Characterization of the Interaction between Heterodimeric αvβ6 Integrin and Urokinase Plasminogen Activator Receptor (uPAR) Using Functional Proteomics

Seong Beom Ahn,†,∇ Abidali Mohamedali,‡,∇ Samyuktha Anand,‡,∇ Harish R. Cheruku,† Debra Birch,‡ Gopichandran Sowmya,‡ David Cantor,† Shoba Ranganathan,‡ David W. Inglis,§ Ronald Frank,∥ Michael Agrez,⊥ Edouard C. Nice,# and Mark S. Baker*†

†Australian School of Advanced Medicine, Faculty of Human Sciences, ‡Department of Chemistry and Biomolecular Sciences, and †Department of Engineering, Faculty of Science, Macquarie University, Sydney, NSW 2109, Australia
∥Department of Chemical Biology, Helmholtz Centre for Infection Research, Inhoffen Strasse, 738124 Braunschweig, Germany
⊥Division of Surgery, John Hunter Hospital, Newcastle, NSW 2310, Australia
#Department of Biochemistry and Molecular Biology, Monash University, Melbourne, VIC 3800, Australia

ABSTRACT: Urokinase plasminogen activator receptor (uPAR) and the epithelial integrin αvβ6 are thought to individually play critical roles in cancer metastasis. These observations have been highlighted by the recent discovery (by proteomics) of an interaction between these two molecules, which are also both implicated in the epithelial−mesenchymal transition (EMT) that facilitates escape of cells from tissue barriers and is a common signature of cancer metastases. In this study, orthogonal in cellulo and in vitro functional proteomic approaches were used to better characterize the uPAR·αvβ6 interaction. Proximity ligation assays (PLA) confirmed the uPAR·αvβ6 interaction on OVCA429 (ovarian cancer line) and four different colon cancer cell lines including positive controls in cells with de novo β6 subunit expression. PLA studies were then validated using peptide arrays, which also identified potential physical sites of uPAR interaction with αvβ6, as well as verifying interactions with other known uPAR ligands (e.g., uPA, vitronectin) and individual integrin subunits (i.e., αv, β1, β3, and β6 alone). Our data suggest that interaction with uPAR requires expression of the complete αβ heterodimer (e.g., αvβ6), not individual subunits (i.e., αv, β1, β3, or β6). Finally, using in silico structural analyses in concert with these functional proteomics studies, we propose and demonstrate that the most likely unique sites of interaction between αvβ6 and uPAR are located in uPAR domains II and III.

KEYWORDS: functional proteomics, uPAR, αvβ6 integrin, proximity ligation assay, peptide array, ovarian cancer, colorectal cancer

INTRODUCTION

A hallmark of epithelial cancer metastasis is the ability of cancer cells to migrate and infiltrate distant organs. Key stages during metastasis include detachment of the tumor cell from neighboring cells and the basement membrane, intravasation of cell(s) to the blood or lymphatic system, invasion of the migrated cell into a new environment, readhesion, and finally angiogenesis.1 At the molecular level, the epithelial−mesenchymal transition (EMT) is thought to be a pivotal biological process that facilitates tissue remodeling and metastatic progression. Normal epithelial cells undergo numerous biochemical alterations during EMT, including loss of cell polarity, loss of cell−cell adhesion, suppression of E-cadherin, and an increase in cell migration and invasiveness.2 EMT is facilitated by degradation of extracellular matrix (ECM) structures, allowing cancer cells to escape and potentially colonize secondary sites in the body.2 Degradation of ECM is now thought to be one of the most complex and important mechanisms that drives EMT, but how this occurs is not yet fully understood. The matrix metalloproteinase (MMP) family and the serine protease plasminogen activation cascade are two major matrix degrading protease families implicated in epithelial cancer metastasis (e.g., breast, endometrial, hepato-
cellular, colorectal, pancreatic, gastric, renal, brain, and lung). Both the MMPs and the plasmin are found as inactive zymogens (pro-MMPs and plasminogen, respectively), which are spatially and temporally (spatiotemporally) activated in a series of steps. Inactive plasminogen can be converted to active plasmin by urokinase plasminogen activator (uPA) on its major receptor the uPA-receptor (uPAR), where it is relatively “shielded” from inhibitors when located on the cell surface. Plasmin degrades many ECM components including fibrin, fibronectin, laminin, and the protein core of proteoglycans, while also activating MMP-1, MMP-3, and MMP-9 among many proteases that consequently degrade additional ECM components. To understand the regulation and consequences of ECM degradation in the tumor microenvironment, it was essential to determine cell surface interacting proteins. Using immunoprecipitation and mass spectrometry, we recently elucidated a cell surface uPAR interactome using an ovarian cancer cell line (OVCA429) with the novel discovery of the interaction of uPAR and integrin αvβ6; subsequently shown as uPAR-αvβ6. This was further validated by Western blot analysis. Interestingly, both of these cell surface proteins have been implicated in many aspects of the biology of epithelial cancer and its progression.

From more than 8000 membrane proteins predicted from the human protein-coding genes, uPAR has been suggested to be one of a few multifunctional multi-interacting cell surface receptors that is known to be involved in, among other things, ECM degradation, growth factor activation, and downstream cellular signaling. A glycosylphosphatidylinositol (GPI) linker anchors the three domains (DI, DII, and DIII) of the mature uPAR protein to the extracellular surface of the plasma membrane. These three domains form a thick-fingered glove-like structure that provides a central pocket for the binding of the cognate ligand protease, uPA. Equally this shape reveals a large contralateral external surface potentially facilitating interactions with other proteins. While initial studies focused exclusively on regulation of plasmin activation by uPAR, 42 proteins (9 extracellular proteins and 33 lateral interacting partners) have now been proposed to interact with uPAR. This exhaustive list suggests that uPAR may have evolved multiple different ligand specificities involved in the regulation of many biologies, like proteolysis, cell migration, proliferation, cell signaling, as well as other yet to be explored cell behaviors. Indeed, in the past decade, extensive evidence has suggested that uPAR is implicated in cell adhesion, proliferation, migration, tissue remodeling, and regulation of signaling pathways (e.g., MAP kinase, Ras pathways), which are important features not only of ubiquitous developmental pathways, but more importantly for cancer metastasis. High expression of the uPAR antigen has been observed in many cancers (including breast, ovarian, colon, and lung). In colorectal cancer (CRC), a high level of uPAR has been suggested as a prognostic factor for poor survival.

Additionally, up-regulation of uPA in metastasis and its subsequent roles in the degradation of the ECM have further suggested uPAR and its interacting partners are central to processes that lead to metastasis, including EMT.

As uPAR possesses no intrinsic intracellular domain, it is commonly thought that downstream cellular signaling pathways influenced by uPAR must be mediated through lateral interactions with transmembrane proteins (e.g., integrins). Indeed, 11 integrins (out of a total of 24) have been suggested to directly interact with uPAR, and many of these studies have implicated these interactions in some role in cancer metastasis. A major function of integrins that relates them directly to cell adhesion in cancer metastasis is in cellular traction, where the β subunit embeds itself across the cell membrane and mechanically links integrins to the cytoskeleton and ECM. Integrins also regulate molecular processes related to cell morphology, proliferation, survival, migration, and invasion, mostly by engaging in crucial intracellular signaling.

This study focuses specifically on the αvβ6 integrin, a transmembrane heterodimer receptor expressed exclusively on the surface of epithelial cells. The αvβ6 integrin is involved in a bidirectional manner in the signal cascade system, sending signals from the cells to the ECM and vice versa via a series of protein binding partners, which include fibronectin, cytactin, tenasin, vitronectin (Vn), and TGFβ1. High expression of αvβ6 has been demonstrated in various cancers including CRC, liver, ovarian, gastric, thyroid, cervical squamous, and endometrial cancer, where its expression is often correlated with poor patient survival. Several studies have implicated αvβ6 in cell proliferation, migration, and invasion, with some reports suggesting the involvement of αvβ6 through activation and up-regulation of various MMP-driven proteolytic pathways. Furthermore, it has been conclusively demonstrated that αvβ6 activates nascent latent transforming growth factor, TGF-β1, which can also up-regulate MMP pathways, leading to similar outcomes.

Our central hypothesis here is that, when coexpressed, uPAR and αvβ6 function cooperatively as a single membrane proteomic machine (as uPAR-αvβ6). In this study, we confirm the originally observed uPAR-αvβ6 interaction by functional proteomics using two orthogonal techniques, proximity ligation assays (PLA) and peptide arrays. In detail, PLA is an in cellulo technique that allows direct detection of protein–protein interactions due to the close proximity of the binding partners, and the in vitro peptide array method was used to locate potential specific interacting sites in uPAR-αvβ6 using an offset 15-mer sequential array of uPAR peptides across the whole protein sequence to find binding sites using HRP-labeled αvβ6 or other ligands (i.e., uPA, Vn, and integrin subunits). Furthermore, using an in silico structural analysis tool (ICM bioinformatics software), we were able to map putative sites of uPAR and αvβ6 interaction. This study not only validates the uPAR-αvβ6 interactions observed by proteomics in CRC and ovarian cancer cells, but also opens significant new avenues for functional targeting of similar interactions that may play key roles in epithelial cancer metastasis and provide unique therapeutic options.

### MATERIALS AND METHODS

#### Antibodies and Recombinant Proteins

Monoclonal antibodies (mAb) against human uPAR (clone R4, IgG1) were purchased from DAKO (Glostrup, Denmark). The mAb against the β6 subunit of the human αvβ6 integrin (clone 6.4B4, IgG1) was obtained from Biogen Idec (Cambridge, MA). Isotype control, IgG1, was purchased from R&D Systems (Minneapolis, MN). The full length recombinant proteins that were used for the peptide array were uPA and integrin αvβ6 (R&D Systems); vitronectin (Mercck Millipore, MA); and integrin αv, β6, β1, and β3 (Abnova, Taipei City, Taiwan).

---

Journal of Proteome Research

**Article**

**Journal of Proteome Research**

**Antibodies and Recombinant Proteins**

Monoclonal antibodies (mAb) against human uPAR (clone R4, IgG1) were purchased from DAKO (Glostrup, Denmark). The mAb against the β6 subunit of the human αvβ6 integrin (clone 6.4B4, IgG1) was obtained from Biogen Idec (Cambridge, MA). Isotype control, IgG1, was purchased from R&D Systems (Minneapolis, MN). The full length recombinant proteins that were used for the peptide array were uPA and integrin αvβ6 (R&D Systems); vitronectin (Mercck Millipore, MA); and integrin αv, β6, β1, and β3 (Abnova, Taipei City, Taiwan).

---

**Antibodies and Recombinant Proteins**

Monoclonal antibodies (mAb) against human uPAR (clone R4, IgG1) were purchased from DAKO (Glostrup, Denmark). The mAb against the β6 subunit of the human αvβ6 integrin (clone 6.4B4, IgG1) was obtained from Biogen Idec (Cambridge, MA). Isotype control, IgG1, was purchased from R&D Systems (Minneapolis, MN). The full length recombinant proteins that were used for the peptide array were uPA and integrin αvβ6 (R&D Systems); vitronectin (Mercck Millipore, MA); and integrin αv, β6, β1, and β3 (Abnova, Taipei City, Taiwan).
Cell Culture

The ovarian and colon cancer cell lines expressing uPAR and varying levels of β6 used for the experiments were: ovarian, OVCA429 (uPAR+, β6+); colorectal, HT29mock (uPAR+, β6+); HT29mock (uPAR+, β6+), HT29mock (uPAR+, β6+), SW480mock (uPAR+, β6+), and SW480mock (uPAR+, β6+). The HT29mock and HT29mock cells were cultured in DMEM (Invitrogen) media supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPEs, and 6 mM l-glutamine. The HT29mock and HT29mock cells were cultured in RPMI media (Invitrogen, San Diego, CA) supplemented with 10% FBS and 2.5 μg/mL puromycin. The SW480mock cells were cultured in DMEM supplemented with 4.5 g/L glucose, 10% FBS, and 500 μg/mL Geneticin G418 (Invitrogen). The cells were seeded at 2 × 10⁵ cells/mL and were grown until ~50% confluence prior to immunofluorescence and PLA experiments. All cells were grown at 37 °C in 5% CO₂ (v/v) in biological triplicates.

Immunofluorescence (IF)

The presence and/or absence of uPAR and β6 in all five cell lines were confirmed using IF. When cell cultures reached ~50% confluence, the cells were fixed using 2% paraformaldehyde for 10 min, washed with 0.1 M glycine in PBS, and incubated with blocking solution (9% goat serum, 1% BSA in PBS) for 1 h at room temperature. The cells were then incubated with anti-uPAR R4 (5 μg/mL) and anti-αvβ6 6.4B4 (5 μg/mL) antibodies for 1 h at 37 °C followed by incubation with Alexa Fluor 488 goat Anti-Mouse IgG (H+L) (Invitrogen) as secondary antibody (4 μg/mL), for 1 h at 37 °C. Cell nuclei were counter stained with the blue fluorescent DAPI (Invitrogen) nucleic acid stain (300 nM) for 10 min and mounted on glass slides in Gelmount (ProSciTech, Australia). The cells were analyzed using a UPLSAPO 40X objective (NA. 0.95) on a fluorescence microscope (BX63, Olympus, Tokyo). All image capture was conducted using a X1010, monochromed cooled CCD camera and CELLSENS dimensions software (Olympus, Tokyo).

Proximity Ligation Assay (PLA)

The assay was performed according to manufacturer’s instructions (Olink Bioscience, Uppsala, Sweden). Briefly, the PLUS oligonucleotide probe was conjugated to anti-uPAR R4 and its isotype control (IgG1), while the MINUS oligonucleotide probe was conjugated to anti-αvβ6 6.4B4 and its corresponding isotype control (IgG1). Cells were fixed using 2% paraformaldehyde in PBS and blocked using blocking solution (9% goat serum, 1% BSA in PBS). Oligonucleotide probe conjugated antibodies were introduced to the cells and incubated for 1 h, followed by incubation with the ligation solution for 30 min, followed by amplification solution (contains Cy5 fluorophore) for 100 min. Cells were counter stained with SYBR Green1 stain and mounted. The PLUS and MINUS oligonucleotide conjugated IgG1 mAbs were used as negative controls.

PLA Imaging

The cells were imaged using an Olympus Fluoview 300 confocal laser scanning system equipped with an inverted microscope (IX70, Olympus Tokyo). A 40X UPLAN APO objective (NA 0.95) was used for analysis of all slides. SYBR Green1 stain was excited using a 488 nm argon laser and the emission signal detected using 510 and 530 nm interference filters. The Cy5 dye was excited using the 633 nm HeNe laser, and the emission signal was detected using a long pass 610 barrier filter. Three sets of images, in the X, Y, and Z dimensions (10 optical slices with a spacing of 0.5 μm), were captured for each replicate and image analysis performed on the extended XYZ images, using Duetlink Image Tool software (Olink Bioscience). The number of protein interaction signals (seen as red spots) per cell was calculated for each image. Aggregated cells were counted manually to avoid miscalculation. A student t test was performed to establish the statistical significance of uPAR-αvβ6 for each cell line.

uPAR Peptide Array

A cellulose-bound array of 108 spots of 15-mer peptides covering the complete uPAR sequence of 331 amino acids with a 3 amino acid shift was synthesized using SPOT synthesis. The uPAR peptide arrays were blocked with 5% skim milk followed by incubation with HRP conjugated recombinant proteins (HRP-RPs) for 4 h. HRP-RPs were prepared by a LighteningLink HRP conjugation kit (Innova Biosciences) as per the manufacturer’s instructions. Unbound HRP-RPs was washed off, and bound HRP-RPs was detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Images were captured using a Fujifilm CS3000 imager in chemiluminescence mode with the intensity adjusted such that the darkest spots were slightly below saturation. The images were then analyzed using MultiGuage software (Fujifilm). A quantitative intensity value for each spot was calculated using the following formula:

\[ \text{intensity} = \frac{(\text{AU} - \text{BG})}{t} \]

where “AU” is the measured intensity of each spot, “BG” is the background, and “t” is the time of exposure of the imaging. The uPAR peptide array with αvβ6 was performed in triplicate to confirm reproducibility.

Bioinformatics Analysis of uPAR Interaction

The known crystal structures (PDB ID: 3BT1) of uPAR, uPA, and Vn complex were analyzed using the ICM bioinformatics software (Internal Coordinate Mechanics). First, the uPAR regions that bound to αvβ6 on the peptide array were graphically visualized using ICM. These regions were then subjected to manual analysis to determine residues with favorable side-chain orientations. The residues with favorable side-chain orientations were then reanalyzed to determine αvβ6 residues potentially accessible to the outer surface of uPAR based on hydrophobicity.

■ RESULTS AND DISCUSSION

Previous proteomics studies using immunoprecipitation, mass spectrometry, and Western blot analysis, using the ovarian cancer cell line OVCA429, demonstrated that uPAR potentially interacts with other membrane associated proteins, including the αvβ6 integrin heterodimer. Many of the proteins identified in that study had been previously implicated in either the biology of cancer metastasis, the regulation of plasminogen activation, or as prognostic indicators of poor cancer patient survival (e.g., a-enolase, αvβ6, uPAR). Specifically, uPAR and αvβ6 have been independently implicated in both cancer biology (e.g., proliferation, TGFβ activation, cell adhesion, migration, proteolysis, and invasion) and poor epithelial cancer patient prognosis (colorectal, breast, prostate, lung, and ovarian cancer). Coexpression of uPAR and αvβ6 in the OVCA429 and other cell lines is now well established. Studies using flow cytometry have also independently confirmed the expression of...
both of these antigens on the cell surface.\textsuperscript{23,28–30} However, correlations of tumor tissue coexpression and relationships with cancer stage, differentiation status, and patient clinical outcomes (including survival) remain to be explored. The confirmation of a direct uPAR-αvβ6 interaction would suggest a novel paradigm that potentially explains how and why these membrane proteins share critical aspects of tumor biology and would assist in the development of novel therapeutics to prevent cancer metastasis.\textsuperscript{29}

\textbf{Figure 1.} A representation of the cell surface expression of uPAR and αvβ6 for five different cell lines as SW480 β6OE, SW480 mock, OVCA-429, HT-29 mock, and HT-29 β6AS each expressing varying levels of β6. The third row represents the antibody control (IgG1). Nuclei were stained with DAPI, while proteins were detected with a secondary antibody conjugated to Alexa 488.

\textbf{Figure 2.} Proximity ligation assay images of the cells shown in (A) where the red spots represent the interaction between uPAR-αvβ6. A signal for the interaction of the uPAR-αvβ6 corresponding to the level of β6 in the cell seems to be observed as compared to the IgG1 isotype control. (B) This observation was quantified by measuring the number of spots per cell. The results showed a significant decrease in interaction when the level of β6 was reduced by 35% (in HT-29 β6AS cells) (\(p < 0.05\)). Similarly, a significant increase in interactions was observed when β6 was up-regulated in SW480 β6OE cells.
The aim of the present study was to functionally validate our previous proteomic studies on IP pull downs of the specific interacting sites of uPAR-αv/β6 by using two diverse orthogonal biochemical techniques: PLA for in cellulo analysis and peptide arrays for in vitro analysis of the specific interacting sites. To validate the uPAR-αv/β6 interaction, ovarian (OVCA429) and four colon cancer cell lines were employed (HT29mock, HT29β6AS, SW480β6OE, and SW480mock). The dysregulation of uPAR and β6 in these cell lines has been previously demonstrated by various techniques not limited to but including flow cytometry, Western blot, and PET analysis.29,31–33

**Immunofluorescence and PLA Confirm the Presence of uPAR-αv/β6 Interactions**

In this study, immunofluorescence (IF) was used to demonstrate the presence of uPAR and αv/β6 on the cell surface using anti-uPAR R4 and anti-αv/β6 6.4B4 mAbs. Consistent with previous studies, these results demonstrated that uPAR was expressed on the cell surface of all cell lines, while αv/β6 was expressed on SW480β6OE, HT29mock, HT29β6AS, and OVCA429, but was not on SW480mock (Figure 1). No binding (no fluorescence) was observed with the negative isotype control IgG1 antibody (Figure 1) as control.

Proximity ligation is an emerging technology that has been used to visualize and simultaneously quantify P–P interactions occurring in situ.34 Proteins in close proximity (30–40 nm) are fluorescently detected using rolling circle amplification of ligatable DNA primers attached to secondary antibodies that bind a pair of epitope-specific monoclonal antibodies.34,35 In our study, primary antibodies were directed against uPAR and αv/β6. Expression of integrin β6 is restricted to epithelial cells, and it is only known to dimerize with the αv subunit.36 Therefore, to identify whether interaction with uPAR could be demonstrated quantitatively, we examined other cell lines in which relative expression levels of the β6 integrin were modulated. The cell lines used expressed uPAR with varying levels of integrin β6 expression. For example, cells that did not express β6 (i.e., SW480mock) were compared to those in which integrin β6 had been engineered to be overexpressed (SW480β6AS). In addition, cells that endogenously expressed β6 (HT29mock) were compared to subclones of the same cell line in which β6 expression had been deliberately and stably reduced by ~80% (i.e., HT29β6AS)29 (Figure 2).

To allow statistical analyses, the assay was performed in biological triplicate for all cell lines, and three images were acquired for each replicate. A significant number of positive spots were observed localized to the cell surface as anticipated (Figure 2). The OVCA429, SW480β6OE, and HT29mock cell lines showed strong signals for the uPAR-αv/β6 interaction, whereas the HT29β6AS cell line showed much weaker signals (p < 0.05) (Figure 2a), which is in agreement with the reduced β6 expression previously reported.29 The SW480mock cell line, where β6 is completely absent, showed no apparent uPAR-αv/β6 PLA signal (Figure 2a). An analysis of the average signal obtained per cell as compared to the corresponding isotype controls demonstrated that the signals obtained from uPAR-αv/β6 were significantly greater (p < 0.05) than the control (Figure 2b).

The results for the OVCA429 cell line were similar to those we had obtained previously. For the colon cancer cell lines, PLA data showed a significant decrease in interaction when the level of αv/β6 was reduced; concordantly, a significant increase in interaction was observed when αv/β6 was up-regulated.

In all cases, our PLA results were in good agreement with previous expression data,29 showing that quantitative uPAR-αv/β6 PLA signal could be altered simply by decreasing or increasing the expression level of β6 present on the cell surface. All isotype controls were negative. However, while collectively these data show close proximity of uPAR and β6 indicative of an interaction, the possibility that other “bridging” proteins may be involved in direct interactions with either partner in uPAR-αv/β6 could not be conclusively excluded. To eliminate this possibility, direct uPAR-αv/β6 was probed using an orthogonal technique, peptide arrays.

**Peptide Arrays Map Potential Sites of uPAR-αv/β6 Interaction**

Peptide arrays are cost-efficient, accurate, and reliable one-dimensional reconstructions that allow mapping of potential peptidyl binding sites of labeled full length interacting proteins.37 They have been widely used to analyze large arrays of synthetic peptides on cellulose membranes, facilitating the rapid screening of diverse biomolecule probes.38 SPOT synthesis24 was used in this study to generate an array composed of 108 sequential overlapping (3 residues) 15-mer peptides (along the linear uPAR expressed protein sequence) arranged successively on a cellulose membrane. This was used to map the potential binding sites of uPAR and the heterodimeric αv/β6 integrin, as well as the individual integrin subunits (αv and β6). While this method involves a reduction of the three-dimensional uPAR structure into single linear overlapping 15-mer peptides, the method has been used...
Six potential binding sites were located on the uPAR sequence from the collective peptide array data. These sites were found to be spread across all three domains of uPAR and covered almost 35% of the uPAR sequence. Interestingly, a number of the sequences found to bind to αvβ6 integrin have previously been implicated in interactions with either Vn and/or uPA (Table 1). To narrow potential docking/binding sites for integrin αvβ6, an in silico structural analysis of where these six sites were located on the uPAR crystal structure was undertaken and mapped using ICM software (Figure 5a). This was followed by a manual identification of uPAR regions with residues containing favorable side-chain orientations (Figure 5b). As observed for individual subunits αv and β6, neither β1 nor β3 (Figure 4c and d) showed any detectable binding to the uPAR peptide array.

Structural Mapping of Interacting Sites Reveals Pockets of uPAR·αvβ6 Interactions

Six potential binding sites were located on the uPAR sequence from the collective peptide array data. These sites were found to be spread across all three domains of uPAR and covered almost 35% of the uPAR sequence. Interestingly, a number of the sequences found to bind to αvβ6 integrin have previously been implicated in interactions with either Vn and/or uPA (Table 1). To narrow potential docking/binding sites for integrin αvβ6, an in silico structural analysis of where these six sites were located on the uPAR crystal structure was undertaken and mapped using ICM software (Figure 5a). This was followed by a manual identification of uPAR regions with residues containing favorable side-chain orientations and then investigated for potential residues that could be accessed on the outer surfaces of uPAR (Table 1). However, uPAR was found to bind to domain I, G22-VS1, G82-R105; domain II, L116-H150, L172-E207; and domain III, G226-N255 (Figure 4b). As observed for individual subunits αv and β6, neither β1 nor β3 (Figure 4c and d) showed any detectable binding to the uPAR peptide array.

Table 1. Potential uPAR and Integrin αvβ6 Interaction Sites

<table>
<thead>
<tr>
<th>uPAR domain</th>
<th>region identified from peptide array</th>
<th>possible surface residues identified</th>
<th>overlapping residues binding to Vn (uPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>61 ELVEKSCTHSEKTNRRTLS 78</td>
<td>E61, V63, K65, S70, E71, N74, T76, S78</td>
<td>S78 (T76)</td>
</tr>
<tr>
<td></td>
<td>82 GLKITSLTEVCGLD 96</td>
<td>I85, S87, T89, V91, L95</td>
<td>857 (T89)</td>
</tr>
<tr>
<td>II</td>
<td>121 GSDDMSCERGRHQLQCRSPE 141</td>
<td>M125, R129, R131, H132, S134, Q136, R138</td>
<td>Q136, R138</td>
</tr>
<tr>
<td></td>
<td>172LPGCPGNSGHNNDTFFH 189</td>
<td>S178, N184, D185, F187, F189</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>191 CNNTKCNNEGPILELE 207</td>
<td>N194, T195, K197, E200, P202, E207, N208</td>
<td>none</td>
</tr>
<tr>
<td>III</td>
<td>229 SEETFLIDCRGPMNQCLATGTHEPKN 255</td>
<td>S229, E230, L234, D236, D238, N242, Q243, V246, T248,</td>
<td>none</td>
</tr>
</tbody>
</table>

*Regions binding to integrin αvβ6 on the peptide array and possible surface residues were identified by manual analysis of the uPAR crystal structure. The last column lists known overlapping binding residues to Vn and uPA (in parentheses). Amino acid residue numbers correspond to full uPAR sequence from UniProt KB (ID: Q03405).*

In this study, a GUI (graphical user interface) was developed to semiquantitatively determine the binding affinity of the labeled species (e.g., HRP-labeled αvβ6) to the uPAR peptide array based on the intensity of positive spots identified (Figure 3a). Overall, our data showed that integrin αvβ6 binds to peptides emanating from all three uPAR domains (DI, DII, and DIII); in particular, positive binding of labeled peptides with the more intense spot (semiquantitatively indicated on the bar chart) indicating a stronger affinity for the heterodimer to the corresponding uPAR peptide. The same peptide array incubated with vitronectin, another known binding partner of uPAR, and its corresponding intensity plot (b) showed that the β1 (c) and β3 (d) integrins separately, neither of which again, as monomers, showed any binding to the array.

Structural Mapping of Interacting Sites Reveals Pockets of uPAR·αvβ6 Interactions

Successful identification of linear specificity involved in many P-P interactions. In this study, a GUI (graphical user interface) was developed to semiquantitatively determine the binding affinity of the labeled species (e.g., HRP-labeled αvβ6) to the uPAR peptide array based on the intensity of positive spots identified (Figure 3a). Overall, our data showed that integrin αvβ6 binds to peptides emanating from all three uPAR domains (DI, DII, and DIII); in particular, positive binding of labeled-αvβ6 was located within the following uPAR amino acid sequences: uPAR DI at E61-R75 and G82-D96, uPAR DII at G121-E141, L172-F189, and C193-E207, and uPAR DIII at S229-N255. In control experiments using identical protein concentrations, the individual integrin protein subunits αv and β6, neither β1 nor β3 (Figure 3c) did not bind to any region of the uPAR peptide array, in contrast to the αvβ6 dimer.

The peptide array was also used to identify the binding sites of other potential uPAR partners, uPAR’s cognate protease ligand uPA and the well-established binding partner Vn. The integrin subunits β1 and β3 were also examined to determine if they were able to bind as individual integrin subunits in contrast to the data observed for β6 (Figure 3C).

These data showed that uPA could bind through domain I, C16-VS1, I85-T108; domain II, S112-H150, C169-P210; and domain III, M226-Y258 and I283-V300 (Figure 4a), while Vn was found to bind to domain I, G22-VS1, G82-R105; domain II, L116-H150, L172-E207; and domain III, G226-N255 (Figure 4b). As observed for individual subunits αv and β6, neither β1 nor β3 (Figure 4c and d) showed any detectable binding to the uPAR peptide array.
Further manual analysis revealed that many of these residues were inaccessible. Only the favorable residues were then subjected to physicochemical (hydrophobicity) analysis (Figure 5). Figure 5b illustrates the hydrophobic nature of the residues identified. It was noted that most of the identified residues had hydrogen (H-) bond acceptor potential (red residues) with some residues having the potential to be H-bond donors (blue residues), while very few residues showed any potential to form H-bonds. Those with acceptor or donor H-bond potentials should prove better binding sites than those with low or no H-bond acceptor potential.

It was clear from this analysis that some residues identified in regions of uPAR domain I (E61 to R75 and G82 to D96) that had been previously suggested to be required for interaction with Vn and/or the receptor’s cognate protease ligand uPA, were buried inside the outer surfaces of uPAR. Residues Q136 and R128, and L172, P173, and H188 in uPAR domain II, which have been previously demonstrated to be required for interaction with Vn and uPA, respectively, were found to be surface accessible.

This study revealed that most of the domain II and III residues identified from the arrays could potentially be sites of αvβ6 integrin interaction. Interestingly, a previous study addressing interactions between integrin α5β1 and uPAR suggested that integrin α5β1 directly interacts with uPAR domain III across the sequence G262-Q270 and the interaction was lost when a single amino acid alanine substitution (S267A) was introduced. Our data suggest that although domains II and III maybe accessible for integrin binding, domain III appears to be a more favorable site, should other ligands be available.

While binding of uPA to its cognate receptor uPAR is a high affinity interaction ($K_d = 4 \times 10^{-10} \text{M}$), significant external regions of uPAR remain available for binding to other potential interacting partners (e.g., Vn and various integrins like α3β1, α5β2, αvβ1, α5β1, αvβ3). The uPA and Vn sites indicated from the peptide array showed ~70% overlap with binding sites already published, including data obtained from alanine scanning mutagenesis experiments. A detailed structural docking study has been performed to recapitulate and confirm these findings on the interaction of uPAR and αvβ6.

### IMPLICATIONS AND FUTURE DIRECTIONS

The most likely binding sites for αvβ6 to uPAR, based on the crystal structure of uPAR (bound to uPA and Vn) coupled with information arising from our peptide array data and a manual analysis of potential binding sites by side-chain orientation and hydrophobicity, appeared to be neighboring adjacent integrin binding sites that were previously identified. An additional advantage of the use of peptide arrays in this study over screening by site directed protein interaction libraries or molecular modeling is that not only are potential binding sites identified, but lead peptide antagonists also determined. These can subsequently be used as tools to address the specific interaction under study. Structural analysis coupled with the previous study on interaction of uPAR with α5β1 suggests that uPAR domain III may be a favorable binding site for “all” uPAR-binding integrins. Experiments using blocking peptides against the domain III region of uPAR to determine the precise binding site of uPAR and integrin αvβ6 are currently ongoing.

For cell motility, invasion, proliferation, and adhesion, it is essential for uPAR to interact with transmembrane proteins for transmission of specific signals across cell membranes to activate appropriate intracellular second messenger systems. Thus, interaction of uPAR with αvβ6 and other integrins not
only couples the proteolytic activation (by binding with uPA) with cell signaling but also localizes the proteolysis to the cell surface.\(^{7}\) Interactions between uPAR and αβ6 could potentially have profound implications on the promotion of cancer cell metastasis by activating a series of specific signaling pathways. For example, uPAR is involved in the Ras-ERK pathway, which is known to directly induce EMT in cells.\(^{7,45}\) The association of uPAR with integrins like α3β1, αvβ3, αβ6 has been studied to varying degrees. It has been shown that uPAR interaction with β1 activates both FAK and ERK/MAPK pathways,\(^ {40}\) while interaction with β3 activates the Rac pathway.\(^ {46}\) Similarly, studies have shown that disruption of a uPAR and αβ3 integrin interaction selectively inhibits Vn-induced cell migration,\(^ {9,47}\) implying that αβ6 might also modulate cell migration in some comparable manner.

High expression of αβ6 is associated with poor prognosis in many cancer types, including colon cancer.\(^ {48}\) Several studies have implicated β6 in cell proliferation, migration, and invasion,\(^ {39–51}\) although the mechanisms by which these processes occur remain unclear. Some reports have suggested involvement of αβ6 in MMP pathways as a means by which ECM degradation is facilitated.\(^ {16,52}\) For example, Fyn kinase, which associates with αβ6, recruits FAK, thereby activating the Rac/ERK/MAPK pathways, which in turn activate MMP3.\(^ {50}\) There is also evidence showing that αβ6 activates transforming growth factor TGFβ1 by a mechanism involving torsional stress (not proteolysis), which leads to up-regulation of MMP pathways.\(^ {53}\) In addition, a direct interaction between αβ6-P-ERK2 has been conclusively established\(^ {29}\) and shown to mediate MMP-9 secretion in colon cancer cells.\(^ {29}\)

It is possible that the pathways activated, seemingly independently by uPAR and αβ6, could indeed be activated collectively with proteins found in membranes forming the uPAR-αβ6 complex. Indeed, in our initial study several other proteins were identified by proteomics to be binding to uPAR.\(^ {5}\) Targeting αβ6 integrin has the additional benefit that it is exclusively expressed in epithelial restricted tumors. It is possible that by therapeutically targeting the uPAR-αβ6, the αβ6 signaling pathway can be uncoupled from the plasmin activity, potentially leading to a disruption of the pathways involved in EMT resulting in decreased metastasis.

This study provides the detailed groundwork for an analysis of the uPAR-αβ6 interaction aimed at using it as a potential novel therapeutic cancer target. Further alternative and complementary techniques could be used to elucidate P-P interactions and to identify significant pathways affected by the interaction. When combined with the approaches taken here, methods like cross-linking mass spectrometry\(^ {24}\) in conjunction with competition studies using peptide arrays and surface plasmon resonance analysis (e.g., BLAcore, Proteon) could be used to analyze the binding kinetics of potential interactors. Indeed, preliminary studies using complementary peptides to block the sites of binding followed by functional assays (migration, proliferation, etc.) on related cell lines have been shown to induce biological and morphological effects (data not shown). The consequences of ablating such interactions can be investigated in mouse models of CRC enabling an in vivo approach.

### References


### Author Information

**Corresponding Author**

Phone: +61 2 9850 8211. Fax: +61 2 9812-3600. E-mail: mark.baker@mq.edu.au.

**Author Contributions**

These authors contributed equally.

**Notes**

The authors declare no competing financial interest.

**Acknowledgments**

We thank Paul H. Weinreb and Sheila M. Violette, from Biogen Idec Inc., Cambridge Center, Cambridge, MA 02142, for kindly providing the 6.4B4 antibody against the integrin αβ6. This study was supported with research project grant funding from the NHMRC (#1010303), Cancer Council NSW (RG10-04 and RG08-16), and a Macquarie University MQSN grant and supported through the Australian School of Advanced Medicine (ASAM), Macquarie University, MQ Biofocus and Biomolecular Frontiers Research Centres. Some of the research described herein was facilitated by access to the Australian Proteome Analysis Facility (APAF) and Monash University Antibody Technology Facility (MATF), both established under the Australian Government’s National Collaborative Research Infrastructure Strategy (NCRIS).