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<b>13. ABSTRACT (Maximum 200 Words)</b> Integrins that bind to vitronectin are highly expressed in neovasculature and play an important regulatory role in angiogenesis. There are at least two cytokine-dependent pathways that lead to angiogenesis in vivo, and there is evidence indicating that they can be distinguished by their dependency on specific $\alpha$ integrins. Here we aim to define what molecules are involved in $\alpha$ v $\beta$ 3- and $\alpha$ v $\beta$ 5- selective angiogenic signaling. We hypothesize that: (i) molecules that associate with either of those integrins after angiogenesis is triggered by defined cytokines are different; (ii) the assembly of specific molecules associating with the $\beta$ 3 or $\beta$ 5 cytoplasmic domains results in selective signaling. The strategy that will be used to approach these questions is based on the panning of phage peptide libraries of $\beta$ 3 and $\beta$ 5 cytoplasmic domains. We will also investigate whether phosphorylation events can modulate the interaction with such integrin cytoplasmic domain-binding peptides. Finally, by using microinjection-based techniques, we will study the effect of integrin cytoplasmic domain binding peptides in cell adhesion, migration and proliferation upon stimulation with factors that activate endothelial cells in vitro. These studies will shed light into molecular basis of selective signal transduction pathways initiated by $\alpha$ v $\beta$ 3 and $\alpha$ v $\beta$ 5. New ways of inhibiting angiogenesis, and ultimately, new strategies to treat breast cancer may result from this work.				
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## INTRODUCTION

Angiogenesis, the formation of new blood vessels, is a requirement for malignant tumor growth and metastasis (1-3). In the absence of angiogenesis, local tumor expansion is suppressed at a few millimeters and cells lack routes for distant hematogenous spread. Clinical studies have demonstrated that the degree of angiogenesis is correlated with the malignant potential of several cancers, including breast cancer and malignant melanoma (4-7).

Integrin function is regulated by cytokines and other soluble factors in a variety of biological systems. Most commonly, exposure to such factors leads to conformational alterations that result in changes in the activation state of the receptors. Changes in integrin-dependent adhesion ultimately activates various complex signal transduction pathways. Cytoplasmic domains are key regulators of integrin function (8-14). Although the cytoplasmic domains of the various  $\beta$  subunits share similar primary structures, they differ in certain functional characteristics, since they are responsible for the regulation of receptor distribution and recruitment to the focal adhesion sites (15,16). Thus,  $\beta$  chain cytoplasmic domains are critical for integrin-mediated signaling into the cell (outside-in signaling) and activation of integrin-ligand binding activity (inside-out signaling) (9, 17-19).

Several lines of evidence have implicated the integrins  $\alpha v\beta 3$  and  $\alpha v\beta 5$  in the angiogenic process. It has been shown that  $\alpha v$  integrins are selectively expressed in angiogenic vasculature but not in normal vasculature (20,21). Moreover,  $\alpha v$  integrin antagonists can block the growth of neovessels through induction of endothelial cell apoptosis (22-26). Concordant with these findings, it appears that two distinct cytokine-dependent pathways that lead to angiogenesis depend on specific  $\alpha v$  integrins. Angiogenesis initiated by bFGF can be inhibited by an anti- $\alpha v\beta 3$  blocking antibody, whereas VEGF-mediated angiogenesis can be prevented by a blocking antibody against  $\alpha v\beta 5$ .

Although both  $\alpha v$  integrins bind to vitronectin, they are likely to mediate different post-ligand binding events. For instance, the integrin  $\alpha v\beta 5$  fails to promote cell adhesion, spreading, migration and angiogenesis in the absence of exogenous soluble factors. On the other hand, the  $\alpha v\beta 3$  can induce such events without additional stimulation by cytokines (27-29). Experiments designed to study the molecular basis for cytokine regulation of  $\alpha v\beta 5$  function have shown that upon binding to immobilized vitronectin, inactivated  $\alpha v\beta 5$  is barely detectable in association with actin,  $\alpha$ -actinin, talin, tensin, p130<sup>cas</sup> and vinculin. In contrast,  $\alpha v\beta 3$  induces the localized accumulation of such molecules. Upon activation of PKC,  $\alpha v\beta 5$  can then behave similarly to  $\alpha v\beta 3$ , but can not recruit talin (28). Moreover, calphostin C, an inhibitor of PKC, appears to block angiogenesis mediated by  $\alpha v\beta 5$ , but not  $\alpha v\beta 3$  (29). These observations suggest that PKC activation probably affects the conformation and/or phosphorylation state of the  $\beta 5$  cytoplasmic domain. Therefore, cellular events mediated by  $\alpha v\beta 3$  or  $\alpha v\beta 5$  are clearly controlled by different mechanisms (reviewed in 30-33).

Little is known about molecules associated to the cytoplasmic domains of  $\beta 3$  or  $\beta 5$  chain. Two-hybrid screenings with the  $\beta 3$  cytoplasmic domain provided evidence for a specific interaction between the  $\beta 3$  cytoplasmic domain and  $\beta 3$ -endonexin (34,35). A few other proteins have been reported to interact with  $\beta$  integrin cytoplasmic domains in general: talin (36), filamin (37),  $\alpha$ -actinin (38,39), focal adhesion kinase (40) and the serine/threonine kinase ILK (41), and skelemin (42). Many studies suggest that the molecules which associate with either  $\beta 3$  or  $\beta 5$  after the angiogenesis switch is triggered are different. We

hypothesize that the assembly of specific molecules associating with the respective cytoplasmic domains may explain their selective signaling properties.

## **PROPOSAL BODY**

**The tasks approved for this proposal are listed below:**

**Task 1.** To select peptides that bind specifically to  $\beta 3$  or to  $\beta 5$  integrin cytoplasmic domains. (Months 1-12)

- Construction of phage display peptide libraries and panning on  $\beta 3$  and  $\beta 5$  cytoplasmic domain fusion proteins.
- Characterization of  $\beta 3$  and  $\beta 5$  cytoplasmic domain-binding phage

**Task 2.** To investigate whether phosphorylation events can modulate the interaction of the selected peptides with integrin cytoplasmic domains. (Months 13-24)

- Biopanning using phosphorylated libraries
- Binding assays to investigate the affinity of novel phosphorylated peptides
- Investigate if the  $\beta 3$  binding peptides already isolated may be specifically phosphorylated *in vitro* by known protein-kinases involved in classical signal transduction pathways.
- Evaluate the specificity and affinity of the phosphorylated peptides

**Task 3.** To determine the biological properties of the cytoplasmic domain-binding peptides. (Months 25-36)

- Microinjection of cells with integrins  $\beta 3$  and  $\beta 5$  cytoplasmic domain-binding peptides after stimulation by selected angiogenic factors.
- Cell adhesion, migration and proliferation studies

***In the following pages, we report in significant progress on each specific aim during the past year.***

We offer experimental evidence to support the rationale and feasibility of this proposal. We introduce the peptide sequences that have been isolated after panning of phage libraries with the cytoplasmic domain of  $\beta 3$  and  $\beta 5$ .

First, we show data related to the specificity and binding properties of the peptides, including the ones isolated from Tyr-containing peptide libraries. Second, we show results from homology searches using the most promising peptides, and describe the development and testing of anti-peptide antibodies.

Third, we demonstrate that the penetratin peptide chimeras are internalized by tumor and endothelial cells. Finally, we present functional data that strongly support the notion that our peptides affect integrin function in a selective, specific and dose-dependent fashion.

*Phage display library screenings produce peptides that interact selectively with integrin cytoplasmic domains*

We have successfully panned phage display peptide libraries to identify peptides that interact with the cytoplasmic domain of  $\beta 3$  and/or  $\beta 5$  subunits.

**Panning of phage peptide libraries on  $\beta 3$  or  $\beta 5$  cytoplasmic domains.**

We have isolated  $\beta 3$  and  $\beta 5$  cytoplasmic domain-binding peptides, by screening multiple phage libraries with recombinant GST fusion proteins that contain either GST- $\beta 3$ cyto or GST- $\beta 5$ cyto coated onto microtiter wells. Immobilized GST was used as a negative control for enrichment during the panning on each cytoplasmic domain. Phage were sequenced from randomly selected clones after three rounds of panning as described elsewhere (43-45). We successfully isolated distinct sequences that interact specifically with the  $\beta 3$  or with the  $\beta 5$  cytoplasmic domains (Tables 1 and 2). Randomly selected clones from rounds II and III were sequenced. Amino acid sequences of the phagemid-encoded peptides were deduced from nucleotide sequences. The most frequent motifs found after panning with the indicated libraries are shown. The ratios were calculated by dividing the number of colonies recovered from  $\beta 3$ -GST-coated wells and those recovered from GST or BSA.

Table 1. Sequences displayed by phage binding to  $\beta 3$  integrin cytoplasmic domain; Table 2 Sequences displayed by phage binding to  $\beta 5$  integrin cytoplasmic domain.

Table 1

PEPTIDE MOTIF	$\beta 3$ /GST RATIO	$\beta 3$ /BSA RATIO
<i>CX<sub>6</sub> Library</i>		
CEQRQTQEGC	4.3	14
CARLEVLLPC	2.8	18.7
<i>X<sub>1</sub>YX<sub>1</sub> Library</i>		
YDWYYPWSW	5.6	163
GLDTYRGSP	4.1	48
SDNRYIGSW	3.3	32
YEWYYSWA	2.2	281
KVSWYLDNG	2.1	20
SDWYYPWSW	2.1	157
AGWLYMSWK	1.8	2.4
<i>Pool Cyclic Libraries</i>		
CFQNRG	3.1	16
CNLSSEQC	2.7	62
CLRQSYSYNC	2.4	3.2

Table 2

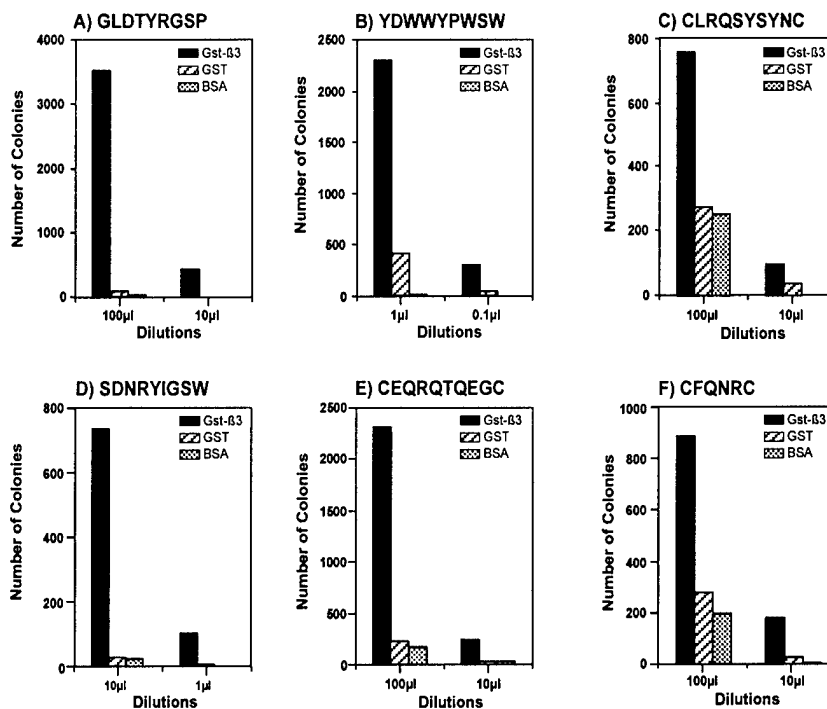
PEPTIDE MOTIF	$\beta 5$ /GST RATIO	$\beta 5$ /BSA RATIO
<i>Pool Cyclic libraries</i>		
CYIWPDSGLC	5.2	193
CEPYWDGWFC	3.1	400
CKEDGWLMTC	2.3	836
CKLWQEDGY	1.8	665
CWDQNYLDDC	1.5	100
<i>X<sub>1</sub>YX<sub>1</sub> Library</i>		
DEEGYMMR	11.5	29
KQFSYRYLL	4.5	8
VVISYSMPD	3.8	28
SDWYYPWSW	2.4	304
DWFSYYEL	1.7	153

selected on GST- $\beta 5$ cyto revealed several peptide motifs, as indicated in Table 3. Ratios were calculated as described.

The specificity of the interaction with  $\beta 3$  or  $\beta 5$  cytoplasmic domains was determined by calculating the ratios by using the number of phage bound to the cytoplasmic domain containing-fusion proteins ( $\beta 3$  or  $\beta 5$ ) and GST alone (the negative control). Figure 1

shows the results from binding assays performed with the GST- $\beta$ 3cyto binding phage. We have tested six phage that display the motifs more frequently found during the second and third rounds of panning. Each panel shows the results from binding assays for the phage displaying different peptides that bind to the  $\beta$ 3 cytoplasmic domain, as indicated. Insertless phage or unselected libraries were used as negative controls and do not show binding above background. Two plating dilutions are shown for each assay.

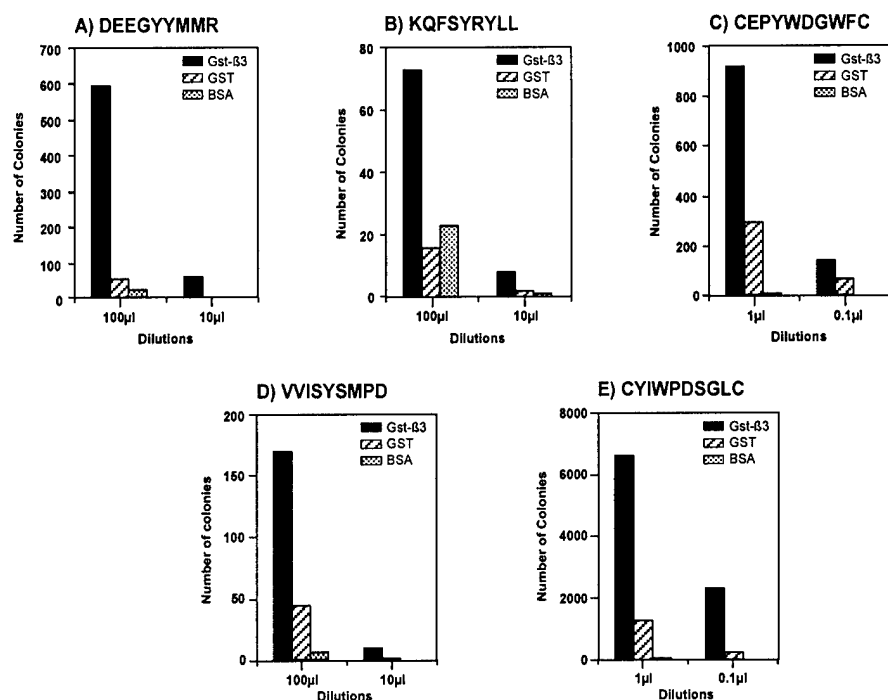
A similar strategy was used to determine the specificity of each phage isolated in the screenings involving the  $\beta$ 5 cytoplasmic domain fusion protein. The binding assays were performed with individually amplified phage. Figure 2 shows the results from binding assays performed with  $\beta$ 5 cyto binding phage. We have tested four phage displaying the motifs that were found more frequently during the second and third rounds of panning. Each panel shows the binding assays for the phage displaying peptides that bind to the  $\beta$ 5 cytoplasmic domain. Insertless phage or unselected libraries were used as negative controls and do not show binding above background in these assays.



**Figure 1. Binding of  $\beta$ 3 cytoplasmic domain-selected phage to immobilized proteins.**

GST fusion proteins or GST alone were coated on microtiter wells at 10  $\mu$ g/ml and used to bind phage expressing the peptides shown in Table 2 (each phage is identified by the peptide sequence it displays, i.e. GLD TYR GSP; YD W W Y P W S W; CLR Q S Y S Y N C; SD N R Y I G S W; CE Q R Q T Q E G C; CF Q N R C). The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

To determine whether the binding of the selected motifs was specific for each cytoplasmic domain, we performed binding assays comparing the interaction of individual phage motifs with  $\beta$ 1,  $\beta$ 3, or  $\beta$ 5 cytoplasmic domain fusion proteins (Table 3). We have determined by ELISA with anti-GST antibodies that the three proteins can be coated onto plastic at equivalent efficiency, and thus that the differences in binding do not reflect differences in coating concentrations. As shown in Figures 3A and 3B, both the  $\beta$ 3- and  $\beta$ 5-selected phage interact preferably with the proteins on which they were originally selected. None of the phage tested seem to bind strongly to the  $\beta$ 1 cytoplasmic domain.



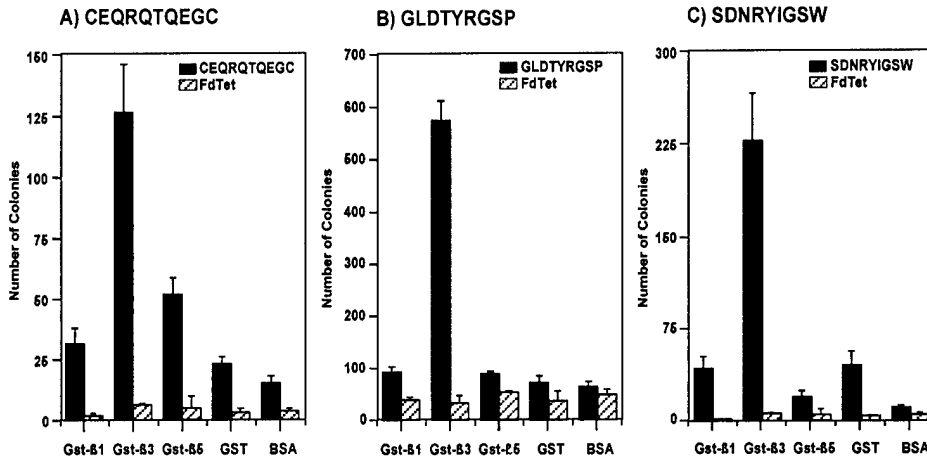
**Figure 2. Binding of  $\beta 5$  cytoplasmic domain-selected phage to immobilized proteins.** GST fusion proteins or GST alone were coated on microtiter wells at 10  $\mu\text{g/ml}$  and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). (A) VVISYSMPD; (B) KQFSYRYLL; (C) CEPYWDGWFC (D) CYIWPDSGLC; (E) DEEGYYMMR. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

**Table 3. Binding of  $\beta 3$  and  $\beta 5$  cytoplasmic domain-selected phage to immobilized proteins.**

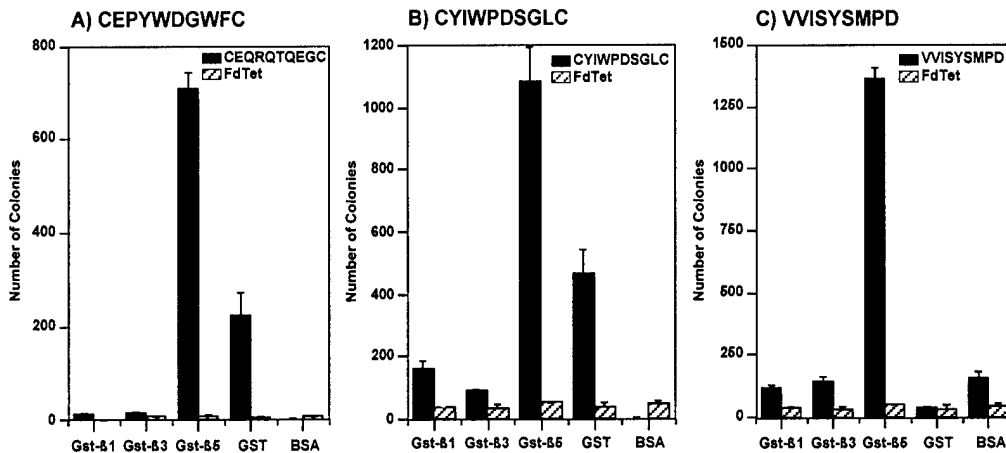
$\beta 3$ MOTIF	$\beta 3/\beta 1$ RATIO	$\beta 3/\beta 5$ RATIO	$\beta 3/\text{GST}$ RATIO	$\beta 5$ MOTIF	$\beta 5/\beta 1$ RATIO	$\beta 5/\beta 3$ RATIO	$\beta 5/\text{GST}$ RATIO
<i>CX<sub>9</sub> Library</i>				<i>Pool Cyclic Libraries</i>			
CEQRQTQEGC	3.6	2.4	8.3	CYIWPDSGLC	7	12	3.2
CARLEVLPC	2.2	1.4	2.8	CEPYWDGWFC	64	54	3.2
<i>X<sub>4</sub>YX<sub>4</sub> Library</i>				<i>X<sub>4</sub>YX<sub>4</sub> Library</i>			
SDNRYIGSW	5.4	9.5	15.1	DEEGYYMMR	1.2	2.7	11.4
GLD TYRGSP	6.4	6.5	10.4	DWFSYYEL	1.7	1.5	1.2
YEWYWSWA	2.1	1.3	2.2	VVISYSMPD	11.4	9.4	36.2
YDWWYPWSW	10.6	1.7	5.6	KQFSYRYLL	1.2	10.2	5.6
SDWYYPWSW	1.9	1.1	2.1				
<i>Pool Cyclic Libraries</i>							
CLRQSYSYNC	1.1	1.5	2.4				
CFQNRC	1.7	8	3.1				

Characterization of the synthetic peptides corresponding to the sequences displayed by the integrin-cytoplasmic domain-binding phage.

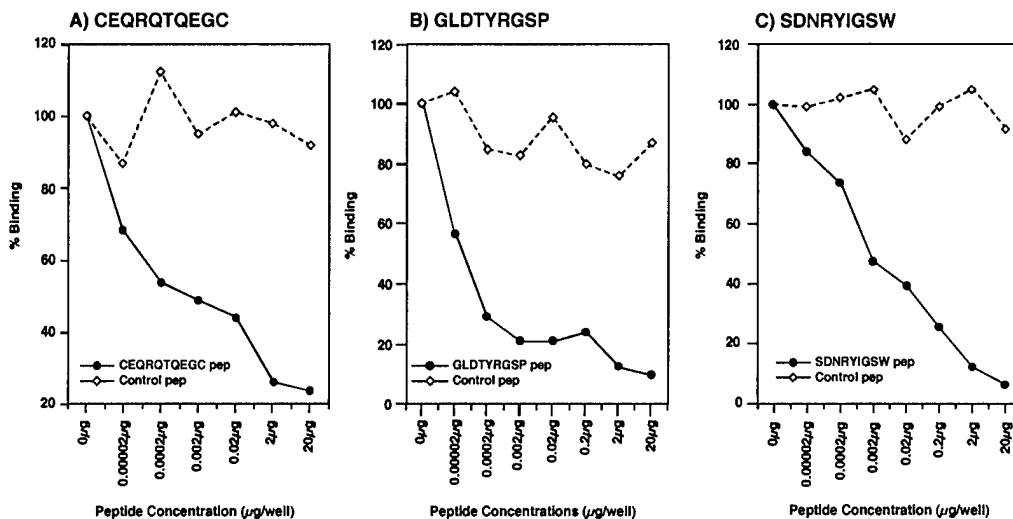
We selected specific phage for further studies on the basis of their binding properties. We used synthetic peptides corresponding to the sequence displayed by the phage to perform inhibitory studies. This assay is important because it determines whether phage binding is entirely mediated by the peptide displayed by the phage. As expected, we found that the synthetic peptides can inhibit the binding of the corresponding phage in a dose-dependent manner (Figures 4A and 4B). A control peptide containing unrelated amino acids had no effect on phage binding when tested at identical concentrations.



**Figure 3A. Binding of  $\beta 3$  cytoplasmic domain-selected phage to  $\beta 1$ ,  $\beta 3$  and  $\beta 5$  immobilized proteins.** GST fusion proteins or GST alone were coated on microtiter wells at  $10 \mu\text{g/ml}$  and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). The data represent the mean colony counts from triplicate wells with standard error less than 10% of the mean. Fd-tet insertless phage was used as a negative control.

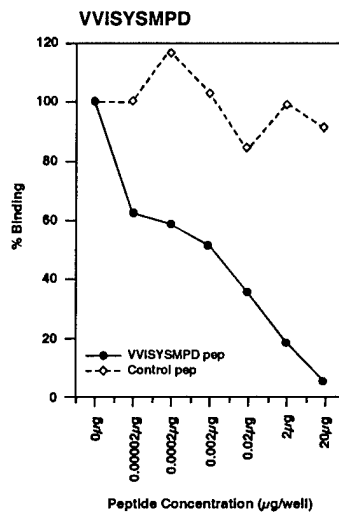


**Figure 3B. Binding of  $\beta 5$  cytoplasmic domain-selected phage to  $\beta 1$ ,  $\beta 3$  and  $\beta 5$  immobilized proteins.** GST fusion proteins or GST alone were coated on microtiter wells at  $10 \mu\text{g/ml}$  and used to bind each phage expressing the peptides shown in Table 3 (each phage is identified by the peptide sequence it displays). The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean. Fd-tet insertless phage was used as a negative control.



**Figure 4A. Binding of the cytoplasmic-domain binding phage to  $\beta 3$  immobilized protein and inhibition with the synthetic peptide.** Phage were incubated on wells coated with GST- $\beta 3$ cyto in the presence of increasing concentrations of the corresponding synthetic peptide or a control peptide. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.

**Figure 4B. Binding of the cytoplasmic-domain binding phage to  $\beta 5$  immobilized protein and inhibition with the synthetic peptide.** Phage were incubated on wells coated with GST- $\beta 5$ cyto in the presence of increasing concentrations of the corresponding synthetic peptide or a control peptide. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.



*Phosphorylation events modulate the interaction of the selected peptides with cytoplasmic domains*

Events involving phosphorylation are important in regulating signal transduction. We used the phage display system to evaluate the effect of tyrosine phosphorylation at two levels: (i) recombinant fusion proteins containing  $\beta 3$  or  $\beta 5$  cytoplasmic domains were used for panning of phage libraries displaying tyrosine-containing peptides or (ii) the cytoplasmic domains themselves were phosphorylated before phage selection was performed. Experiments were performed to investigate the capacity of specific tyrosine kinases to modulate the interaction of the selected peptides with the cytoplasmic

domains. This strategy is interesting because it can reveal the effect of phosphorylation on their binding properties at different levels.

The results obtained in the panning of phage libraries displaying tyrosine-containing peptides are shown in Table 4.

**Table 4. Sequences displayed by phosphorylated phage binding to integrin cytoplasmic domains.**

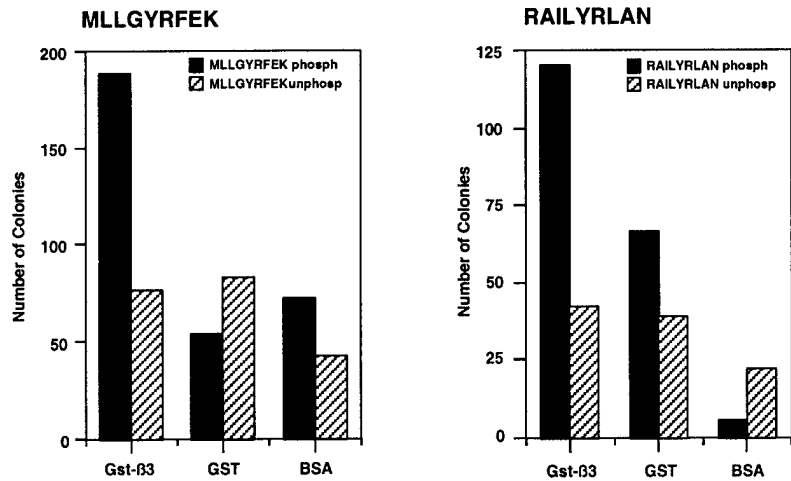
PEPTIDE MOTIF	Phosp/Unphosp	$\beta 3$ or $\beta 5$ /GST	$\beta 3$ or $\beta 5$ /BSA
<i><math>\beta 3</math> cytoplasmic domain</i>			
GGGSYRHVE	13.2	1.5	5.3
RAILYRLAN	2.8	1.3	20
MLLGYRFEK	2.5	3.5	2.7
<i><math>\beta 5</math> cytoplasmic domain</i>			
TMLRYTVRL	14.3	3.4	2.2
TMLRYFMFP	4.2	2.3	3.8
TLRKYFHSS	3.8	3.8	15.2
TLRKYFHSS	1.8	5.6	7.3

Randomly selected clones from rounds III and IV were sequenced from X4YX4 phosphorylated library with Fyn. Amino acid sequences of the phagemid-encoded peptides were deduced from nucleotide sequences. Table 5 shows the motifs found most frequently after the indicated libraries were panned with  $\beta 3$  or  $\beta 5$ . The ratio of binding to  $\beta 3$  or  $\beta 5$  was calculated by dividing the number of  $\beta 3$  or  $\beta 5$  colonies by GST or BSA colonies found after panning. The ratio of binding to  $\beta 3$  or  $\beta 5$  with phosphorylated phage by Fyn versus unphosphorylated phage was calculated by dividing the number of colonies found after the panning.

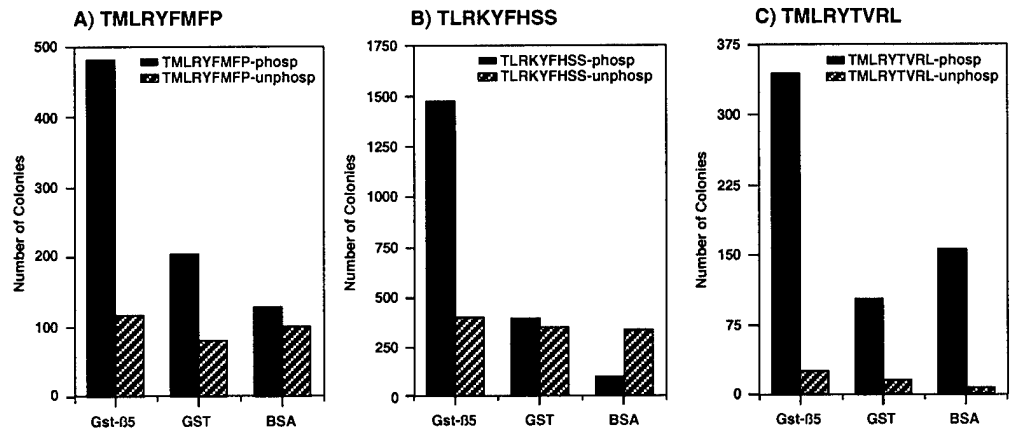
We also have begun to evaluate the capacity of specific tyrosine kinases to phosphorylate isolated cytoplasmic domain-binding peptides. We investigated the effect of phosphorylation on the affinity and specificity of the cytoplasmic domain-binding. Phage displaying the  $\beta 3$  and  $\beta 5$  cytoplasmic domain-binding peptides were phosphorylated *in vitro* as described previously (56-58). We then investigated whether phosphorylation of the phage affected their interaction with the respective cytoplasmic domain in a phage-binding assay. For these experiments, phage were specifically phosphorylated *in vitro* by Fyn kinase. Specific phosphorylation of the tyrosine-containing peptide on the surface of the phage was confirmed by using  $^{32}\text{P}$ -gamma dATP in the kinase reaction and by separating the phage pIII protein by SDS-PAGE.

After phosphorylating the phage *in vitro*, we found that their phosphorylation state increased the binding affinity and specificity to the  $\beta 3$  integrin cytoplasmic domain (Figures 5A and 5B). The TLRKYFHSS phage was also tested in assays that included other GST-cytoplasmic domain fusion proteins to determine specificity (Figure 5C).

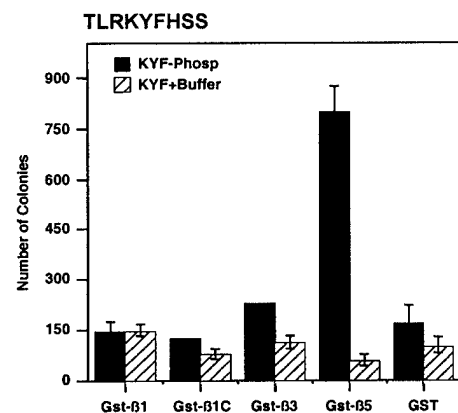
**Figure 5A. Binding of phage to immobilized  $\beta$ 3-GST after phosphorylation.** Phage were phosphorylated with Fyn kinase. Insertless phage were used as a control. Phage were incubated on wells coated with GST- $\beta$ 3cyto. The data represent the mean colony counts from triplicate wells, with standard error less than 10% of the mean.



**Figure 5B. Binding of phage to immobilized  $\beta$ 5-GST after phosphorylation.** Phage were phosphorylated with Fyn kinase. Insertless phage were used as a control. Phage were incubated on wells coated with GST- $\beta$ 3cyto. The data represent the mean of colony counts from triplicate wells, with standard error less than 10% of the mean.

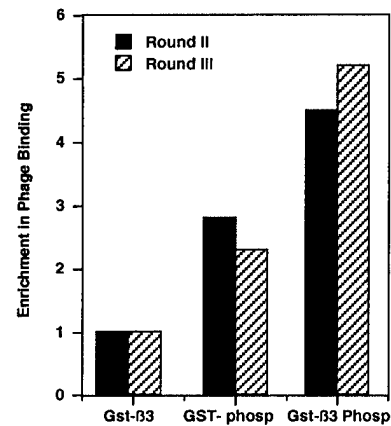


**Figure 5C. Binding of phage to  $\beta$ 1,  $\beta$ 3 and  $\beta$ 5 immobilized GST fusion proteins after phosphorylation.** Phages were phosphorylated with Fyn kinase. Insertless phage was used as a control. Phage were incubated on wells coated with GST-cytoplasmic domains. The data represent the mean of colony counts from triplicate wells, with standard error less than 10% of the mean.



Peptides that bind to the  $\beta$ 3 or  $\beta$ 5 cytoplasmic domains after phosphorylation in vitro are likely to be useful tools in understanding signal transduction regulation in the context of intact cells. We have performed screenings using that system. We have generated interesting data suggesting that it is possible to obtain peptides that will recognize the  $\beta$ 3 cyto domain exclusively after phosphorylation (Figure 6).

**Figure 6. Panning on phosphorylated GST- $\beta$ 3 cyto.** A GST fusion protein containing the  $\beta$ 3 cytoplasmic domain was immobilized onto microtiter wells at 10  $\mu$ g/ml before and after phosphorylation with Fyn kinase. The CX7C phage library was used. Bound phage were recovered after infection with K91 bacteria. Results illustrate the % of enrichment on phosphorylated GST- $\beta$ 3 cyto versus that of phosphorylated GST after the second and third round of selection. The unphosphorylated GST- $\beta$ 3 cyto was used as a control. The data represent the mean of duplicate wells, with standard error less than 10% of the mean.



*Sequence similarity of integrin binding peptides with known cytoskeletal and signaling proteins.*

Searching for sequence homology between these peptides and protein sequences found in the database, we found that the peptides displayed by integrin cytoplasmic domain-binding phage are similar to certain regions found within cytoskeletal proteins and proteins involved in signal transduction. At this point, despite the difficulty of evaluating what these similarities might mean, we plan to investigate whether they represent potentially relevant interactions. The similarity of some of the isolated peptides to a region of mitogen-activated protein kinase 5 (MAPK5, amino acids 227–234) is particularly interesting. Although the molecular mechanisms involved are not firmly identified as yet, a connection involving the MAPK cascade, cell adhesion, migration, and proliferation has been proposed (54). We plan to pursue studies with the most promising peptides

## KEY RESEARCH ACCOMPLISHMENTS

- **We have selected peptides that bind specifically to  $\beta$ 3 or to  $\beta$ 5 integrin cytoplasmic domains.** We used recombinant fusion proteins containing  $\beta$ 3 or  $\beta$ 5 cytoplasmic domains for biopanning of phage peptide libraries.
- **We have investigate whether phosphorylation events can modulate the interaction of the selected peptides with integrin cytoplasmic domains.** We used recombinant fusion proteins containing  $\beta$ 3 or  $\beta$ 5 cytoplasmic domains for biopanning of phage libraries displaying tyrosine-containing peptides. The libraries were phosphorylated *in vitro* using different kinases. We also studied the capacity of specific tyrosine kinases to phosphorylate isolated cytoplasmic domain binding peptides. The effect of phosphorylation on their binding properties were investigated.
- **We have determined some of the biological properties of the cytoplasmic domain-binding peptides.**

## REPORTABLE OUTCOMES

A manuscript is in preparation to report the results of this project.

## CONCLUSIONS

We have clearly demonstrated certain critical results that will contribute to the success of this project.

(i) evidence of successful introduction of peptides into cells and (ii) demonstration that the introduced peptides can affect cell function. Data addressing these issues are compiled in a manuscript in preparation. We also developed improved and more direct experimental strategies to complete the project.

Functional data are now available showing that the cytoplasmic domain-binding peptides selected on  $\beta 3$  or  $\beta 5$  can indeed interfere with integrin-mediated signaling and subsequent cellular responses (i.e., endothelial cell proliferation, and migration). We made a major investment to commercially obtain a panel of "internalizable" versions of our synthetic motifs found by phage screenings. These complex chimeric peptides consist of the most selective of the  $\beta 3$  or  $\beta 5$  cytoplasmic domain-binding peptides coupled to penetratin plus a biotin moiety to allow the peptides to be tracked once they are inside intact cells. Our results show that these membrane-permeable forms of the peptides (i) are indeed internalized (ii) may affect  $\beta 3$  and  $\beta 5$  post-ligand binding cellular events and (iii) can clearly induce massive apoptosis.

Identifying signaling events characteristic of tumor cells and angiogenic vasculature will improve our understanding of integrin biology and may suggest new therapeutic strategies for cancer and other diseases involving angiogenesis.

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## APPENDICES

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