10), $\alpha8^t\beta1$-AP heterodimer (lanes 2, 4, 7, 9, 11 and 12), and conditioned medium containing nonfused secreted AP (lanes 5 and 13) were separated by SDS−PAGE in 6% polyacrylamide gels in reducing (with 2-mercaptoethanol) or nonreducing (without 2-mercaptoethanol) conditions. Then gels were either silver-stained (lanes 1−5) or transferred to nitrocellulose membranes and probed with anti-GST-α8 antiserum (lanes 6−9), anti-$\beta1$ mAb 9EG7 (lanes 10 and 11), or anti-AP mAb (lanes 12 and 13). Position of native AP is indicated with an arrow. Positions of protein standards of known molecular mass are indicated in kilodaltons.

while the rest appeared to have been secreted as monomer. The monomers and heterodimers were recovered, respectively, in the flow-through and retained fractions of the YM100 membrane filtration at the last step of the purification. The final yield of heterodimers varied in each transfection, but was typically approximately 100 μg from 10 dishes (15 cm diameter). Once purified, the $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP heterodimers were stable during prolonged storage at 4 or −80 °C. The secreted $\alpha8^t$ not associated with a β subunit seems most likely to have been monomeric and not aggregated since it passed through a YM100 membrane with a 100 kDa cut off, which was used to concentrate the $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP heterodimers.

Purified $\alpha8^t\beta1$-AP Binds to FN and VN in Solid Phase Binding Assays. To determine whether purified $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP interact specifically with ECM ligands, we performed solid phase binding assays with highly purified heterodimers on purified ECM substrates. The binding properties of the purified heterodimers were directly compared to the ligand specificity of cell surface expressed $\alpha8\beta1$ heterodimers as assessed using cell adhesion assays. For this purpose, adhesion assays were carried out in parallel with K562 cells and KA8 cells. K562 cells express the integrin $\alpha5\beta1$; KA8 is a derivative of K562 cells, transfected with the chicken α8 subunit, that expresses both the $\alpha5\beta1$ and $\alpha8\beta1$ heterodimers. In addition, both KA8 and K562 cells have been shown to express low levels of αv-containing integrins (e.g., ref *5*).

In solid phase binding assays, both purified $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP bound to FN, FN120, and VN, but not to FN40 and several other ECM molecules, including laminin-1, collagen I, collagen IV, recombinant entactin, and thrombospondin. Figure 4A shows the data obtained using $\alpha8^t\beta1$-AP in the presence of Mn^{2+}; similar data were obtained using $\alpha8^t\beta1^t$ (not shown). Increasing of coating concentrations of substrate proteins resulted in similar data (not shown). Binding was observed in the presence but not absence of Mn^{2+} (not shown). Binding was inhibited by an RGDS-containing peptide and by a function-blocking mAb specific for the $\beta1$ subunit, indicating that binding was mediated by the ligand-recognition domain of the integrin heterodimer (Figure 4B). A virtually identical binding specificity was observed for the integrin $\alpha8\beta1$ expressed on the cell surface of KA8 cells (Figure 4C). As K562 cells express $\alpha5\beta1$ as the major integrin and very low amount of $\alpha v\beta5$ or other αv-integrins (*5*), the adhesion to FN and FN120 has been saturated in this assay and there is no detectable difference between KA8 and K562 cell adhesion to FN and FN120. These cells have been shown previously to bind to full-length FN and FN120 via the integrins $\alpha5\beta1$ and $\alpha8\beta1$ in an RGDS-peptide inhibitable manner (*5*). Some K562 cells adhere to VN in our assay conditions, reflecting the presence of low levels of $\alpha v\beta5$ and known VN receptor on these cells (*5*). KA8 cells which express also $\alpha8\beta1$ exhibit enhanced attachment to VN, consistent with our evidence that purified $\alpha8^t\beta1$-AP binds VN. KA8 cell adhesion to VN is mediated by $\alpha8\beta1$ plus αv-containing integrins and is also sensitive to RGDS peptide competition.[2] We observed no significant $\alpha8\beta1$-mediated adhesion of KA8 cells to any of the substrates to which the purified $\alpha8^t\beta1$-AP chimera did not bind and vice versa.

To summarize, we conclude that the truncated $\alpha8^t\beta1^t$ and $\alpha8^t\beta1$-AP heterodimers show similar ligand-binding properties to those of normal $\alpha8\beta1$ expressed on the surface of K562 cells. This shows that neither subunit truncation nor fusion of AP to the C-terminus of the $\beta1$ subunit perturbs ligand−receptor interactions. Direct comparison revealed that the detection sensitivity and reproducibility of the detection assays were much higher with $\alpha8^t\beta1$-AP than with $\alpha8^t\beta1^t$ (not shown), presumably because $\alpha8^t\beta1^t$ had to be chemically modified by biotinylation prior to use in solid phase binding assays. This may lead to variations in the degree of biotinylation and binding activity among different preparations (see also Discussion). Consistent with previous observations with other monomeric integrin α subunits, purified $\alpha8^t$ monomer did not bind detectably to FN120 (not shown).

$\alpha8\beta1$-Mediated Cell Adhesion and Spreading on Tenascin-C: Binding to Fragments Containing the Third FN-Type III Repeat. In contrast to previous reports (*6*, *8*), using native chicken or human TN-C as a substrate, we did not observe significant $\alpha8\beta1$-dependent interactions mediated by either

[2] U. Müller, unpublished experiments.

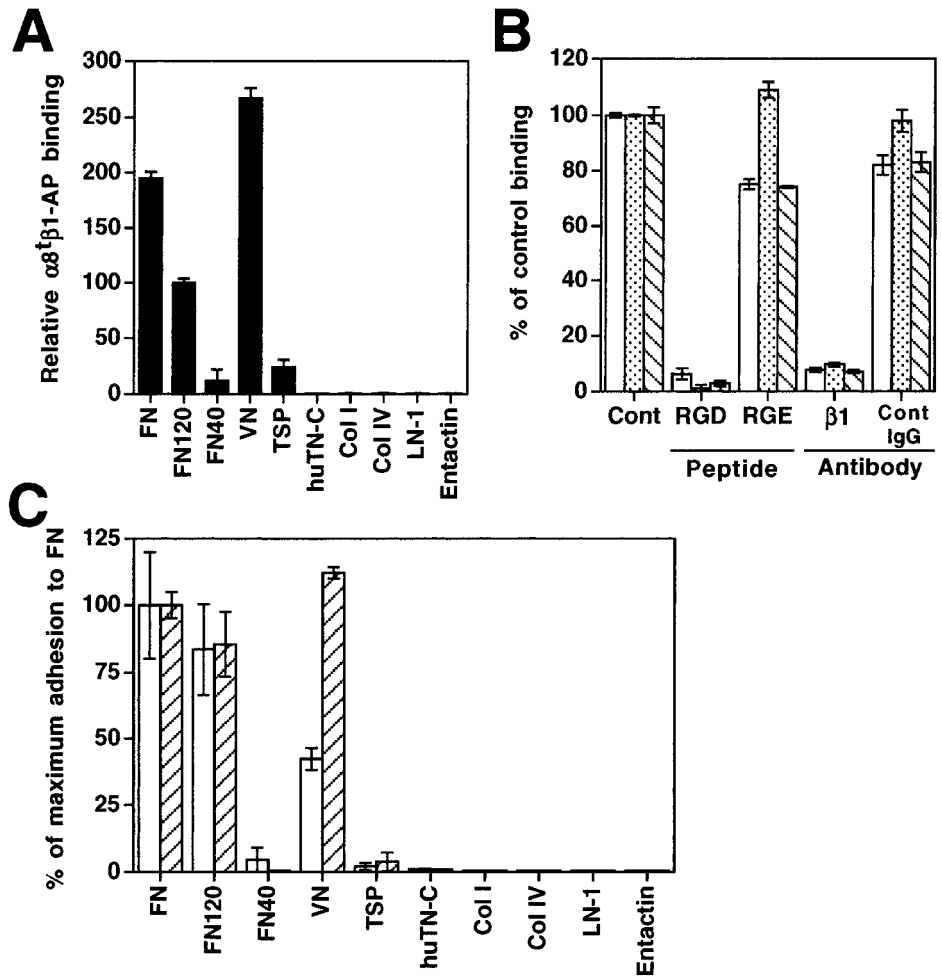

FIGURE 4: Comparisons of the binding specificities of the purified α8^{t}β1-AP chimera and cellular α8β1 to different purified ECM proteins. (A, B) Solid phase binding assays were performed with α8^{t}β1-AP in the presence of 1 mM $MnCl_2$. (A) Plates were coated with each ECM protein at 10 μg/mL. The absorbance values were normalized using the absorbance value (A405 nm, typically 0.3) for binding to substrates coated with 10 μg/mL FN120. Abbreviations: TSP, thrombospondin-1; hu TN-C, human tenascin-C; Col I, collagen type I; Col IV, collagen type IV; LN-1, laminin-1. (B) Effects of peptides and an anti-β1 mAb on α8^{t}β1-AP binding. Wells coated with 10 μg/mL of FN (open bars), FN120 (dotted bars), and VN (hatched bars) were incubated with α8^{t}β1-AP with additions indicated: (Cont) no addition; (RGD) 1 μg/mL GRGDSP peptide; (RGE) 1 μg/mL GRGESP peptide; (β1) 150 μg/mL anti-integrin β1 mAb HA2/11; (Cont IgG) 150 μg/mL control IgG. (C) Cell adhesion assays were performed in the presence of 1mM $MnCl_2$, using the same substrata as in Figure 4A, with K562 cells (open bars) and KA8 cells (transfected K562 cells expressing the chicken α8, human β1 heterodimer; hatched bars). The absorbance was quantified as the percentage of maximum adhesion to FN (typical A540 nm was 1.2). Data represents mean ±SD of triplicate wells.

KA8 cells or the purified α8β1 integrin chimera (Figure 4, panels A and C). Similar results were seen using various human and chicken TN-C preparations (including the same preparation as used in ref *8*) coated either directly onto plastic or onto nitrocellulose-coated plastic. However, the same TN-C preparations actively promoted neurite outgrowth of embryonic chicken sensory neurons as described (*8* and data not shown), demonstrating that the TN-C preparations employed here were biologically active. Since interactions of α8β1 are inhibited by RGDS-containing peptides and both human and chicken TN-C contain a single RGD site within the third FN type III-like repeat, purified fragments of chicken and human TN-C were assayed for possible interactions with this integrin. These fragments are illustrated in Figure 5. Results of binding assays using the purified integrin chimera and cell adhesion assays using α8β1-expressing KA8 cells are presented in Figure 6. In these assays, α8^{t}β1-AP was shown to bind strongly to fragments containing the third FN-type III repeat of either chicken or human TN-C the TNfn3, weakly to a fragment containing the third through fifth FN-type III repeats of human TN-C the TNfn3−5, but not detectably to native human TN-C or other TN-C fragments (Figure 6A and data not shown). Increasing the concentration (up to 100 μg/mL) of α8^{t}β1-AP in the assays did not result in specific binding. The binding of α8^{t}β1-AP to chicken TNfn3, human TNfn3, and human TNfn3−5 was inhibited by an RGDS peptide and by an anti-β1 mAb (Figure 6B). Similar results were obtained in assays of KA8 cell adhesion (Figure 6, panels C and D). Results in Figure 6C show that, in the presence of Mn^{2+}, KA8 cells, but not K562 cells, adhered to chicken TNfn3 and human TNfn3 in a dose-dependent manner but adhered only weakly to human TNfn3−5. Neither cell line exhibited significant adhesion to other TN-C fragments and full-length TN-C (Figure 4C and data not shown). Neither Mg^{2+} nor Ca^{2+} promoted adhesion to TNfn3 in the absence of Mn^{2+} (not shown). Because α5β1 does not recognize TN-C, and probably because very low amount of αv integrins are not sufficient to support the cell adhesion, K562 cell adhesion was not detected in this assay. Consistent with α8^{t}β1-AP-mediated interactions, KA8 cell adhesion to chicken TNfn3 or human TNfn3 was inhibited by an RGDS-

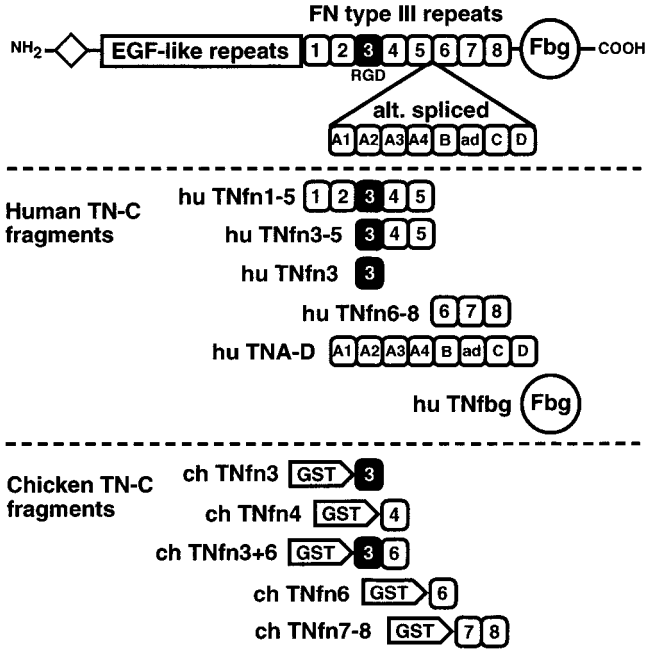

FIGURE 5: Schematic representation of TN-C and recombinant fragments used in the experiments for Figure 6. The primary structure of the human TN-C monomer is shown at the top of the figure. TN-C is a hexamer, and the monomer is composed of an N-terminal domain, an EGF-like repeat domain, a FN type III repeat domain including alternatively spliced repeats which are different among species, and a C-terminal fibrinogen-like domain (Fbg). Both human and chicken TN-C contains an RGD site within the third FN type III repeat. Chicken TN-C fragments are GST fusion proteins.

containing peptide and by a function-blocking anti-β1 mAb, but not by anti-α5 or anti-$\alpha v\beta$5 integrin mAbs (Figure 6D). In addition, KA8 cells, but not K562 cells, were observed to spread as effectively on human TNfn3 or chicken TNfn3 as on FN (Figure 7). We thus conclude that human and chicken TN-C have an RGD site in the third FN-type III repeat, which functions as a binding site for the integrin $\alpha 8\beta 1$. However, this site does not serve as an efficient binding site for this integrin in native TN-C. Instead it appears to be cryptic, only exposed in small fragments containing the third FN-type III repeat. In a search for possible additional cryptic binding sites in other ECM proteins, we examined interactions of $\alpha 8^t\beta 1$-AP and KA8 cells with recombinant entactin fragments, one of which contains an RGD site. We did not detect significant interactions with any of these fragments (not shown).

DISCUSSION

In this report, we described the efficient production and utilization of a soluble functional integrin-AP chimera for biochemical characterizations of integrin-ligand binding properties. The $\alpha 8^t\beta 1$-AP chimera was found to mimic the cellular $\alpha 8\beta 1$ in its "activation" and ligand-binding specificities. As previously reported for other integrins (*22*−*27*), the extracellular domains of $\alpha 8$ and $\beta 1$ can be secreted as a functional heterodimer. Neither subunit association nor ligand binding require the presence of the cytoplasmic or transmembrane domains. In the previous reports using stably transfected cell lines, the amounts of integrins secreted was low (*22*, *24*), and it was not practical to purify large amounts of the secreted integrins using antibody columns. Furthermore, to detect the integrins in biochemical assays, the proteins had to be further modified by either radiolabeling (*22*−*27*) or biotinylation (*21*), which can affect the ligand binding properties of the integrin (e.g., ref *21*). We have overcome these difficulties by transient expression and affinity purification utilizing a $(His)^6$-tag and Ni-affinity chromatography. With this approach, we were able to purify hundreds of micrograms of the secreted integrin; the AP-tagged integrin subunit allowed sensitive and quantitative detection of interactions with ligands in solid phase binding assays (this paper), in Far Western blotting, and in tissue staining (*9*, *44*).

We have previously shown that integrin $\alpha 8\beta 1$, when expressed on the surface of human K562 cells, can be activated to bind FN by either Mn^{2+} or an activating anti human β1 mAb (*5*). In the present paper, we have observed that this integrin, expressed on the surface of K562 cells, can also mediate binding to chicken or human TNfn3 fragments after activation by Mn^{2+} (Figure 6C). We have also found that the truncated heterodimer is initially inactive, but can be activated by Mn^{2+} to bind to FN, VN, and TNfn3 fragments. Unfortunately, we could not determine whether activating anti-β1 mAb is effective on the soluble, truncated integrin because the activating antibodies recognize only the human β1 subunit (expressed in K562 cells), but not the murine β1 subunit present in the secreted heterodimer. Nevertheless, we conclude that the binding specificity of the soluble integrin heterodimer closely reflects the binding properties of cell surface-expressed $\alpha 8\beta 1$. This is further supported by observations that ligand binding by both soluble and cell-surface-associated $\alpha 8\beta 1$ can be prevented by RGDS-containing peptides.

Although $\alpha 8\beta 1$ is not constitutively activated as a soluble heterodimer or in the cells used for these experiments, it almost certainly is activated by physiological stimuli. In previous work, the activation state of an integrin heterodimer has been shown to be determined by the cell-type in which it is expressed and by intracellular signaling pathways (e.g., refs *28*−*32*). Similar to other integrins, activation of cellular $\alpha 8\beta 1$ is likely to require the presence of the transmembrane and cytoplasmic domain of each integrin subunit.

We characterized carefully interactions of the integrin $\alpha 8\beta 1$ with human and chicken TN-C. Using both solid phase binding assays and cell adhesion assays, our data have confirmed previous experiments showing that this integrin binds to the TN-C domain TNfn3 (*6*). Bacterial expression proteins containing either human or chicken TNfn3 were effectively bound by the $\alpha 8\beta 1$ integrin. Remarkably, binding was reduced when TNfn3 was included in a larger segment. TNfn1−5 supported no detectable binding, nor did native TN-C or any other fragments containing TNfn2. Joshi et al. (*34*) noted that the RGD segment is very close to the N-terminus of TNfn3 in the three-dimensional structure and suggested that the RGD is partially buried when TNfn2 is connected to TNfn3. They found that endothelial cells adhered strongly (probably through $\alpha v\beta 3$) to TNfn3 and TNfn3−5, which have the RGD exposed at the N-terminus, but only weakly to TNfn1−3, TNfn1−5, and larger fragments containing TNfn2. Our results with $\alpha 8\beta 1$ are very similar, except that we also observed reduced binding to TNfn3−5 relative to TNfn3. The reduced binding to TNfn3−5 suggests that the $\alpha 8\beta 1$ integrin interacts with the

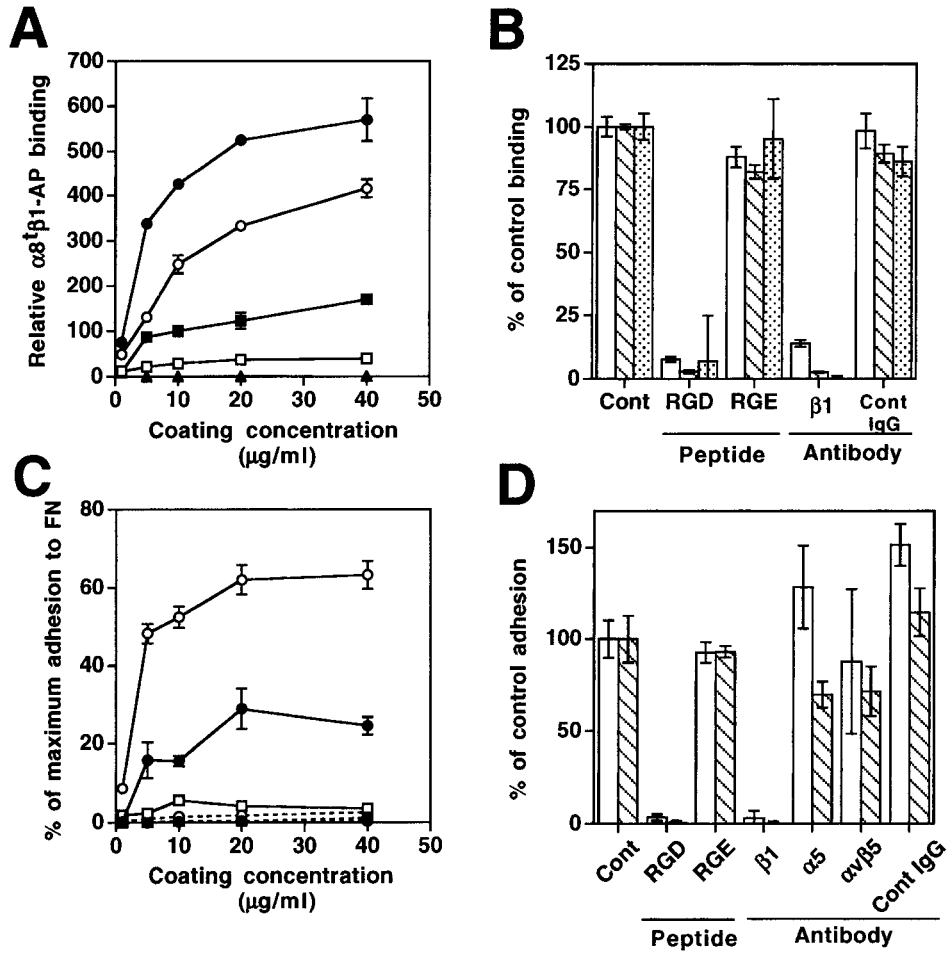

FIGURE 6: Mapping of the α8β1 binding site in TN-C: the third FN type III repeat containing RGD site is accessible only in short fragments. (A, B) Solid phase binding assays were performed with α8^{t}β1-AP in the presence of 1 mM $MnCl_2$. (A) α8^{t}β1-AP binding to TN-C fragments. Plates were coated with indicated concentrations of purified proteins: ch TNfn3 (closed circles); hu TNfn3 (open circles); FN120 (closed squares); hu TNfn3−5 (open squares); hu TN-C (closed triangles). The absorbance values were normalized as in Figure 4A. Results demonstrate that α8^{t}β1-AP binds to RGD-containing fragments (hu TNfn3, ch TNfn3, and hu TNfn3−5) but not detectably to native TN-C. (B) Effects of peptides and an anti-β1 mAb on α8^{t}β1-AP binding to TN-C fragments. Wells coated with 20 μg/mL of ch TNfn3 (open bars), hu TNfn3 (hatched bars), and hu TNfn3−5 (dotted bars) were incubated with α8^{t}β1-AP with additions indicated: (Cont) no addition; (RGD) 1 μg/mL GRGDSP peptide; (RGE) 1 μg/mL GRGESP peptide; (β1) 150 μg/mL anti-integrin β1 mAb HA2/11; (Cont IgG) 150 μg/mL control IgG. (C) Cell adhesion to TN-C fragments. Cell adhesion assays were performed with KA8 (solid lines) and K562 (dotted lines) cells in the presence of 1 mM $MnCl_2$ on plates coated with indicated concentrations of purified proteins: ch TNfn3 (closed circles); hu TNfn3 (open circles); hu TNfn3−5 (open squares). The absorbance was quantified as in Figure 4C. Note that only KA8 cells adhered to the TN-C fragments. (D) Effects of peptides and antibodies on KA8 cell adhesion to TN-C fragments. KA8 cell adhesion assays were performed on ch TNfn3 (open bars) and hu TNfn3 (hatched bars) coated at 20 μg/mL, in the presence of 1 mM $MnCl_2$ with additions indicated: (Cont) no addition; (RGD) 0.3 μg/mL GRGDSP peptide; (RGE) 0.3 μg/mL GRGESP peptide; (β1) anti-β1 mAb AIIB2; (α5) anti-α5 mAb B1E5; (αvβ5) anti-αvβ5 mAb P1F6; (Cont IgG) 100 μg/mL control IgG. Note that KA8 cell adhesion to TN-C fragments was RGDS-sensitive and was almost completely prevented by the anti-integrin β1 mAb. Data represents mean ±SD of triplicate wells.

C-terminal part of TNfn3 as well as the RGD sequence.

For integrins α8β1 and αvβ3, the binding site on TNfn3 appears to be cryptic, in that it is largely or completely masked in larger fragments and native TN-C. The cryptic-binding site might be exposed if the TN-C were proteolytically cut in TNfn2, or alternatively if the tenascin arms were mechanically bent. A sharp bend at the interface of TNfn2 and TNfn3, and possibly also at TNfn3 and TNfn4, could expose the full cell adhesion site on TNfn3. There is no evidence for proteolysis in this region of TN-C, so mechanical bending may be a more attractive hypothesis for exposing the cryptic adhesion site. This is still only a speculation, since it is not known that the site is actually exposed in tissues.

Schnapp et al. (*6*) previously reported that α8-transfected 293 cells adhered and spread on human TN-C. The absorbance value for adhesion to native TN-C was only 25% of that to chicken TNfn3, a quantitative difference that was not discussed but is suggestive of weak adhesion. Varnum-Finney et al. (*8*) reported adhesion of KA8 cells to chicken TN-C purified from sterna, using a centrifugation assay that can detect very weak, transient cell adhesion. We could not demonstrate binding of purified integrin or cell adhesion in our present assays or her centrifugation assay using two preparations of human TN-C. The adhesion reported by Varnum-Finney et al. (*8*) may have been a very weak adhesion peculiar to chicken TN-C and the centrifugation assay. One possible explanation for the discrepancies between our results and those of these other workers is that a low level of proteolysis of TN-C, either during purification, storage, or assay, can expose the binding site present in the third FN-type III repeat.

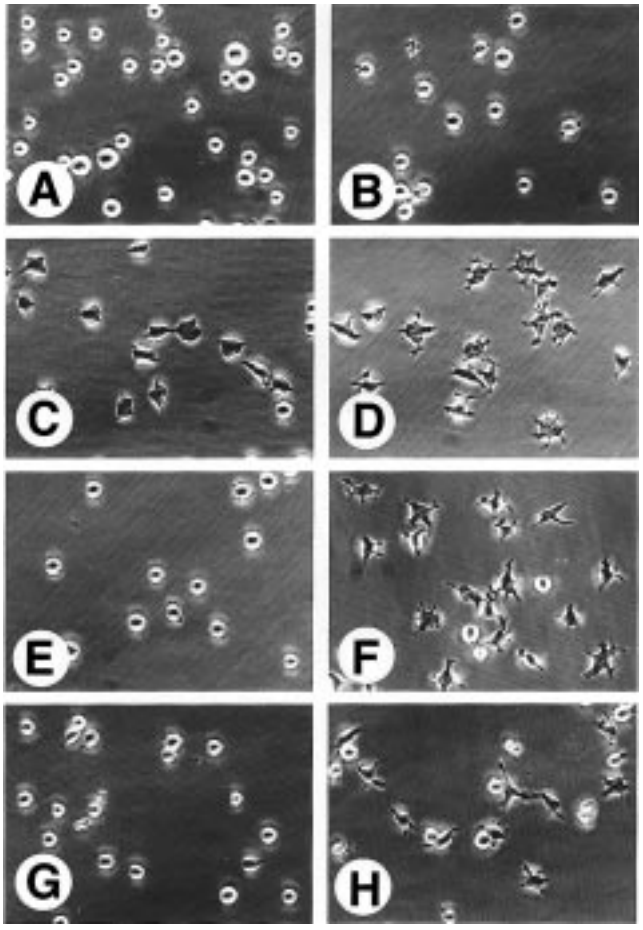

FIGURE 7: Cell spreading assay. In the presence of 1 mM $MnCl_2$, K562 cells (A, C, E, G) and KA8 cells (B, D, F, H) were plated onto different substrata: (A, B) BSA; (C, D) FN (coated at 5 μg/mL); (E, F) hu TNfn3 (5 μg/mL); (G, H) ch TNfn3 (20 μg/mL). Bar represents 100 μm.

What is the significance of the existence of a cryptic-binding site on TN-C for $\alpha 8\beta 1$? As one possibility, this site may be exposed in physiological conditions by proteolysis or interactions with other proteins. TN-C has been shown to be expressed at many sites of tissue remodeling, both during development and in response to injury (e.g., refs *35*−*38*), so fragments of TN-C that can be recognized by cells expressing $\alpha 8\beta 1$ may well be generated during tissue remodeling. Activation of cryptic-binding sites appears likely to be of general significance in understanding interactions of cells with ECM constituents. Many ECM proteins have been found to contain such cryptic-binding sites for particular integrins, including fibrinogen and laminin. Laminin-1, for example, has been shown to contain cryptic-binding sites for both $\alpha 1\beta 1$ and $\alpha v\beta 3$ which are activated by heat or proteolysis (e.g., ref *39*).

The importance of interactions of the integrin $\alpha 8\beta 1$ with FN, VN, and TN-C in vivo remains uncertain. $\alpha 8$ mutant homozygotes have severe defects in kidney formation which result in early postnatal lethality (*9*). In contrast, the VN and TN-C genes have been mutated, but animals lacking each protein develop normally (*41*, *42*). With the $\alpha 8^t\beta 1$-AP chimera, it may be possible to identify the unidentified ligand(s) crucial for kidney morphogenesis and map histochemically regions of potential $\alpha 8\beta 1$ function in vivo (*9*). There is additional evidence that both TN-C and $\alpha 8$ are expressed in smooth muscle cells and some neurons (*7*, *35*, *42*) and that TN-C expression is especially upregulated at these sites after injury (e.g., ref *43*). Since various proteases are also upregulated and ECM components and tissue morphology change rapidly and dynamically after injury, the interaction of $\alpha 8\beta 1$ with the RGD site of TN-C may modulate the tissue remodeling.

In summary, findings in this paper have characterized biochemical interactions of the integrin $\alpha 8\beta 1$ with known ligands. The integrin-AP chimeras are easy to prepare, do not require further modification, and appear to be more sensitive and specific as probes of ligand−receptor interactions than biotinylated or iodinated integrins. Integrin-AP chimeras will be generally applicable for characterizing interactions of integrins with known ligands and for identifying additional receptor−ligand interactions with important physiological or developmental roles.

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