

Rapid report

Interaction of ACE2 and integrin β 1 in failing human heart

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Abstract

ACE2 purified from failing human heart was found to form a complex with integrin β 1 by immunoprecipitation, Western blotting, activity assay, and ESI tandem mass spectroscopy. The ACE2/integrin complex showed a K_m of 6.8 μ M and a V_{max} of 2.13 pmol/min/ μ l purified enzyme. Activity was optimal at pH 7.5 with Ang II substrate.

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The recently described homologue of angiotensin converting enzyme (ACE), ACE2 or ACEH [1,2], was shown to hydrolyze Ang I to Ang-(1–9) and Ang II to Ang-(1–7) when expressed as a recombinant protein. We previously demonstrated a significant increase in ACE2 activity in failing human heart ventricles, and found that ACE2 was a major pathway for Ang-(1–7) formation from Ang II in the intact human heart [3,4]. The ACE2 knockout mouse was shown to have impaired cardiac contractility suggesting a potential counter-regulatory role for ACE2 in the pathogenesis of heart failure [5]. In this study we purified ACE2 from failing human heart and discovered its unanticipated association with integrin β 1. The protein complex showed high catalytic activity with Ang II as substrate, but, in contrast to recombinantly expressed ACE2, did not hydrolyze Ang I.

ACE2 was purified from a solubilized failing human heart left ventricular membrane fraction with ammonium sulfate precipitation followed by a series of chromatographic steps including Q-sepharose, Phenyl-Sepharose, DEAE-sepharose, SP-Sepharose and Suprose HR. ACE2 activity was tracked as previously described [3]. The ACE2 specific inhibitor, compound 16 (C16), was a kind gift of Dr. Natalie

Dales [6]. Protein concentration was measured with the Bio-Rad DC protein assay kit. The purification strategy is summarized in Table 1. The pH effect of the purified enzyme complex was measured with a universal buffer containing 50 mM Tris–Maleic acid, 50 mM NaCl, and 1 μ M ZnCl₂ with pH range from 5.4 to 8.6. Michaelis–Menten kinetics were determined with ³H-Ang II. This study was performed under the auspices of an Institutional Review Board approved protocol.

Sequencing with tandem mass spectroscopy was performed at the University at Albany Mass Spectroscopy Facility (Rensselaer, NY). The tryptic peptides were analyzed and sequenced on an LC-ESI-MS-MS system (Micro-mass Q-Tof 2, Micromass, UK).

Immunoprecipitation was performed as follows: Protein G sepharose beads (Amersham) were washed with assay buffer (50 mM Tris–Cl pH 7, 50 mM NaCl, 1 μ M ZnSO₄). Protein G (50 μ l) was added to 400 μ l of the Q-sepharose ACE2 fraction or solubilized membrane preparations of failing human heart to “clear” the samples. Each sample was centrifuged at 12k \times g for 30 s and the supernatant transferred to a new 1.5-ml Eppendorf centrifuge tube. Integrin β 1 monoclonal antibody (P5D2, 20 μ g, 1 μ g/5 μ l, Santa Cruz Biotechnology) was added to the cleared supernatant. Incubation with gentle mixing at 4 °C was performed for 2 h. Protein G was added and the sample mixed gently for 2 h at 4 °C. The sample was centrifuged at

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Table 1
Purification of the ACE2/integrin complex from failing human heart

Step	Total protein (mg)	Activity (fmol/min)	Specific activity (fmol/min/ μ g)	Yield (%)	Fold enrichment
0.6%TX100 extraction	512	0.683	0.0958	100	1
AS Pellet	198	1.350	0.294	38.7	3.1
Q-sepharose	39.2	1.420	0.579	7.7	6
First Phenyl-sepharose	7	2.230	1.730	1.4	18
DEAE	1.84	0.923	4.40	0.36	45.8
Second Phenyl-sepharose	0.928	2.800	6.00	0.18	62.6
SP-sepharose	0.01	1.300	86.80	0.002	906
SEC	0.003	1.400	311	0.0006	3247

12,000 \times g for 30 s. The supernatant was removed and the pellet washed three times with 1-ml assay buffer to ensure that there was no residual non-precipitated material from the

supernatant. The supernatant and pellet were saved for immunoblotting and for measurement of ACE2 activity. To measure ACE2 activity in the pellet, the assay was performed as usual except that the incubation tube was vortexed every 2 min during the 20-min incubation to keep the protein G-immunocomplex in suspension.

After each purification step, an aliquot of the preparation was analyzed by 10% SDS-PAGE gel according to the method of Laemmli [7]. Blotting was with either the anti ACE2 antibody (a kind gift of Dr. Susan Acton, Cambridge, MA) or the anti-integrin β 1D antibody (a kind gift from Dr. Joseph C. Loftus, Scottsdale, AZ). Bound antibody was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, NJ). In the case of ACE2, Western blot incubations were performed with scrambled peptide, TFDDPSVELVAHSHEPNYCP, or blocking peptide, EPVPHDETYCDPASLFHVSN, to prove the specificity of the antibody (Biopeptide, San Diego, CA).

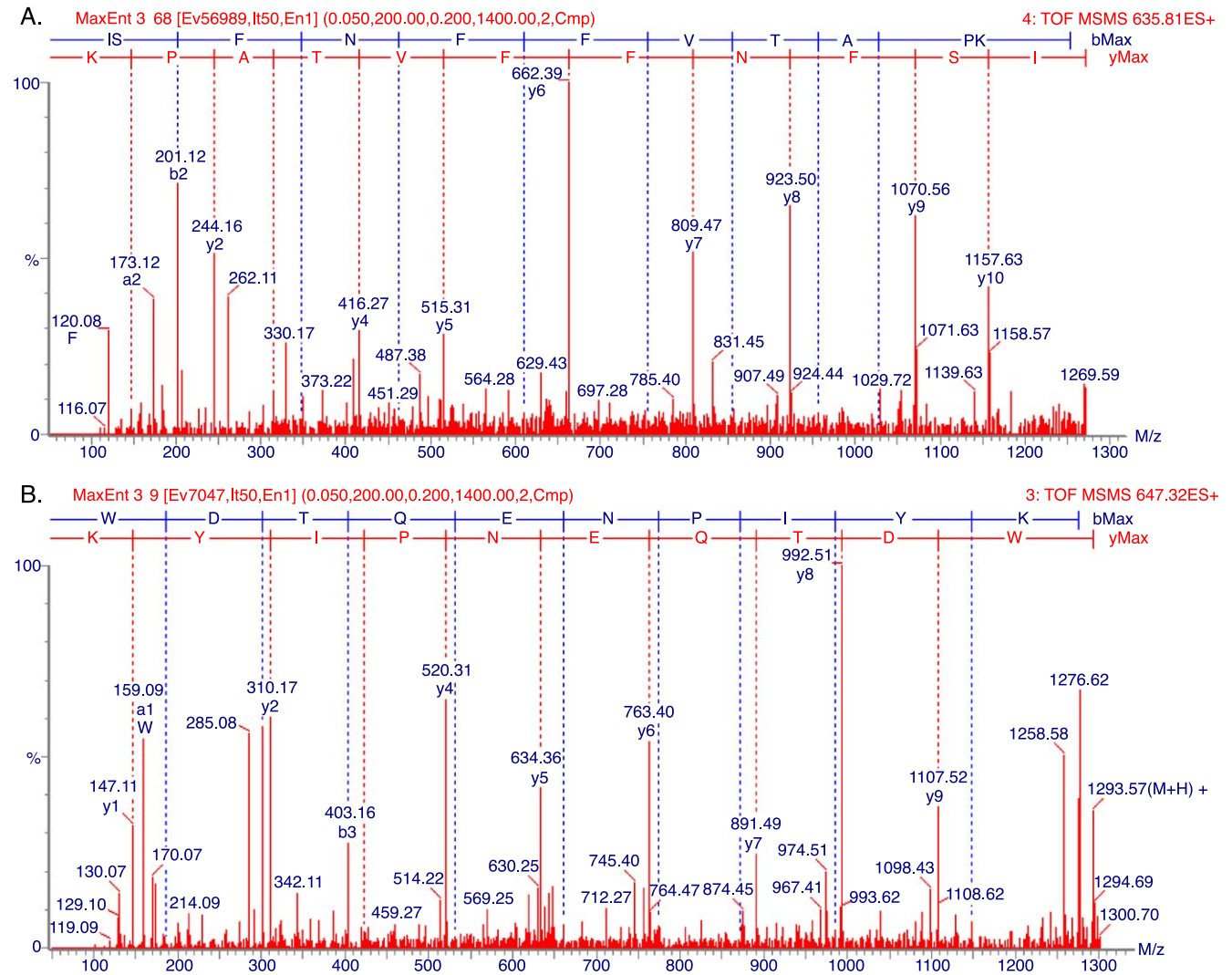


Fig. 1. Tandem mass spectrometry performed on the SEC fraction containing 3000-fold purified ACE2 complex demonstrated the presence of integrin β 1 in addition to ACE2. (A) CID fragmentation of tryptic peptide demonstrating the sequence ISNFFVTAPK from ACE 2 (AB046569). (B) CID fragmentation of tryptic peptide demonstrating the sequence WDTQENPIYK from integrin β 1 (NM_033668).

As shown in Table 1, more than 3000-fold activity enrichment was achieved for the ACE2 complex. When fractions from each step were analyzed by Coomassie blue-stained SDS-PAGE, a band of 130 kDa was the major band remaining at the end of the purification. ESI tandem mass spectroscopy confirmed the presence of ACE2 as well as integrin $\beta 1$ in this band (Fig. 1). Western blot analysis with the anti-ACE2 antibody and the anti-integrin $\beta 1D$ antibody demonstrated the co-purification of ACE2 and integrin $\beta 1D$ (Fig. 2). Immunoprecipitation experiments confirmed that ACE2 and integrin $\beta 1D$ formed a protein complex (Fig. 3). In addition, ACE2 activity could be measured in the immunoprecipitate that used an anti-integrin $\beta 1$ antibody to capture the protein complex (Fig. 4). Kinetic analysis with the purified ACE2/integrin complex demonstrated an apparent K_m of 6.12 μM for [tyrosyl-3,5- 3H] Angiotensin II and a V_{max} of 2.13 pmol/min/ μl . The enzyme activity was maximal at pH 7.5. Interestingly, when [^{125}I]-Angiotensin I and [^{125}I]-Bradykinin were used as substrates, there was no measurable peptidase activity. Instead, Ang I inhibited the enzyme with an IC_{50} of 43.6 μM . Bradykinin was a less potent inhibitor (IC_{50} = 2.64 mM).

The major findings of this study are that ACE2 purified from failing human heart interacted with integrin $\beta 1$, and hydrolyzed Ang II, but not Ang I. While there are previous reports of interactions of matrix metalloproteinases and ADAMS with integrins, this study is the first to report an interaction between ACE2 and an integrin protein [8–13].

Historically integrins were first shown to play an important role in cell–matrix adhesion, and function to transduce changes in physical strain in the extracellular matrix to intracellular signals [14]. Several integrin binding motifs have been described, the most well known being the RGD motif.

ACE2 contains two integrin binding domains: an RGD at position 204–206, and the sequence RKKKNKAR in the cytoplasmic tail at its C-terminus, which is highly homologous to the integrin binding domain of the HIV Tat protein (RKKRRQRRR) [15]. Collectrin, a truncated form of ACE2, has no intrinsic angiotensinase activity but has been found to play a role in renal organogenesis. Collectrin also contains an RKNK motif [16]. It is possible that both

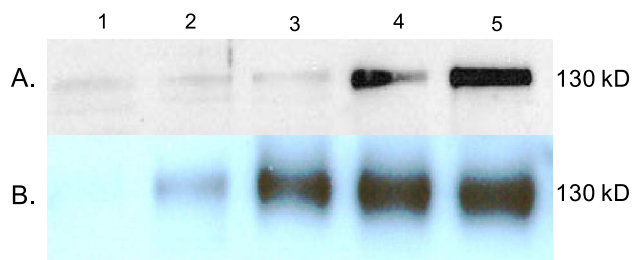


Fig. 2. Western blots of the ACE 2/integrin complex. (A) Western blot with the ACE2 antibody. (B) Western blot with the anti-integrin $\beta 1 D$ antibody. Lane 1, extraction; lane 2, Q-sepharose; lane 3, phenyl-sepharose; lane 4, DEAE-sepharose; lane 5, SP-sepharose. Each lane was loaded with 2.5 μg of protein.

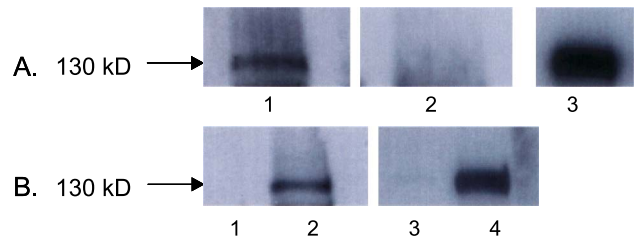


Fig. 3. Immunoprecipitation of the ACE2/integrin complex purified from human heart. The immunoprecipitation was performed with the anti-integrin $\beta 1$ antibody, P5D2. (A) Q-sepharose-purified ACE2 was immunoprecipitated with P5D2. Lane 1: Western blot with the anti-ACE2 antibody in the presence of scrambled peptide; 2: Western blot with anti-ACE2 antibody in the presence of blocking peptide showing specificity of the ACE2 antibody; 3: Western blot with the anti-integrin $\beta 1D$ specific antibody showing the presence of integrin $\beta 1D$ in the immunoprecipitate. (B) Direct immunoprecipitation of a solubilized membrane preparation from a failing human heart right ventricle. Lanes 1 and 3: supernatant after immunoprecipitation. Lanes 2 and 4: immunoprecipitate with the P5D2 antibody. Western blots were performed with either the ACE2 antibody (lanes 1 and 2) or the anti-integrin $\beta 1D$ antibody (lanes 3 and 4). Immunoprecipitation with the anti-integrin antibody pulled down both ACE2 and integrin $\beta 1D$ from a failing human heart membrane preparation.

collectrin and ACE2 have effects on integrin signaling independent of angiotensinase activity. Interactions of Ang II and integrins have been previously reported. Ang II has been shown to induce $\beta 1$ -integrin-mediated adhesion and spreading in human vascular smooth muscle cells [17]. Function blocking antibodies to $\alpha v \beta 3$ and $\alpha 5 \beta 1$ prevented Ang II-induced contraction of aortic rings [18]. However, there are no previous reports of a direct or indirect interac-

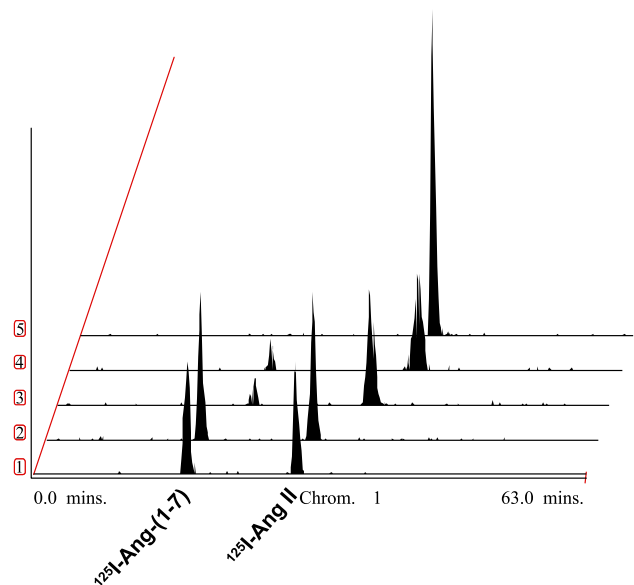


Fig. 4. HPLC chromatograms showing ACE2 activity in the anti-integrin $\beta 1$ antibody immunoprecipitate (lanes 1 and 2 are duplicate experiments). Lanes 3 and 4 show chromatograms of residual ACE2 activity in the supernatant after immunoprecipitation. Lane 5 shows that all [^{125}I]-Ang-(1-7) forming activity in the immunoprecipitate was inhibited by the ACE2 specific inhibitor C16. These data indicate that ACE2 formed a complex with integrin $\beta 1D$, and that ACE2 in this complex was catalytically active.

tion between integrins and an angiotensin metabolizing enzyme.

ACE2 was first reported to hydrolyze Ang I to Ang-(1–9). Subsequently, it was found that ACE2 hydrolyzed Ang II to Ang-(1–7) at a much higher rate than Ang I to Ang-(1–9) [19]. The enzyme kinetics we found for the ACE2/integrin complex purified from human heart were similar to those reported for the recombinantly expressed form of ACE2 when Ang II was used as substrate. However, the purified ACE2 integrin protein complex did not metabolize Ang I. It is important to keep in mind that the recombinantly expressed form of ACE2 was specifically engineered to result in a truncated form of ACE2 that did not contain the transmembrane or cytosolic domains of the protein. This fact may explain some of the differences in enzyme kinetics and substrate specificities that we observed between ACE2 purified from human heart and the recombinantly expressed ACE2. It is also possible that some of the observed differences were the result of the interaction between ACE2 and integrins. Because the $\alpha 5\beta 1A$ and $\alpha 7\beta 1D$ integrin heterodimers have differential expression in myocytes vs. non-myocytes, the relative affinity of ACE2 for each of these heterodimers may have important implications for the localization of ACE2 in cardiac cells [14].

In conclusion, this study is the first to report the novel interaction between ACE2 and an integrin protein. It is proposed that the interaction between ACE2 and integrin $\beta 1$ is a regulatory mechanism for ACE2 activity and localization.

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