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HYPOTHESIS

p56^{lck} CONTROLS PHOSPHORYLATION OF FILAMIN (ABP-280) AND REGULATES FOCAL ADHESION KINASE (pp125^{FAK})

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Transformation of cells by *src*-like kinases leads to altered cell morphology associated with the disassembly of focal contacts and concomitant increase in tyrosine phosphorylation of pp125^{FAK}. p56^{lck} is a lymphocyte-specific member of the *src* family of protein tyrosine kinases that associates with cell surface glycoproteins such as CD4 and CD8. It phosphorylates and activates pp125^{FAK} and increases its autokinase activity, thus pretreatment of pp125^{FAK} with protein kinase C (PKC) markedly attenuates its phosphorylation and activation, suggesting a potential regulatory pathway of pp125^{FAK} activation in focal contacts. p56^{lck} further phosphorylates and activates actin binding protein (ABP-280; filamin) and controls its association with cell surface receptors such as β-2 integrins, actin filament cross-linking, and possibly lipid membrane insertion.

KEYWORDS: tyrosine phosphorylation; p56^{lck}; pp125^{FAK}; PKC; filamin (ABP-280).

INTRODUCTION

The mechanism by which signals are transmitted from the cell surface to the nucleus is largely unknown despite a large body of evidence that suggests that the phosphorylation of several cellular proteins is involved (Hall, 1998; Ktistakis, 1998). Cell-surface receptors either have intrinsic tyrosine kinase activity, i.e. EGF receptors, or are associated with kinases that are wholly cytoplasmic or membrane-bound (Klagsbrun and D'Amore, 1996). Several of these kinases belong to the *src* family because of their structural homology with the oncogenic product, pp60$^{v\text{-}src}$, which is encoded by the Rous sarcoma virus. These are plasma membrane-associated enzymes consisting of nine well-characterized members, including p56^{lck} (Ziegler *et al.*, 1989; Burgess *et al.*, 1992; Schieven *et al.*, 1992; Sleckman *et al.*, 1992; Venkitaraman and Cowling, 1992; Pleiman *et al.*, 1993; Levin *et al.*, 1993; Wechsler and Monroe, 1995; Henning and Cantrell, 1998). This *src* kinase is expressed in high amounts in T-lymphocytes and plays a vital role in T-cell activation. It has a unique N-terminal sequence that is involved in the physical association with specific cell receptors such as CD4 and CD8. p56^{lck} further interacts with IL-2 receptors and indirectly associates with GPI-anchored surface receptors (Torigoe *et al.*, 1992; Tosello *et al.*, 1998).

The cytoplasmic kinase that is concentrated in focal adhesions, pp125^{FAK}, has been identified as one of the major tyrosine-phosphorylated proteins in *src*-transformed chicken embryo fibroblasts (Reynolds *et al.*, 1989; Kanner *et al.*, 1990, 1991; Harder *et al.*, 1998). cDNA sequences of avian, murine, and human pp125^{FAK} have been characterized and found to be highly conserved among different species (Schaller *et al.*, 1992). It is

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structurally distinct from other known kinases and lacks the SH-2 and SH-3 domains that associate with other cellular proteins for biological functions (Kanner *et al.*, 1991). It is tyrosine-phosphorylated in response to the clustering of integrins mediated by cross-linking with monoclonal antibodies or interaction of integrins with the extracellular matrix (Guan and Shalloway, 1992). Although pp125^{FAK} was identified in pp60$^{v\text{-}src}$ transfected cells and is thought to be a substrate of pp60$^{v\text{-}src}$, so far it has not been shown to be an *in vitro* substrate of pp60$^{v\text{-}src}$. Since both pp125^{FAK} and *src*-kinase are localized in focal contacts, it is important to investigate whether the activation of *src*-like kinases phosphorylates and activates pp125^{FAK}.

Despite recent progress in the elucidation of how surface receptors interact with p56^{lck}, little is known about the substrate specificity of this tyrosine kinase either *in vitro* or *in vivo.* Thus, one substrate has been identified as the ζ-subunit of the T-cell receptor (TCR) complex, which when phosphorylated, strengthens its interaction three- to fourfold after cross-linking with CD4 (August and Dupont, 1996). Other substrates identified by *in vitro* p56^{lck} kinase reaction, p21*ras* GTPase activating protein (GAP) and mitogen activated protein kinase (MAPK) have been found to phosphorylate specifically at tyrosine residues within one tryptic peptide (Brenner *et al.*, 1996). Despite these identified substrates, the downstream signal via cytoskeletal proteins from the surface to the nucleus has only recently been addressed (Meyer *et al.*, 2000; Goldmann, 2002). Previously, Sharma *et al.* (1995) focused on whether actin-binding protein (ABP-280; filamin) is a probable substrate for p56^{lck}. Their assumption is based on the reported function and localization of ABP-280 in lymphocytes (Loo *et al.*, 1998). They found this protein in the periphery of the cell under the plasma membrane and attached p56^{lck} to the cytoplasmic face of the plasma membrane through the myristoylated N-terminal glycine. The investigation into utilizing immunopurified ABP-280 and purified p56^{lck} by *in vitro* kinase reaction showed that ABP-280 is a major substrate of p56^{lck} that binds specifically to immunopurified ABP-280 (Sharma *et al.*, 1998). Further studies showed that purified p56^{lck} also phosphorylates pp125^{FAK} *in vitro* and increases its autokinase activity. Thus, when first phosphorylated by protein kinase C (PKC) and then followed by p56^{lck}, the tyrosine phosphorylation of pp125^{FAK} decreased significantly (Sharma *et al.*, 1999).

p56^{lck} INTERACTION WITH ACTIN-BINDING PROTEIN (ABP-280; FILAMIN)

It has been reported that p56^{lck} associates with CD4 and that this complex plays an integral role in generating signals that lead to T-cell activation by inducing the phosphorylation of a number of transmembrane and intracellular proteins (Zhuang *et al.*, 1984; Kadena *et al.*, 1997). The major tyrosine kinases expressed in human T-lymphocytes include p56^{lck} and to date, several surface receptors have been shown to interact directly or indirectly with p56^{lck} (Torigoe *et al.*, 1992). Several studies have indicated that the *in vitro* activity of p56^{lck} increased manifold after cross-linking with CD4 and that the phosphorylated substrate is the ζ-subunit of the T-cell antigen receptor (Koyasu *et al.*, 1992; Parolini *et al.*, 1996). Sharma *et al.* (1995) addressed the question of whether ABP-280 can serve as a substrate for p56^{lck}, given its location in the cell. Utilizing immunopurified ABP-280 and recombinant p56^{lck} in *in vitro* kinase assays they demonstrated that ABP-280 specifically phosphorylates tyrosine residue(s) and that the phosphorylation is restricted to a single tryptic or V8 peptide. Given this result, they proposed a possible function for the *in vivo* phosphorylation of ABP-280 by p56^{lck}, i.e. that it regulates the association with surface receptors such as β2-integrins, and/or cross-linking of actin filaments into networks (Yada *et al.*, 1990). Phosphorylation and dephosphorylation events of ABP-280 have been thought to play a dynamic role in the organization of the cytoskeleton (Kovacsovics and Hartwig, 1996), which could account for the increased avidity observed in lymphocytes. This view is supported by the finding that phosphorylation of ABP-280 by p56^{lck} is highly effective when ABP-280 isolated from cells is treated with phorbol myristate acetate (PMA). It is therefore intriguing to consider a mechanism whereby the majority of the p56^{lck} molecule is associated through its N-terminal peptide with ABP-280. This region contains cysteine residues with T-cell surface glycoproteins CD4, which also contain cysteine residues in the cytoplasmic domains to form a bimolecular complex. Its association leaves the catalytic domains of p56^{lck} (present in the C-terminal half of the molecule) free to phosphorylate its substrate, thus making this molecule less mobile. This interaction between CD4 and p56^{lck} is probably responsible for the inability of p56^{lck} to co-immunoprecipitate with ABP-280, despite the fact that p56^{lck} phosphorylates ABP-280 as described by Sharma *et al.* (1998). In all,

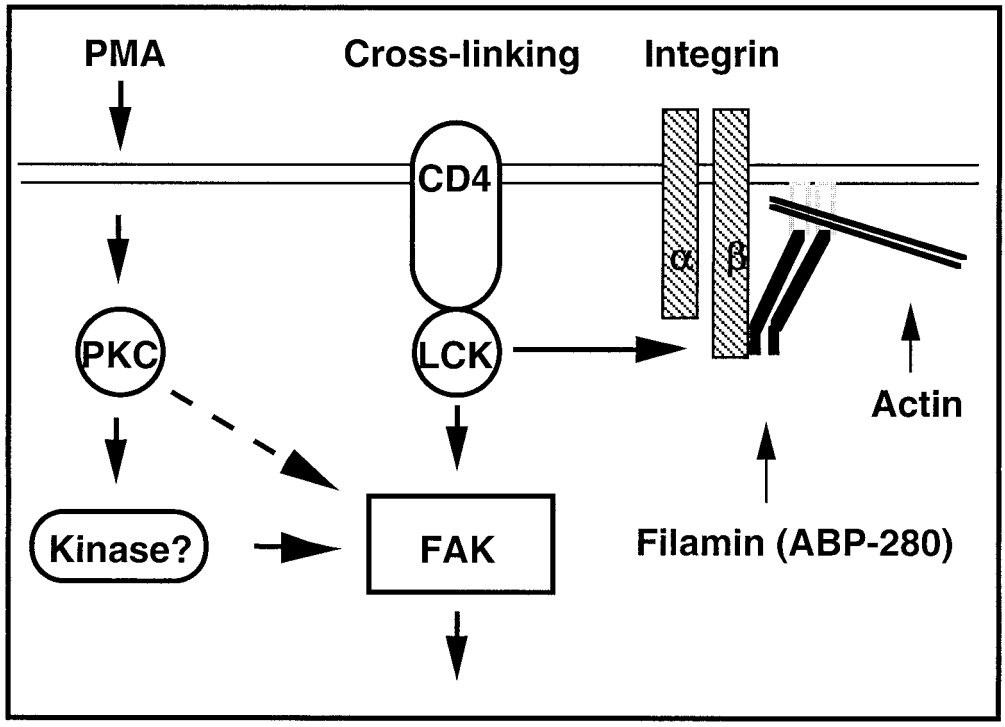

Fig. 1. A minimum scheme of a potential co-stimulatory pathway that might regulate pp125^{FAK} phosphorylation.

these findings suggest conformationally induced regulation which could be indicative of novel downstream signalling through the receptor-p56^{lck} ABP-280-cytoskeleton linkage (Goldmann, 2001).

p56^{lck} REGULATION OF FOCAL ADHESION KINASE (pp125^{FAK})

It has also been suggested that during T-cell activation, p56^{lck} undergoes a complex series of post-translational modifications that is the consequence of biochemical alterations induced by the interaction of antigens with TCR. Amrein *et al.* (1992) expressed the SH-2 domain of p56^{lck} as a GST-p56^{lck} fusion protein utilizing the SH-2 affinity matrix for the identification of interacting proteins. From cell lysates of 3T3 (NIH) cells, a protein of ~120 kDa was found to associate with the SH-2 domain of p56^{lck}, which was identified by Western blot as pp125^{FAK}. Sharma *et al.* (1999) further addressed the question of whether the phosphorylation and autocatalytic sites of p56^{lck} were identical and how they might regulate the activity of pp125^{FAK}. They showed that activated pp125^{FAK} serves as a better substrate than inactivated for pp56^{lck}. That is, p56^{lck} not only increases tyrosine phosphorylation of activated pp125^{FAK}, but also increases its *in vitro* activation. Small levels of phosphorylation of inactivated pp125^{FAK} by p56^{lck} indicated the specificity of the reaction, suggesting that either the phosphorylation site of pp125^{FAK} is different from the autocatalytic site or p56^{lck} does not have access to the autocatalytic site in the inactivated state of pp125^{FAK}. It could also be argued that activation of pp125^{FAK} by pp60$^{v\text{-}src}$ transformed cells is due to the activation of other kinases that activate pp125^{FAK}. This view is supported by the observation that the cellular transformation of pp125^{FAK} by pp60$^{v\text{-}src}$ leads to increased tyrosine protein phosphorylation (Harder *et al.*, 1998). It is therefore tempting to speculate, since p56^{lck} phosphorylates activated pp125^{FAK} that these kinases can be co-immunoprecipitated after activation of pp125^{FAK}. Sharma *et al.* (1999), however, were unable to co-immunoprecipitate pp125^{FAK} and p56^{lck} even at high stoichiometry, which might be due to the transient association of these molecules as well as CD4 and TCR, during T-cell activation. This suggests that during T-cell activation p56^{lck} undergoes a complex series of post-translational modifications which are the consequence of biochemical alterations induced by the interaction of antigens with TCR.

Sharma *et al.* (1999) also investigated the activation of peripheral blood lymphocytes (PBLs) with PMA and found that it activates and tyrosine-

phosphorylates pp125^{FAK} in a similar way to Swiss 3T3 and Chinese hamster ovary cells (Dunlop and Clark, 1993; Wang *et al*., 1997). These researchers also observed *in vitro* phosphorylation of pp125^{FAK} by PKC, which is contrary to results by Vuori and Ruoslahti (1995). The reason for this might be that during the phosphorylation of pp125^{FAK} by PKC only the PKC-a isoform was used, whereas in Sharma's *et al.* (1999) investigation a mixture of all PKC sub-isoforms was used. (Note: inhibitors of PKC like GF 109203 X, calphostin C, and staurosporin can specifically block the PMA activation of pp125^{FAK}). Although PKC phosphorylates pp125^{FAK} *in vivo*, its phosphorylation by PKC does not influence its tyrosine phosphorylation and autocatalytic activity *in vitro*. It can be speculated that phosphorylation of pp125^{FAK} by PKC might make it a better substrate for another kinase, or that PKC activates another kinase(s) that in turn tyrosine-phosphorylates and activates pp125^{FAK}. However, at least in the case of p56^{lck} PKC does not make pp125^{FAK} a better substrate as PKC decreases the tyrosine phosphorylation of pp125^{FAK} by p56^{lck}. These results support the notion that the role of PKC in the activation of pp125^{FAK} may be indirect, i.e. it activates another kinase that in turn phosphorylates and activates pp125^{FAK} (Fig. 1). It provides further evidence that PKC is essential but not sufficient in regulating the tyrosine phosphorylation of pp125^{FAK}. Sinnett-Smith *et al.* (1993) showed that pp125^{FAK} is activated both by PMA and certain neuropeptides like bombesin, vasopressin and endothelin in Swiss 3T3 cells. A selective inhibitor of PKC (GF 109203 X) markedly inhibited the activation of pp125^{FAK} by PMA, but had little effect on the response to bombesin, vasopressin, and endothelin. Calcium seemed not to be important because its mobilization from intracellular stores by bombesin was blocked by the tumour promoter thapsigargin, and it had no effect on the activation of pp125^{FAK} by bombesin. These findings suggest that neither PKC nor Ca^{2+} is responsible for the activation of pp125^{FAK}. The results by Sharma *et al.* (1999) indicate that although PKC may not have a direct role in the activation of pp125^{FAK}, it certainly phosphorylates pp125^{FAK} and regulates its phosphorylation by p56^{lck} or any other unknown kinase(s). It is quite possible that pp125^{FAK} is activated by many independent pathways and that blocking one pathway does not influence the others. It would be intriguing to identify a downstream substrate that is phosphorylated by pp125^{FAK}, and then test whether pp125^{FAK} is the converging point for many pathways. Finally, signals generated by the interaction of ligands with the surface receptors in T-cells or cross-linking of surface receptors still need further detailed elucidation as do changes induced in the level and activity of critical regulatory proteins through tyrosine phosphorylation.

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