Definition of an Unexpected Ligand Recognition Motif for αvβ6 Integrin*

(Received for publication, June 9, 1998, and in revised form, October 15, 1998)

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Integrin interactions with extracellular matrix proteins are mediated by brief oligopeptide recognition sequences, and synthetic peptides containing such sequences can inhibit integrin binding to the matrix. The RGD peptide motif is recognized by many integrins including αvβ6, a specific receptor for fibronectin thought to support epithelial cell proliferation during wound healing and carcinoma progression. We report here the discovery of an unexpected non-RGD recognition motif for integrin αvβ6. We compared the recognition profiles of recombinant αvβ6 and αvβ3 integrins by using phage display screening employing 7-mer and 12-mer peptide libraries. As predicted, phages binding strongly to αvβ3 contained ubiquitous RGD sequences. However, on αvβ6, in addition to RGD-containing phages, one-quarter of the population from the 12-mer library contained the distinctive consensus motif DLXXL. A synthetic DLXXL peptide, RTDLDSLKRTYTL, selected from the phage sequences (clone-1) was a selective inhibitor of RGD-dependent ligand binding to αvβ6 in isolated receptor assays (IC50 = 20 nM), and in cell adhesion assays (IC50 = 50 μM). DLXXL peptides were highly specific inhibitors of αvβ6-fibronectin interaction as synthetic scrambled or reversed DLXXL peptides were inactive. NH2- and COOH-terminal modifications of the flanking amino acids suggested that the preceding two and a single trailing amino acid were also involved in interaction with αvβ6. The DLXXL sequence is present in several matrix components and in the β chain of many integrins. Although there is as yet no precise biological role known for DLXXL, it is clearly a specific inhibitory sequence for integrin αvβ6 which has been unrecognized previously.

Integrins are a family of heterodimeric class I transmembrane receptors involved in numerous cell-matrix and cell-cell adhesion phenomena (1). They can be grouped roughly into three classes: the β1 series, which are ubiquitous receptors for extracellular matrix (2); the β2 series, which are activatable on leukocytes and are triggered during the inflammatory response (3); and the α series, which bind and mediate the cell response to provisional extracellular matrices found during wound repair and other pathological processes (4).

The integrins α5β1 (5), αIIbβ3 (6), α8β1 (7), αvβ1 (8), αvβ3 (9), and αvβ6 (10) all bind the Arg-Gly-Asp (RGD) peptide sequence in fibronectin, where it is presented in a constrained loop (11). Soluble RGD-containing peptides can inhibit the interaction of each of these integrins with fibronectin. However, to analyze the function of individual fibronectin receptors in a particular cellular environment it is useful to have more specific inhibitors, and a variety of inhibitory antibodies, modified peptides, and non-peptidic substances has been developed. However, as yet no inhibitor has been discovered specific for αvβ6. αvβ6 is a rare integrin, induced during repair processes in epithelia (10, 12). Its only known specificities are for fibronectin (10), where it can be the dominant receptor mediating cell adhesion (13), and for tenasin (14). αvβ6 is believed to be involved in supporting the proliferation of epithelia during repair processes (15), and it can promote the proliferation of carcinoma cells (16).

Phage display technology has proved useful for identifying novel specific peptide sequences that act as ligand mimetics (17, 18). Accordingly, both constrained cyclic and linear peptide libraries have been used to discover novel peptides that interact with integrins (19–23). But, with few exceptions, these peptides contain RGD sequences, whereas the non-RGD sequences found have only bound weakly.

Here we have used recombinant αvβ6 expressed as a transmembrane truncated soluble receptor to screen phage libraries displaying peptides of 7 or 12 amino acids. This revealed a strong and previously unpredicted recognition motif for αvβ6 integrin which we describe here. Similar motifs are displayed in several extracellular matrix molecules.

EXPERIMENTAL PROCEDURES

Ligands—The ligands fibronectin (24), vitronectin (25), and fibrinogen (26) were purified from human blood and biotinylated as described previously (27, 28).

Preparation of Integrins—Human integrins were extracted from native sources (αvβ5, αIIbβ3) or expressed as recombinant soluble receptors using the baculovirus expression system (αvβ3, αvβ6).

αIIbβ3 was prepared from outdated human platelet concentrates (6) as detailed previously (27) by affinity chromatography on GRGDS-PK-conjugated Sepharose CL-4B. The column was eluted with the GRGDSPK, the peak containing αIIbβ3 was concentrated ~5-fold, dialyzed, and stored at ~80 °C.

αvβ6 was purified from human placenta (29). Term placenta was minced in ~2 volumes of ice-cold solution A (0.05% w/v digentin, 2 mM CaCl2, 2 mM Pefabloc (Merck), pH 7.4), then washed twice in solution A by centrifugation (12,000 × gmax, 45 min, 4 °C) and resuspension. The final pellet was extracted by resuspension in ~4 volumes of ice-cold buffer B (100 mM octyl β-D-glucopyranoside, 1 mM CaCl2, 2 mM Pefabloc, in phosphate-buffered saline) and centrifuged (12,000 × gmax, 45 min, 4 °C). The supernatant was reincubated over a PIP6 antibody column (16 h, 4 °C) (13). After washing with buffer C (0.1% Nonidet P-40 in phosphate-buffered saline; ~10 bed volumes) and buffer D (0.1% Nonidet P-40, 2 mM CaCl2, 10 mM sodium acetate, pH 4.5; ~10 cv), bound material was eluted with buffer E (buffer D adjusted to pH 3.1). The
eluant was neutralized with 3 M Tris, pH 8.8, dialyzed against buffer C, and concentrated ~10× by using Amicon II (Calbiochem). The purified receptor was stored at ~80 °C.

αvβ3 was purified in a soluble transmembrane truncated form from a baculovirus expression system as detailed previously (28) with minor modifications using 14D9.F8 antibody affinity chromatography. Briefly, the extracellular domains of αv and β3 human integrin chains were cloned into the pBacPAK expression system, the resulting recombinant baculoviruses containing both chains were used to coinfect High Five insect cells, and the soluble receptor was harvested from the culture supernatant at 48–72 h of culture by passing the supernatant over 14D9.F8 antibody affinity columns, washing, and eluting at pH 3.1. Peak fractions were neutralized, concentrated, and dialyzed before shock freezing and storage at ~80 °C. The soluble human receptor (αvβ3-ΔTM) had ligand binding specificities indistinguishable from the native receptor isolated from placenta (27).

αvβ6 was purified in a soluble transmembrane truncated form (13) from a baculovirus expression system as detailed previously for αvβ3 (28) using 14D9.F8 antibody affinity chromatography (27). The β6 cDNA clone pCDNNeoβ6 was the generous gift of Dr. D. Sheppard (University of California, San Francisco). The procedure and the cloning will be detailed elsewhere.1 In brief, the transfer vector pAcreU31 (CLONTECH Laboratories, Inc.) allowed simultaneous expression of two different target cDNAs and was used to make recombinant baculoviruses by transfecting Sf9 insect cells with HindIII-linearized pAcreU31 containing truncated αv and truncated β6 cDNA, which was excised from pCDNNeoβ6 (13) using EcoRI and XbaI and cloned into the EcoRI site of pAcreU31 downstream of the polyhedrin promoter by blunt end ligation. Transmembrane truncated β6 cDNA was excised from pCDNNeoβ6 with EcoRI and XbaI and cloned into the BsrHI site of pAcreU31 downstream of the polyhedrin promoter by blunt end ligation. The tandem vectors containing truncated αv and truncated β6 were used to prepare recombinant baculovirus as described (28). The recombinant baculoviruses were used to infect High Five insect cells, and the soluble receptor was harvested from the culture supernatant at 48–72 h of culture by passing the supernatant over 14D9.F8 antibody affinity columns, washing, and eluting at pH 3.1. All processes were carried out at room temperature and in the absence of detergents. Peak fractions were neutralized, concentrated, and dialyzed at 4 °C before shock freezing and storage at ~80 °C. The recombinant soluble human receptor (αvβ6-ΔTM) is biologically active and retains ligand specificity (13).

The integrin αvβ3-ΔTM, αvβ5, and αvβ6-ΔTM preparations were ~95% pure as judged by anti-integrin ELISA1 using α and β chain–specific monoclonal antibodies (data not shown) and by SDS-polyacrylamide gel electrophoresis against molecular weight standards (Bio-Rad).

Peptides—Peptides were synthesized, purified, and analyzed in house as described (30) using an Fmoc (N-(9-fluorenylethoxycarbonyl) strategy with acid-labile side chain protection on acid-labile lysyl (Bio-Rad). A 500-nmol scale was carried out with the Fmoc (above). The purity of the synthesized peptides was monitored routinely by fluorescence-activated cell sorter and showed the expected purity of >95%.

Antibodies—17E6 and 14D9.F8 (anti-αv), LM609 (anti-αβ3), P4C10 (anti-β1), and P1F6 (anti-αvβ5) all inhibit cell adhesion mediated by their respective integrins and have been described in detail elsewhere (27).

Selection of Integrin-binding Phages—M13 phage display libraries displaying linear peptides of 7 or 12 amino acids (PHD system from New England Biolabs) were used to select integrin-binding phages. Panning was performed as described in the product manual with the M13 phage display library, which is abbreviated as c(RGDfV).

RESULTS

Recombinant soluble human integrins αvβ3 and αvβ6 and native placental αvβ5 and platelet αIIbβ3 were purified by antibody or ligand affinity chromatography. On SDS gels each preparation showed the two major bands corresponding to the α and the β chains (Fig. 1A). The chains migrated at the molecular mass predicted for the intact and transmembrane truncated recombinant chains (αv full-length, 150 kDa; αv-ΔTM, 125 kDa; αvIIb full-length, 145 kDa; β3 full-length, 105 kDa; β5 full-length, 100 kDa; β3-ΔTM, 85 kDa; β6-ΔTM, 90 kDa). Each integrin was biologically active and showed ligand binding and divalent cation requirements predicted from the

1 B. Diefenbach, R. J. Mehta, A. Brown, E. Cullen, J. Adams, D. Sheppard, R. Dunker, S. L. Goodman, and D. Gussow, manuscript in preparation.
2 The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; BSA, bovine serum albumin.
literature and also as demonstrated independently by us (13, 27, 28, 30).

Recombinant αvβ6 expressed as a transmembrane truncated soluble receptor bound two distinct classes of phages in phage display panning experiments from a linear 12-mer library as determined from the sequences of more than 100 clones: those containing RGD sequences (51%) and those containing an XX-DLXXLX motif (27%). Some phages contained RGD and also continued with the motif as RGDXXL (9%), whereas others displayed the sequence RGDL (38%); the remaining phages that were bound often contained DLXXL-related sequences. A selection of representative displayed sequences is shown (Table I).

In the non-RGD sequences, the amino acid distribution at X within X1X2DLX3X4L5 appeared nonrandom. Arg was favored at X1 and X5, Thr/Ser/Asp/Gly at X2, whereas at X3X4 Ser/Thr were often paired with a charged amino acid. These characteristics were typified by a dominant clone with the sequence RTDLDLSLRTYTL (clone 1). Non-RGD, DLXXL-containing peptides were represented in only 5% of clones isolated from the 7-mer phage display library, suggesting that sequences COOH-terminal to DLXXL are also involved in integrin binding (Table I). Indeed, the sequence COOH-terminal to the peptide insertion site in the phage pIII protein continues Gly-Gly-Gly (i.e., the 7-mer library inserts would read XDDLXXLGGG), and related sequences were not isolated from the 12-mer library. Pro at X4 was never found in a DLXXL motif, and it was similarly excluded from the 4 amino acids COOH-terminal of the DLXXL sequence, although not from these positions in RGD-containing sequences. One sequence was found where the motif was concatenated as GDLDLLKLRLTR. To investigate whether the presence of DLXXL sequences was a library artifact, we also screened on αvβ3. Here mainly RGD-containing phages were bound, the DLXXL sequence was absent, and RGDL was present in fewer than 10% of clones. This distribution was distinct from that of the αvβ6 phage display library screen (Table I) and similar to that reported for linear 15-mer library screens (23).

To eliminate the possibility that the differences in phage interactions seen between αvβ3 and αvβ6 were artifacts caused by differential adsorption of integrins during the phage screen, we estimated the amounts of each integrin adsorbed to the screening plates under the conditions of the screen using an indirect ELISA technique (Fig. 1B). The ELISA titration curves derived using the 17E6 antibody, which recognizes the αv chain, were similar in form, in saturation level of antibody binding, and in the amount of antibody needed to titrate 50% of the receptors. This indicated that similar amounts of the αv integrins were adsorbed to the plates.

To test the specificity of interaction of phage clones we used a quantitative ELISA to measure the binding of representative phages from the αvβ6 screen to immobilized purified integrins (Fig. 2). DLXXL-containing phages bound only to αvβ6, whereas RGD phages bound both αvβ6 and αvβ3 and weakly to αvβ5 and to αIIbβ3. This indicated that the XDDLXXL clones isolated in the screen were specific for αvβ6.

RGD-containing peptides inhibit the interaction of RGD-containing ligands with their integrin receptors. To test whether DLXXL peptides were able to inhibit integrin-ligand interaction, we synthesized the clone-1 peptide. We compared the effects of clone-1 peptide and RGD-containing peptides on ligands binding to integrins αvβ3, αvβ5, and αvβ6 (Fig. 3). The clone-1 peptide specifically inhibited fibronectin binding to αvβ6 with an IC50 of 20 nM but did not affect ligand binding to αvβ3, αvβ5, or αIIbβ3 (IC50 > 50 μM). By contrast, the peptide GRGDSPK inhibited ligand binding to all four integrins with IC50 = 1 μM. A cyclic peptide inhibitor c(RGDfV) showed specificity for αvβ3 (IC50 = 10 nM) over αvβ6 and αIIbβ3 (IC50 = 1 μM). αvβ3 also binds to fibronectin. To test whether the effects of DLXXL peptides were a result of the ligand used with αvβ6, fibronectin, we also examined the effect of clone-1 peptide on fibronectin binding to αvβ3 (Fig. 4). The DLXXL peptide also had no effect on fibronectin binding, whereas the GRGDSPK peptide inhibited (IC50 = 200 nM). Thus, the results for vitronectin and fibronectin on αvβ6 were similar.

We also examined whether the assay geometry was producing an artifactual binding of DLXXL to αvβ6. In vivo, αvβ6 binds to insoluble fibronectin in the extracellular matrix rather than to the soluble form used in the receptor assay here. In addition, adsorption to plastic might change the conformation of the αvβ6 integrin and so alter its specificity. We therefore tested the effect of DLXXL peptides on αvβ6 binding to immobilized fibronectin (Fig. 5). Once again, the RTDLDLSLRTYTL peptide strongly inhibited the αvβ6-fibronectin interaction (IC50 = 100 nM). Both c(RGDfV) and GRGDSPK peptides were also effective in this assay configuration. Thus, the effect of DLXXL peptide on αvβ6 was probably not an artifact of integrin adsorption to plastic.
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Integrin binding clones from phage display library screens

Representative displayed peptide sequences from clones isolated by low pH elution from recombinant αvβ6 and αvβ3. Sequences have been selected to approximate the distribution of features found. RGD sequences have been underlined and DL motifs highlighted bold and underlined.

| 12-mer/αvβ6 RGD motif | 12-mer/αvβ6 DLXXX motif | 7-mer/αvβ6 | 12-mer/αvβ3 |
|------------------------|------------------------|------------|------------|
| RGDLKQGELTWW           | RDLDLSLRTYTL           | RDLLSM     | RGDLILPLFML|
| SARGDGLVQTP            | GDDLQILTR             | RDLDHL     | RGDIFHLAPRN|
| ILARGLDSTLA            | RDLDILSTRH            | RDLDLHT    | RGDLPV    |
| NARGLDFPPR             | RSDLQMLGLM            | SSDDLKQKRYG | RDLDTSQ   |
| RDVMLPFPWRL            | TSDDLKRLRLH           | RDLDLPP    | TGRDHHMPWKNG|
| RDGLPHQYRPS            | RDDLTYNRYAT           | RDLDLPY    | VRGALDPFAIG|
| RDGLYPFRSLTL           | RDDLRLRTTR            | RDLDLRHS   | IPGRDNYLMWQI|
| RDGLMLPFPWGL           | HPRDLSLAKRK           | RDLDLS     | AMGRDGYLHPTK|
| RDGLAAPHRTGA           | QRDLSALKTL            | RDLDLYV    | WLRQGRDGFQNL|
| RDGLNNLYMLML           | VTDDDLVKYRIK          | RDGIPPL    | AFN1RDGWFD|
| RDGLPVPMARE            | RDDLRLNRGL            | RGDILPR    | TEISRGDNFIN|
| RDGLPQFRHYPR           | RDDLRLNARQ           | RGDILMP    | NTHAFAKSGYPA|
| RDGLNASAKAS            | OSDLQVLYNRS           | RGDQMP     | NTHAFAKSGYPA|
| RGDAPSNIFIRL           | KGDLATLIRTN           | RGDMLTH    | VTCRGLDFCS|
| QSAHGRDPFPNVL          | RGDAYPS             | RGDHLSH    |             |
| VAPRDQRFILF           | ASDISALGARLA         | RGDAMFP    | DGRSYSTRKWFM|
| RGDYANLANNL           | RDPQALNVRQ            | RGDTSAL    | GRIPHTGPSER|
| HARGGDSWilP             | VSDVYDLTERL           | RGDQMP     | GRMFPFNSPHG|
| RGDFAQLLITWQ           | AGDILTFKLRLH         | LLRGDM     | GRFPFIASALPP|
| RGDIFPSMTLPR           | RHELSELARLM          | LLRGDM     |             |

**Fig. 2.** Binding to integrins of phages displaying different peptides. Phage binding to immobilized integrins was detected using an anti-M13 antibody. Phages displaying RTLDLSLRTYTL (solid circles), GDDLQILTR (solid squares), RGDAPSNIFIRL (triangles up), QSAHGRDPFPNVL (triangles down) binding to αvβ6 (panel A), αvβ3 (panel B), αvβ5 (panel C), αIIbβ3 (panel D). Note that the DLXXL-containing phages bind only αvβ6.

Clone-1 peptides were also tested in cell attachment assays. HT-29 carcinoma cell attachment to fibronectin was strongly inhibited by clone-1 peptide and by GRGDSPK (Fig. 6A). This attachment was also suppressed completely by the 17E6 (anti-αv) antibody, showing that it was dependent on αv integrins. HT-29 attachment to fibronectin has been shown to be mediated by αvβ6. M21-L melanoma cells attached to fibronectin, and this was inhibited by GRGDSPK but was little affected by the clone-1 peptide (Fig. 6B). M21-L attachment was suppressed by either P4C10 (anti-β1) or P1D6 (anti-α5) antibodies, indicating that it was mediated by the α5β1 integrin. Together, these data indicated that the peptide displayed by clone-1 was an active and specific inhibitor of αvβ6 independent of the RGD sequence.

We next investigated which elements of the X3X2DLXX3X4L5 motif were important for its inhibitory activity. NH2- and COOH-terminal truncated forms of the peptide were synthesized and tested for their activity to block fibronectin binding to αvβ6. The data are summarized in Table II. Truncation of the COOH terminus of DLXXL had little effect on inhibitory activity until the group at X3 was deleted, when activity diminished by 30-fold. Removal of the groups at X3X2 also abolished the activity, indicating that the core motif was the 8-amino acid...

**Fig. 3.** Effect of peptides on integrin-ligand interaction. Biotinylated ligand binding to immobilized integrins in the presence of peptides was detected using an anti-biotin antibody. Values were converted to percentage of control (no peptide). Solid circles, RTLDLSLRTYTL; open circles, GRGDSPK; open squares, cRGD(P)3; open diamonds, AGDV. Panel A, fibronectin binding to αvβ6; panel B, vitronectin binding to αvβ3; panel C, vitronectin binding to αvβ6; panel D, fibrinogen binding to αIIbβ3.
sequence $\chi^1\chi^2\chi^3\chi^4\chi^5$.

Although the specific selection of DLXXL sequences from a highly degenerate $(2 \times 10^8$ clones) display library implies specificity of interaction, we examined this more directly by investigating the effect of reversed (TRLSDLTTR) and scrambled (LDTRTRLSD) peptides on $\alpha v \beta 6$-fibronectin interaction. These peptides were >4 orders of magnitude less active than the corresponding DLXXL peptide (Fig. 7).

The high specificity of the clone-1 sequence for $\alpha v \beta 6$ suggested that it might represent a sequence in a native $\alpha v \beta 6$ ligand. Indeed, a FASTA search of the GEMBL data bases revealed several extracellular matrix components with related consensus sequences (Table III) including fibrinogen $\gamma$ chain, tenascin, laminin $\gamma 1$, $\beta 3$, and $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains. With the exception of tenascin, which has been reported to bind in an $\alpha v$-inhibitory $\gamma$ chain, $\beta 3$, and $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains.

Interestingly, the sequence DLYYLMDL is strongly conserved in human integrin $\beta$ chains and may interact directly with ligands, hinting that the clone-1 sequence might function by disturbing the interaction from the side of the receptor rather than as a ligand mimetic (e.g. like an RGD peptide). To test this possibility, we examined the synthetic DLXXL peptide sequence derived from the $\beta 6$ chain, $\text{P}^{122}\text{DLYYLMDL}$, for its effect on fibronectin-$\alpha v \beta 6$ interaction (Fig. 7). Compared with the corresponding 10-mer derived from clone-1, RTLDL-

**Fig. 4.** Effect of peptides on fibronectin binding to $\alpha v \beta 6$. Fibronectin binding to $\alpha v \beta 6$ was measured in the presence of increasing amounts of $\text{RTDLDSLRTYTL}$ (solid circles) or GRGDSPK (open circles). Values were converted to percentage of control (no peptide).

**Fig. 5.** Inverted integrin-ligand competition assay. Biotinylated $\alpha v \beta 6$-ATM binding to fibronectin was detected in the presence of the indicated concentrations of test peptides. Bound integrin was detected with anti-biotin antibody. Solid circles, $\text{RTDLDSLRTYTL}$; open circles, GRGDSPK; open squares, cRGDKV; open diamonds, AGDV.

**Fig. 6.** Effect of peptides on cell attachment to fibronectin. Cell attachment on fibronectin of HT-29 cells (panel A) and M21-L cells (panel B) in the presence of peptides and inhibitory antibodies is shown. Solid circles, $\text{RTDLDSLRTYTL}$; open circles, GRGDSPK; open squares, open triangles up, $\text{P4C10}$ ($\beta 1$-inhibitory); open triangles down, P1D6 ($\alpha 5$-inhibitory). Values were converted to percentage of control (control cell attachment in the absence of peptide for HT-29 and M21-L was 54 and 47% of cells added, respectively). HT-29 attachment to fibronectin is dependent on $\alpha v$ integrins. M21-L attachment to fibronectin is dependent on $\alpha v \beta 1$.

**Table II**

| Peptide | $\text{IC}_{50}$ FN/\text{$\alpha v \beta 6$} ($\mu$M) |
|---------|---------------------------------|
| $\text{RTDLDSLRTYTL}$ | 0.02 |
| $\text{RTDLDSLRTY}$ | 0.04 |
| $\text{RTDLDSLRT}$ | 0.05 |
| $\text{RTDLSDLR}$ | 0.1 |
| $\text{RTDLSDL}$ | 3 |
| $\text{DSLRTYTL}$ | >10 |
| $\text{DLDSLRTY}$ | >10 |
| $\text{DLDSLRT}$ | >10 |

**Fig. 7.** Effect of peptides on fibronectin binding to $\alpha v \beta 6$. Fibronectin binding to $\alpha v \beta 6$ was measured in the presence of increasing amounts of $\text{RTDLDSLRTY}$ (solid circles), $\text{RTDLYYLRTY}$ (solid squares), $\text{RTDLYVLMDL}$ (solid triangles), $\text{PVDLYVLMDL}$ (solid diamonds), GRGDSPK (open circles), TRLSDLTTR (open triangles), or LDTRTRLSD (open squares).

SLRTY, the $\beta$ peptide was >4 orders of magnitude less active as an inhibitor of this interaction. Once again, this supported the concept that the sequences selected from the phage display library were highly specific. We examined the structural basis...
of the inhibition by exchanging the flanking sequences from the clone-1 10-mer with those of the β6 DLLX peptide. Experiments of the DLYYL core gave a highly active inhibitor, as did the core present we believe that the binding sequences that we characterize here provide a unique pharmacological tool from which to investigate αβ6 biological function further.

Acknowledgments—We thank E. Rosell (Merck LBI) for discussion and introduction to the phage display technology, D. Sheppard (UCSF) for discussion and access to β6 cDNA, and R. Dunker and I. Remitschka (Merck, kgAa) for biotechnological support.

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J. Biol. Chem. 1999, 274:1979-1985.
doi: 10.1074/jbc.274.4.1979

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