Molecular Requirements for Assembly and Function of a Minimized Human Integrin αIIbβ3

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Integrin subunit compatibility within and between species plays a major role in heterodimer assembly and ligand specificity. As an example, human αIIb pairs only with human β3 and does not assemble a heterodimer with β3 from other species. We use interspecies subunit chimeras to identify molecular requirements for subunit compatibility and show that species-restricted heterodimer assembly depends on a unique hexapeptide VGDSDKN in an extended loop of the hypothetical human β3 MIDAS domain. This allows us to express αIIb(1–233) and β3(111–318) as a soluble, mini-integrin that retains RGD-dependent ligand recognition. Thus, in the case of one integrin, αIIbβ3, the molecular requirements for integrin subunit compatibility and ligand recognition are intimately related.

αβ subunit compatibility is fundamentally important to integrin biology since it determines the success of heterodimer assembly and is a predominant factor influencing receptor specificity (1, 2). In this regard, human αIIb is highly restrictive, pairing exclusively with human β3 (3). Heterodimer assembly between analogous subunits from different species is subject to the same compatibility restrictions. For example, human αIIb does not form a functional heterodimer with β3 subunits from other species, including Xenopus and avian species. While the expression of membrane-associated αIIbβ3 depends upon intracellular subunit processing or trafficking that is regulated to a certain extent by cytoplasmic sequences (4), heterodimer assembly and function are retained by soluble subunits composed entirely of extracellular domains (5, 6). Moreover, soluble heterodimers retain the same subunit compatibility restrictions characteristic of the full-sized integrin. Thus, the molecular cues for both selective heterodimer formation and function must reside within extracellular sequences peculiar to each integrin subunit.

In this study, we sought to ascertain the sequences of each subunit that are required for heterodimer assembly in the case of the well characterized integrin αIIbβ3.

EXPERIMENTAL PROCEDURES

Synthesis of Interspecies β3 Subunit Chimeras—Extracellular domains of human αIIb and β3 were expressed by co-infection of Trichoplusia ni (High Five) insect cells with recombinant baculoviruses (7–9). Soluble human αIIb (residues 1–964) was produced from a 3-kilobase cDNA fragment encoding the extracellular domains, and the signal sequence was isolated as an EcoRI fragment from a Bluescript clone (gift of Dr. David R. Phillips, COR Therapeutics, Inc., South San Francisco). RI fragment from a Bluescript clone (gift of Dr. David R. Phillips, COR Therapeutics, Inc., South San Francisco) was ligated into pVL1392 (Invitrogen), 192 nucleotide (nt) sequence of recombinant αIIb (clone IIb.5) was confirmed using Sequenase 2.0 (US Biochemical Corp.). β3 clones represent residues 1–690, with respect to the numbering of the mature human sequence. Chimera 2.10 was produced from 2.9 (9) and inserted into pVL1392. To create 2.11, 2.12, and 2.13, an EcoRI-Sau I 812-base pair fragment of 2.9 was reconstructed by splice-overlap extension polymerase chain reaction. For each construct, primers were designed so as to exchange the avian coding sequence with the analogous human sequence (sites A and B). cDNA constructs were cloned into pVL1392 or pVL1393, purity of recombinant viral clones was confirmed by polymerase chain reaction, and sequence accuracy was established using Sequenase 2.0. Individual integrin subunits and heterodimeric complexes were expressed in T. ni High Five cells (Invitrogen) grown to 1 × 10^6 cells/ml in serum-free EXCell 400 medium (JRH Biosciences, Lenexa, KS). Cells were collected in culture flasks with clone IIb.5 at a multiplicity of infection of 15 and one of the β3 chimeras (multiplicity of infection = 1.5). Culture media were harvested 4 days after infection, cellular debris was removed by centrifugation, and protease inhibitors were added (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.01 mM leupeptin, 1 mM pepstatin A, and 5 mM N-ethylmaleimide) prior to storage at 4 °C for up to 2 weeks.

Expression of Functional Integrin Heterodimers—Assembly of the human αIIb subunit into αIIbβ3 heterodimers requires that specific human sequences be represented within the β3 subunit. The αIIb subunit is secreted at concentrations ranging from 0.05 to 0.2 μg/ml, and each of the β3 subunits is secreted in (several)-fold excess of αIIb, i.e. at concentrations ranging from 0.5 to 1.0 μg/ml. When co-infection results in secretion of heterodimers, the concentration of these typically ranges from 0.1 to 0.2 μg/ml.

Aliquots of media from cells infected with recombinant viruses encoding integrin proteins were subjected to native acrylamide gel electrophoresis in the absence of SDS to separate αIIbβ3 heterodimers from other proteins. Protein samples were added in 50 mM Tris-HCl, 15% glycerol (v/v), 0.01% bromphenol blue, 2 μM CaCl2, pH 6.8, to polyacrylamide slab gels (5% resolving; 3% stacking) cast without SDS. Cast gels and electrophoresis chambers were chilled to 4 °C for at least 4 h prior to use. The running buffer (20 mM Tris-HCl, 192 mM glycine, 2 mM CaCl2, pH 8.3, at room temperature) was cooled to 4 °C. Protein samples were loaded (20 μl per lane), and electrophoresis was conducted for 3–5 h at 10 mA/3 mm of gel, maintaining the gel chamber and buffer at 4 °C. Resolved proteins were transferred to polyvinylidene filters (PVDF) by electrophoresis in fresh buffer at 4 °C, maintaining a constant voltage (30 V) for 18 h. Unreacted PVDF sites were blocked by incubating the membrane in 10% nonfat milk for 30 min at ambient temperature with gentle agitation. The membrane was then incubated

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Printed in U.S.A.

Vol. 271, No. 48, Issue of November 29, pp. 30544–30547, 1996

THE JOURNAL OF BIOLOGICAL CHEMISTRY

DOI: 10.1074/jbc.271.48.30544

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in 10 ml of 5 µg/ml AP2 or OPG2, diluted in 50 mM Tris, 150 mM NaCl, 2 mM CaCl$_2$, 0.1% (v/v) Tween, pH 7.4 (TBST), for 2 h at 22°C. These murine monoclonal IgG antibodies, AP2 and OPG2, bind epitopes expressed only in the context of the process and serve to detect heterodimers. Moreover, OPG2 is an RGD mimetic that contains the RYD sequence in an extended loop of its antigen binding site (10, 11). Individual α$_{\text{in}}$, or β$_3$ subunits and the individual fragments derived from them, regardless of species origin, are never bound by AP2, OPG2, or the RGDW matrix. Unbound primary antibody was removed by three successive rinses with TBST, and membranes were exposed to an excess of horseradish peroxidase-conjugated donkey anti-murine IgG (1:5000 dilution; Jackson Immunologicals, Westgrove, PA). Following 1 h at 22°C, unbound secondary antibody was removed by three TBST rinses, and bound antibody was detected by the ECL method (Amerham) according to the manufacturer’s directions. No protein bands were visualized using monoclonal antibody 12F1, specific for the integrin α$_3$β$_2$ (hybridoma cell line is a generous gift from Dr. V. Woods, University of California, San Diego, La Jolla, CA), or AP1, specific for glycoprotein Ib heavy chain (7) (not shown).

Affinity Purification of Heterodimers by RGDW-Sepharose Chromatography—Culture media from cells co-infected with viruses containing human α$_{\text{in}}$, and β$_3$ subunits were incubated for 24 h at 4°C with RGDW-coupled Sepharose 4B beads (Pharmacia, Uppsala, Sweden). Buffer was then aspirated, and packed beads were thrice rinsed in 20 mM CaCl$_2$ and 1.5% (v/v) glycerol. The stacking gel (2.5% acrylamide) was cast in the presence of 2 mM CaCl$_2$ and 3% (v/v) glycerol. Acrylamide mixture was applied to a 7.5% polyacrylamide gel using a 3% stacking gel. Proteins in each fraction were separated by standard (denaturing) electrophoresis using a 6% acrylamide separating gel cast in the presence of 2 mM CaCl$_2$ and 1.5% (v/v) glycerol. The stacking gel (2.5% acrylamide) was cast in the presence of 2 mM CaCl$_2$ and 3% (v/v) glycerol. Acrylamide gels and apparatus were chilled to 4°C. Electrophoresis was conducted for 5 h at 25 mAmp per 3 mm maintaining a fixed temperature of 4°C. Separated proteins were transferred to PVDF membranes (Immobilon-P$^\text{TM}$; Bedford, MA) using constant voltage (85 V) for 1 h at 4°C.

RESULTS AND DISCUSSION

A comparison of interspecies β$_3$ chimeric subunits (Fig. 1) identifies a restricted human sequence that is required for heterodimer assembly with human α$_{\text{in}}$. In Western blots of proteins separated by native polyacrylamide gel electrophoresis (in the absence of SDS), only β$_3$ subunits containing human sequence 133–323 form heterodimers (Fig. 2). For example, the β$_3$ subunit 2.3, which contains predominantly human sequence, but avian sequence within residues 133–323, is not incorporated into a heterodimer, while the inverse construct, β$_3$ subunit 2.10, containing human sequence as residues 133–323 within an avian background, is isolated in heterodimers that are detected by either AP2 or OPG2 (Fig. 2) and bind to RGDW-Sepharose. Regardless of the extent to which the β$_3$ subunit is incorporated into heterodimers, equivalent amounts of α$_{\text{in}}$, and β$_3$ molecules are secreted, based on the binding of PMI-1, specific for α$_{\text{in}}$, 844–860 (12), and AP5 or AP6, specific for β$_3$1–6 and β$_3$214–219, respectively (7, 13) (not shown).

Thus, the human β$_3$ sequence necessary for subunit compatibility is confined to residues 133–323 within the putative β$_3$ MIDAS domain (14). A comparison of this sequence between diverse species (Fig. 3) reveals isolated single amino acid differences and, more strikingly, two relatively long stretches of nonhomologous sequence that we designate site A (residues 179–183) and site B (residues 278–280). This finding compelled us to test the relevance of each of these sequences to subunit compatibility.

β$_3$ subunit 2.11, containing human site A alone, does not associate with human α$_{\text{in}}$ (Fig. 2). On the other hand, β$_3$ subunit 2.12, containing human site B alone, forms a complex with the human α$_{\text{in}}$ that is detected by AP2 or OPG2 (Fig. 2). β$_3$ subunit 2.13, containing both human sites A and B, behaves exactly as does β$_3$ subunit 2.12. Thus, the hexapeptide sequence VGSNDH (site B of human β$_3$) is necessary for species-related association of the β$_3$ subunit with human α$_{\text{in}}$. In every instance, heterodimer assembly results in a receptor that is capable of binding specifically to the RGDW matrix. The results with the human α$_{\text{in}}$/human β$_3$ heterodimer are depicted in Fig. 4, but identical findings were made with human α$_{\text{in}}$ coexpressed with each of the chimeric β$_3$ subunits 2.10, 2.12, or 2.13.

The β$_3$ MIDAS domain is structurally analogous to integrin α subunit I domains from α$_{\text{in}}$ and d$_{\text{H}}$, which can be expressed as isolated entities (15–17), so it is not surprising that it too can be expressed as an isolated domain, as we describe below. However, our parallel objective was to identify a short segment of the α$_{\text{in}}$ subunit that is also necessary for heterodimer assem-
The human β3 subunit that sequences required for function and heterodimer assembly are intimately associated if not identical. Thus, we focused first on those regions of αIIb considered to be important to receptor function. Ligand binding specificity of β3, sequences corresponding to defined structures (α-helices or β-strands) of the integrin αIIb subunit (25) are indicated. Extended loop regions designated A and B, with high nonidentity between species, were further investigated as potential determinants of species-restricted heterodimer formation.

FIG. 4. Affinity purification of heterodimers by RGDW-Sepharose chromatography. Proteins are detected by silver staining. Lane 1 represents an aliquot of nonfractionated culture media. Lane 2 represents proteins that were not adsorbed after incubation with the RGDW-Sepharose beads (flow-through). Aliquots of each of three successive fractions are depicted in lanes 3–5, respectively. No integrin subunits were detected in control samples, including buffer alone (lane 6) or media from cells infected with nonrecombinant virus (not shown). The relative mobility of molecular mass standards are shown to the left of the gel: 200 kDa (a), 97 kDa (b), and 62 kDa (c). Protein bands corresponding to eluted integrin subunits are indicated: closed arrowhead, αIIb; open arrowhead, β3.

This strategy was successful. High Five cells co-infected with viruses encoding αIIb1–233 and β3111–318 secrete heterodimers formed by these short segments that can be isolated from culture media by RGDW-Sepharose affinity chromatography and then resolved in eluate fractions by the binding of OPG2 (Fig. 5) or AP2 (not shown). Thus, these limited subunit segments retain sufficient structural integrity to facilitate assembly of a functional receptor. This represents the strongest proof of the importance of these domains to subunit compatibility and receptor functions.

FIG. 5. Synthesis and secretion of a mini-integrin heterodimer. Media from High Five cells co-infected with recombinant viruses encoding αIIb1–233 and β3111–318 were adsorbed to RGDW-Sepharose. Eluted heterodimers were separated by native gel electrophoresis, transferred to nitrocellulose membranes, and detected with OPG2. The following samples are depicted: lane 1, nonadsorbed media; lane 2, adsorbed media; lanes 3–5, three successive buffer rinses; lanes 6–9, four consecutive elution volumes with buffer containing 3 mM RGDW; lane 10, buffer alone; lane 11, purified, native αIIbβ3; and lane 12, media from cells infected with nonrecombinant virus.
interacts specifically with this flexible peptide segment and that this association influences the overall tertiary structure of both the αIIb and the β₃ subunits, thereby permitting heterodimer assembly to occur. While it is tempting to speculate that comparable sequences regulate subunit compatibility and heterodimer assembly of all integrins, in general, we are compelled to point out that our findings have been made only with one integrin, α₁β₁₃, and may thus reflect properties unique to this integrin.

We show that molecular compatibility between otherwise complex polypeptides like αIIb or β₃ can depend upon a short sequence, such as VGDSDNH in β₃. It is not coincidental that this key sequence represents an extended loop of the β₃ MIDAS domain, thought to be involved in divalent cation coordination in a manner critical to receptor function. There must be an intimate relationship between structural cues that regulate heterodimer assembly and ligand recognition. We propose that during receptor assembly, the initial contact between key sequences of each subunit initiates conformational changes in the opposite subunit resulting in the formation of an effective ligand recognition pocket. Thus, these key sequences on each subunit initially occupy at least a portion of the recognition pocket. Our findings validate the proposal (3) that subunit interactions, influenced by cation coordination, would then be disrupted or displaced by ligand binding.

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