☆ インテグリンスーパーファミリーは創薬のターゲットである.
☆ インテグリンノックアウトマウスはすべて致死的か表現型に異常をもつ.

表❶ インテグリン各サブユニットノックアウトマウスの結果（筆者作成）

	αサブユニット	βサブユニット
総数	18	8
除去により致死的となるもの	7	4
除去により病的となるもの	7	4
未発表	4	0

る炎症所見をポスドク Xiaouzhu Huang が述べていたころ，筆者は大きな興味を残して日本へ帰国した．その歴史を知るものとして，この論文に関し，費やされた多くの努力とともに紹介したい．

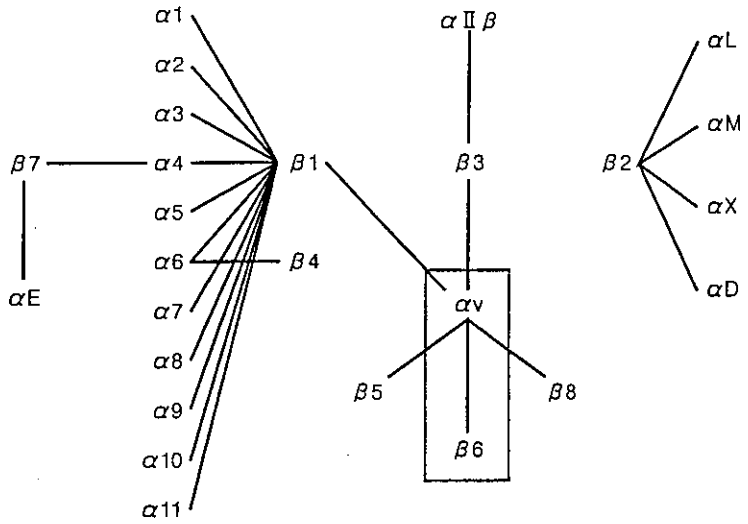
1. インテグリン

分子を除去されたマウスが肺気腫に至った標的分子は，接着分子インテグリンである．インテグリンは薬剤開発の標的分子[2)3)]として，プロテアーゼ，キナーゼ，七回膜貫通蛋白，核内レセプター，イオンチャンネルなどと並び注目されている．原核生物には存在しないが後生生物では海綿，ヒドラなどにも存在し，多細胞生物において生命現象の基本的な部分に役割を果たしていると考えられる．細胞外マトリックス，イムノグロブリンスーパーファミリーのレセプターとして細胞の接着を仲介するのがプライマリーな役割であるが，リガンド結合によるシグナル伝達が明らかになって以来，リガンド結合後の研究が精力的におこなわれ，アポトーシスの抑制と細胞周期の進行に必要であることがほぼ確かめられている[3)]．がん細胞の特徴とされる足場非依存性増殖に関して，インテグリンからのシグナルが細胞の増殖に必須であるのに対して，がん細胞ではこのシグナルががん遺伝子，あるいはがん抑制遺伝子（機能喪失）により補われているためと説明されている．また最近では，細胞膜表面で他の分子と相互作用をおこなう[4)]ことやトロンビンの炎症制御を仲介する[5)]など，第三の役割がみえ隠れしている．

2. インテグリンノックアウトマウス全体像

つぎつぎと誕生するノックアウトマウスの解析結果は，長年謎であった病態を一気に解決することがあり，興味が尽きない．呼吸器領域では GM-CSF ノックアウトにより生じた肺胞蛋白症に関してその病因がわが国で解明されている[6)]．一方で，疾患に関わっている証拠が多く集積している遺伝子や生命現象の維持にとって基本的存在と考えられている遺伝子（サイトカイン，テロメラーゼなど）が除去されても，意外にもマウスは致死的ではなくまた表現型にも大きな異常を示さない場合も多くみられる．もちろんこのことが直接，対象遺伝子が疾患制御に役割を果たしていないこととは結びつかないにせよ，逆に一見予備的に複数が存在しているように思われていたファミリーのメンバーが実際には一つでも欠失するといずれも致死的あるいは表現型が異常となるファミリーがある．その一つがインテグリンスーパーファミリーである．インテグリンは 18 の α，8 つの β サブユニットにより形成される，24 種類のヘテロダイマーからなる．一つのリガンドに重複するレセプターも多く，一見してリダンダントな感じを

☆ インテグリン欠損マウスのなかでは αVβ6 および α9β1 欠失マウスに肺の障害がみられる.
☆ αVβ6 は創傷治療で強く発現するが，その欠失マウスの創傷治療は完璧である.

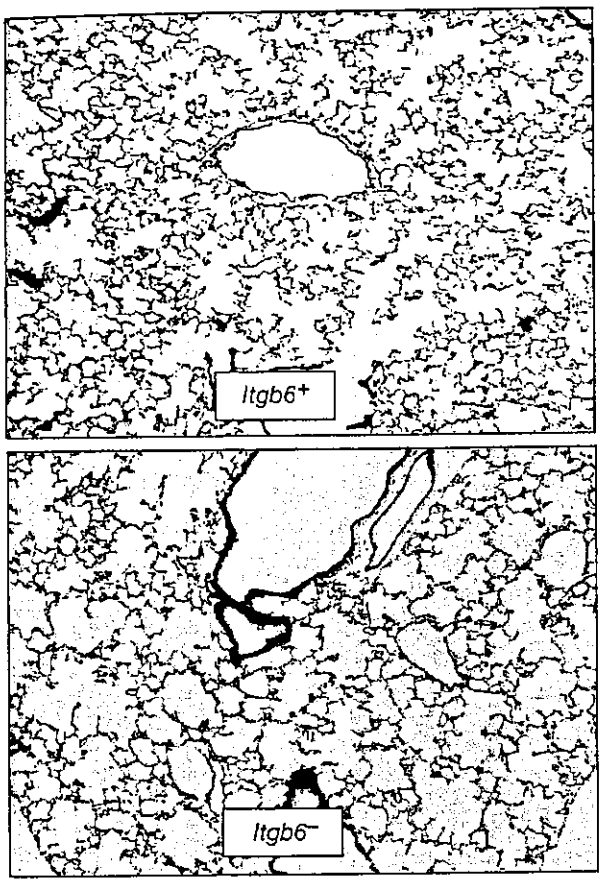


図❶ インテグリンスーパーファミリー（筆者作成）
インテグリンの18のαサブユニットと8つのβサブユニットとの関係．一つのサブユニット遺伝子をノックアウトしても，欠失するインテグリンの数は異なる.

受ける.しかしノックアウトの結果がほぼ出そろい，ほとんどのサブユニットが生存あるいは少なくとも健康の維持に必要な，ほかで補われない特異的なはたらきをしていることが判明した(表❶)．気道上皮に発現がみられるインテグリンは α2β1，α3β1，α5β1，α6β4，α9β1，αvβ5，αvβ6，αvβ8 である．これらのなかで肺の病変が報告されているノックアウトマウスは αvβ6 あるいは α9β1 を欠くもののみである．しかし，ほかのインテグリンに関しても，注意深い診療？ により何らかの異常が発見できるものかもしれない．ちなみに α9β1，αvβ6 の発現を欠くマウスはいずれも Lung Biology Center で誕生したもので，スタッフの呼吸器臨床医としての観察眼があったことも無関係とは言えないであろう.

3. インテグリンβ6サブユニットノックアウトマウス

このマウスが heterozygote として最初に世に誕生したのは1994年である．Lab ではじめてのノックアウトマウスの誕生にメンバーの期待とその表現型への好奇が集まっていたが，その homozygote は1995年一見正常に生まれた．αvβ6 は胎生期に強く発現されるため，homozygote の誕生は困難かあるいは明らかな奇型をともなうのではないかと考えられていたにもかかわらず，図❶からもわかるようにインテグリンβ6サブユニットを欠くと，αvβ6 の発現が欠失する．αvβ6 はまた，組織傷害の場で強く発現されるため，マウスの皮膚は何度も傷つけられ試されたが，その修復は完璧であった．しかし，やがてはじめての異常が体毛に見つかった．生後間もない時期に皮膚の一部に体毛の脱落がみられたの



図❷ インテグリンβ6ノックアウトマウスの肺気腫像（筆者作成）
生後14ヵ月のマウス．β6ノックアウトでwild typeにくらべて肺胞腔の拡大が認められる．(×10)
(Nature 422：169-173, 2003より引用)

である．部分欠失の理由がしばらく謎であったが，それが子を運ぶ母親にくわえられる部位に一致していることが判明し，皮膚の病理組織像が観察されたところ全体的に炎症細胞の浸潤が認められ，脱毛は皮膚の炎症の結果と考えられた．つぎに，インテグリンαvβ6は皮膚同様気道上皮にも発現されまた，病理組織像も炎症細胞浸潤を示唆していたため気管支肺胞洗浄がおこなわれ，はたして細胞数は増加していた．上皮のインテグリンを除去すると肺と皮膚に炎症が生じていた事実は[7]，このインテグリンが炎症を制御していることを示していたが，その機序はしばらく不明であった．

つぎに，肺に慢性炎症が存在すれば肺線維症につながるのではないかと，ブレオマイシンの投与がおこなわれたが，投与60日後の病理像はwild typeにくらべβ6ノックアウトで明らかに線維化が軽度であった．これらの機序解明は，Dean Sheppardがアメリカ胸部疾患学会（ATS）ミーティングで見つけた一つの演題が大きな局面を与えた．そこには潜在型TGFβがインテグリンαvβ1のリガンドになることが発表されており，早速共同研究が精力的に進められていった．潜在型TGFβは活性型がlatency associated protein (LAP)という蛋白に覆われたものであるが，インテグリンαvβ6はαvβ1同様LAPに結合し，しかもこれを潜在型TGFβからはずし活性型に変化させることがわかりCell誌に報告されている[4]．

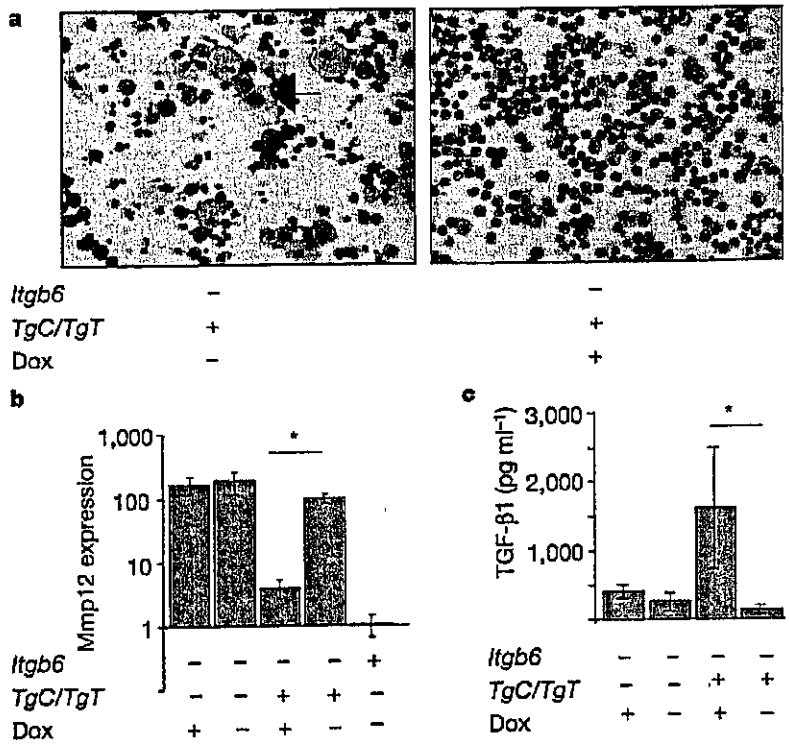
さて，このノックアウトマウスを長期間観察しているとさらに意外なことが発見された．生後8週ではほとんど変化がないものの，半年から1年あまりの長期間観察により肺胞腔が拡大して来たのである[1]（図❷）．DNA chipを用いた遺伝子解析がすでにおこなわれており，肺においてβ6ノックアウト

表❷ 論文中で用いられるマウスの表記の解説（筆者作成）

a.	ノックイン発現蛋白	発現部位	TGFβ活性化	細胞増殖（3次元ゲル）	気腫化
Itgb6-Tg（ITGB6^{FL}）	αvβ6	肺のみ	＋	＋	−
Itgb6-Tg（ITGB6^{777T}）	変異αvβ6	肺のみ	＋	−	−
Itgb6-Tg（ITGB6^{D140A}）	変異αvβ6	肺のみ	−	−	＋

b.	ノックイン発現蛋白	発現部位	発現調節	気腫化
Itgb6-Tg（CCSP-rt TA） Tg（tetO-Tgfb1Cys-Ser）	活性化TGFβ	肺のみ	ドキシサイクリン	−

☆ 気道上皮インテグリン αvβ6 が欠損すると肺胞マクロファージの MMP 12 発現が上昇する.



図❸ インテグリン β6 ノックアウトマウスにおける TGFβ の遺伝子導入とその発現誘導による肺胞マクロファージ像の正常化.
a. 気管支肺胞洗浄像 (×40), b. MMP 12 発現量, ドキシサイクリン投与後 21 日目 c. 気管支肺胞洗浄液中の活性化型 TGFβ 量, ドキシサイクリン投与後 14 日目. (Nature 422 : 169-173, 2003 より引用)

で wild type にくらべ発現が亢進している分子が検討されていた[8]. 前述のごとく肺には細胞浸潤がみられるためそれらの病態への関与を考え, 白血球に限られる遺伝子に焦点をあてて検討されたところ, 遺伝子の多くに発現亢進がみられたが, 白血球数の増加を反映したと考えられる 2 倍程度の増加にすぎないものがほとんどであった. しかし, 唯一, マクロファージに存在するメタロプロテアーゼ MMP 12 は 18 倍にも亢進していた. さらに肺胞マクロファージにおけるこの分子の発現を定量 PCR で比較したところ, 228 倍にも達していた. 実際 MMP 12 ノックアウトマウスは, タバコ煙暴露によっても, 肺の気腫化を生じないことが報告されている[9]. インテグリン αvβ6 は気道上皮のインテグリンである. この発現を欠くことと肺胞マクロファージの MMP 12 の発現上昇は一体どのようにつながっているのであろうか. インテグリン αvβ6 には現在までに 2 種類の機能が知られている. 一つには細胞増殖, もう一つは TGFβ の活性化である. MMP 12 亢進がどちらを介するものか, β6 ノックアウトマウスにこれらの機能の異なる 2 種類に加え, wild type も加えた 3 種類の αvβ6 が肺のみに

☆ αvβ6-TGFβ-MMP 12経路が，ヒトの病態にあてはまるのか，検討が待たれる．

発現されるようにCC 10プロモーター存在下で遺伝子導入された（表❷a）．その結果細胞増殖能は失っていてもTGFβは活性化できる変異型αvβ6ノックインマウスではMMP 12活性は上昇しておらず気腫化を生じなかった．一方，TGFβを活性化することのできない変異型αvβ6ノックインマウスは気腫化も生じなければ，MMP 12活性の上昇もみられた．以上は，活性型TGFβの存在がMMP 12の抑制につながることを強く示唆するが，このことをさらに直接的に観察するため，つぎにドキシサイクリン投与により活性化TGFβを肺に発現するモデルが二つの遺伝子をβ6ノックアウトマウスに導入することにより作成されている（表❷b）．これによればドキシサイクリンを投与されつづけた場合は気管支肺胞洗浄液中に活性化TGFβを認めていることを確かめたうえで（図❸c），肺胞マクロファージの空胞化もMMP 12の活性化もみられない（図❸a，b）ことが報告されている．さて，ここまでではαvβ6の欠損がTGFβの活性化障害につながりそれがMMP 12の発現亢進を引き起こしていることが確かめられた．しかし，インテグリンαvβ6欠損マウスにおいて，MMP 12の亢進が肺気腫を生じさせていることを最終的に確かめるためには，αvβ6を欠くがMMP 12の亢進はないマウスで確認することが必要である．そのためβ6ノックアウトとMMP 12ノックアウトが掛け合わされた結果，ダブルノックアウトマウスは肺の細胞浸潤や皮膚の炎症所見は認められたものの，肺気腫を生じないことが確かめられている．

以上の結果はインテグリンαvβ6欠損マウスが肺気腫を生じる分子病態においてはMMP 12の亢進が必要かつ十分であることを示している．ただしαvβ6-TGFβ-MMP 12経路がartificialなものでなく，ヒトの肺気腫の成因であるか否かは今回の結果からだけでは不明であり，今後確かめられなければならない問題である．また，αvβ6とTGFβ活性化関連は良く検討されているが，TGFβ活性化とMMP 12の発現亢進に関しての分子レベルでの経路はあいだにいくつかの分子を介しており，こちらも今後の課題であろう．良く練られた臨床検体の比較で，これらの経路を構成する遺伝子のいずれかに肺気腫患者と健常肺者のあいだで差が確認されることに期待したい．また，αvβ6-TGFβ-MMP 12経路がヒトの病態に当てはまったとしても，それがすべての肺気腫がこの経路により生じることを意味するのではないことも忘れてはならない．また，興味深いことに，αvβ6の気道上皮における発現は喫煙者で亢進する[10]．この理由に関して皆さんはどのようにお考えであろうか．

4. 何がこの発見と証明を可能にしたか

わが国でも多くの臨床医が大変なエネルギーを実験，研究に費やしているが，なかなか疾患の分子病態の本質を明らかにすることは容易ではない．その点で，本論文は出色のものである．UCSF Lung Biology Centerは1988年にDean SheppardをDirectorとして創設された．それまで職業性喘息の肺生理で成果を挙げていたが，分子生物学の経験はなかったDean Sheppardは自らのセンターの中のlabをサバティカルの場所に選び，自分がリクルートしたPhDの下で分子生物学を学んだ．

今回の成果に不可欠であったものはなんであろうか．まず，やはり分子生物学テクノロジーの進歩とそれを積極的に取り入れたことに尽きるであろう．第二には，Dean Sheppardの呼吸器疾患の本質解明をゴールとして掲げるためには分子機能の解析がま

ず不可欠であるという信念であったように思われる．1990年頃しばらくはホモロジーPCRで新たなインテグリンをクローニングすることに明け暮れていた．未知の分子を「気道上皮細胞から得る」ことにより呼吸器病学との接点は保っていたが，その分子が呼吸器疾患に関与しているという保証はまったくなかった．彼はときどき“superficial”という言葉を口にしていたが，臨床との結びつきのみに目を向けすぎるあまり，自らの発展性を制限してしまわないという意味であったことを，1999年のCell誌，2003年のNature誌の論文が語っているように筆者には思われる．

また忘れてならないのは，この仕事を支えてきた多くのfellowの努力とそれを見事に統合したDean Sheppardの統率力であろう．この論文はこれまでの多くの仕事の積み重ねのうえにある．Deanの大きな声は誰の紹介もなく飛び込んだ筆者には緊張を強いるものであった．彼は最低1日2回ベンチサイドにやってきて，進行の具合について声をかけた．休憩時間以外はfellowたちは無駄話をせず，窓のない実験室にピペットやPCRの音だけが響いていた．その雰囲気に合わずに去っていった者も何人かいる．しかしDeanは結果が出たときは心から喜び，大げさに見えるくらいに嬉しそうであった．また，そこで仕事をしていると次第にわかってきたのであるが，結果を出した者に対しては必ず報いてくれる[11]．以下，この論文につながった結果を挙げてみる．さまざまなコンストラクトが論文中で使用されているが，まずItgb^{777t}は1994年にオーストラリアから留学していた外科医Michael Agrezが$\alpha v\beta 6$の増殖能を報告した論文のなかで用いられている[12]．実際のmutantの作成はミャンマーから移住してきていたAileen ChenがUCBerkley卒業後医学部に入るまでの2年間をLBCでテクニシャンとして過ごした際に作成したものである．(彼女は結局UC Irvineの医学部に進学した．) Itgb^{D140A}はDominant negative mutantとしてXiaouzhu Huangにより作成され1995年報告された[13]．またノックアウトマウスの誕生はほとんど彼女の努力に負うものである．Wild typeのコンストラクトは現在Stanford大学のPulmonary and Critical Care Medicineのfacultyである Ann Weinackerにより1994年報告されたものである[14]．また，この論文は筆頭著者である呼吸器科医David Morrisの着眼点と解析力によるところが大きいが，その基礎となっているのが，現在Pittsburgh Medical CenterのNaftali Kaminskiによりおこなわれていたマイクロアレイによるインテグリン$\beta 6$ノックアウトの解析であり[8]，KawakatsuによるTGFβアッセイのlabでの確立[4]である．さらにずっと遡れば，まず$\beta 6$ cDNAのクローニングがDean Sheppard自身により41歳のときおこなわれ[15]，リガンドの決定[16]，細胞質ドメインの役割[17]，などが一つずつ積み重ねられてきた．今回の論文ではこれら作成された遺伝子が実にうまく使用されており，努力をDean Sheppardは念入りにintegrateしている．これらのなかでもとくにノックアウトマウスを作成したXiaouzhu Huangの役割は大きい．彼女は1992年に渡米するまで細胞培養もおこなったことがなかったが，物怖じせず淡々と，しかも前向きに実験手技を修得した．あっというまに実験の腕を上げ，Itgb^{D140A}遺伝子を導入したトランスジェニックマウスを作成，つぎにノックアウトマウスの作成に取りかかった．かといって，7時以降にはたらいていたのを見たことはなく，決して悲壮ではなかった．これらの仕事は当時子守り当番だったエンジニアの夫もlabに連れてきて手伝わせ研究した1993年から1995年の2年の間におこなわれた．彼女の成長も時間ではなく成果を問い，彼女のペースで仕事をさせたDean Sheppardのdirectionと無縁ではないと思われる．

おわりに

UCSFは現在大規模な移転をおこなっている．築100年のレンガ造りの建物のなかで窓のなかったLung Biology Centerもやっと新築ビルに移行する．(SF Giantsの試合がDean Sheppardの部屋から見えるそうです)また，Xiaouzhuは郊外に庭付き一戸建てを購入し，2台の車を持っている．

これまで，肺の炎症は何らかの刺激に引き続き，白血球やサイトカインが主導すると考えられ，気道上皮は炎症に関してはむしろ二次的，三次的な役割が想定されていたように思われる．しかし気道上皮

インテグリンによるTGFβ活性化は，上皮細胞が積極的に炎症制御に関与していることを示すものである．また，文中にも述べたように，インテグリン$\alpha v\beta 6$欠損マウスはブレオマイシン肺線維症抵抗性である．一連の結果は肺気腫と肺線維症が対局にある疾患であるとする概念にTGFβという座標軸を与えたものかも知れない．これらの成果が，ヒトの特発性間質性肺炎，肺気腫の病態解明，さらに治療・予防へとつながってゆくことに期待したい．

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Distinct structural requirements for binding of the integrins αvβ6, αvβ3, αvβ5, α5β1 and α9β1 to osteopontin

Yasuyuki Yokosaki[1,5,*], Kumi Tanaka[3,5], Fumiko Higashikawa[1,5], Keisuke Yamashita[4,5], Akira Eboshida[2]

From [1]Molecular Division, [2]Department of Public Health, [3]Department of Orthopedics, [4]Department of Anatomy and Developmental Biology, Graduate School of Biomedical Sciences, [5]Integrin Therapeutic Frontier Laboratory, Hiroshima University, 1-2-3 Kasumi, Minamiku, Hiroshima, 734-8551, Japan

Running title: Structural requirements of osteopontin receptors

Key words: integrin, osteopontin, thrombin, matrix metalloprotease

*Address correspondence to: Yasuyuki Yokosaki M.D., Ph.D., Department of Public Health, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minamiku, Hiroshima, 734-8551, Japan, Telephone: +81-82-257-5166, Fax: +81-82-257-5169, e-mail: yokosaki@hiroshima-u.ac.jp

Abstract

The extracellular matrix protein, osteopontin, is a ligand for several members of the integrin family, including α5β1, αvβ3, αvβ5 and α9β1. Osteopontin is a substrate for a number of extracellular proteases, including thrombin and the metalloproteases MMP-3 and MMP-7, which cleave osteopontin at sites close to or within the mapped integrin binding sites. Using affinity chromatography and cell adhesion assays, we now identify the integrin αvβ6 as an additional osteopontin receptor. Utilizing a series of recombinant forms of osteopontin we compared the structural requirements for αvβ6 binding with those for the 4 other osteopontin-binding integrins. Like α5β1, αvβ3 and αvβ5 (but not α9β1), αvβ6 binds to the RGD site in osteopontin, since RGD peptide or mutation of this site to RAA completely inhibits αvβ6-mediated cell adhesion. For both α9β1 and α5β1 the N-terminal fragment generated by thrombin cleavage is a much better ligand than full length osteopontin, whereas thrombin-cleavage does not appear to be required for optimal adhesion to αvβ3, αvβ5 or αvβ6. A recombinant fragment predicted to be generated by MMP cleavage no longer supported α5β1 or α9β1-mediated adhesion, but adhesion mediated by αvβ5 or αvβ6 was unaffected. Finally, adhesion of αvβ5 or αvβ6 was inhibited by mutation of two aspartic acid residues upstream of the RGD site, whereas adhesion mediated by αvβ3, α5β1 or α9β1 was unaffected by these mutations. These results suggest that the hierarchy of integrin interactions with osteopontin can undergo complex regulation at least in part through the action of extracellular proteases.

INTRODUCTION

Osteopontin is an acidic phosphorylated glycoprotein with versatile functions, including roles in tissue remodeling and regulation of immunity and inflammation (Denhardt and Chambers, 1994; Denhardt et al., 2001; Weber and Cantor, 1996). Principal ways osteopontin can affect cellular behavior is through interactions with integrins or CD44 (Ashkar et al., 2000). Integrins are heterodimeric cell surface glycoproteins that mediates cell response to extracellular matrix proteins (Danen and Sonnenberg, 2003; Hynes, 2002; Sheppard, 2000). Osteopontin contains the canonical integrin recognition sequence, arginine-glycine-aspartic acid (RGD) and the integrins αvβ1 (Hu et al., 1995; Liaw et al., 1995), αvβ3 (Miyauchi et al, 1991), αvβ5 (Hu et al., 1995; Liaw et al., 1995), α5β1 (Barry et al., 2000; Nasu et al., 1995) and α8β1 (Denda et al., 1998) have all been reported to bind to osteopontin through this sequence. Two other integrins, α4β1 and α9β1, bind to non-RGD sites of osteopontin. We have recently mapped the binding site of α9β1 in osteopontin and shown that it recognizes the sequence 162SVVYGLR168 immediately adjacent to the RGD (Yokosaki et al., 1999). According to recent microarray studies, osteopontin is dramatically up-regulated in response to various environmental insults in many tissues (Kang et al., 2003; Boeshore et al., 2004; Ye et al., 2003). Expression of the αvβ6 integrin is also upregulated in response to environmental insults. Interestingly, osteopontin itself appears to be induced at least in part by an αvβ6-dependent pathway, since induction of osteopontin in response to lung injury by bleomycin is markedly attenuated in β6-subunit knockout mice (Kaminski et al., 2000). Because αvβ6 binds to RGD sites in each of its known ligands, we sought to determine whether this integrin is also a receptor for osteopontin. In the extracellular space osteopontin has been shown to be a substrate for proteolytic cleavage by thrombin (Senger, 1994) and the matrix metalloproteases MMP-3 and MMP-7 (Agnihotri et al., 2001). Thrombin cleaves osteopontin between 168R and 169S, and this cleavage event is required for α9β1 interaction with the resultant N-terminal fragment (Smith et al., 1996). MMP-3 and MMP-7 have been reported to cleave osteopontin between 166G and 167L, within the α9β1 binding site (Yokosaki et al., 1999) and adjacent to the RGD sequence recognized by most of the other osteopontin-binding integrins. Integrin binding to RGD sites in ligands has been shown to be modulated by the amino acid sequences adjacent to the RGD, it is thus

conceivable that the specificity of integrin interactions with osteopontin could be regulated, at least in part by proteolytic cleavage of osteopontin. To explore this possibility, we generated recombinant forms of osteopontin to map the binding requirements for several integrins that bind to osteopontin and to determine the likely effects of proteolytic cleavage by MMPs and thrombin on the specificity of integrin binding.

RESULTS

αvβ6 integrin directly binds an N-terminal fragment of osteopontin

To determine whether the αvβ6 integrin is a receptor for osteopontin, we performed affinity chromatography by passing [^{35}S]methionine- and [^{35}S]cysteine-labeled secreted αvβ6 over Sepharose cross-linked to thrombin-cleaved N-terminal osteopontin fragment (nOPN)(or BSA as a control). Bound αvβ6 was eluted by EDTA. There were no bands detected in the eluant from the BSA column, whereas each lane of the eluted fraction from nOPN column showed bands (Fig. 1) corresponding to truncated αv (130 kDa) and β6 (85 kDa). These results indicate integrin αvβ6 binds to nOPN.

Adhesion of SW480 cells to nOPN

We tested four cell lines, mock-, β3-, β6- and α9-transfected SW480 cells for the expression of integrins, αvβ3, αvβ5, αvβ6, α5β1 and α9β1 to use for adhesion assays to various recombinant osteopontin fragments. Although levels of expression were not exactly the same among the four cell lines, all cell lines including mock-transfected cells expressed integrins αvβ5 and α5β1. β3-, β6- and α9-transfectants also expressed αvβ3, αvβ6 and α9β1 respectively (Fig. 2). These 4 cell lines were analyzed in adhesion assays to nOPN first. In mock-transfected cells, anti-α5β1 monoclonal antibody, P3D10, and anti-αvβ5 antibody, P1F6 each partially inhibited adhesion to nOPN when used separately and completely inhibited adhesion used in combination (Fig. 3A). These data confirm that α5β1 and αvβ5 are the principal osteopontin receptors on mock-transfected SW480 cells. However, these two antibodies were not sufficient to abolish adhesion of any of the other 3 transfectants. For β3-, β6-

and α9-transfectants the further addition of anti-αvβ3, anti-αvβ6, or anti-α9β1 blocking antibodies, respectively, did result in complete inhibition of adhesion to nOPN. But each of these antibodies, when used alone, could not inhibit the adhesion completely (Fig. 3B, C, D). Therefore, in the presence of P3D10 and P1F6, these 3 transfectants adhered to nOPN utilizing a single integrin receptor.

αvβ6 binds to the RGD site in osteopontin

Since αvβ6 has not been reported as an osteopontin receptor, we further tested this interaction of β6-transfected SW480 cells with osteopontin. In the presence of blocking antibodies against α5β1 and αvβ5, β6-transfectants adhered to nOPN in a concentration-dependent fashion at concentrations within a range of 20 to 600 nM, which was completely abolished by the αvβ6-blocking antibody 10D5. Under these conditions, mock transfectants did not adhere at any concentration of nOPN (Fig. 4A). To determine if this αvβ6-mediated adhesion was RGD-dependent, β6-transfected cells were incubated with GRGDSP peptide before plating, which completely abolished adhesion to nOPN. β6-transfected SW480 cells were also plated on mutant nOPN in which the RGD sequence was mutated to RAA, and no adhesion was detected. These results demonstrate that αvβ6 binds to the RGD site in osteopontin (Fig. 4B). To confirm this interaction, we next tested adhesion of UCLA P3 lung cancer cell line that naturally express αvβ6 to nOPN. Unlike SW480 cells UCLA P3 cells do not express α5β1, but express αvβ5 (Fig. 4C). UCLA P3 cells adhered to nOPN well, which was only partially blocked by anti-αvβ5, P1F6. The residual adhesion was prominently blocked by an addition of anti-αvβ6, 10D5, although adhesion of UCLA P3 to nOPN was only partially blocked by10D5 alone (Fig. 4D). The blocking effect of 10D5 was obvious in the presence of P1F6, indicating that UCLA P3 adhered to osteopontin mediated at least in part by integrin αvβ6.

Structural requirements for binding of integrins αvβ6, αvβ3, αvβ5, α5β1 and α9β1 to N-terminal fragment of osteopontin

Several integrins overlap their binding to the region of osteopontin containing the RGD site. Sequences around the RGD site can provide specificity for binding of integrins (Ruoslahti, 1996). We therefore examined whether αvβ6 has distinct structural requirements from other osteopontin receptors that recognize RGD,

αvβ3, αvβ5 and α5β1, in comparison with α9β1 that recognizes a non-RGD site (Yokosaki et al., 1999). The structure of the RGD-containing region in osteopontin was modified by substitution of two upstream asparatic acid residues by alanine (D154A and D157A) and by substitution of a downstream tyrosine residue that is critical for binding of α9β1 (Y165A) (Fig. 5) (Yokosaki et al., 1999) . To examine the role of individual integrins in binding to each mutant, we performed adhesion assays in the presence of blocking antibodies to each of the other integrins present in our various cell lines. Thus, binding of integrin αvβ3 was observed as adhesion of β3-transfected SW480 cells in the presence of antibodies against α5β1 and αvβ5, αvβ5 as adhesion of mock-transfectant in the presence of antibodies against α5β1, αvβ6 as adhesion of β6-transfectant in the presence of antibodies against α5β1 and αvβ5, α5β1 as adhesion of mock transfectant in the presence of antibodies against αvβ5, and α9β1 as α9-transfectants in the presence of antibodies against α5β1 and αvβ5. Adhesion of αvβ3 was the same for wild type nOPN and all mutants examined. In contrast, αvβ6 adhered poorly to the D154A mutant and did not bind at all to the D154,157A double mutant. The αvβ5 adhesion pattern was similar to αvβ6, but αvβ5-mediated adhesion was less sensitive to these mutations. α5β1-mediated adhesion was only minimally affected by mutation of D154 and D157 and α9β1 mediated adhesion was not affected at all by these mutations. However the, Y165A mutation dramatically inhibited α9β1- and α5β1-mediated adhesion, with no effect on adhesion mediated by αvβ3, αvβ5 or αvβ6 (Fig. 6). These results suggest that these integrins have different structural requirements for interaction with osteopontin. αvβ3-mediated adhesion appears to depend principally on the RGD sequence itself, αvβ6- and αvβ5-mediated adhesion is also sensitive to amino acids upstream of the RGD site, α5β1–mediated adhesion depends on the RGD site and is sensitive to amino acids downstream, and α9β1-mediated adhesion is completely dependent on the SVVYGLR sequence downstream of RGD.

Differential effects of thrombin- or MMP-3, 7-cleavage on cell adhesion mediated by αvβ6, αvβ3, αvβ5, α5β1 and a9β1

Since integrin mediated adhesion to osteopontin appeared to be affected by conformational changes close to or within the integrin binding site (Fig. 5), we next compared adhesion mediated by each integrin to full length osteopontin (fOPN) and

recombinant forms mimicking two naturally occurring cleavage forms that are produced by cleavage by proteases, MMP-3 or MMP-7 (nOPN-dLR), or thrombin (the nOPN form used above) (Fig. 5). The MMP-3, 7-cleaved form (nOPN-dLR) was made by deletion of 2 residues, LR, of nOPN. fOPN was generated from full length cDNA. Integrins αvβ6, αvβ3 or αvβ5 each bound equally well to nOPN, nOPN-dLR and fOPN, indicating that these 2 cleavages do not influence their binding to osteopontin. In contrast, α5β1-mediated adhesion was dramatically affected by these cleavages. Although α5β1 mediated robust adhesion to nOPN, α5β1–mediated adhesion to fOPN or nOPN-dLR was substantially reduced (Fig. 7A), indicating that adhesion of α5β1 to fOPN was enhanced by thrombin cleavage, but that osteopontin cleavage by MMPs would be inhibitory. To confirm these results, we enzymatically cleaved fOPN by thrombin or MMP-3 (Fig. 7C) and used these cleaved fragments for adhesion assays. As expected, thrombin cleaved osteopontin in two overlapping fragments of essentially the same molecular mass, which were also the same mass as nOPN. This band was separated in 15% polyacrylamide gel (data not shown). In addition to the 166G-167L cleavage site, MMP-3 cleaves two other sites in the C-terminal fragment of osteopontin (Agnihotri et al., 2001). MMP-3 cleavage was incomplete and generated several fragments, one of which was the same molecular mass as nOPN-dLR. Wells of cell adhesion plate were coated with these protease-treated fragments. Assays with these cleaved-fragments demonstrated the same findings as we observed for recombinant fragments (Fig. 7A, B).

DISCUSSION

In the present study, we have identified a new osteopontin receptor, the integrin αvβ6, that, like the integrins αvβ3, αvβ5 and α5β1 recognizes the RGD site in osteopontin. At least one other integrin, α9β1 also binds to osteopontin, but recognizes a distinct sequence adjacent to the RGD site. By utilizing a variety of recombinant fragments of osteopontin, we were able to identify specific sequence requirements for each of these 5 integrin osteopontin receptors. αvβ3 recognized all of the mutant fragments we generated, as long as the RGD sequence remained intact. αvβ5 and αvβ6 were both more sensitive to mutations in the sequence adjacent to the RGD site, but all

three of these αv-integrins could bind equally well to intact osteopontin or to the N-terminal fragments generated by thrombin or MMP-mediated cleavage. Interestingly, proteolytic cleavage had important effects on osteopontin binding of both α5β1, which recognizes the same RGD site as the αv-integrins and α9β1, which does not bind to this site. In both cases, adhesion was minimal to intact full-length osteopontin, was greatly enhanced by thrombin-mediated cleavage and was inhibited by MMP-mediated cleavage. These results suggest that proteolytic cleavage of osteopontin can substantially impact the specific integrin receptors that cells use to detect and respond to osteopontin.

The thrombin cleavage site is downstream from the RGD sequence by 7 residues, SVVYGLR (Fig. 4) (Senger, 1994). We have previously reported that either deletion of 2 residues, LR (nOPN-dLR), or alanine-replacement of the tyrosine in the SVVYGLR sequence (nOPN-Y165A) of nOPN abolishes integrin α9β1 mediated adhesion (Yokosaki et al., 1999). After we reported the SVVYGLR sequence, osteopontin was found to be a substrate for MMP-3 and MMP–7 (Agnihotri et al., 2001). A cleaved fragment of either MMP-3 or MMP-7 was identical to nOPN-dLR. Although this MMP-cleavage did not affect binding of αv-integrins, α5β1-mediated adhesion was inhibited either by the nOPN-dLR deletion or nOPN-Y165A, like α9β1. Thus the osteopontin SVVYGLR sequence appears to be required for optimal α5β1 binding. α5β1 has been most extensively characterized as a receptor for fibronectin (Mould et al., 2000; Obara et al., 1988; Pierschbacher and Ruoslahti, 1984). In that case, interaction with an RGD site is also necessary, but not sufficient for optimal α5β1-mediated adhesion. In addition to the classical synergy site, PHRSN (Aota et al., 1994), other sites that enhance α5β1-mediated binding, including TVRYYR (SVRYYR in mouse) (Redick et al., 2000) have been described. Interestingly, α5β1 mediated adhesion was reduced when fOPN was used as a substrate. This is consistent with a previous report that α5β1 on K562 cells binds to thrombin-cleaved but not to full length osteopontin (Barry et al., 2000). The integrin α9β1 recognizes the SVVYGLR sequence in osteopontin and we have previously reported that it does not bind to full length osteopontin, suggesting that the SVVYGLR site is cryptic in full length osteopontin and exposed upon thrombin cleavage (Yokasaki and Sheppard, 2000). The enhancement of α5β1 binding to osteopontin by thrombin-cleavage and reduction by MMP-cleavage within the SVVYGLR support this idea that the SVVYGLR sequence serves as a

synergy site for α5β1 binding to osteopontin.

Integrin αvβ6 is abundant in developing epithelial organs, but expression is limited in healthy adult epithelia (Breuss et al., 1995). In response to tissue injury or inflammation αvβ6 is commonly highly induced (Hakkinen et al., 2004; Miller et al., 2001; Sawada et al., 2004). Osteopontin is also expressed at low levels in healthy adult organs, but dramatically induced in the setting of injury (Iguchi et al., 2004; Isoda et al., 2002; Takahashi et al., 2004; Wang et al., 2000). There is some evidence that expression of osteopontin and αvβ6 might be coordinately regulated in response to injury. For example, treatment of mice with intratracheal bleomycin, a drug that causes acute lung injury and inflammation, dramatically induces pulmonary expression of both osteopontin (Kaminski et al., 2000) and αvβ6 (Munger et al., 1999). However, whereas osteopontin was among the most highly induced genes in wild type mice treated with bleomycin, osteopontin was not induced in mice homozygous for a null mutation in the β6 gene, suggesting that induction of osteopontin may, in some cases, be regulated by the αvβ6 integrin. At the very least, osteopontin and αvβ6 are often coordinately expressed in developing, injured or inflamed epithelial organs.

Osteopontin has been suggested to contribute to a wide array of biological and pathological responses (Denhardt et al., 2001; Diao et al., 2004; Gravallese, 2003; Khan and Kok, 2004; Kyriakides and Bornstein, 2003; O'Regan, 2003). From this paper and several others, it is now clear that several members of the integrin family can serve as osteopontin receptors, including αvβ1, αvβ3, αvβ5, αvβ6, α4β1, α5β1, α8β1 and α9β1. Thus, some of the diversity of osteopontin function might be regulated by the integrin repertoire of the responding cells. In addition, as we have shown, the two proteolytic cleavage sites within the region of osteopontin containing all of the apparent integrin recognition sequences is likely to allow for further regulation of osteopontin function through extracellular processing by proteases. Finally, osteopontin is a highly flexible molecule (Fisher et al., 2001; Helluin et al., 2000), which might be able to adopt different conformations depending on interactions with additional proteins in the extracellular space. It is thus conceivable that the distinct structural requirements we have identified for interaction with several members of the integrin family could provide an additional level for regulation of osteopontin function.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies and Reagents

Stably transfected SW480 cells (human colon carcinoma) with either the expression plasmids pcDNAIneoβ6 (Weinacker et al., 1994), pcDNAIneoβ3 (Yokosaki et al., 1996), pcDNAIneoα9 (Yokosaki et al., 1994) or the empty vector pcDNAIneo, CHO cells that secrete truncated integrin αvβ6 (Weinacker et al., 1994) and human lung cancer cells UCLA P3 cells were from Dean Sheppard (UCSF, San Francisco, CA). Anti-αvβ5 mAb P1F6 (Weinacker et al., 1994), anti-αvβ6 E7P6 (Weinacker et al., 1994), 10D5 (Huang et al., 1998) anti-α5β1 P3D10 (Setty et al., 1998) and anti-α9β1 Y9A2 (Wang et al., 1996) were also gifts from Dean Sheppard. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 1 mg/ml of the neomycin analog, G418 (Invitrogen, Grand Island, NY). GRGDSP peptide was purchased from Invitrogen (Grand Island, NY), thrombin from Amersham Biosciences (Piscataway, NJ), MMP-3 from Sigma (St. Louis, MO). Antibody LM609 against αvβ3 was from Chemicon (Temecula, CA). Phycoerythrin-conjugated anti-α5, IIA1, was from BD Biosciences (San Jose, CA). cDNA encoding osteopontin (Saitoh et al., 1995) was from Yoshiki Saitoh (Kumamoto University, Kumamoto, Japan). [^{35}S]methionine and [^{35}S]cysteine cell labeling mixture was purchased from Amersham Biosciences (Piscataway, NJ).

Cell adhesion assays

Wells of non-tissue culture treated polystyrene 96-well flat-bottom microtiter plates (Nunc Inc., Naperville, IL) were coated by incubation with 100 µl of osteopontin in phosphate buffered saline at 37°C for 1 hour. For blocking experiments cells were incubated in the presence or absence of soluble peptide or monoclonal antibody on ice for 15 minutes before plating. Wells were washed with phosphate buffered saline, then blocked with 1% bovine serum albumin in DMEM. 50,000 cells were added to each well in 200 µl of serum-free DMEM containing 0.5% bovine serum albumin. Plates were centrifuged at 10 x *g* for 1 minute, then incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed by centrifugation topside down at 48 x *g* for 5 minutes. The attached cells were fixed with 1% formaldehyde, stained with 0.5% crystal violet, and excess dye was washed off with phosphate

buffered saline. The cells were solubilized in 50 ml of 2% Triton-X-100 and quantified by measuring the absorbance at 595 nm in a Microplate Reader (TECAN, Maennedorf, Switzerland). In each experiment wells were coated with BSA only of which absorbance values were subtracted from those of test wells. The subtracted values were not over 0.08 throughout the experiment.

Expression of recombinant osteopontin fragments

Recombinant osteopontin fragments were produced as previously described (Yokosaki et al., 1998). Briefly, cDNA encoding thrombin-cleaved N-terminal osteopontin fragment (nOPN) was amplified from the full length cDNA in pCRII by Polymerase Chain Reaction with restriction site-tagged primers that amplify the same region as previously described (Smith et al., 1996), and then cloned between the *Bam*H1 and *Eco*RI sites within the multiple cloning site of pGEX6P2 plasmid (Amersham Bioscience, Piscataway, NJ). cDNA for full length osteopontin (fOPN) was amplified with the same forward primer as above and reverse encompassing the coding region, and cloned between the *Bam*H1 and *Xho*1 sites of the pGEX6P2. One isoform, OPNa, that contains each exon (Yokosaki et al., 1999) was used throughout this study. Wild type or variant recombinant osteopontin proteins were prepared by bacterial expression as recommended by the manufacturer. Briefly, competent DH5α cells were transformed by heat shock and grown on ampicillin-containing plates. Individual colonies were picked and propagated overnight in 2 ml of 2 x YT medium with 100 μg/ml of ampicillin at 37°C. 100 ml of 2 x YT medium was inoculated with 1 ml of the bacteria, and incubated at 30°C until OD600 reached 0.5-2, at which time isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. Cultures were grown for several more hours, cells were collected and sonicated, and glutathione S-transferase fusion proteins were affinity purified with glutathione Sepharose 4B beads and then cleaved off from glutathione S-transferase with PreScission protease (Amersham Bioscience, Piscataway, NJ) into Tris-buffer or phosphate buffered saline (PBS). Concentrations of recombinant proteins were determined by the Bradford assay (Pierce, Rockford, IL) using Bovine serum albumin (BSA) as a standard. Purity of the product was confirmed by 12.5% SDS-Polyacrylamide gel electrophoresis followed by Comassie Blue staining.

Mutagenesis

Site directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as previously described (Yokosaki et al., 1998). Both strands of the expression plasmid were replicated by PCR using pfuDNA polymerase with two complementary primers designed to introduce the desired mutation. The amplification product was treated with *Dpn*I endonuclease, specific for methylated DNA, to digest the parental DNA template. Then DH5α competent cells were transformed with the PCR-generated nicked plasmid. Plasmids from several isolated colonies were prepared by QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and inserts were sequenced by ABI3100 sequencer (Applied Biosystems, Foster City, CA) with primers flanking the polylinker of the pGEX vector. The verified mutated inserts were subcloned into pGEX vector that had not been amplified by PCR.

Affinity Chromatography

BSA or recombinant nOPN was coupled to Sepharose beads (Amersham Bioscience, Piscataway, NJ) for 4 hours at 4 °C. Secreted αvβ6 was metabolic-labeled and produced by CHO cells transfected with truncated αv and β6 cDNAs as described (Weinacker et al., 1994). After affinity matrices were blocked with 0.5 M monoethanolamine and 0.5 M NaCl buffer with PH 8.3, and washed with 0.1 M sodium acetate and 0.5 M NaCl, followed by PBS, culture medium of the αvβ6-secreting CHO cells was passed thorough the affinity columns. The bound protein was washed with column buffer then eluted with 20 mM ethylenediamine tetraacetic acid (EDTA) in PBS. The eluted fraction was run on the 7.5 % polyacrylamide gel followed by a fluorography.

Flow cytometry

Expression of integrins αvβ3, αvβ5, αvβ6, α5β1 or α9β1 was analyzed by flow cytometry. Cells were incubated with goat serum and washed with PBS. Then cells were incubated with either antibody LM609, P1F6, E7P6 or Y9A2 for 20 minutes at 4 °C for staining αvβ3, αvβ5, αvβ6, or α9β1, respectively, followed by incubation with secondary phycoerythrin-conjugated goat anti-mouse IgG. α5β1 was stained with phycoerythrin-conjugated IIA1 for 20 minutes at 4 °C. After cells were washed with

PBS, the expressions were then quantified on 10^5 cells with a FACSCalibur (BD Biosciences, San Jose, CA).

Enzyme cleavage of osteopontin

fOPN was subjected to enzyme reaction with thrombin or MMP-3 at 37°C for 15 to 120 minutes in cleavage buffer (200 mM of NaCl, 50 mM of Tris-HCl, pH7.6, 5mM $CaCl_2$) as previously described (Agnihotri et al., 2001). The products were separated by SDS-PAGE and stained with Comassie Blue.

ACKNOWLEDGEMENTS

We are grateful to Dr. Dean Sheppard for his generous support of cell lines and antibodies as in experimental procedures. We thank the Research Center for Molecular Medicine and Analysis Center of Life Science, Hiroshima University for the use of their facilities. This work was supported in part by Health and Labour Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labour and Welfare (to KY and YY), and by a grant for integrin therapeutic research from Astellas Pharma Inc (to YY), a grant from Ministry of Education, Science, Sports and Culture (to FY).