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Methods for Identifying Novel Integrin Ligands

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Abstract

Integrins are cell adhesion receptors that have many important roles in organ development and tissue integrity, functioning to mediate interactions between cells and the ECM. The entire repertoire of integrins is vast, and the specific roles of each are determined by unique integrin–ligand interactions. These interactions allow for dynamic regulation of multiple processes. Despite intense efforts to elucidate individual integrin ligands, existing methods have been limiting. In this chapter, we describe methods developed in our laboratory to identify new integrin ligands that should be useful for characterizing novel integrin functions. These methods are applicable for studies on a variety of integrins, and may be extended to other cell surface receptors as well.

1. Introduction

Integrins comprise a family of heterodimeric cell surface receptors that play important roles in embryonic development, wound healing, hematopoiesis, hemostasis, and immunity (Geiger *et al*., 2001; Hynes, 2002). Each consists of an α-subunit and a β-subunit, and in many cases, an individual α-subunit is able to pair with more than one β-subunit. To date, more than 20 different integrin heterodimers have been identified. In addition to their promiscuous partnering, most integrins have more than one extracellular ligand. Conversely, many matrix proteins, such as laminin, collagen, fibronectin, and vitronectin, have been shown to bind more than one integrin. This vast and intricate network of receptor–ligand complexes allows integrins to participate in a variety of cellular processes, such as cellular adhesion, migration, proliferation, differentiation, and survival (Geiger *et al*., 2001; Howe *et al*., 1998).

Integrins and their ligands have the capability of mediating bidirectional signaling (Takagi *et al*., 2002). In outside-in signaling, an extracellular matrix (ECM) ligand or other ligand binds to its integrin receptor, transducing a signal to cytoskeletal and other cytoplasmic proteins to activate intracellular signaling pathways. In inside-out signaling, signals from cell surface receptors transduce intracellular signaling that changes the affinity of integrin for its extracellular ligand (Han *et al*., 2006). Many of the signaling molecules have been identified (for review see Giancotti and Tarone, 2003), although the complex signaling pathways are still being elucidated.

While ECM proteins are one class of integrin ligands, the other major category of ligands are members of the immunoglobulin superfamily, such as vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecules (ICAMs), and mucosal addressin cell adhesion molecule (MadCAM-1). In addition to these two ligand classes, E-cadherin, ADAM family members, and TGF-β have all been identified as integrin ligands. E-cadherin has been shown to be a ligand of αEβ7 in that an E-cadherin-Fc fusion protein binds to recombinant αEβ7 and

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solubilized αEβ7 from T lymphocytes (Higgins *et al*., 1998). Several members of the ADAM (a disintegrin and metalloprotease) family of proteins have been shown to bind integrins via their disintegrin domains. Integrins known to interact with ADAM disintegrin-like domains include α4β1, α4β7, α5β1, α6β1, α9β1, and αvβ3 (White, 2003). Much of these data have been elucidated through cell-binding assays. The *in vivo* significance of these interactions remains unclear, although recently, cell culture experiments have demonstrated that specific ADAM–integrin interactions can alter integrin-mediated cell migration (Huang *et al*., 2005). It has been postulated that ADAM–integrin interactions may promote the ectodomain shedding function of ADAMs, or alternatively, perturb integrin–ECM ligand interactions (White, 2003).

Several families of cell surface proteins also interact with integrins in *cis*, regulating their adhesive functions. Most notably, several tetraspanin proteins have been shown to associate with integrins and regulate their affinities (Lammerding *et al*., 2003). In addition to regulating integrin affinity for ligands, the tetraspanins function as scaffolds for recruitment of cytoplasmic signaling proteins, including PKC (Chattopadhyay *et al*., 2003). Urokinase-type plasminogen activator receptors (uPARs) have also been shown to bind multiple integrins in *cis*, including αMβ2 (Mac-1), α3β1, and α5β1. As with ADAMs proteins, uPARs have been shown to alter integrin-mediated cell migration in a nonproteolytic fashion (Kugler *et al*., 2003).

The process of identifying physiologically relevant integrin ligands has been arduous. Subsequent determination of the individual biological functions of each integrin–ligand pair has also been difficult, and in many instances cell culture models have substituted for *in vivo* models. Several factors have served as obstacles for ligand identification. First, in many conditions, integrins exist in multiple conformational states, with low-, intermediate-, and high-affinity conformations coexisting (Takagi *et al*., 2002). Structural studies have shown that an integrin existing as a bent conformer in the extracellular domain has a low affinity for ligands, an extended conformer with a closed headpiece has an intermediate affinity, and an extended conformer with an open headpiece has a high affinity for ligand. Even in the presence of Mn^{2+}, a known activator of integrins, all three conformers of soluble αvβ3 are present and exist in equilibrium. However, the presence of Mn^{2+} does shift the equilibrium to the high-affinity state so that a higher percentage of conformers bind ligand. In addition to Mn^{2+}, several other molecules and proteins, such as talin, are known to modulate the affinity state of integrins (Han *et al*., 2006; Tadokoro *et al*., 2003). Overexpression of talin in cells producing LFA-1 (αLβ2) increases the ability of LFA-1 to bind its ICAM ligand (Kim *et al*., 2003), a prime example of inside-out signaling. Talin also has been shown to increase the proportion of cells in the high-affinity state, inducing separation of the cytoplasmic α_L and α_2 domains (Kim *et al*., 2003). Another example is the uPAR-α5β1 interaction. This interaction with uPAR changes α5β1 integrin conformation and the site at which α5β1 binds fibronectin from an RGD site to a non-RGD site (Wei *et al*., 2005). Thus, integrin affinity and specificity for its ligand are modulated by multiple factors.

Since an integrin's conformation may equilibrate from high affinity to low affinity during purification or other procedures, it is possible that a bound ligand will be released. If only a fraction of ligand remains bound to its integrin, this will hinder *in vivo* ligand identification. Furthermore, even in the high-affinity state, many ligand-binding assays may not be sensitive enough to detect bound ligand. For example, the known dissociation constant of αLβ2 for ICAM is 133 n*M* for the high-affinity state and 10,000-fold lower for the low-affinity state (Labadia *et al*., 1998; Shimaoka *et al*., 2001).

Given these limitations, most ECM integrin ligands have been identified using *in vitro* assays for *presumed* ligands rather than taking an unbiased approach. For example, integrin ligands have been identified using cell attachment assays in which cells expressing a particular integrin

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are tested for adherence to ECM-coated wells at increasing concentrations of purified ECM components (Tomaselli *et al*., 1987). Specificity in this assay is often determined by blocking cell attachment with an integrin-blocking antibody or RGD peptide, or by using cells that either lack or contain a mutated integrin. Second, in cell-spreading assays, cells expressing an integrin heterodimer are assessed for their ability to extend processes on various ECM ligands (Denda *et al*., 1998b). Third, gel filtration has been used to identify fibronectin, fibrinogen, and other proteins as ligands. For example, purified integrin and fibronectin are incubated together and then shown to co-elute from a gel filtration column (Horwitz *et al*., 1986). Fourth, affinity purification over columns coupled with either ligand or integrin has been successful in providing evidence for direct interactions between several integrins and specific ligands. For example, this approach has been used to demonstrate direct binding of collagen to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Pfaff *et al*., 1994) and of fibronectin to $\alpha 5\beta 1$ (Cheng and Kramer, 1989). Fifth, surface plasmon resonance—in which purified soluble, truncated integrin is injected into flow cells that contain a particular purified ECM protein—has been used to characterize several previously identified integrin–ligand pairs (Pfaff *et al*., 1994). Finally, in some instances, the affinity of integrin–ligand interactions is high enough to survive immunoprecipitation and washing procedures. For example, we have co-immunoprecipitated integrin $\alpha 8\beta 1$ from kidney extracts using an antibody to one of its ligands, nephronectin (Marciano, unpublished results).

All of these approaches have the limitation of testing only proposed ligands, and thus may overlook ligands that an unbiased approach would reveal. There are several instances in which identification of an integrin ligand has been delayed by the lack of an unbiased approach. For example, the identification of latent TGF-β as a ligand for integrins was very surprising as the TGF-β receptor complexes had been well characterized as ligand-activated protein kinases (Derynck and Zhang, 2003; Massague, 1987). Since latent TGF-β contains an RGD sequence and is located in the ECM, cell adhesion assays with a variety of integrins were performed. Surprisingly, the N-terminus of TGF-β (also known as the latency-associated peptide, or LAP) binds to $\alpha v\beta 6$ in the adhesion assay, a finding that was confirmed using LAP-affinity column chromatography (Munger *et al*., 1998). This interaction was shown later to be crucial in keratinocytes and airway epithelial cells for latent TGF-β activation, serving to induce matrix deposition and downregulate the inflammatory process after injury (Munger *et al*., 1999).

As with TGF-β, the presence of an RGD sequence in a particular protein has stimulated studies to investigate the possibility that it functions as an integrin ligand. However, not all integrins recognize the RGD sequence, and some integrins recognize sites consisting of amino acids from more than one protein subunit, such as sequences in the triple helical domain of collagen (Sacca and Moroder, 2002). In addition to overlooking ligands, several of the abovementioned assays may identify ligands that are not relevant at physiological concentrations of integrin and ligand.

Several unbiased approaches could be used to discover novel protein– protein interactions that have not yet been used for integrin–ligand identification. One approach is to immunoprecipitate a specific integrin, and then evaluate co-eluted ligands by SDS-PAGE and subsequent mass spectrometry of gel fragments (Coon *et al*., 2005). In this manner, an integrin–ligand interaction could be identified with only trace amounts of ligand (approximately at femtomole level). A variant of this approach, using mass spectrometry to identify ligands from gel filtration peaks, might also yield novel interactions. Another approach, peptide display by filamentous phage, has been used successfully to identify peptide ligands, such as integrin–ligand mimetics (Koivunen *et al*., 1994, 1995), but has not identified physiologically relevant ligands. However, this may not be an ideal approach since the binding site of ligands is often derived from multiple segments of the protein that may not be contiguous (Sacca and Moroder, 2002).

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We have developed a unique, unbiased approach to identify integrin ligands that has resulted in the discovery of two novel $\alpha 8\beta 1$ integrin ligands, nephronectin and osteopontin. Nephronectin was not known previously to be an ECM constituent (Brandenberger *et al*., 2001; Denda *et al*., 1998b). Because many more ECM proteins exist than have been characterized biochemically (Lander *et al*., 2001), there is a pressing need to develop methods to identify their receptors and other interactions. Using a soluble, truncated integrin heterodimer, we employed an expression cloning strategy and overlay assays to identify ligands. This approach had been used previously in tyrosine kinase receptors, namely c-kit, Mek-4, and Sek, to successfully identify ligands (Cheng and Flanagan, 1994; Flanagan and Leder, 1990), but had not been used with heterodimeric receptors such as integrins. In this approach, we engineered a soluble, truncated integrin heterodimer, consisting of the $\beta 1$-subunit extracellular domain genetically fused to placental alkaline phosphatase, and the $\alpha 8$-subunit extracellular domain genetically fused to a His6-myc tag. The His6 tag was inserted for ease of purification, and the alkaline phosphatase tag allowed us to quantify and trace the protein. When co-expressed in cultured cells, this heterodimeric probe ($\alpha 8^t\beta 1$-AP) yielded a soluble protein that retained its ability to bind known ligands, and could be used to detect and characterize novel ligands.

Once expressed, we purified $\alpha 8^t\beta 1$-AP heterodimer via its His6 tag with Ni^{++}-affinity chromatography and determined the functionality and specificity of $\alpha 8^t\beta 1$-AP using binding assays with known $\alpha 8\beta 1$ ECM ligands such as fibronectin and vitronectin. Binding was inhibited in the presence of RGD peptide and $\beta 1$ monoclonal antibody, demonstrating its specificity.

After determining that the chimeric protein could bind known ligands, we used this soluble probe in an overlay assay (Far Western) to look for novel ligands, and found that that $\alpha 8^t\beta 1$-AP bound an additional band migrating at 70 to 90 kD that did not correspond to known ligands. To elucidate its identity we screened a cDNA expression library. We selected a lambda phage expression library (UNIZAP®) from murine embryonic heart since it lacks vitronectin, another $\alpha 8\beta 1$ ligand. Two million plaques were screened with $\alpha 8^t\beta 1$-AP, identifying both fibronectin and a novel protein, nephronectin.

To confirm that nephronectin was indeed a ligand for $\alpha 8\beta 1$, we performed cell adhesion assays and overlay assays. In the overlay assays, $\alpha 8^t\beta 1$-AP bound to blots containing kidney extract immunoprecipitated with anti-nephronectin antibody. There was no $\alpha 8^t\beta 1$-AP binding to blots that had been depleted of nephronectin. Our *in vivo* data from nephronectin null mice indicates that these mice have a similar phenotype as the $\alpha 8$ null mice, further supporting the biological interaction of $\alpha 8\beta 1$ and nephronectin (Linton, J. *et al*., 2007; Muller *et al*., 1997).

The $\alpha 8^t\beta 1$-AP fusion protein also was used to localize sites of ligand binding directly in murine tissue using a procedure that has been named RAP (receptor affinity probe or receptor alkaline phosphatase) *in situ* by Cheng and Flanagan (1994). This is essentially an overlay assay on histological sections. $\alpha 8^t\beta 1$-AP binding in embryonic kidney was colocalized with nephronectin by anti-nephronectin antibody staining.

The ease with which $\alpha 8^t\beta 1$-AP was used to identify a novel ligand is due in part to its solubility, in comparison to full-length integrins, which are integral membrane proteins. Another factor may be the absence of cytoplasmic domains. Based on structural studies, it is well known that the α and β cytoplasmic tails are in close apposition in the low-affinity state, and they separate from each other in the high-affinity state (Kim *et al*., 2003). It is possible, although we have not confirmed this by experimentation, that the absence of these membrane and cytoplasmic domains affects the conformational equilibrium, favoring the high-affinity state.

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Discussed in subsequent sections are the materials and methods for $\alpha 8^t\beta 1$-AP construction, purification, cDNA expression cloning screen, and overlay assays for blots and histochemistry. We also describe methods for using intact, detergent-solubilized integrins for ligand identification and suggest possibilities for extension of these methods.

2. Production of Soluble $\alpha 8^T\beta 1$-AP

2.1. Construction of secreted integrin expression vectors

cDNA clones encoding the murine $\alpha 8$ and $\beta 1$ integrin subunits, including signal sequences, were modified by PCR reactions to produce soluble protein subunits lacking their cytoplasmic and transmembrane domains. Specific details, including primer sequences, have been published (Denda *et al*., 1998a). The truncated $\alpha 8$ subunit was fused to six histidine residues followed by a Myc epitope followed by a stop codon (VIWATPNVSHHHHHHGEQKLISEEDL-stop). This was expressed in pCR3 (Invitrogen). A truncated integrin $\beta 1$ subunit ($\beta 1^t$) was generated by introducing a stop codon after the end of the extracellular domain (amino acid sequence number 728) by PCR. An integrin $\beta 1$ extracellular domain–alkaline phosphatase (AP) chimera ($\beta 1$-AP) was generated by isolating a modified pCR3-$\beta 1^t$ in which the stop codon at the end of the extracellular domain of $\beta 1^t$ was eliminated by PCR. This was fused with a fragment containing the alkaline phosphatase sequence from Aptag-1 and was expressed in pCR3 (Flanagan and Leder, 1990). Although very successful for expressing the $\alpha 8\beta 1$ heterodimer, these truncations theoretically could prevent stable association of other α and β subunits because interactions between their transmembrane and cytoplasmic domains are lost. In such instances, fusion of the AP tag to the cytoplasmic domain of either subunit, followed by purification of the detergent-solubilized intact integrin heterodimer, may provide a useful reagent (see methods described in following sections).

2.2. 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 ($\alpha 8^t$ and $\beta 1^t$ to express $\alpha 8^t$, β^t, $\alpha 8^t$, and $\beta 1$-AP to express $\alpha 8^t\beta 1$-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 to 4 days for 1 wk. After addition of $MgCl_2$ (at final concentration of 1 m*M*), phenylmethanesulfonyl fluoride (PMSF, 1 m*M*), and sodium azide (0.02%), the conditioned medium was filtered (paper number 1, Whatman, Maidstone, UK) and concentrated 10- to 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 m*M*), imidazole (10 m*M*), and PMSF (0.5 m*M*), and was then incubated at 4° for 2 h in batch with Ni-NTA beads (Qiagen).

The beads were transferred into an empty column and washed with buffer A (20 m*M* Tris-Cl, pH 7.5, 300 m*M* NaCl, 1 m*M* $MgCl_2$, 0.02% sodium azide, 20 m*M* imidazole, 0.5 m*M* PMSF). After washing, bound proteins were eluted with elution buffer (20 m*M* Tris-Cl, pH 7.5, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 0.02% sodium azide, 100 m*M* imidazole, 0.5 m*M* PMSF). To remove imidazole, $\alpha 8^t$ 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 mo when stored at either 4° or −80°.

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3. Solid-Phase Binding Assays with Soluble Integrin Heterodimers

Ninety-six–well plates (Maxisorp, Nunc, Rochester, NY) were coated with indicated concentrations of substrate proteins in TBS (25 m*M* Tris-Cl, pH 7.5, and 100 m*M* NaCl) at 4° overnight, blocked with 1% BSA (RIA grade, Sigma, St. Louis, MO) in TBS, and washed with TBS containing 1 m*M* $MnCl_2$ (TBS-Mn). Extracellular matrix ligands, including thrombospondin, laminin, fibronectin, osteopontin, and vitronectin were coated in the presence of 1 m*M* $CaCl_2$. As negative and positive controls, wells were coated with 1% BSA or 10 μg/ml FN120, respectively. One hundred microliters of $\alpha 8^t\beta 1$-AP (5 μg/ml in TBS-Mn) were then added to each well, and then incubated for 2 h at room temperature. After washing wells five times with TBS-Mn^{2+}, 100 μl of AP substrate (12 m*M* *p*-nitrophenyl phosphate, 0.5 m*M* $MgCl_2$, and 1 *M* diethanolamine, pH 9.8) were added and incubated at room temperature for an appropriate time. Integrin binding was quantified by measuring absorbance at 405 nm. $\alpha 8^t\beta 1^t$ biotinylated with NHC-LC-biotin (Pierce, Rockford, IL) was also used in the binding assay, and binding was detected as described above (DeFreitas *et al*., 1995).

When working with scarce proteins or protein fragments, the plates were coated first with nitrocellulose and then with proteins as described (Lagenaur and Lemmon, 1987). The presence of nitrocellulose on the plate results in capture of virtually all of the protein in solution. In this case, we recommend first coating the Linbro Titertek 96-well plastic dishes with nitrocellulose (Lagenaur and Lemmon, 1987). In this procedure, 5 cm^2 of nitrocellulose type BA 85 (Schleicher and Schuell) is dissolved in 6 ml of methanol. Aliquots are spread over the surface of microwells and allowed to dry under a laminar flow hood. Test protein samples are applied at 50 μl per well at concentrations approximately 100-fold lower than the concentrations used for standard substrate preparation. Our laboratory has used this procedure to examine integrin-mediated interactions of neurons with tenascin and purified fragments of tenascin (Varnum-Finney *et al*., 1995).

4. Histochemistry with Soluble Integrin Heterodimers

C57Bl/6 mouse embryos were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 7 μm, stained with antibodies, and counterstained as described (Jones *et al*., 1994). Staining with $\alpha 8^t\beta 1$-AP was performed as described (Cheng and Flanagan, 1994) with some modifications. Sections were (1) blocked with 1% BSA, 25 m*M* Tris-Cl, pH 7.5, and 100 m*M* NaCl; (2) incubated for 4 to 12 h at room temperature with 7 μg/ml $\alpha 8^t\beta 1$-AP in 20 m*M* Tris-Cl, pH 7.5, 50 m*M* NaCl, 1 m*M* $MnCl_2$, 0.05% BSA, and 0.02% sodium azide; (3) washed with the same buffer; fixed with 60% acetone, 3% formaldehyde, and 20 m*M* HEPES, pH 7.0; washed with 20 m*M* HEPES, pH 7.0, and 150 m*M* NaCl; (4) heated at 65° for 1 h in this buffer; (5) rinsed in AP buffer (100 m*M* Tris-Cl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$); and (6) incubated at room temperature with AP substrate solution (0.33 mg/ml nitroblue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indoylphosphate in AP buffer). Sections were counterstained with methyl green (Zymed, South San Francisco, CA) and mounted with GVA mount (Zymed).

5. Far Western Blotting Using Integrin Heterodimers

Protein extracts were obtained by homogenizing mouse embryos and kidneys in ice-cold extraction buffer (50 m*M* Tris-Cl, pH 7.5, 50 m*M* octylglucoside, 20 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* sodium vanadate, 1 m*M* sodium molybdate, 1 m*M* PMSF, 10 mg/ml leupeptin, 3 mg/ml pepstatin A) followed by centrifugation to remove debris. Protein concentrations were determined by Coomassie assays (Pierce). Far Western blotting was performed as described (Hildebrand *et al*., 1995) with some modifications. Proteins were separated by electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and renatured. Membranes were blocked at 4° with 10% BSA, 20 m*M* HEPES, pH 7.5, 75 m*M* KCl, 0.1

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m*M* EDTA, 2.5 m*M* $MgCl_2$, and 0.05% NP40 for 1 h, and then 1% BSA, 25 m*M* Tris-Cl, pH 7.5, 50 m*M* NaCl, and 0.05% NP-40 for 1 h. Membranes were (1) washed in 0.1% BSA, 25 m*M* Tris-Cl, pH 7.5, 50 m*M* NaCl, 1 m*M* $MnCl_2$, and 0.05% NP-40; (2) incubated with 0.3 mg/ml $\alpha 8^t\beta 1$-AP in the same solution for 2 h at room temperature, and washed with this solution; (3) rinsed in AP buffer containing 0.5 m*M* $MnCl_2$; and (4) incubated with AP-substrate solution containing 0.5 m*M* $MnCl_2$.

In our experience of analyzing serum samples, the presence of fibronectin could obscure the presence of other proteins of similar molecular weight. When this was a problem, extracts were depleted of fibronectin by passing them three times through an anti-fibronectin antibody column. Alternatively, when we wished to assay interactions with a known protein, the extracts were immunoprecipitated with antiserum specific for this protein and protein A beads before Far Western analysis to detect binding to this protein in the absence of fibronectin (Denda *et al*., 1998a).

6. Ligand Detection Using Intact Integrin Receptors

As described in the introduction, we used a lambda UNIZAP embryonic heart library. The lambda phage contains inserts whose expression is under the control of an isopropyl-β-D-thiogalactoyranoside (IPTG)–inducible promoter, and IPTG was used to screen for expressed proteins in infected bacteria. Numerous (2×10^6) plaques were screened with the $\alpha 8^t\beta 1$-AP.

Although the methods described above have worked exceedingly well in our laboratory, it is not clear that they provide methodology useful for all integrin heterodimers. The association of the two subunits of an integrin heterodimer is promoted not only by extracellular interactions mediated by the integrin α-and β-subunit head domains, but also by interactions mediated through their transmembrane and cytoplasmic domains. For many heterodimers, deletion of these domains, as was done in construction of the $\alpha 8\beta 1$ chimeras described above, may result in subunit dissociation, making ligand detection impracticable (Hemler *et al*., 1987). Although the introduction of interaction motifs, such as leucine zippers, may, in principle, stabilize integrin heterodimers, integrin activation is mediated through changes in the relationship between the α and β subunit stalks (Takagi *et al*., 2002). In at least one instance, disulfide bonding of the integrin stalks has frozen it in its inactive form, which is clearly not appropriate for detecting functional interactions (Luo *et al*., 2004). In section 7, we describe protocols used successfully by our laboratory to characterize a novel interaction between the integrin $\alpha 3\beta 1$ and thrombospondin, which utilized an intact integrin solubilized in detergent (DeFreitas *et al*., 1995). This approach seems likely to be more generally useful for characterizing integrin receptor ligands and can be further modified using recently developed procedures for incorporating membrane proteins into nanodiscs (Civjan *et al*., 2003; Leitz *et al*., 2006). It does require identification of a reasonable source for purification of the integrin, but this can be from a tumor or other cell line modified to ensure high expression of the integrin of interest. Alternatively, the assay can be modified by fusion of AP to the cytoplasmic C-terminus of the $\beta 1$ subunit.

7. $\alpha 3\beta 1$ Immunolabeling and Purification

As a first step, we label integrins on the surface of cells using biotinylation, and purify the integrin using an anti-cytoplasmic peptide antibody coupled to Sepharose. For integrin biotinylation on the surfaces of intact cells, cells were grown to confluency in 75-cm^2 tissue culture flasks. Rugli cells were used as a source for purification of the integrin $\alpha 3\beta 1$. Biotinylation was performed by a modification of the procedure described (Miyake *et al*., 1991). Briefly, confluent 75-cm^2 tissue culture flasks of Rugli cells were washed twice with warm PBS, after which 2 ml of PBS/0.1 *M* Na-HEPES, pH 8.0, was added to each flask or well with 80 μl of 10 mg/ml sulfo-NHS-biotin (Pierce Chemical Company, Rockford, IL) in

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PBS. After rocking for 1 h at room temperature, cells were washed twice with PBS, removed from the flask or well with CMF-PBS with 10 m*M* EDTA, washed again with CMF-PBS with 10 m*M* EDTA, and washed once with PBS to remove unreacted sulfo-NHS-biotin.

To immunopurify $\alpha 3\beta 1$, we used an affinity-purified antibody to the integrin α3A cytoplasmic domain (CYEAKGQKAEMRIQPSETERLIDDY), which was coupled via the N-terminal cysteine residue to keyhole limpet hemocyanin (KLH), using the water-soluble hetero-bifunctional cross-linking reagent m-maleimidobenzoyl sulphosuccinimide ester according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Rabbit antisera to the peptide–KLH conjugates were raised in New Zealand white rabbits by a commercial vendor. The specific antibodies were affinity purified from the serum using as an affinity reagent the same cytoplasmic peptide coupled to thiopropyI-Sepharose (Pharmacia, Piscataway, NJ) via its amino-terminal cysteine residue. Antibodies were eluted with 0.1 *M* glycine-CI, pH 2.3, and dialyzed against CMF-PBS. Ten milligrams of affinity-purified, anti-α 3 cytoplasmic domain antibody was cross-linked to 3 ml of protein A-Sepharose (Pharmacia) with 20 m*M* dimethylpimelimidate (Pierce) in 0.2 *M* Na-borate, pH 9.0, as described (Harlow and Lane, 1988), and blocked with 0.2 *M* ethanolamine, pH 8.0. Biotinylated or nonbiotinylated Rugli cells from four confluent flasks were extracted in buffer B (50 m*M* N-octyl-β-D-glucopyranoside, 50 m*M* Tris-Cl, pH 7.5, 15 m*M* NaCI, 1 m*M* PMSF, CLAP) with 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, centrifuged at 10,000×*g* for 15 min, and incubated with CL-4B Sepharose (Pharmacia). The protease inhibitor cocktail, CLAP, was prepared as a 1000× stock with chymostatin, leupeptin, antipain, and pepstatin, each at 7 mg/ml in dimethylsulfoxide. The supernatant was then incubated with anti-α3-Sepharose for at least 4 h. Following six washes in buffer B, $\alpha 3\beta 1$ was eluted for 1 h with the α3A cytoplasmic domain peptide (1 mg/ml in buffer B).

While our laboratory has used this procedure only to purify the integrin $\alpha 3\beta 1$, we have prepared specific antibodies to almost all of the cytoplasmic domains of the integrins and believe this procedure is generally useful for purification of individual integrin heterodimers. Our laboratory has also solubilized these receptors through use of the detergent N-octyl-β-D-glucopyranoside. Recently, exciting procedures for incorporating membrane proteins into soluble nanoscale nanodiscs have been described that prevent aggregation and preserve function through use of a membrane scaffold protein (Chan *et al*., 2003; Leitz *et al*., 2006). This methodology seems likely to be particularly useful for examination of integrin functions.

8. Receptor-Binding Assays

For receptor-binding assays, substrate (75 μl per well) was incubated in 96-well dishes overnight at 4°, and then blocked with 1% BSA in PBS for 1 h at room temperature. Receptor purified as described above was diluted in buffer B with 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ at 1:10 to 1:50, respectively. The solutions were added to the substrata-blocked dishes and allowed to bind at room temperature for 2 h. The wells were then washed five times with buffer C (25 m*M* N-octyl-β-D-glucopyranoside, 50 m*M* Tris-CI, pH 7.5, 15 m*M* NaCl, 1 m*M* PMSF, CLAP) with 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ and incubated with streptavidin-conjugated horseradish peroxidase (Zymed) diluted in the same buffer, for 1 h at 40 °. After washing five times more with buffer C with 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, binding was assayed by developing with 3,3′,5,5′-tetramethylbenzidine reagent (Kirkegaard and Perry, Gaithersburg, MD) and stopping the reaction with an equal volume of 1 *M* H_3PO_4. Absorbance at 450 nm was read in a microtiter plate reader (Flow Laboratories). Results were zeroed to wells with no receptor added. For experiments testing various divalent cations, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$ were replaced by the divalent cation tested. For experiments with antibodies added, receptor was used at a 1:50 dilution with antibody at 150 μg/ml. The receptor and antibody were incubated at room temperature for 1 h before the binding assay.

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REFERENCES

Brandenberger R, Schmidt A, Linton J, Wang D, Backus C, Denda S, Müller U, Reichardt LF. Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin alpha8beta1 in the embryonic kidney. J. Cell Biol 2001;154:447–458. [PubMed: 11470831]

Chan CS, Weeber EJ, Kurup S, Sweatt JD, Davis RL. Integrin requirement for hippocampal synaptic plasticity and spatial memory. J. Neurosci 2003;23:7107–7116. [PubMed: 12904471]

Chattopadhyay N, Wang Z, Ashman LK, Brady-Kalnay SM, Kreidberg JA. alpha3beta1 integrin-CD151, a component of the cadherin-catenin complex, regulates PTPmu expression and cell–cell adhesion. J. Cell Biol 2003;163:1351–1362. [PubMed: 14691142]

Cheng HJ, Flanagan JG. Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell 1994;79:157–168. [PubMed: 7522971]

Cheng YF, Kramer RH. Human microvascular endothelial cells express integrin-related complexes that mediate adhesion to the extracellular matrix. J. Cell. Physiol 1989;139:275–286. [PubMed: 2469686]

Civjan NR, Civjan NR, Bayburt TH, Schuler MA, Sligar SG. Direct solubilization of heterologously expressed membrane proteins by incorporation into nanoscale lipid bilayers. Biotechniques 2003;35:556–560. 562–563. [PubMed: 14513561]

Coon JJ, Syka JEP, Shabanowitz J, Hunt DF. Protein identification using sequential ion/ion reactions and tandem mass spectrometry. Proc. Natl. Acad. Sci. USA 2005;102:9463–9468. [PubMed: 15983376]

DeFreitas MF, Yoshida CK, Frazier WA, Mendrick DL, Kypta RM, Reichardt LF. Identification of integrin alpha 3 beta 1 as a neuronal thrombospondin receptor mediating neurite outgrowth. Neuron 1995;15:333–343. [PubMed: 7544141]

Denda S, Müller U, Crossin KL, Erickson HP, Reichardt LF. Utilization of a soluble integrin-alkaline phosphatase chimera to characterize integrin alpha 8 beta 1 receptor interactions with tenascin: Murine alpha 8 beta 1 binds to the RGD site in tenascin-C fragments, but not to native tenascin-C. Biochemistry 1998a;37:5464–5474. [PubMed: 9548928]

Denda S, Reichardt LF, Müller U. Identification of osteopontin as a novel ligand for the integrin alpha8 beta1 and potential roles for this integrin-ligand interaction in kidney morphogenesis. Mol. Biol. Cell 1998b;9:1425–1435. [PubMed: 9614184]

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425:577–584. [PubMed: 14534577]

Flanagan JG, Leder P. The kit ligand: A cell surface molecule altered in steel mutant fibroblasts. Cell 1990;63:185–194. [PubMed: 1698555]

Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane crosstalk between the extracellular matrix—cytoskeleton crosstalk. Nat. Rev. Mol. Cell Biol 2001;2:793–805. [PubMed: 11715046]

Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. Annu. Rev. Cell Dev. Biol 2003;19:173–206. [PubMed: 14570568]

Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuent,e EM, Boussiotis VA, Shattil SJ, Ginsberg MH. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr. Biol 2006;16:1796–1806. [PubMed: 16979556]

Harlow, E.; Lane, D. “Antibodies: A Laboratory Manual.”. Cold Spring Harbor Press; Cold Spring Harbor, NY: 1988.

Hemler ME, Huang C, Takada Y, Schwarz L, Strominger JL, Clabby ML. Characterization of the cell surface heterodimer VLA-4 and related peptides. J. Biol. Chem 1987;262:11478–11485. [PubMed: 3497921]

Higgins JM, Mandlebrot DA, Shaw SK, Russell GJ, Murphy EA, Chen YT, Nelson WJ, Parker CM, Brenner MB. Direct and regulated interaction of integrin alphaEbeta7 with E-cadherin. J. Cell Biol 1998;140:197–210. [PubMed: 9425167]

Hildebrand JD, Schaller MD, Parsons JT. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. Mol. Biol. Cell 1995;6:637–647. [PubMed: 7579684]

Horwitz A, Duggan K, Buck C, Beckerle MC, Burridge K. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature 1986;320:531–533. [PubMed: 2938015]

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Howe A, Aplin AE, Alahari S, Juliano RL. Integrin signaling and cell growth control. Curr. Opin. Cell Biol 1998;10:220–231. [PubMed: 9561846]

Huang J, Bridges LC, White JM. Selective modulation of integrin-mediated cell migration by distinct ADAM family members. Mol. Biol. Cell 2005;16:4982–4991. [PubMed: 16079176]

Hynes RO. Integrins: Bidirectional, allosteric signaling machines. Cell 2002;110:673–687. [PubMed: 12297042]

Jones KR, Fariñas I, Backus C, Reichardt LF. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 1994;76:989–999. [PubMed: 8137432]

Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science 2003;301:1720–1725. [PubMed: 14500982]

Koivunen E, Wang B, Ruoslahti E. Isolation of a highly specific ligand for the alpha 5 beta 1 integrin from a phage display library. J. Cell Biol 1994;124:373–380. [PubMed: 7507494]

Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: Ligand specificities of the RGD-directed integrins. Bio./Technol 1995;13:265–270.

Kugler MC, Wei Y, Chapman HA. Urokinase receptor and integrin interactions. Curr. Pharm. Des 2003;9:1565–1574. [PubMed: 12871068]

Labadia ME, Jeanfavre DD, Caviness GO, Morelock MM. Molecular regulation of the interaction between leukocyte function-associated antigen-1 and soluble ICAM–1 by divalent metal cations. J. Immunol 1998;161:836–842. [PubMed: 9670961]

Lagenaur C, Lemmon V. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA 1987;84:7753–7757. [PubMed: 3478724]

Lammerding J, Kazarov AR, Huang H, Lee RT, Hemler ME. Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. Proc. Natl. Acad. Sci. USA 2003;100:7616–7621. [PubMed: 12805567]

Lander, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860–921. [PubMed: 11237011]

Leitz AJ, Bayburt TH, Barnakov AN, Springer BA, Sligar SG. Functional reconstitution of Beta2-adrenergic receptors utilizing self-assembling Nano-disc technology. Biotechniques 2006;40:601–602. 604, 606. [PubMed: 16708760]passim

Luo BH, Takagi J, Springer TA. Locking the beta3 integrin I-like domain into high and low affinity conformations with disulfides. J. Biol. Chem 2004;279:10215–10221. [PubMed: 14681220]

Massague J. Identification of receptors for type-beta transforming growth factor. Methods Enzymol 1987;146:174–195. [PubMed: 2891018]

Miyake K, Weissman IL, Greenberger JS, Kincade PW. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J. Exp. Med 1991;173:599–607. [PubMed: 1997648]

Muller U, Wang D, Denda S, Meneses JJ, Pedersen RA, Reichardt LF. Integrin alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell 1997;88:603–613. [PubMed: 9054500]

Munger JS, Harpel JG, Giancotti FG, Rifkin DB. Interactions between growth factors and integrins: Latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. Mol. Biol. Cell 1998;9:2627–2638. [PubMed: 9725916]

Munger JS, Harpel JG, Giancotti FG, Rifkin DB. The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999;96:319–328. [PubMed: 10025398]

Pfaff M, Göhring W, Brown JC, Timpl R. Binding of purified collagen receptors (alpha 1 beta 1, alpha 2 beta 1) and RGD-dependent integrins to laminins and laminin fragments. Eur. J. Biochem 1994;225:975–984. [PubMed: 7525287]

Sacca B, Moroder L. Synthesis of heterotrimeric collagen peptides containing the alpha1beta1 integrin recognition site of collagen type IV. J. Peptide Sci 2002;8:192–204. [PubMed: 12043994]

Shimaoka M, Ferzly M, Oxvig C, Takagi J, Springer TA. Reversibly locking a protein fold in an active conformation with a disulfide bond: integrin alphaL I domains with high affinity and antagonist activity *in vivo*. Proc. Natl. Acad. Sci. USA 2001;98:6009–6014. [PubMed: 11353828]

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Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, Ginsberg MH, Calderwood DA. Talin binding to integrin beta tails: A final common step in integrin activation. Science 2003;302:103–106. [PubMed: 14526080]

Takagi J, Petre BM, Walz T, Springer TA. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 2002;110:599–611. [PubMed: 12230977]

Tomaselli KJ, Damsky CH, Reichardt LF. Interactions of a neuronal cell line (PC12) with laminin, collagen IV, and fibronectin: Identification of integrin-related glycoproteins involved in attachment and process outgrowth. J. Cell Biol 1987;105:2347–2358. [PubMed: 3500175]

Varnum-Finney B, Venstrom K, Muller U, Kypta R, Backus C, Chiquet M, Reichardt LF. The integrin receptor alpha 8 beta 1 mediates interactions of embryonic chick motor and sensory neurons with tenascin-C. Neuron 1995;14:1213–1222. [PubMed: 7541634]

Wei Y, Czekay RP, Robillard L, Kugler MC, Zhang F, Kim KK, Xiong JP, Humphries MJ, Chapman HA. Regulation of alpha5beta1 integrin conformation and function by urokinase receptor binding. J. Cell Biol 2005;168:501–511. [PubMed: 15684035]

White JM. ADAMs: Modulators of cell–cell and cell–matrix interactions. Curr. Opin. Cell Biol 2003;15:598–606. [PubMed: 14519395]