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Award Number: DAMD17-00-1-0363

TITLE: Elucidation of Pertussis Toxin-Sensitive Migration  
Signaling in Human Breast Cancer Cells

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REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20010926 138

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

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<b>1. AGENCY USE ONLY (Leave blank)</b>	<b>2. REPORT DATE</b> May 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 May 00 - 30 Apr 01)
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<b>4. TITLE AND SUBTITLE</b> Elucidation of Pertussis Toxin-Sensitive Migration Signaling in Human Breast Cancer Cells	<b>5. FUNDING NUMBERS</b> DAMD17-00-1-0363
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<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>
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<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012
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<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>
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<b>11. SUPPLEMENTARY NOTES</b>
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<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited
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<b>12b. DISTRIBUTION CODE</b>
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<b>13. ABSTRACT (Maximum 200 Words)</b>  The long range goal of this laboratory is to identify integrin-associated signaling events that contribute to the constitutive migration of human breast cancer cells on the laminin extracellular matrix proteins. This project has shown that the heterotrimeric G-protein $G\alpha i3$ may link integrin activation and migration of MCF-10A cells via a cAMP signaling pathway. The focus of the remaining work on this project is to identify how each integrin subtype which binds laminin-1 contributes to the regulation of this and other pro-migratory signaling pathways.
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<b>14. SUBJECT TERMS</b> Metastasis, Extracellular Matrix, signal transduction
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<b>15. NUMBER OF PAGES</b> 15
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<b>16. PRICE CODE</b>
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<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified
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<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified
---

<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified
--

<b>20. LIMITATION OF ABSTRACT</b> Unlimited
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## Table of Contents

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>5</b>
<b>Reportable Outcomes.....</b>	<b>6</b>
<b>Conclusions.....</b>	<b>6</b>
<b>References.....</b>	<b>N/A</b>
<b>Appendix A.....</b>	
<b>Appendix B.....</b>	

Award Number DAMD17-00-1-0363

Annual Report for the period 5/00-4/01

Title: Elucidation of pertussis toxin-sensitive migration signaling in human breast cancer cells.

Author: William L. Rust

5/17/01

### **Introduction:**

The long range goal of our lab is to describe cellular mechanisms that contribute to the metastatic potential of breast cancer cells for the purpose of identifying targets for chemotherapy development. The specific focus is to identify signaling events that contribute to the migration of breast cancer cells on the extracellular matrix laminin proteins. Cells interact with laminins primarily through integrin receptors. The objectives of the supported project are to identify the role of pertussis toxin sensitive heterotrimeric G-proteins in integrin-associated signaling that leads to enhanced migration of normal and malignant breast cells on laminin. While the focus and goals of the project remain the same, the tasks of the work have changed to reflect the incorporation of a more informative model system.

### **Body:**

The first objective of the project, to identify the heterotrimeric G protein  $G\alpha i3$  as a component of the migration signaling pathway stimulated by engagement of  $\beta 1$  integrin to laminin-5 was accomplished (appendix A). We demonstrated that stimulation of the  $\beta 1$  integrin by the activating antibody TS2/16 both enhanced migration of non-migratory breast cells on laminin-5, and induced a concurrent rise in cAMP production. The enhanced migration could be mimicked by application of non-hydrolyzable cAMP analogs, and could be blocked by inhibitors of cAMP dependent protein kinase or the  $G\alpha i$  and  $G\alpha s$  classes of heterotrimeric G-proteins. Furthermore, we demonstrated that pertussis toxin, an inhibitor of the  $G\alpha i$  class, specifically ADP ribosylates  $G\alpha i3$  in our model cell line (MCF-10A).

The tasks outlined in the original project focused on examining the effects of wild-type and dominant-negative mutant  $G\alpha i3$  over-expression on migration, cAMP turnover, and mitogen activated protein kinase activity in MCF-10A cells plated on laminin-5. Although we demonstrated that  $\beta 1$  integrins were responsible for the observed migration and signaling (Appendix A), it is possible that differences in signaling and migratory behavior of cells on laminin may be due to the specific subset of  $\beta 1$  integrins engaged by a particular cell type. Four  $\beta 1$  integrin subtypes ( $\alpha 1\beta 1, \alpha 2\beta 1, \alpha 3\beta 1$  and  $\alpha 6\beta 1$ ) are known to bind laminins. We therefore decided that a model which incorporates the activation of specific  $\beta 1$  integrin subtypes individually by laminins would be more informative for studying the intracellular signaling responses stimulated by integrin-laminin engagement. To that end, the tasks outlined in the original proposal have been changed from producing  $G\alpha i$  transfected cell lines to that described below.

Task 1: Identify the integrins subtypes used by MCF-10A cell to engage laminin-1. Time frame = 1 month.

Task 2: Design, produce, and purify candidate domains of laminin-1 that are likely binding partners for the integrins identified in Task 1. Time frame = 1 year.

Task 3: Identify the domains that are bound by the individual integrins identified in Task 1. Time frame = 1 month.

Task 4: Identify the contribution of each particular integrin/domain combination to cell binding morphology. Cell binding morphology is defined as strength of adhesion, cell spreading, focal adhesion formation, and stress fiber formation. Time frame = 3 months.

Task 5: Quantify the contribution of each integrin/domain combination to cAMP signaling through G $\alpha$ i, and other known signaling events stimulated by integrin engagement. Specifically, the phosphorylation of focal adhesion kinase and intracellular calcium flux. Time frame = 1 year.

#### **Key Research Accomplishments:**

1. Identification of the heterotrimeric G protein G $\alpha$ i3 as a component of the migration signaling pathway stimulated by engagement of  $\beta$ 1 integrin to laminin-5. Demonstration that cAMP modulation through G $\alpha$  proteins is required and sufficient for enhanced migration of MCF-10A cells on laminin-5 (Appendix A).

2. Identification of the integrins subtypes used by MCF-10A cell to engage laminin-1. The integrins  $\alpha$ 1 $\beta$ 1,  $\alpha$ 3 $\beta$ 1, and  $\alpha$ 6 $\beta$ 1 contribute to adhesion of MCF-10A cells on laminin-1 (Appendix B, figure 1).

3. Design, production, and purification of candidate domains of laminin-1 that are likely binding partners for  $\alpha$ 1 $\beta$ 1,  $\alpha$ 3 $\beta$ 1, and  $\alpha$ 6 $\beta$ 1 integrins. Seven domains of laminin-1 were recombinantly produced in a baculovirus expression system. The late viral promoter gp64 was used, and each domain was appended with a secretion signal peptide and a 6X polyhistidine tag. Purification was accomplished with metal-chelate anion chromatography. (Appendix B, Table 1). The major problem encountered in achieving this task was the low yield of protein in our expression system. While the baculovirus system generally achieves a yield of 1-2 mg/L of protein, the yield of our proteins ranges from 30-300  $\mu$ g/L. This result is a combination of the following factors: secreted proteins generally provide lower yield, late viral promoters provide lower yield than very late viral promoters, small protein size.

**Reportable Outcomes:**

1. Publication of a manuscript describing cAMP-dependant migration of breast cells on laminin-5 (Plopper G.E., Huff, J.L., Rust, W.L., Schwartz, M.A., Quaranta, V. (2000) *Molecular Cell Biology Research Communications* 4, 129-135, Appendix A)
2. Production of seven novel recombinant proteins (Appendix B, Table 1).
3. Employment opportunities based on experience and training supported by this award. I anticipate graduating with my Ph.D. within one year, and must decide between two post-doctoral job opportunities already offered to me. These are with the Harvard Institutes of Medicine in Boston, Massachusetts and the Pasteur Institute in Paris, France.

**Conclusions**

The objectives and training goals of the project have not changed and have been met according to the originally proposed summary table. The original tasks have been changed to reflect the incorporation of a more informative model system. Instead of describing G protein-linked signaling events that are a consequence of pan-specific  $\beta 1$  integrin binding to laminin, I will now describe G-protein-linked signaling events that are a consequence of specific  $\beta 1$  integrin subtype binding to laminin. The model has been constructed and I am on the verge of establishing the contribution of three distinct integrins to G protein-linked signaling.

Cell migration is an integral part of tumor cell metastasis. Cell migration is largely controlled by, and dependant upon, tumor cell interaction with extracellular matrix proteins via integrin receptors. The completion of the outlined project is likely to identify novel targets for chemotherapy design. The incorporation of the integrin-specific model system also has the advantage of suggesting mechanisms for differences in migratory potential between cell lines and differences in metastatic target tissues between cell lines. In addition, these studies are relevant to the pre-doctoral training I require to prepare me for an industrial career in developing new anti-cancer therapies.

## Antibody-Induced Activation of $\beta 1$ Integrin Receptors Stimulates cAMP-Dependent Migration of Breast Cells on Laminin-5

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Received January 8, 2001

**The  $\beta 1$  integrin-stimulating antibody TS2/16 induces cAMP-dependent migration of MCF-10A breast cells on the extracellular matrix protein laminin-5. TS2/16 stimulates a rise in intracellular cAMP within 20 min after plating. Pertussis toxin, which inhibits both antibody-induced migration and cAMP accumulation, targets the Gai3 subunit of heterotrimeric G proteins in these cells, suggesting that Gai3 may link integrin activation and migration via a cAMP signaling pathway.** © 2000 Academic Press

**Key Words:** extracellular matrix; metastasis; signal transduction.

Laminins are a diverse group of heterotrimeric extracellular matrix proteins that constitute a major component of the basement membrane of epithelial tissues. The laminin-5 isoform, consisting of the  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  subunits, is abundantly expressed in the basement membrane of breast tissue [1] where it plays a role in mammary branching morphogenesis, and adhesion and migration of breast epithelial cells [2].

Evidence from both *in vitro* and *in vivo* studies support a functional role for laminin-5 in cell migration of both normal and malignant breast epithelial cells. Our laboratory has previously shown that *in vitro*, laminin-5 is the preferred adhesive substrate for breast epithelial cells [1]. In haptotactic migration assays, nontumorigenic breast cell lines fail to migrate significantly on laminin-5, whereas laminin-5 supports migration of highly malignant breast cell lines. *In vivo*, laminin-5 expression is enhanced in invading regions of metastatic breast tumors [3]. In addition, an altered conformation of laminin-5, resulting from proteolytic cleavage of the  $\gamma 2$  chain by matrix metalloprotease 2, is

found at sites of tissue invasion, and this cleavage stimulates migration of otherwise nonmigratory breast cells *in vitro* [4]. Laminin-5 may contribute to the progression of tumorigenic breast cells from the stationary to malignant phenotype by stimulating enhanced migration of these cells.

Cells interact with laminins primarily through integrin receptors [5]. Ligand induced signal transduction by integrin/laminin binding regulates intracellular pH, tyrosine phosphorylation, inositol lipid metabolism, and calcium ( $\text{Ca}^{2+}$ ) oscillations [6]. Signaling molecules known to associate with integrins receptors include protein tyrosine kinases, serine/threonine kinases, phospholipid kinases and lipases, ion channels, and members of the rho family of small molecular weight GTP binding proteins [6]. Laminin-5 is recognized by the  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 6\beta 4$  integrin receptors in a number of cell types, and the functional consequence of these interactions depend on the integrin receptor engaged. For example, ligation of laminin-5 with the  $\alpha 6\beta 4$  integrin receptor supports branching morphogenesis and hemidesmosome formation in breast epithelial cells [2], while interaction with  $\alpha 3\beta 1$  integrin supports migration of these same cells *in vitro* [7]. Little information is currently available on the specific signaling pathways triggered during these events.

While investigating the role of the  $\alpha 3\beta 1$  integrin in motility of breast epithelial cells, we observed that haptotactic migration of the immortalized breast epithelial cell line MCF-10A on laminin-5 was stimulated by direct activation of the  $\beta 1$  integrin receptor with the  $\beta 1$ -activating monoclonal antibody TS2/16. Migration was dependent on intracellular cAMP signaling, and TS2/16-promoted a rise in intracellular cAMP levels that occurred 20 min after plating on laminin-5. Migration and cAMP accumulation were inhibited by treatment of the cells with pertussis toxin, a compound that inactivates the  $\alpha$  subunit of the inhibitory class of heterotrimeric G proteins via ADP-ribosylation. We

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show that the *Gai3* isoform is a target for ribosylation by pertussis toxin in these cells. Together these data present evidence that the  $\beta 1$  integrin participates in the regulation of MCF-10A cell migration on laminin-5 through a cAMP-signaling pathway involving *Gai3*. This is the first description linking integrin activation to signaling through heterotrimeric G proteins.

## MATERIALS AND METHODS

### Cells

MCF-10A cells were maintained in DFCI medium according to Band and Sager [8]. MDA-MB-231 cells were cultured as described [1]. Rat 804G cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum and  $1 \times$  Glutamine Pen-Strep solution (Irving Scientific). 804G cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500g.

### Reagents

Mouse monoclonal antibodies against human integrin  $\alpha 3$  (clone PIB5) and  $\beta 1$  (Clone P4C10) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody clone P5D2 against human  $\beta 1$  integrin was purchased from Chemicon (Temecula, CA). Purified rat anti-mouse  $\beta 1$  antibody 9EG7 was purchased from Pharmingen (San Diego, CA), and dialysed against PBS to remove sodium azide. Mouse monoclonal anti-human, activating  $\beta 1$  integrin antibody TS2/16 (in ascites form) was generously provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-rat laminin-5 monoclonal antibody TR1 was produced in this laboratory [9]. Both TS2/16 and TR1 were purified with a protein G affinity chromatography kit (Pierce, Rockland, IL). SQ22536 was purchased from Biomol (Plymouth Meeting, PA) and pertussis toxin from List Biological Laboratories, Inc. (Campbell, CA). 8-Bromo-cAMP, dibutryl cAMP, H-89, and forskolin were purchased from Calbiochem (San Diego, CA).

### Adhesion and Migration Assays

Adhesion and migration assays were performed as previously described [1]. For anti-integrin antibody blocking experiments, antibodies were incubated with cells for 30 min before adding to assay wells, and were present throughout the assays.

### cAMP Determination

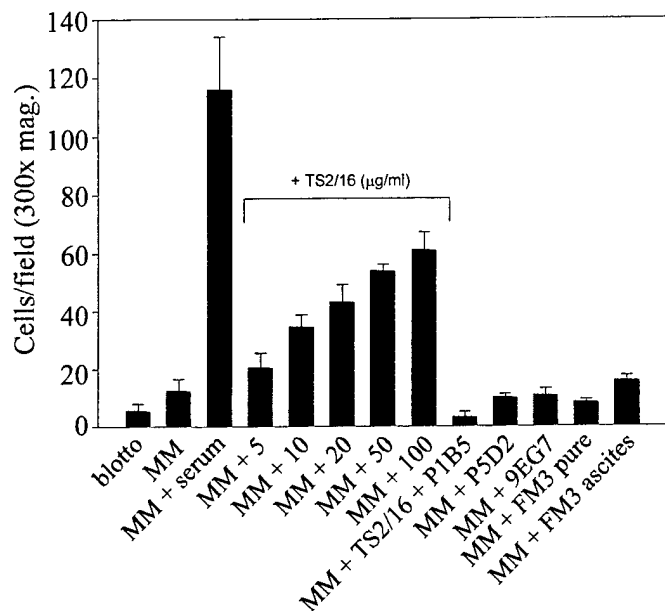
Cells were collected by brief trypsinization, blocked with trypsin inhibitor, washed in DMEM, counted, re-suspended at  $1 \times 10^6$  cells/ml, and incubated at 37°C in migration medium/1 mM isobutylmethylxanthine (Sigma) to block phosphodiesterase activity. After 30

min, anti-integrin antibodies (TS2/16 or P5D2) were added, and cells were incubated at 37°C for an additional hour. Control cells were suspended in DMEM/1 mM isobutylmethylxanthine alone during this time. Cells ( $1 \times 10^6$ /plate) were then plated on 35-mm dishes coated with affinity-captured laminin-5 [1] and incubated at 37°C for the indicated times. Cells representing the 0 time point were immediately retrieved from the dishes, collected by centrifugation, and lysed in cold cAMP extraction solution (95% ethanol, 5% 0.1 N HCl). After 10, 20, 30, and 90 min nonadherent cells were aspirated, plates were washed with PBS, and cAMP extraction buffer was added to the adherent cells. The PBS washes from each plate were centrifuged to collect loosely adherent cells, and these were added back to the appropriate extraction. All samples were kept on ice in cAMP extraction buffer for 2 h, then centrifuged to pellet precipitated protein. Protein was dissolved in 0.1 N NaOH and concentrations were determined with the BCA microassay (Pierce). Supernatants were evaporated and cAMP measured using a cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge, MA) as directed by the manufacturer. cAMP amounts were normalized to total protein in each sample and expressed as fmol/ $\mu$ g protein.

### ADP Ribosylation Assay

**Membrane preparation.** Membranes were isolated from MCF-10A cells by lysis in ice-cold 10 mM Hepes pH 7.5, 3 mM  $MgCl_2$ , 2 mM EDTA containing 10  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, and 0.5 mg/ml Pefabloc SC (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were scraped, centrifuged to pellet nuclei, and the supernatant was collected. Membranes were pelleted from supernatant by centrifugation at 13,000g for 30 min at 4°C; and the pellets were resuspended in lysis buffer. Protein concentrations were determined by BCA protein assay (Pierce).

**ADP-ribosylation and immunoprecipitation.** ADP-ribosylation reactions were performed as described [10]. Final reaction conditions were as follows: 100  $\mu$ g membrane protein was suspended in 20 mM thymidine, 1 mM ATP, 1 mM GTP, 1 mM EDTA, 20 mM Hepes, pH 7.5 with or without 7.5  $\mu$ g pertussis toxin (activated prior to experiment by incubation for 10 min at 37°C in 20 mM DTT, 20 mM Hepes, pH 7.5) and 25  $\mu$ Ci  $^{32}$ P-NAD (Specific activity = 30 Ci/mM, New England Nuclear catalog #BLU023). Reactions proceeded for 45 min at 30°C and were stopped by chilling to 4°C followed by a wash with 20 mM Hepes pH 7.5, 1 mM EDTA and 1 mM DTT. For SDS-PAGE analysis, membranes were solubilized in 50  $\mu$ l Laemmli sample buffer (LSB), heated for 5 min at 100°C and separated on a 12% SDS-polyacrylamide gel.  $^{32}$ P-labeled proteins were detected by autoradiography of dried gels using Kodak X-Omat AR film with intensifying screens. For



**FIG. 1.** The integrin activating antibody TS2/16 stimulates migration of MCF-10A breast cells on laminin-5. Indicated concentrations of TS2/16 were added to MCF-10A cells in a minimal medium lacking serum or other growth factors (MM) 15 min prior to plating in laminin-5 migration assays, and migrated cells were counted 18 h later. As controls, cells were plated in the presence of 10% serum, irrelevant mouse ascites (FM3 ascites), antibody purified from irrelevant ascites (FM3 pure), or nonfat dried milk (blotto). Results are expressed as the mean of eight measurements on two filters using 300 $\times$  magnification,  $\pm$  standard deviation.

immunoprecipitations, ribosylated membrane proteins were solubilized in RIPA buffer containing protease inhibitors and were incubated with the following G-protein  $\alpha$  subunit-specific peptide antibodies: I-20, specific for Gai1; C-10, specific for Gai3 (Santa Cruz Biotechnology). Immune complexes were captured by incubation with A/G agarose (Santa Cruz Biotechnology), solubilized by boiling in LSB and analyzed by SDS-PAGE as described.

## RESULTS AND DISCUSSION

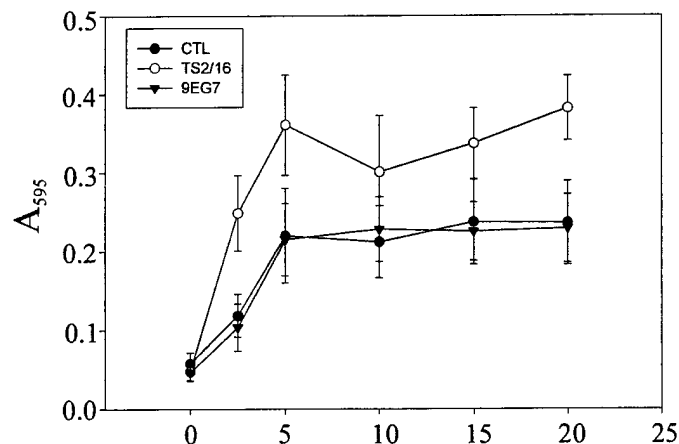
### *The $\beta$ 1 Integrin-Activating Antibody TS2/16 Stimulated MCF-10A Migration on Laminin-5*

The nontumorigenic breast cell MCF-10A remains statically adherent to laminin-5 via the  $\alpha$ 3 $\beta$ 1 integrin [1]. In haptotactic Transwell filter migration assays, these cells demonstrated only modest migration towards laminin-5. When preincubated with TS2/16, however, MCF-10A cells increased their migration in a dose-dependent manner towards laminin-5 (Fig. 1). TS2/16-treated cells also exhibited increased adhesion to laminin-5 (Fig. 2). These effects are not observed with other  $\beta$ 1 targeting antibodies (P5D2, 9EG7, Fig. 1; 9EG7, Fig. 2) or with TS2/16 on other substrates (data not shown). TS2/16 therefore stimulated a signaling

pathway that, concurrent with laminin-5 binding, led to enhanced cell migration. This pathway is dependent upon binding of the  $\alpha$ 3 $\beta$ 1 integrin, as pretreatment of the cells with the  $\alpha$ 3 integrin-blocking antibody P1B5 completely blocked TS2/16-stimulated migration on laminin-5 (Fig. 1).

In each experiment, maximum stimulation of cell migration was observed when cells were allowed to migrate towards a gradient of fetal calf serum. This control was included in each migration assay to indicate the dynamic range of migration response in each population of cells. It is likely that this chemotactic migration was stimulated by the growth factors present in fetal calf serum, as serum-induced migration was inhibited by greater than 80% when cells were preincubated with antibodies that block the function of the epidermal growth factor receptor or drugs that inhibit tyrosine kinases (G. E. Plopper, unpublished data). It is therefore likely that serum was a stronger promigratory stimulant than TS2/16 because it activated several signaling pathways stimulated by soluble growth factors, while TS2/16 targeted integrin-associated signaling pathways.

The strength of cell adhesion to extracellular matrix ligands varies over a wide range and is under the control of both intracellular and extracellular cues. Work by Lauffenburger [11] suggests that very tight or very loose cell adhesion to matrix proteins will not support cell migration, and that migration occurs only when a medium-strength of adhesion is achieved. Thus, varying the potency of adhesion of integrin receptors for their ligands may be a critical step for



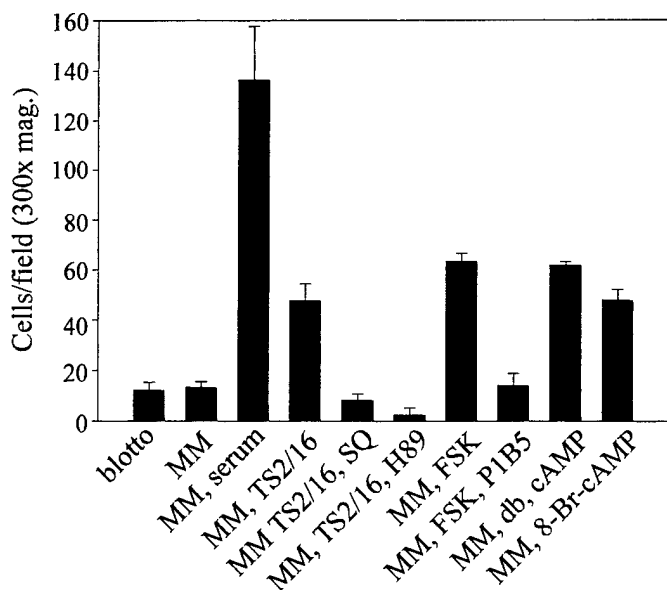
**FIG. 2.** TS2/16 antibody increases adhesion of MCF-10A cells to laminin-5. Cells were incubated in MM with 50  $\mu$ g/ml of TS2/16 or 9EG7 antibodies for 15 min, then were plated on affinity-captured laminin-5 for 30 min and adhesion quantified by measuring absorbance of crystal violet-dyed cells at 595 nm. Affinity capture was accomplished by successive addition of indicated concentrations of TR1 antibody, blotto, and 804G-conditioned medium containing soluble laminin-5. As a control (CTL), cells were incubated with no antibodies prior to plating. Results expressed as statistical mean  $\pm$  standard deviation ( $n = 8$ ).

regulating cell migration. It is possible that TS2/16 stimulated migration in these cells by changing the strength of adhesion between  $\alpha 3\beta 1$  integrin and laminin-5, either directly or via activation of internal signaling pathways.

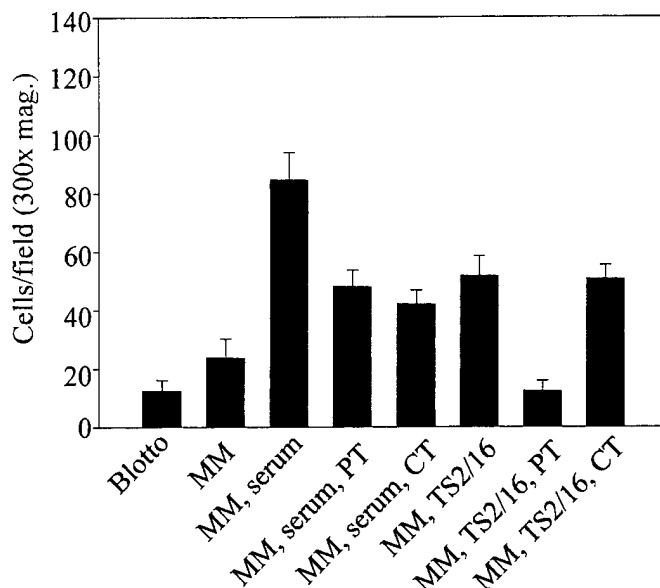
Alternatively, it is plausible that TS2/16 induced a conformational change in the  $\beta 1$  integrin that mimicked binding to a promigratory form of laminin-5, such as those created through proteolytic processing. For example, cleavage of the  $\gamma_2$  subunit of laminin-5 creates a conformation on which MCF-10A cells migrate constitutively [4, 12]. A promigratory laminin-5 can be converted to one that inhibits cell migration through cleavage of the  $\alpha 3$  chain [13]. In both instances it is assumed that proteolytic processing masks or unmasks a promigratory domain on the intact laminin-5 trimer. This theory is also supported by studies showing that integrin activation by TS2/16 will rescue the growth of MCF-10A cells inhibited by treatment with laminin-5 blocking antibodies [14].

#### MCF-10A Cell Migration on Laminin-5 Is Modulated by cAMP

To define the mechanisms by which TS2/16 stimulated MCF-10A cell migration on laminin-5, we added inhibitors of known signaling molecules to antibody-stimulated cells in haptotaxis migration assays. We found that SQ22536, an inhibitor of adenylate cyclase,



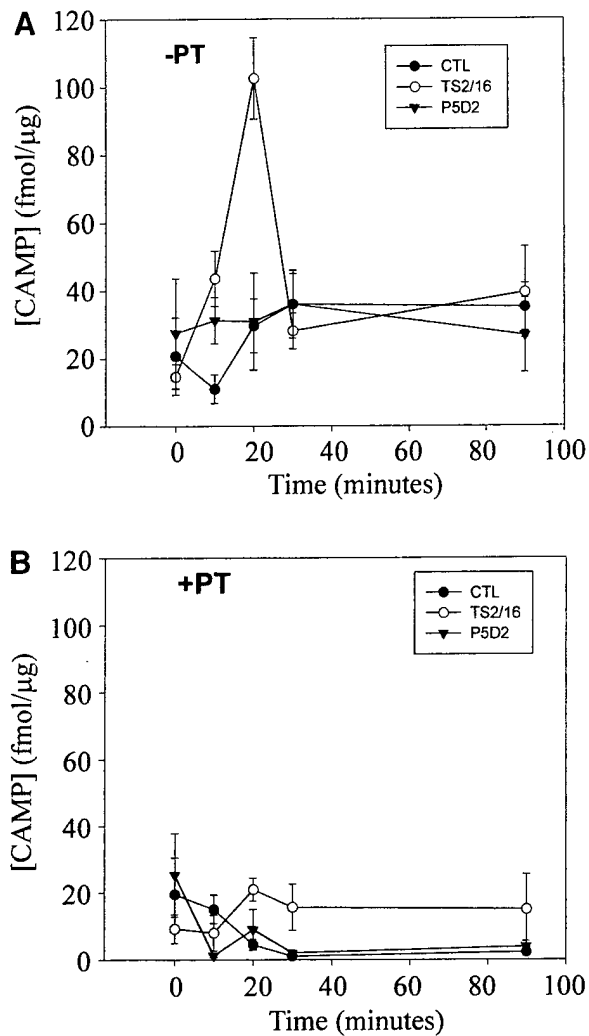
**FIG. 3.** Enhanced cAMP levels induce migration of MCF-10A cells on laminin-5. Cells were incubated in MM supplemented with 50  $\mu\text{g/ml}$  TS2/16, 50  $\mu\text{g/ml}$  PIB5, 250 mM SQ22536 (SQ), 4  $\mu\text{M}$  H89, 5 nM forskolin (FSK), 500  $\mu\text{M}$  dibutyryl cAMP (db cAMP), or 500  $\mu\text{M}$  8-bromo-cAMP (8-Br-cAMP) for 15 min prior to adding to laminin-5 migration assays. As a control, cells were plated in the presence of serum or in MM on filters lacking laminins (blotlo). Results are expressed as in Fig. 1.



**FIG. 4.** Pertussis toxin inhibits TS2/16-stimulated migration on laminin-5. MCF-10A cells were suspended for 30 min in MM supplemented with either 10% serum or 50  $\mu\text{g/ml}$  TS2/16. 100 ng/ml pertussis toxin (PT), 100 ng/ml cholera toxin (CT), were added 15 min prior to plating cells in laminin-5 migration assays. As a control, cells suspended in MM were added to filters coated with blotlo alone. Results expressed as in Fig. 1.

and H-89, an inhibitor of cAMP dependent protein kinase, completely blocked TS2/16 stimulated migration on laminin-5 (Fig. 3). In addition, pharmacological enhancement of cAMP levels with either forskolin or the nonhydrolyzable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP were sufficient to enhance migration of MCF-10A cells on laminin-5 to levels stimulated by TS2/16 (Fig. 3). Prolonged (18 h) exposure to pertussis toxin, a compound that inhibits the cAMP signaling pathway mediated by the  $G_{\alpha i}$  class of signaling proteins, abolished migration on laminin-5 (Fig. 4) and reduced cAMP levels in MCF-10A cells over the same time course (Fig. 5B). These data established that cAMP was required for enhanced migration of MCF-10A cells on laminin-5.

Because adenylate cyclase activity is governed by different classes of heterotrimeric G proteins we exposed MCF-10A cells to pertussis toxin (an inhibitor of the  $G_{\alpha i}$  class) and cholera toxin (an inhibitor of the  $G_{\alpha s}$  class). While both pertussis and cholera toxin partially blocked serum stimulated migration of MCF-10A cells (approximately 50%), only pertussis toxin blocked TS2/16 stimulated migration on laminin-5 (Fig. 4). These data demonstrated that the specific pathway triggered by TS2/16 and laminin-5 was susceptible to regulation by  $G_{\alpha i}$  rather than  $G_{\alpha s}$  proteins, and again suggest that serum-stimulated migration resulted from activation of multiple signaling pathways, some of which utilize cAMP as a second messenger.



**FIG. 5.** Pertussis toxin inhibits a cAMP peak in TS2/16-stimulated cells. MCF-10A cells were suspended in MM supplemented with 20  $\mu\text{g/ml}$  TS2/16 or P5D2 antibodies and plated on laminin-5 for the indicated time, then lysed and assayed for total cAMP content by ELISA assay. As a control, cells were plated in the absence of antibodies (CTL). The experiments were performed (A) in the absence (-PT) or (B) presence (+PT) of 100 ng/ml pertussis toxin. Results are normalized to total cell protein for each time point and represent the mean of triplicate measurements for four experiments,  $\pm$  the standard error of the means.

Not all compounds that induced a rise in cAMP stimulated migration on laminin-5. Cholera toxin induced a transient rise in cAMP in MCF-10A cells (G. E. Plopper, unpublished observations), and stimulated growth of these cells when used in small quantities (ng/ml) in the low-serum media for these cells (DFCI medium) [8]. It is generally thought that small quantities of cholera toxin stimulate growth in these cells by activating signaling pathways used by G protein-linked chemokines (Gary Bokoch, Department of Immunology, The Scripps Research Institute, personal communication). Higher concentrations of cAMP ( $\mu\text{g/ml}$ ) typically used to irreversibly activate *G $\alpha$ s* did not

stimulate haptotactic migration of MCF-10A cells on laminin-5 (G. E. Plopper, unpublished observations), suggesting that while cholera toxin does affect signaling in these cells, the pathways it affects do not play a role in integrin activated migration on laminin-5.

Chemotactic migration of many cell types is inhibitable by cholera and pertussis toxins [15, 16]. While pertussis toxin allows for unchecked cAMP production in the short term, prolonged pertussis toxin exposure suppressed cAMP levels in our cells, likely because of long-term desensitization of this pathway [17]. Although O'Conner *et al.* [18] reported that  $\alpha\beta4$  expression suppressed cAMP levels in migrating breast cancer cells, no evidence has been published linking cholera and pertussis-sensitive signaling pathways to integrin-activated signaling.

These findings are consistent with our observation that numerous chemokines that modulate cAMP through *G $\alpha$ s* (bombesin, bradykinin, adrenaline) raised cAMP levels but failed to stimulate migration in our cells (G. E. Plopper, unpublished data). Each of these compounds exerts very distinct responses in breast cells, suggesting that while they share cAMP as a second messenger, they must generate specificity elsewhere in their signaling pathways. The specificity necessary to modulate haptotactic migration may be generated by localizing cAMP bursts to specific times and/or locations within a cell, by targeting specific isoforms of adenylate cyclase, or by integrating cAMP bursts with other integrin-associated behaviors (e.g., formation of focal adhesions, generation of cellular tension, activation of signaling pathways linked to migration in other cell types [e.g., those that utilize rho/ras G proteins or focal adhesion kinase]) [19].

Indeed, such integration appears to take place in smooth muscle cells, which exhibit increased migration on collagen upon activation of a cAMP signaling pathway linked to integrin associated protein and  $\alpha2\beta1$  integrin; this activation also stimulates the mitogen activated protein kinase ERK, and is inhibited by pertussis toxin. [20]. In this study, migration is stimulated upon a reduction in cAMP levels and is inhibited by analogs of cAMP. The differences between these findings and ours may be attributed to differences in cell type, migratory stimulus, migratory substrate, and/or integrin receptor involved: we have observed that inhibition of  $\alpha2\beta1$  integrin stimulates haptotactic  $\alpha3\beta1$ -mediated migration in our cells, for example (G. E. Plopper, unpublished findings).

#### *TS2/16 Stimulated a Rise in Intracellular cAMP via a Pertussis Toxin-Sensitive Signaling Pathway*

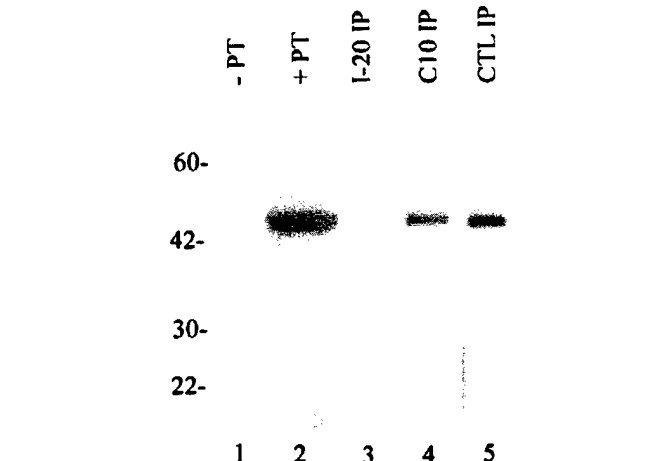
Since pertussis toxin alters intracellular cAMP levels, and cAMP modulation was sufficient to enhance migration in our cells, we examined the levels of cAMP in TS2/16-stimulated cells plated on

laminin-5. Within 20 min after plating, cAMP levels were raised approximately fourfold in TS2/16 treated cells. This peak occurred within the time frame of integrin signaling [6]. Enhanced cAMP accumulation was specific to TS2/16, and not a product of integrin clustering, as neither cells treated with the nonactivating  $\beta 1$  antibody P5D2 nor cells plated on laminin-5 without antibodies exhibited enhanced cAMP production (Fig. 5A). Preincubation with pertussis toxin completely eliminated this peak but did not significantly affect basal cAMP levels (Fig. 5B). Concurrent stimulation by laminin-5 adhesion and TS2/16 are required, as cAMP levels did not change in suspended cells treated with TS2/16 (G. E. Plopper, unpublished). It appeared, therefore, that the combination of intact laminin-5 and TS2/16 pretreatment stimulated a signaling pathway involving cAMP that was specifically blocked by pertussis toxin.

#### *Pertussis Toxin ADP-Ribosylated Gai3 in MCF-10A Cells*

Pertussis toxin ADP ribosylates the Gai class of heterotrimeric G proteins. To determine the repertoire of Gai subunits expressed in MCF-10As we performed Western blot analysis of whole cell lysates and isolated membrane fractions using polyclonal antibodies raised against specific G protein subunits. These studies revealed that MCF-10A cells expressed Gai1 and Gai3, but not Gai2 (data not shown). To establish the targets of pertussis toxin in these cells we carried out ADP-ribosylation assays in the presence of  $^{32}\text{P}$ -NAD. Addition of pertussis toxin specifically induced the ribosylation of a 43-kDa protein (Fig. 6, lane 2). No  $^{32}\text{P}$  labeled proteins are detectable without addition of pertussis toxin (Fig. 6, lane 1). The molecular weight of the ribosylated protein was consistent with that of the  $\alpha$  subunits of heterotrimeric G proteins. The identity of this protein was determined by immunoprecipitation of the ribosylated membrane proteins with Gai1 and Gai3 antibodies. Antibody C10, which reacts primarily with Gai3, immunoprecipitated a band of 43 kDa (Fig. 6, lane 4). The anti-Gai1 antibody I-20 failed to precipitate any ADP ribosylated proteins in MCF-10A cells (Fig. 6, lane 3), but did precipitate a 43-kDa band from a control cell line, MDA-MB-231 (Fig. 6, lane 5). Therefore, pertussis toxin ribosylated Gai3, but not Gai1 in MCF-10A cells.

In addition to controlling adenylate cyclase activity, Gai3 is associated with and activates amiloride-sensitive  $\text{Na}^+$  channels [21], which are expressed in many epithelial cells including breast. These channels are also regulated by the actin cytoskeleton [22] and cAMP dependent protein kinase [23], suggesting that Gai3 may link integrin-mediated actin polymerization, cAMP signaling, cAMP dependent protein kinase ac-



**FIG. 6.** Pertussis toxin specifically ADP ribosylates Gai3 in MCF-10A cells. 100  $\mu\text{g}$  of cell membranes were incubated with 25  $\mu\text{Ci}$   $^{32}\text{P}$ -NAD in the presence (lanes 2–4) or absence (lane 1) of 7.5  $\mu\text{g}$  activated pertussis toxin. Pertussis toxin-treated lysates were immunoprecipitated with anti-Gai1 (I-20, lane 3) or anti-Gai3 (C10, lane 4) antibodies. As a control, Gai1 was immunoprecipitated from  $^{32}\text{P}$ -NAD labeled lysates of pertussis toxin-treated MDA-MB-231 cells (CTL, lane 5). Migration of molecular weight standards is shown at left.

tivity, and amiloride-sensitive channel activation. Curiously, amiloride also suppresses lung metastases from breast tumors [24]; our data suggest that it may do so, at least in part, by inhibiting tumor cell migration.

In conclusion, we report that the  $\beta 1$  integrin-stimulating antibody TS2/16 induced migration of MCF-10A cells on laminin-5 that was dependent upon cAMP linked signaling. TS2/16 also stimulated a rise in intracellular cyclic AMP within 20 min after plating on laminin-5. Both the enhanced migration and cAMP peak were inhibited by pertussis toxin. Pertussis toxin targeted the Gai3 subunit of heterotrimeric G proteins in these cells. This evidence suggests that the  $\beta 1$  integrin participates in the control of MCF-10A cell migration on laminin-5 via a cAMP signal pathway regulated by Gai3. This form of signaling, beginning with an external stimulus of the integrin receptor, is referred to as "outside-in signaling" to differentiate it from changes in integrin function resulting from activation of internal signaling pathways [6]. We propose that TS2/16 mimics the effects of proteolytic processing of laminin-5 by forcing the  $\alpha 3\beta 1$  integrin into a conformation formed by binding promigratory forms of laminin-5. We are currently examining the effect of these proteolytic modifications on intracellular signaling activities in MCF-10A cells. Because acquisition of a migratory phenotype is required for malignant progression of tumorigenic breast cells, elucidating pathways involved in enhanced migration of breast may lead to discovery of novel targets for anticancer therapies.

## ACKNOWLEDGMENTS

This work was supported by the following grants: U.S. Army Breast Cancer Research Program Career Development Award BC971342, grants from the UNLV Office of Sponsored Programs to G.E.P., and National Institutes of Health (GM46902) to V.Q.

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## Appendix B: Figures and Tables

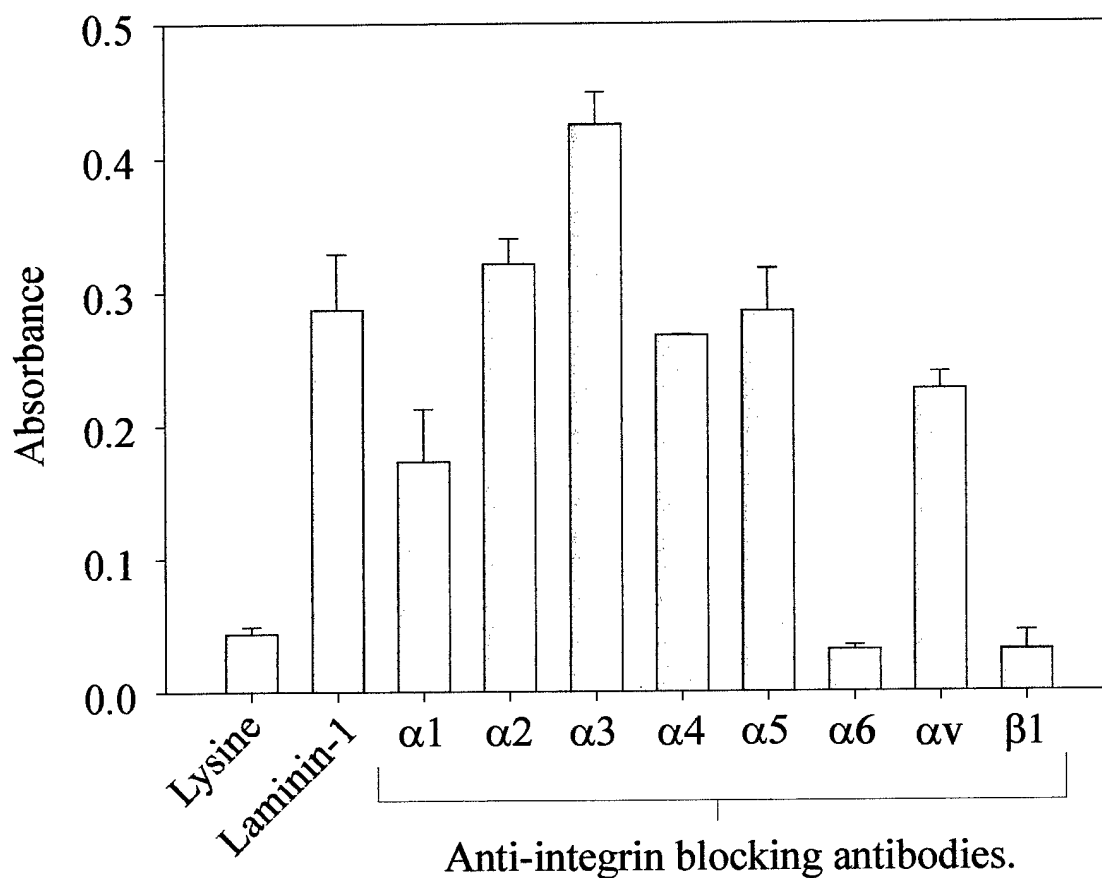


Figure 1. MCF-10A adhesion to laminin-1 in the presence of function blocking anti-integrin antibodies. MCF-10A cells engage laminin-1 with integrins  $\alpha1\beta1$ ,  $\alpha3\beta1$ , and  $\alpha6\beta1$ .

**Table 1. Laminin-1 domains produced in Baculovirus expression system.**

<b>Laminin-1 chain</b>	<b>Domain</b>	<b>Location (bp)</b>	<b>promoter</b>	<b>His tag</b>	<b>M.W. (kD)</b>	<b>Glycosylation</b>
$\alpha$	VI	111-723	gp64	C-term	32	yes
$\alpha$	IVb	1572-2326	gp65	C-term	42	yes
$\alpha$	G1	6640-7056	gp66	N-term	18	no
$\alpha$	G3	7377-8105	gp67	N-term	16	no
$\alpha$	G4	8079-8781	gp68	N-term	45	yes
$\alpha$	G5	8763-9321	gp69	N-term	28	yes
$\beta$	VI	96-907	gp70	C-term	unkown	unknown