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13. ABSTRACT ***(Maximum 200 Words)***

During the third year of this career development award, we have continued to make progress in determining the contribution of the α6 integrin receptors to breast carcinoma progression. In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases. In addition, we had demonstrated that expression of the β4 integrin subunit in breast carcinoma cell lines that lack this integrin subunit increases their invasive potential. Since submitting the initial proposal, we have demonstrated that the ability of the α6β4 integrin to promote carcinoma invasion involves its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac. We have also identified PKC-epsilon as a critical effector for invasion and we have determined that it contributes to cell motility through the regulation of lamellae organization and function. We have identified IRS-1 and IRS-2 as intermediate adapter proteins in the activation of PI3K by the α6β4 integrin and we have identified specific domains of the IRS proteins that are involved. We have also established that the α6β4 receptor can cooperate with the IGF-1R to activate the IRS proteins and promote IGF-1R signalling.

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Introduction

Alterations in integrin expression and function during transformation are likely to have multiple consequences on tumor progression because of their adhesive and signaling properties (1,2). Our research has focused on the possible involvement of the α6 integrins, α6β1 and α6β4, receptors for the laminin family of matrix proteins, in breast cancer progression. This attention was triggered by the finding that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (3). In addition, a recent report also revealed a correlation between expression of the β4 subunit and poor prognosis (4). In order to take full advantage of the α6 and β4 subunits as markers for predicting the prognosis of breast cancer, it is necessary to understand mechanistically how these integrins promote aggressive tumor behavior. Until this is established, the full potential of α6 for diagnosis, or as a target for therapeutic development, will not be known. In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases (5). In addition, we had demonstrated that de novo expression of the integrin β4 subunit in colon and breast carcinoma cell lines that lack this integrin subunit increases their invasive potential (6,7). The aims of this career development award were designed to investigate these α6-dependent functions in more molecular detail. In this regard, since submitting the initial proposal we have demonstrated that the ability of the α6β4 integrin to promote carcinoma invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the small GTP-binding protein Rac (7). We have recently identified the IRS proteins, IRS-1 and IRS-2 as intermediates in the activation of PI3K by the α6β4 integrin. The involvement of the IRS proteins in α6β4 signaling is intriguing given the importance of these proteins for signaling by other receptors, such as the IGF-1 and prolactin receptors, which have been implicated in breast carcinoma progression. In fact, we now have data to support a signaling cooperativity between the IGF-1 and α6β4 receptors that involves the IRS proteins. Finally, as reported in the previous annual report, we have identified Protein Kinase C-epsilon as a downstream effector of PI3K that is required for promoting carcinoma motility and invasion.

Body

Activation of PI3K by the α6β4 integrin.

We previously demonstrated that the α6β4 integrin can promote carcinoma invasion through its ability to activate phosphoinositide 3-OH kinase (PI3K) and its downstream effectors, the small GTP-binding protein Rac and PKC-epsilon (7). The involvement of a PI3K-dependent signaling pathway in invasion is supported by other studies (8) and adds to previous data that have implicated PI3K in tumor promoting functions including transformation (9), cell survival (10, 11), anchorage-independent growth (12), and motility (13). Taken together, these findings support a central role for PI3K and its lipid products in carcinoma progression and highlight the need to investigate in more detail how this pathway is regulated. Although we have demonstrated that α6β4 can activate PI3K, a direct binding motif for the p85 regulatory subunit of PI3K is not present in the β4 cytoplasmic domain (14), suggesting that the activation of this lipid kinase is through intermediate signaling molecules. To identify these signaling intermediates, we sought to identify tyrosine phosphorylated proteins that associatiate with PI3K after ligation of the α6β4 receptor. As shown in Figure 1A , ligation of cells expressing the α6β4 receptor resulted in the association of PI3K with a 180kD phosphoprotein. These results suggest that the α6β4 integrin activates PI3K through a mechanism involving a 180kD intermediate.

To understand further the mechanism of α6β4 activation of PI3K, we identified the 180kD protein with which PI3K associates after α6β4 ligation. As shown in Fig. 1B, we have identified this phosphoprotein as IRS-2. IRS-2 belongs to the IRS family, a group of cytosolic adapter molecules that organize signaling complexes downstream of surface receptors, many of which have been implicated in mammary tumorigenesis (15,16). For example, the IRS proteins

are essential downstream signaling intermediates of the Insulin-like Growth Factor Receptor (IGF-1R) which has been linked to increased tumor recurrence and reduced survival of breast cancer patients (17,18). MDA-MB-435/mock and β4 transfectants were clustered with α6- or β4-specific antibodies and cell extracts were immunoprecipitated with antibodies that recognize IRS-2. The immune complexes were immunoblotted with phosphotyrosine or p85 (PI3K) specific antibodies. Ligation of the α6β4 receptor, but not the α6β1 receptor, increased IRS-2 tyrosine phosphorylation (upper panel). More importantly, PI3K associated with IRS-2 after α6β4 ligation (lower panel). Addition of IGF-1 to the cells increased the level of IRS-2 phosphorylation and association with PI3K.

Mechanism of IRS-2 phosphorylation in response to α6β4 ligation.

The α6β4 receptor does not contain an intrinsic kinase domain and therefore it must activate an intermediate kinase to promote IRS-2 phosphorylation. The src family of non-receptor kinases has been shown previously to be capable of phosphorylating IRS-2. However, an inhibitor of the src kinases, PP2, did not prevent IRS-2 phosphorylation in response to α6β4 ligation (Fig. 2), although it did significantly diminish total levels of tyrosine phosphorylation in the cells. We are presently investigating alternative pathways for the α6β4-dependent phosphorylation of IRS-2. These possibilities include members of the JAK kinase family of kinases and the IGF-1 receptor kinase. Identification of the kinase that is activated by α6β4 to promote IRS phosphorylation will provide a target for disrupting the α6β4-dependent promotion of invasion and survival.

To investigate further the mechanism of IRS phosphorylation in response to α6β4 ligation, we have begun to identify the specific domains of IRS that are essential for α6β4-dependent phosphorylation. The IRS proteins must be recruited to the plasma membrane to be functional. Several domains in the N-terminus of IRS are known to be required for their recruitment to the insulin and IGF-1 receptors. We have transiently transfected the MDA-MB-435/β4 transfectants with a series of IRS deletion mutants. As shown in Figure 3, the wildtype IRS and the ΔSAIN deletion mutant were phosphorylated in response to β4 clustering. In contrast, the ΔPH, ΔPTB, and ΔPH/PTB deletion mutants were not phosphoryated under the same conditions. These results indicate that the PH and PTB domains are essential for α6β4-dependent phosphorylation. Using this information we will now use these minimal domains to identify the upstream proteins that interact with the IRS proteins in response to α6β4 ligation.

The α6β4 integrin promotes phosphorylation of IRS-1 and IRS-2.

IRS-2 belongs to a family of signaling adapters that includes IRS-1, IRS-2, IRS-3, and IRS-4 (15,16). To determine if other IRS homologs could also be activated by α6β4, we examined the expression of the IRS family members in the MDA-MB-435 cells. As shown in Fig. 4, MDA-MB-435 cells expressed relatively low levels of IRS-1 (A) when compared with their level of IRS-2 expression (B). In contrast, another breast carcinoma cell line, T47D, expressed high levels of IRS-1 (Fig. 4A) and very low levels of IRS-2 (Fig. 4B). Neither of these cell lines expressed IRS-3 or IRS-4, which are known to be restricted in their tissue. The differences in the ability of the MDA-MB-435/mock and MDA-MB-435/β4 transfectants to promote IRS-2 phosphorylation is not due to a lack of IRS-2 expression in the mock transfectants because these subclones express equivalent levels of IRS-2 (Fig. 4B).

IRS-2 is tyrosine phosphorylated in response to α6β4 ligation in the MDA-MB-435 cells. Given that these cells express very low levels of IRS-1, T47D cells were used to examine the ability of α6β4 to promote IRS-1 phosphorylation. The α6β4 integrin was ligated with either α6 or β4-specific antibodies and cell extracts were immunoprecipitated with antibodies that recognize IRS-1 and immunoblotted with phosphotyrosine-specific antibodies. As shown in Fig. 4C, IRS-1 is tyrosine phosphorylated in response to α6β4 ligation.

The α6β4 integrin and the IGF-1R cooperate to promote IRS-2 phosphorylation and PI3K activation.

Growth factor/hormone and integrin receptors are both necessary to promote normal mammary epithelial and carcinoma functions. However, it is not clear how these receptors cooperate to promote signaling and to alter cellular function. To examine the influence of α6β4-mediated IRS phosphorylation on IGF-1 signaling, we examined the ability of IGF-1 to promote tyrosine phosphorylation in the presence or absence of α6β1 or α6β4 ligation. As shown in Fig. 5, addition of IGF-1 to cells maintained in suspension resulted in a small increase in total cellular tyrosine phosphorylation. When α6β1 was ligated in the MDA-MB-435/mock transfectants, the stimulation of tyrosine phosphorylation by IGF-1 was not significantly increased. In contrast, ligation of the α6β4 integrin in the presence of IGF-1 in the MDA-MB-435/β4 transfectants resulted in a marked increase in the level of total cellular tyrosine phosphorylation. In addition, PI3K association with IRS-2 was significantly increased when α6β4 was ligated in the presence of IGF-1 in the MDA-MB-435/β4 cells (lower panel).

These results are important because the IGF-1R has been previously implicated in promoting breast carcinoma progression. The ability of the α6β4 integrin to promote IGF-1 signaling supports further the involvement of this integrin receptor in breast cancer.

Mutational analysis of the β4 cytoplasmic domain.

To investigate the mechanism by which the α6β4 receptor activates downstream signaling pathways to promote carcinoma invasion, we identified several tyrosine residues that are potential binding sites for downstream effectors. We have established stable transfectants of the MDA-MB-435 cells that express each of these β4 point mutants and we are presently characterizing these cell lines (Figure 6).

Key Research Accomplishments:

-The α6β4 integrin promotes PI3K activation through the IRS proteins IRS-1 and IRS-2.

-The src kinase is not involved in the α6β4-dependent phosphorylation of IRS-2.

-The PH and PTB domains of the IRS proteins are essential for α6β4-dependent phosphorylation.

-The α6β4 integrin cooperates with the IGF-1 receptor to promote IRS phosphorylation and activation of PI3K.

-Construction of β4 tyrosine mutants and establishment of MDA-MB-435 cells expressing these mutant β4 subunits.

Reportable Outcomes:

1. Manuscripts

Chen, M.S., E.A.C. Almeida, A.-P.J. Huovila, Y. Takahashi, **L.M. Shaw**, A.M. Mercurio, and J.M. White. 1999. Evidence that different 'states' of the integrin α6β1 interact with laminin and an ADAM. J. Cell Biol.*144*:549-561.

Zrihan-Licht, S., Y. Fu, J. Settleman, K. Schinkmann, **L. Shaw**, I. Keydar, S. Avraham, and H. Avraham. 2000. RAFTK/Pyk2 tyrosine kinase mediates the association of p190 RhoGAP with RasGAP and is involved in breast cancer cell invasion. Oncogene *19:*1318-1328.

Guan, R.J., H.L. Ford, Y. Fu, **L.M. Shaw**, and A.B. Pardee. 2000. Overexpression of DRG 1 induces cell differentiation and suppresses colon cancer metastasis. Cancer Res. *60:*749-755.

2. Submitted RO1 application (6/1/00) based on the work supported by this award.

3. Established new laboratory in the Department of Pathology at the Beth Israel Deaconess Medical Center.

Conclusions

The overall goal of this career development award is to understand the contribution of the α6 integrins to breast carcinoma progression. To date, we have made significant progress toward identifying α6β4-dependent signaling pathways that are involved in promoting breast carcinoma invasion. Activation of PI3K and the downstream effectors Rac and PKC-epsilon are essential for carcinoma invasion. We have identified the IRS proteins, IRS-1 and IRS-2, as intermediates in the activation of PI3K by the α6β4 integrin. In addition, we have established a cooperative signaling mechanism for α6β4 and the IGF-1R. We will continue to investigate the mechanism of α6β4 signaling so that we can increase our understanding of how these pathways can be manipulated for therapeutic intervention in the future.

On a personal level, the work performed during this career development award has contributed to my establishment of an independent research program in the Department of Pathology at the Beth Israel Deaconess Medical Center.

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Appendices:

1. Figures

Figure 1

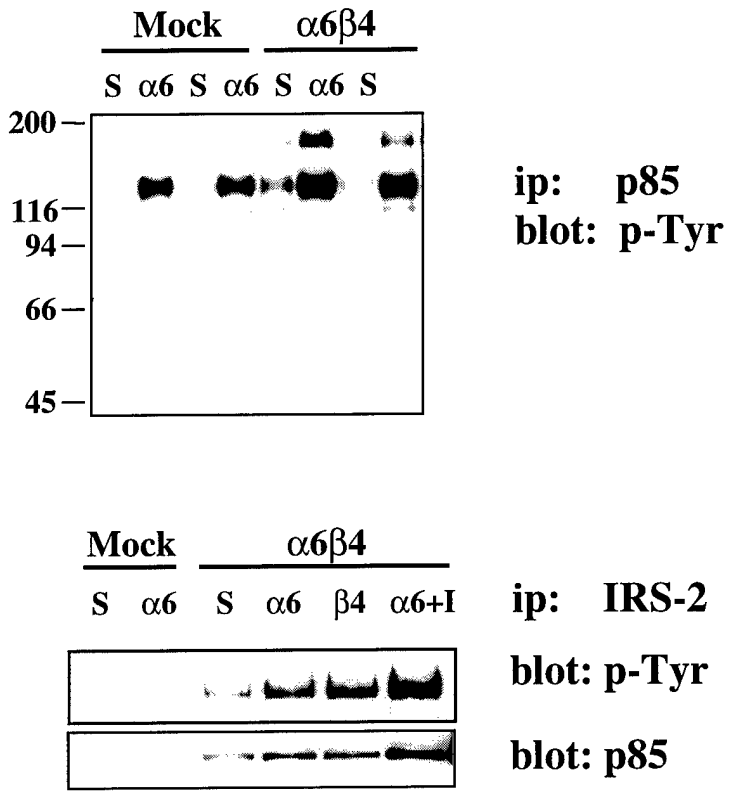

Figure 1: Tyrosine phosphorylated proteins associated with PI3K after ligation with α6β1 and α6β4. The α6β1 and α6β4 integrins activate PI3K through distinct mechanisms. MDA-MB-435 transfectants were maintained in suspension or incubated with α6- or β4-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. A) Aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated with p85-specific antibodies and the immune complexes were immunoblotted with phosphotyrosine-specific (p-Tyr) antibodies. Ligation of α6β1 results in the association of PI3K with a 120kD phosphoprotein whereas ligation of α6β4 results in the association of PI3K with a 180 kD phosphoprotein. B) Aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated with IRS-2-specific antibodies and the immune complexes were immunoblotted with phosphotyrosine-specific (p-Tyr) or PI3K specific (p85) antibodies. Mock, MDA-MB-435 cells transfected with vector alone; α6β4, MDA-MB-435 cells transfected with the β4 subunit; S, cells maintained in suspension; α6, cells clustered with an α6-specific antibody; β4, cells clustered with a β4-specific antibody; α6+I, cells clustered with α6-specific antibodies in the presence of IGF-1.

Figure 2

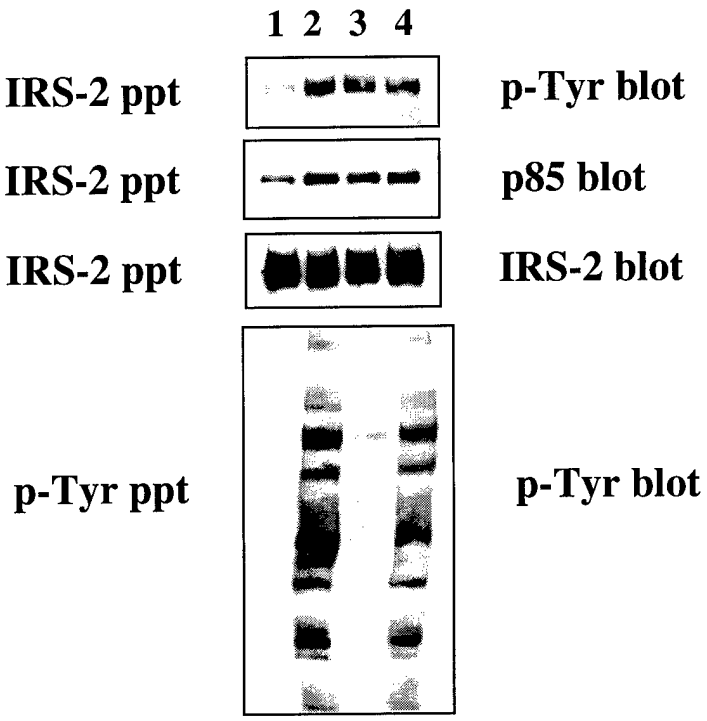

Figure 2: pp60-src is not involved in the phosphorylation of IRS-2 in response to α6β4 ligation. MDA-MB-435/β4 transfectants were maintained in suspension (Lane 1) or incubated with β4-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes in the absence (Lane 2) or presence of the src kinase inhibitor PP2 (Lane 3) or the inactive inhibitor PP3 (Lane 4). Aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated with IRS-2- or p-Tyr-specific antibodies and the immune complexes were immunoblotted with phosphotyrosine-specific (p-Tyr), PI3K-specific (p85), or IRS-2-specific antibodies. 1, cells maintained in suspension; 2, cells clustered with a β4-specific antibody; 3, cells clustered with a β4-specific antibody in the presence of PP2; 4, cells clustered with a β4-specific antibody in the presence of PP3.

Figure 3

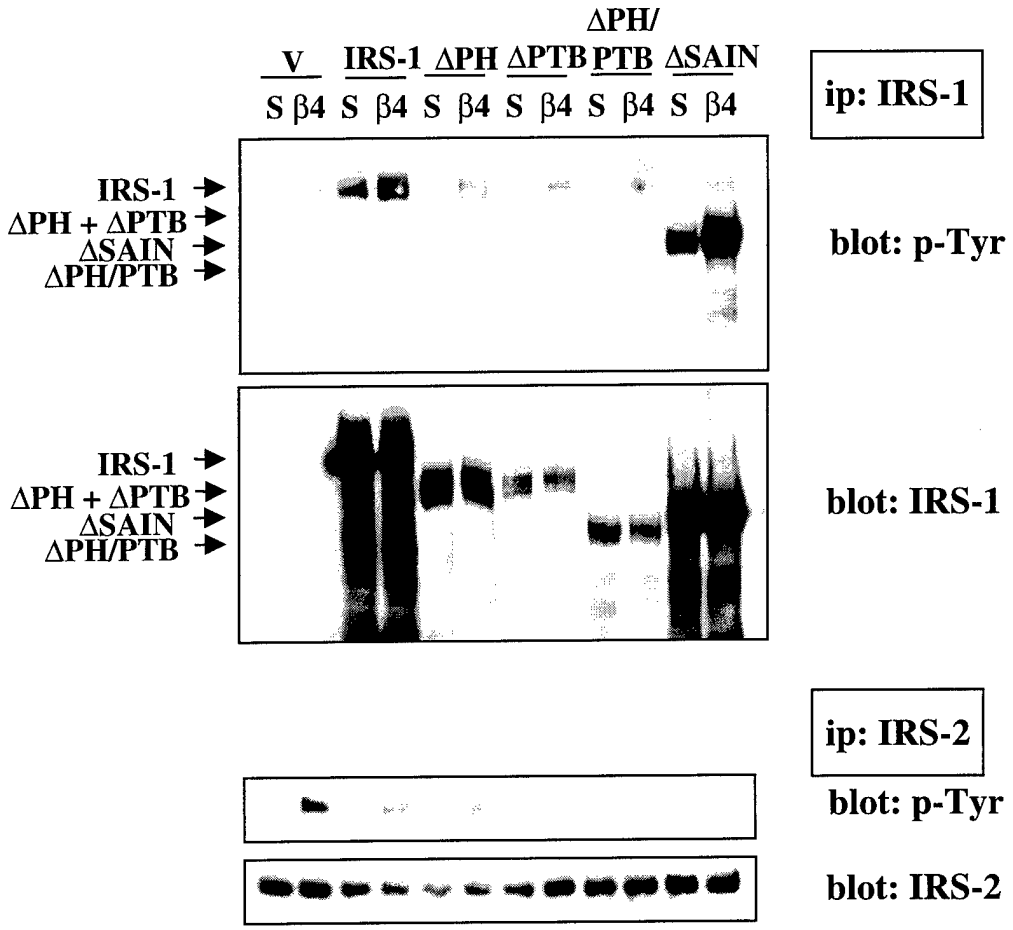

Figure 3: Identification of the IRS domains that are essential for phosphorylation by the α6β4 integrin. MDA-MB-435/β4 cells were transiently transfected with wild type IRS-1 or deletion mutants of IRS-1 that lack the PH, PTB, and SAIN domains. 24 hours after transfection, the cells were either incubated in suspension or clustered with β4 specific antibodies. Cell extracts containing equivalent amounts of total protein were immunoprecipitated with either IRS-1- or IRS-2-specific antibodies and the immune complexes were resolved by SDS-PAGE and immunoblotted with phosphotyrosine-specific antibodies. The blots were subsequently stripped and re-probed with IRS-1 and IRS-2 antibodies to confirm expression. S, suspension; β4, clustered with β4-specific antibodies; V, vector alone; ΔPH, IRS lacking the PH domain; ΔPTB, IRS lacking the PTB domain; ΔPH/PTB, IRS lacking both the PH and PTB domains; ΔSAIN, IRS lacking the SAIN domain.

Figure 4

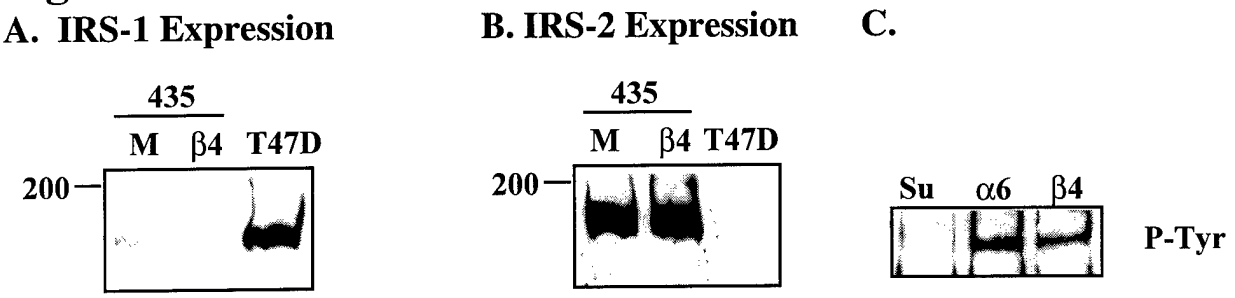

Figure 4: IRS-1 and IRS-2 expression in MDA-MB-435 and T47D cells. A and B) Total cell lysates containing equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies specific for IRS-1 and IRS-2. C) T47D cells were maintained in suspension or incubated with α6- or β4-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated with IRS-1-specific antibodies and the immune complexes were immunoblotted with phosphotyrosine-specific (p-Tyr) antibodies. M, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the β4 subunit; 435, MDA-MB-435 cells; SUS, cells maintained in suspension; α6, cells clustered with an α6-specific antibody; β4, cells clustered with a β4-specific antibody.

Figure 5

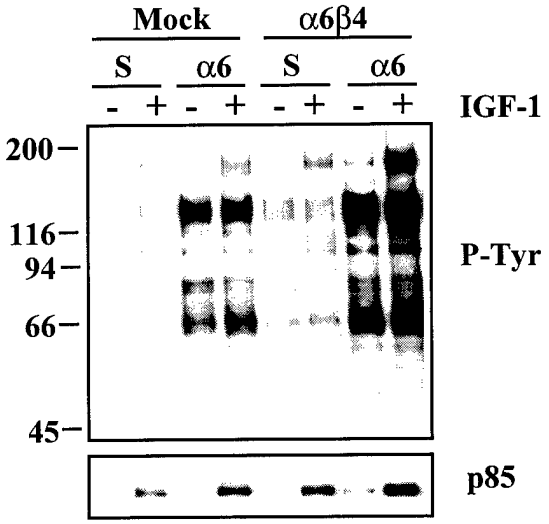

Figure 6: Cooperative signaling between the α6β4 integrin and the IGF-1 receptor. MDA-MB-435 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. IGF-1 (50 ng/ml) was added to the cells for the final 5 minutes of the clustering (+). Aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated with phosphotyrosine-specific antibodies and the immune complexes were immunoblotted with either phosphotyrosine-specific (p-Tyr) or PI3K specific (p85) antibodies. Mock, MDA-MB-435 cells transfected with vector alone; α6β4, MDA-MB-435 cells transfected with the β4 subunit; S, cells maintained in suspension; α6, cells clustered with an α6-specific antibody; β4, cells clustered with a β4-specific antibody; +, incubated with 50 ng/ml IGF-1.

Figure 6

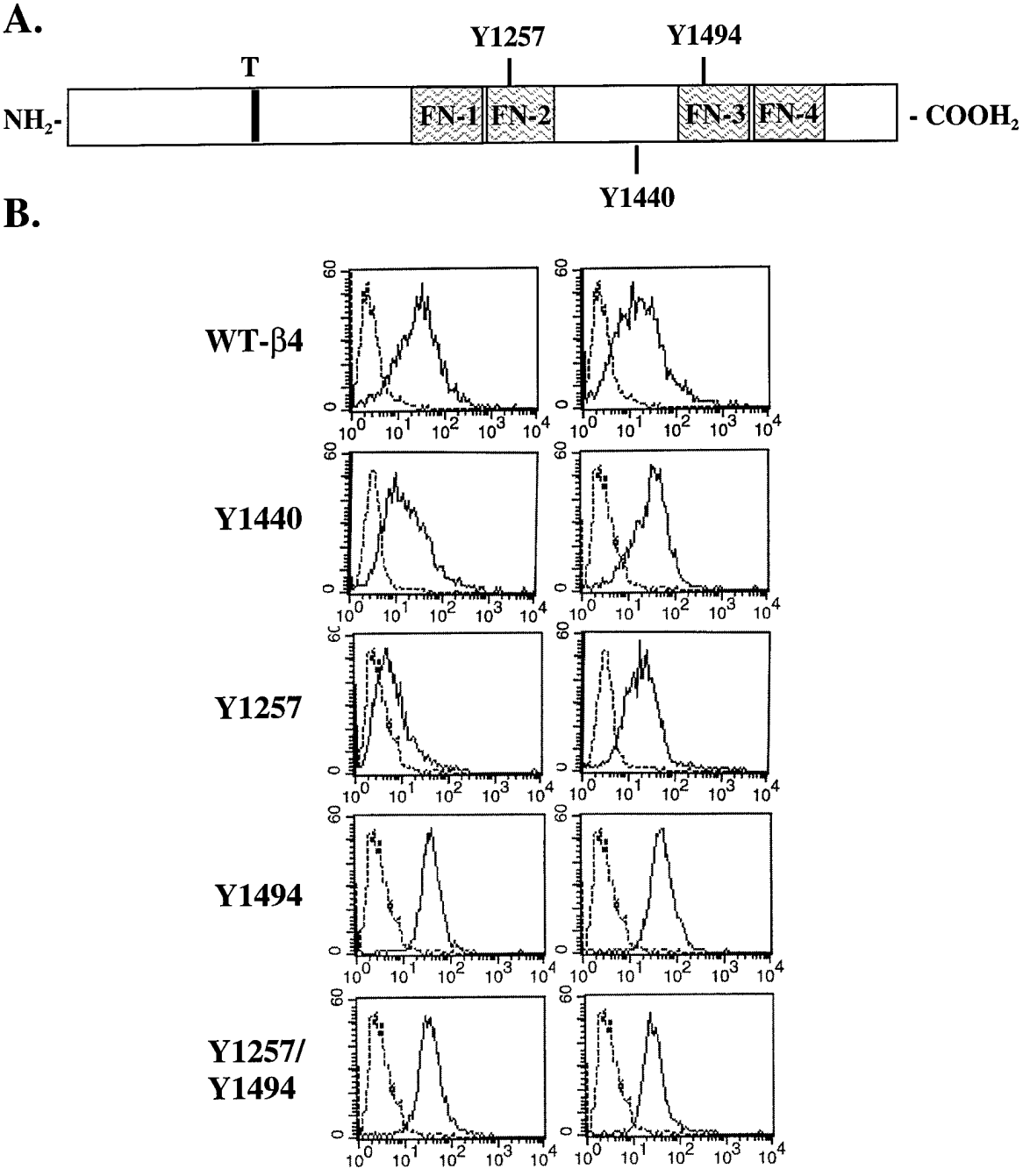

Figure 6: β4 Site-directed Mutagenesis. A) Schematic of the β4 integrin subunit showing the tryosine residues that we have mutated to phenylalanine. B) FACS analysis of surface expression of the β4 tyrosine mutants.