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Involvement of the Paxillin Pathway in JB6 Cl41 Cell Transformation

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Abstract

Paxillin is a substrate of the Src tyrosine onco-kinase and is involved in cell transformation, cell spreading, migration and cancer development mediated through the mitogen-activated protein (MAP) kinase signaling cascades. Here, we demonstrated that paxillin plays a key role in skin cell transformation induced by epidermal growth factor (EGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). To investigate the mechanism of paxillin's role in cell transformation, we established a paxillin knock-down stably transfected cell line by introducing siRNA-paxillin (si-paxillin). The si-paxillin cells displayed a dramatic suppression of cell proliferation and anchorage-independent cell transformation induced by EGF or TPA compared with si-mock control cells. In si-paxillin cells, decreased activator protein-1 (AP-1)-dependent luciferase activity corresponded with suppressed AP-1 DNA binding activity. Importantly, knock down of paxillin inhibited EGF- or TPA-induced c-Jun phosphorylation at serines 63 and 73. Furthermore, total c-Jun protein level was dramatically decreased in si-paxillin cells and was dependent on serum deprivation time. The down-regulation of c-Jun was restored in si-paxillin cells by treatment with the proteasome inhibitor lactacystin, but not by the lysosome inhibitor leupeptin. These results clearly provided evidence that paxillin regulates c-Jun protein level and plays a key role in cell transformation most likely through the regulation of c-Jun stability.

Keywords

paxillin; mouse epidermal JB6 Cl41 cells; cell transformation; AP-1; c-Jun

Introduction

Paxillin is a multidomain-containing adaptor molecule consisting of LD (LDXLLXXL) motifs and LIM (Lin-11, Isl-1, Mec-3) domains, which provide binding sites for various proteins at focal adhesions (1–5). Paxillin has been found at the interface between the plasma membrane and the actin cytoskeleton where it plays an important role in providing a platform for the integration and processing of adhesion and growth factor-related signals (6). Paxillin is a highly conserved protein with 90% amino acid homology between chicken and humans (7). It is widely distributed in all tissues and cells (7,8), suggesting that paxillin may have a unique function in cell shape change and actin cytoskeleton reorganization (9,10).

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Cell adhesion is mediated mainly through engagement of transmembrane integrin molecules by their extracellular-matrix ligands, including fibronectin, vitronectin, collagen, and laminin (11–14). This interaction leads to activation resulting in a series of intracellular responses such as protein phosphorylation, changes in intracellular pH and calcium levels, and stimulation of mitogen-activated protein (MAP) kinase signaling pathways (11–14). Coordination or integration of adhesion and growth factor signals is facilitated by the close physical proximity of key molecules in the signaling cascades (12). Indeed, integrins co-localize and co-precipitate with several growth factor receptors such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (12,14). Paxillin is a scaffold protein functioning to recruit signaling proteins, including focal adhesion kinase (FAK), p210BCR/ABL or pp60v-Src, to the plasma membrane (7,15,16). At the membrane, these proteins are activated by engagement of integrins with the extracellular matrix or by growth factor stimulation of receptor tyrosine kinases (RTK) (6). This results in MAP kinase family activation, cell proliferation and cytoskeleton rearrangement through the phosphorylation of paxillin (6). Moreover, paxillin is selectively up-regulated in certain tumor cell lines such as HPV-18 immortalized cells and in cervical carcinoma cell lines (17), strongly suggesting that paxillin might have a role in cell transformation or tumor development. However, no report has yet implicated paxillin in these processes. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and epidermal growth factor (EGF) are well known tumor promotion agents used to study malignant cell transformation in cell and animal models of cancer (18). The JB6 Cl41 (promotion sensitive) mouse epidermal cell line is a well-developed cell culture system for studying genetic susceptibility to transformation promotion at the molecular level (19). Moreover, we previously found that activator protein 1 (AP-1) activity and cell transformation were stimulated by TPA and EGF in the JB6 mouse epidermal cell line (20). The components of AP-1, including c-Jun, are activated by the MAP kinases (21). Paxillin was first identified as a substrate for the oncogenic tyrosine kinase v-Src in v-Src transformed cells (22). It was also shown to act as a transducer for activation signals from RTKs to downstream MAP kinase such as JNKs (23). Although these results have suggested an involvement of paxillin in cell transformation and tumor development, the molecular basis of signaling or related mechanism(s) are clearly not understood yet.

Here, we demonstrated that paxillin plays a key role in skin cell transformation induced by EGF or TPA in JB6 Cl41 cells through regulation of c-Jun stability. The phosphorylation of c-Jun at serines 63 and 73 was suppressed in siRNA-paxillin stably transfected cells when stimulated with EGF or TPA. In addition, total c-Jun protein level was dramatically decreased in siRNA-paxillin cells dependent on serum deprivation time. The down-regulation of c-Jun was restored in siRNA-paxillin cells by treatment with the proteasome inhibitor lactacystin, but not by the lysosome inhibitor leupeptin. These results clearly provided evidence showing that paxillin has a role in regulating c-Jun protein stability and cell transformation.

Materials and Methods

Reagents and antibodies

Eagle's minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, MA). Taq DNA polymerase was from Qiagen, Inc. (Valencia, CA) and the DNA ligation kit (Version 2.1) was purchased from TAKARA Bio, Inc. (Otsu, Shiga, Japan). EGF was from BD Biosciences (San Jose, CA); TPA, cycloheximide, lactacystin, and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO); the luciferase assay substrate was from Promega (Madison, WI). Antibodies for Western blotting were purchased from Cell Signaling Technology, Inc.

(Beverly, MA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), or Upstate Biotechnology, Inc. (Charlottesville, VA).

Cell culture

The mouse skin epidermal cell line, JB6 Cl41, was cultured with MEM supplemented with 5% FBS and incubated in a 5% CO₂ atmosphere at 37°C. The human immortalized keratinocyte cell line, HaCaT, and the human skin epidermoid carcinoma cell line, A431, were cultured with DMEM supplemented with 10% FBS incubated in a 5% CO₂ atmosphere at 37°C. The mouse embryo fibroblast cell line, NIH/3T3, was cultured with DMEM supplemented with 10% fetal calf serum (FCS) incubated in a 5% CO₂ atmosphere at 37°C.

Construction of small interfering RNA (siRNA) paxillin and establishing the stable expression cell line

To construct the siRNA paxillin cells, the pU6pro vector was digested with *Xba*I and *Bbs*I. The sense oligonucleotide of paxillin that was used for siRNA was 5'-TTT GGA GCC CTC ACC TAC CGT CAT TCA AGA GAT GAC GGT AGG TGA GGG CTC TTT TT-3' (*Bbs*I cohesive end underlined), and the antisense was 5'-CTA GAA AAA GAG CCC TCA CCT ACC GTC ATC TCT TGA ATG ACG GTA GGT GAG GGC TC-3' (*Xba*I cohesive end underlined). Annealing, ligation, and colony screening were done as described previously (24). The oligonucleotide synthesis and sequencing of the inserted sequences in the mU6pro vector were performed by Sigma. The plasmid siRNA-paxillin and pcDNA3.1 were stably transfected into JB6 Cl41 cells using the Lipofectamine™ 2000 reagent. The transfected cells (si-mock or si-paxillin) were selected with G418, and paxillin expression level was confirmed by a Western blot assay using anti-paxillin (Upstate Biotechnology, Inc., VA).

Cell proliferation assay

Si-mock or si-paxillin stably transfected cells were suspended in 5% FBS/MEM at a density of 1.5×10^3 cells/ml. The cell suspension (2 ml) was placed in each well of a 6-well plate and cultured in a 5% CO₂ incubator at 37°C. After incubation for various time periods, cell number was determined using a microscope.

Anchorage-independent cell transformation assay

EGF- or TPA-induced cell transformation was investigated in si-mock or si-paxillin stably transfected cells. Cells (8×10^3 /ml) were exposed to EGF (0.1, 1 ng/ml) or TPA (10, 20 ng/ml) in 1 ml of 0.33% basal medium Eagle agar containing 10% FBS. The cultures were incubated in a 5% CO₂ atmosphere at 37°C for 10 days (EGF) or 18 days (TPA) and the colonies were scored as described by Colburn *et al.* (25).

Assay of AP-1 activity

Si-mock or si-paxillin stably transfected cells were transiently transfected with 2 µg of the AP-1 luciferase reporter plasmid together with 0.1 µg of the pRL-SV-40 plasmid. The cells were starved in 0.1% FBS/MEM for 24 h and then were exposed with EGF (10, 50 ng/ml) or TPA (5, 20 ng/ml). The cells were disrupted with lysis buffer 12 h later, and the luciferase activity was measured according to the manufacturer's instructions (Promega, Madison, WI) using a luminometer (Monolight 2010, San Diego, CA). The results are expressed as relative AP-1 activity (26).

Electrophoretic mobility shift assay

AP-1 DNA binding was determined by using the electrophoretic mobility shift assay (EMSA) as described previously (27). Nuclear protein extracts were prepared from si-mock or si-

paxillin stably transfected cells treated for 12 h with EGF (5 ng/ml), TPA (10 ng/ml), UVB (4 kJ/m²), or vehicle (DMSO) as a control. An AP-1 oligonucleotide (5'-CGC TTG ATG ACT CAG CCG GAA-3') was synthesized and labeled with [³²P]dATP using the T4 polynucleotide kinase. Nuclear protein (3 µg) was incubated with 100,000 cpm ³²P-labeled oligonucleotide probe, 1 µg of polydeoxyinosinicdeoxycytidylic acid, 3 µg of BSA on ice for 10 min followed by incubation at room temperature for 20 min. To determine the binding specificity, a 5-fold excess of cold oligonucleotide was incubated in a similar reaction. The DNA-protein complexes were resolved in a 5% nondenaturing polyacrylamide gel. The gel was dried and scanned using the Storm 840 Phosphor-Image System (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting

Samples containing equal amounts of protein were resolved in an 8, 10 or 12% SDS-polyacrylamide gel and transferred onto PVDF (polyvinylidene difluoride) membranes. The membranes were blocked and probed with phospho-specific antibodies against JNKs, p38, ERKs, or c-Jun (Ser63 and Ser73; Cell Signaling Technology, Beverly, MA) or with a mouse monoclonal (mAb) paxillin antibody. The Western blots were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences Corp., Piscataway, NJ).

Results

Paxillin is expressed in skin epidermal cell lines

Paxillin is widely expressed in most adult human tissues with the exception of brain (8). Furthermore, paxillin is up-regulated in several types of malignant tumors (28–30). We examined paxillin expression level in mouse embryo fibroblast (NIH/3T3) cells, mouse skin epidermal cells (JB6 Cl41), human immortalized keratinocytes (HaCaT), and human skin epidermoid carcinoma cells (A431) (Fig. 1A). Notably, the expression of paxillin was highest in the A431 human skin epidermoid carcinoma cells compared with the other epidermal cell lines.

Serine phosphorylation of paxillin is induced by EGF or TPA in JB6 Cl41 cells

Recently, JNK1 has been shown to directly phosphorylate paxillin (Ser178), which was implicated in the regulation of EGF-stimulated cell migration (31). We have previously demonstrated that EGF or TPA is a well known skin cancer promoter *in vivo* and *in vitro* and the effects are mediated through the MAP kinase signaling cascades (20,32–36). We examined the effect of EGF or TPA activation on phosphorylation of paxillin (Ser178) in the mouse skin epidermal JB6 Cl41 cell line. EGF treatment induced strong phosphorylation of paxillin (Ser178) at 15 min and the phosphorylation was maintained for at least 3 h (Fig. 1B). On the other hand, TPA induced phosphorylation of paxillin (Ser178) at 15 min and the phosphorylation increased gradually up to 12 h (Fig. 1B). These results indicated that phosphorylation of paxillin (Ser178) was induced by treatment with tumor promoters such as EGF or TPA.

siRNA knock-down of paxillin inhibits cell proliferation

To further delineate the role of paxillin in cell proliferation and transformation, an siRNA against paxillin (si-paxillin) was constructed by replacing the green fluorescence protein (GFP) region of the pU6pro vector with si-paxillin primers (Fig. 2, A and B). We confirmed the efficiency of siRNA in knocking down paxillin protein level in stably transfected cells by Western blotting. Paxillin protein level was dramatically decreased in si-paxillin stably transfected cells compared with si-mock stably transfected cells (Fig. 2C). Furthermore, we found that si-paxillin stably transfected cells delayed the rate of spreading agreeing with others who observed similar results in paxillin-deficient cells (data not shown) (37). To examine the

effect of paxillin on cell proliferation, we analyzed a cell growth curve for si-mock and si-paxillin stably transfected cells. The results indicated that cell proliferation of si-paxillin stably transfected cells was significantly suppressed compared with si-mock stably transfected cells (*, $p < 0.01$; Fig. 2D). These results suggested that paxillin is involved in cell proliferation.

siRNA knock-down of paxillin inhibits anchorage-independent tumor promoter-induced cell transformation

The mouse skin epidermal JB6 Cl41 cell system is a well-developed model for studying tumor promotion under anchorage independent growth conditions (20). This model was therefore used to determine the role of paxillin in neoplastic cell transformation. Si-mock or si-paxillin stably transfected cells were stimulated with EGF (0, 0.1, 1.0 ng/ml) or TPA (0, 10, 20 ng/ml) as described in "Materials and Methods." The number of colonies formed after treatment with EGF (Fig. 3, A and B) or TPA (Fig. 3, C and D) was significantly (*, $p < 0.01$) decreased in the si-paxillin stably transfected cells compared with the si-mock stably transfected cells. These results indicated that paxillin plays an important role in promoting neoplastic cell transformation in JB6 Cl41 cells exposed to EGF or TPA.

siRNA knock-down of paxillin inhibits EGF- or TPA-induced AP-1 activation and DNA binding activity

AP-1 transactivation has been shown to be required for neoplastic transformation in JB6 Cl41 cells (20). To test whether the inhibition of cell transformation by si-paxillin involves suppression of AP-1 activity, we transfected an AP-1 linked luciferase promoter plasmid into si-mock or si-paxillin stably transfected cells and stimulated the cells with EGF or TPA. The results indicated that AP-1 activation was induced by EGF or TPA in si-mock stably transfected cells, but not in si-paxillin stably transfected cells (Fig. 4, A and B). To further study the molecular basis of paxillin's inhibitory effect on AP-1 activation, AP-1 DNA binding activity was analyzed by EMSA. Our results indicated that EGF, TPA or UVB-induced AP-1 DNA binding activity was decreased in si-paxillin stably transfected cells compared to si-mock stably transfected cells (Fig. 4C). Taken together, these results suggested that AP-1 activation might be mediated through the paxillin pathway.

si-Paxillin inhibits EGF- or TPA-induced phosphorylation of JNKs and c-Jun

MAP kinases contribute to the activation of AP-1 in response to a broad range of extracellular stimuli (38,39). To test whether the paxillin pathway is associated with activation of the MAP kinases, we used Western blotting to detect EGF- or TPA-induced phosphorylation of ERKs, p38 or JNKs in si-mock or si-paxillin stably transfected cells. Our results indicated that EGF- or TPA-induced phosphorylation of JNKs was suppressed in si-paxillin stably transfected cells (Fig. 5, A and B). Furthermore, phosphorylation of c-Jun and total c-Jun expression were also decreased in si-paxillin stably transfected cells (Fig. 5, A and B). On the other hand, our results indicated that EGF- or TPA-induced phosphorylation of ERKs and p38 were similar in both si-mock and si-paxillin stably transfected cells (data not shown). These results strongly suggested that paxillin is required for EGF- or TPA-induced cell transformation mediated through the activation and phosphorylation of JNKs and c-Jun.

si-Paxillin is involved in c-jun protein down-regulation

The c-Jun protein is degraded through the ubiquitin pathway, which is also responsible for regulating the stability of other short-lived proteins such as c-Fos, c-Myc and p53 (40,41). Phosphorylation of c-Jun by JNKs can protect c-Jun from ubiquitination (42). Our results indicated that the knock-down of paxillin caused a dramatic decrease in c-Jun protein expression without the addition of EGF or TPA (Fig. 5, A and B). We also observed that TPA-, EGF-, or UVB-induced c-Jun protein expression levels were decreased in si-paxillin stably

transfected cells (Fig. 6A), which further agrees with the observation of decreased AP-1 activation and DNA binding activity. This result suggested that paxillin also might play a role in stabilizing c-Jun protein expression, which is independent of EGF- or TPA-induced signal transduction. To examine whether paxillin is essential for c-Jun protein stability, si-mock or si-paxillin stably transfected cells were starved by replacing the medium with 0.1% FBS/MEM and culturing for the indicated time period (Fig. 6B). Our results indicated that compared to si-mock stably transfected cells, total c-Jun protein level was dramatically decreased in si-paxillin stably transfected cells and was dependent on length of serum deprivation (Fig. 6B). c-Jun has been shown to be ubiquitinated and rapidly degraded *via* the proteasome pathway (40). Therefore, we hypothesized that paxillin may play a key role in maintaining c-Jun protein stability. Si-mock or si-paxillin stably transfected cells were treated for 6 h under serum-deprived conditions, with cycloheximide, an inhibitor of protein synthesis (10 µg/ml), and either lactacystin (10 µM), an inhibitor of the proteasome degradation pathway, or leupeptin (100 µg/ml), an inhibitor of the lysosomal degradation pathway. Our results indicated that expression of the c-Jun protein in si-paxillin stably transfected cells was at least partially restored by treatment with the proteasome inhibitor, lactacystin, but not by the lysosome inhibitor, leupeptin (Fig. 6C). β-actin protein levels were not affected under these conditions. Overall these results indicate that paxillin is required for cell transformation and AP-1 transactivation mediated through the JNKs pathway.

Discussion

Paxillin has been classified as a molecular adaptor or scaffold protein because it contains many protein binding modules such as LIM domains and LD motifs. These domains and motifs are important for binding with signaling molecules such as FAK, vinculin, C-terminal Src kinase (CSK), Src, and papilloma virus E6 oncogene (15,43–47), and serve as phosphorylation targets of intracellular protein kinases (15,31,43,48). Importantly, these phosphorylations are induced after stimulation with angiotensin II, transforming growth factor (TGF)-β or EGF, which are well known growth factors and tumor promoters (5,49,50). The stimulatory signaling of these factors has essential roles in cell proliferation, cell transformation and cell migration mediated through the MAP kinase signaling pathways (5,49,50). Furthermore, JNK1, which is one of the most well studied members of the MAP kinase family, phosphorylates paxillin at serine 178 following EGF stimulation (31). Our results also showed that paxillin is phosphorylated at serine 178 following stimulation by EGF or TPA. Therefore, we hypothesized that paxillin may have functions in addition to cell migration. In our experiments, we showed that knockdown of paxillin by siRNA resulted in decreased proliferation, a delayed rate of cell spreading, and a substantial decrease in EGF- or TPA-induced neoplastic cell transformation. These changes were associated with a suppression of EGF- or TPA-induced AP-1 activation and DNA binding and a down regulation of JNKs and c-Jun phosphorylation and c-Jun protein expression levels. These results clearly indicated that paxillin is involved in mediating cell proliferation and transformation through the regulation of protein stability including JNKs and c-Jun.

There are two possibilities for the observed increase in paxillin protein level with stimulation by EGF and TPA. EGF and TPA stimulate MAPK signaling cascades and activate downstream target transcription factors, resulting in increased target gene expression. One of the most well known downstream targets is the AP-1 transcription factor complex, which most commonly exists as a dimer consisting of c-Jun and c-Fos proteins. In RT-PCR analysis, we found that EGF and TPA increased paxillin mRNA level in a time dependent manner (data not shown). Although paxillin can be phosphorylated by JNK1 and involve cell migration (31), we found that knockdown of paxillin decreased phosphorylation level of the JNKs (Fig. 5A and 5B). This result suggested that paxillin can affect upstream signaling pathway of the JNKs. Overexpression of wildtype FAK induces phosphorylation of p130^{cas} and paxillin, however,

it did not activated ERKs and JNKs (51). COOH-terminal truncated form of the myr-FAK has an ability to associate with paxillin-associate complexes *in vivo* and activates JNK. Conversely, overexpression of COOH-terminal domain of myr-FAK fails to association of paxillin and activation of JNK, suggest that NH₂-terminal of FAK involves paxillin binding, result in JNK activation (52). These FAK-paxillin-JNK signaling pathways may through protein kinase PAK, which is an upstream component of JNK pathway (53,54), or PKC (55).

To date, although a function of paxillin in cell transformation and tumor development has been suggested, no direct evidence or specific molecular mechanism has yet been published. In the present study, the function of paxillin as an adaptor or “protein stabilizer” is a novel mechanism for understanding how proteins can be protected from the ubiquitination-dependent degradation pathway during the cell transformation process. Therefore, paxillin may be a good target molecule for new chemopreventive and therapeutic drugs.

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References

1. Tumbarello DA, Brown MC, Turner CE. The paxillin LD motifs. *FEBS Lett* 2002;513:114–8. [PubMed: 11911889]
2. Gill GN. The enigma of LIM domains. *Structure* 1995;3:1285–9. [PubMed: 8747454]
3. Brown MC, Perrotta JA, Turner CE. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J Cell Biol* 1996;135:1109–23. [PubMed: 8922390]
4. Brown MC, Perrotta JA, Turner CE. Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. *Mol Biol Cell* 1998;9:1803–16. [PubMed: 9658172]
5. Brown MC, Turner CE. Characterization of paxillin LIM domain-associated serine threonine kinases: activation by angiotensin II in vascular smooth muscle cells. *J Cell Biochem* 1999;76:99–108. [PubMed: 10581004]
6. Turner CE. Paxillin and focal adhesion signalling. *Nat Cell Biol* 2000;2:E231–6. [PubMed: 11146675]
7. Salgia R, Li JL, Lo SH, et al. Molecular cloning of human paxillin, a focal adhesion protein phosphorylated by P210BCR/ABL. *J Biol Chem* 1995;270:5039–47. [PubMed: 7534286]
8. Mazaki Y, Hashimoto S, Sabe H. Monocyte cells and cancer cells express novel paxillin isoforms with different binding properties to focal adhesion proteins. *J Biol Chem* 1997;272:7437–44. [PubMed: 9054445]
9. Brown MC, Turner CE. Paxillin: adapting to change. *Physiol Rev* 2004;84:1315–39. [PubMed: 15383653]
10. Schaller MD. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene* 2001;20:6459–72. [PubMed: 11607845]
11. Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol* 1996;12:463–518. [PubMed: 8970735]
12. Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999;285:1028–32. [PubMed: 10446041]
13. Schwartz MA, Baron V. Interactions between mitogenic stimuli, or, a thousand and one connections. *Curr Opin Cell Biol* 1999;11:197–202. [PubMed: 10209147]
14. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 1996;135:1633–42. [PubMed: 8978828]
15. Schaller MD, Parsons JT. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol Cell Biol* 1995;15:2635–45. [PubMed: 7537852]

16. Weng Z, Taylor JA, Turner CE, Brugge JS, Seidel-Dugan C. Detection of Src homology 3-binding proteins, including paxillin, in normal and v-Src-transformed Balb/c 3T3 cells. *J Biol Chem* 1993;268:14956–63. [PubMed: 8325872]
17. McCormack SJ, Brazinski SE, Moore JL Jr, Werness BA, Goldstein DJ. Activation of the focal adhesion kinase signal transduction pathway in cervical carcinoma cell lines and human genital epithelial cells immortalized with human papillomavirus type 18. *Oncogene* 1997;15:265–74. [PubMed: 9233761]
18. Hunter T, Karin M. The regulation of transcription by phosphorylation. *Cell* 1992;70:375–87. [PubMed: 1643656]
19. Bernstein LR, Colburn NH. AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* 1989;244:566–9. [PubMed: 2541502]
20. Dong Z, Birrer MJ, Watts RG, Matrisian LM, Colburn NH. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc Natl Acad Sci U S A* 1994;91:609–13. [PubMed: 8290571]
21. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 1993;268:14553–6. [PubMed: 8325833]
22. Glenney JR Jr, Zokas L. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. *J Cell Biol* 1989;108:2401–8. [PubMed: 2472406]
23. Turner CE. Paxillin interactions. *J Cell Sci* 2000;113(Pt 23):4139–40. [PubMed: 11069756]
24. Yu JY, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* 2002;99:6047–52. [PubMed: 11972060]
25. Colburn NH, Wendel EJ, Abruzzo G. Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: mitogen-resistant variants are sensitive to promotion. *Proc Natl Acad Sci U S A* 1981;78:6912–6. [PubMed: 6947266]
26. Dong Z, Lavrovsky V, Colburn NH. Transformation reversion induced in JB6 RT101 cells by AP-1 inhibitors. *Carcinogenesis* 1995;16:749–56. [PubMed: 7728951]
27. Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. *Cancer Res* 1997;57:4414–9. [PubMed: 9331105]
28. Salgia R, Li JL, Ewaniuk DS, et al. Expression of the focal adhesion protein paxillin in lung cancer and its relation to cell motility. *Oncogene* 1999;18:67–77. [PubMed: 9926921]
29. Vadlamudi R, Adam L, Tseng B, Costa L, Kumar R. Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells. *Cancer Res* 1999;59:2843–6. [PubMed: 10383144]
30. Kuroda N, Guo L, Toi M, et al. Paxillin: application of immunohistochemistry to the diagnosis of chromophobe renal cell carcinoma and oncocytoma. *Appl Immunohistochem Mol Morphol* 2001;9:315–8. [PubMed: 11759057]
31. Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. *Nature* 2003;424:219–23. [PubMed: 12853963]
32. Liu G, Bibus DM, Bode AM, Ma WY, Holman RT, Dong Z. Omega 3 but not omega 6 fatty acids inhibit AP-1 activity and cell transformation in JB6 cells. *Proc Natl Acad Sci U S A* 2001;98:7510–5. [PubMed: 11416221]
33. Liu G, Bode A, Ma WY, Sang S, Ho CT, Dong Z. Two novel glycosides from the fruits of *Morinda citrifolia* (noni) inhibit AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line. *Cancer Res* 2001;61:5749–56. [PubMed: 11479211]
34. Huang C, Ma WY, Young MR, Colburn N, Dong Z. Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. *Proc Natl Acad Sci U S A* 1998;95:156–61. [PubMed: 9419345]
35. Dong Z. Effects of food factors on signal transduction pathways. *Biofactors* 2000;12:17–28. [PubMed: 11216482]
36. Dong Z, Huang C, Ma WY. PI-3 kinase in signal transduction, cell transformation, and as a target for chemoprevention of cancer. *Anticancer Res* 1999;19:3743–7. [PubMed: 10625951]

37. Hagel M, George EL, Kim A, et al. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 2002;22:901–15. [PubMed: 11784865]
38. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* 1996;74:589–607. [PubMed: 8912180]
39. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 1995;270:16483–6. [PubMed: 7622446]
40. Treier M, Staszewski LM, Bohmann D. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell* 1994;78:787–98. [PubMed: 8087846]
41. Ciechanover A, DiGiuseppe JA, Bercovich B, et al. Degradation of nuclear oncoproteins by the ubiquitin system in vitro. *Proc Natl Acad Sci U S A* 1991;88:139–43. [PubMed: 1846034]
42. Musti AM, Treier M, Bohmann D. Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* 1997;275:400–2. [PubMed: 8994040]
43. Bellis SL, Miller JT, Turner CE. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J Biol Chem* 1995;270:17437–41. [PubMed: 7615549]
44. Turner CE. Paxillin. *Int J Biochem Cell Biol* 1998;30:955–9. [PubMed: 9785458]
45. Sabe H, Hata A, Okada M, Nakagawa H, Hanafusa H. Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. *Proc Natl Acad Sci U S A* 1994;91:3984–8. [PubMed: 7513429]
46. Tong X, Howley PM. The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. *Proc Natl Acad Sci U S A* 1997;94:4412–7. [PubMed: 9114003]
47. Vande Pol SB, Brown MC, Turner CE. Association of Bovine Papillomavirus Type 1 E6 oncoprotein with the focal adhesion protein paxillin through a conserved protein interaction motif. *Oncogene* 1998;16:43–52. [PubMed: 9467941]
48. Bellis SL, Perrotta JA, Curtis MS, Turner CE. Adhesion of fibroblasts to fibronectin stimulates both serine and tyrosine phosphorylation of paxillin. *Biochem J* 1997;325 (Pt 2):375–81. [PubMed: 9230116]
49. Riedy MC, Brown MC, Molloy CJ, Turner CE. Activin A and TGF-beta stimulate phosphorylation of focal adhesion proteins and cytoskeletal reorganization in rat aortic smooth muscle cells. *Exp Cell Res* 1999;251:194–202. [PubMed: 10438585]
50. Terfera DR, Brown MC, Turner CE. Epidermal growth factor stimulates serine/threonine phosphorylation of the focal adhesion protein paxillin in a MEK-dependent manner in normal rat kidney cells. *J Cell Physiol* 2002;191:82–94. [PubMed: 11920684]
51. Igishi T, Fukuhara S, Patel V, Katz BZ, Yamada KM, Gutkind JS. Divergent signaling pathways link focal adhesion kinase to mitogen-activated protein kinase cascades. Evidence for a role of paxillin in c-Jun NH(2)-terminal kinase activation. *J Biol Chem* 1999;274:30738–46. [PubMed: 10521463]
52. Schaller MD, Borgman CA, Parsons JT. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Mol Cell Biol* 1993;13:785–91. [PubMed: 8423801]
53. Bagrodia S, Derijard B, Davis RJ, Cerione RA. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* 1995;270:27995–8. [PubMed: 7499279]
54. Brown JL, Stowers L, Baer M, Trejo J, Coughlin S, Chant J. Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. *Curr Biol* 1996;6:598–605. [PubMed: 8805275]
55. Goel HL, Dey CS. PKC-regulated myogenesis is associated with increased tyrosine phosphorylation of FAK, Cas, and paxillin, formation of Cas-CRK complex, and JNK activation. *Differentiation* 2002;70:257–71. [PubMed: 12190987]

Figure 1.

Paxillin is expressed in skin cells and phosphorylation is induced by EGF or TPA. *A*, NIT/3T3 mouse embryo fibroblast cells, JB6 Cl41 mouse skin epidermal cells, HaCaT human immortalized keratinocyte cells, or A431 human skin epidermoid carcinoma cells were cultured in 60-mm dishes and harvested when cells reached 80% confluence. Total cell lysates from the indicated cell lines were used to analyze paxillin protein levels by Western blotting using a paxillin monoclonal antibody (mAb). Equal protein loading was confirmed using a β -actin antibody on the same membrane. The protein level was normalized with β -actin protein level and quantified by comparison with NIH/3T3 cells. *B*, JB6 Cl41 cells were cultured, starved, and stimulated with 1 ng/ml EGF or 10 ng/ml TPA for the indicated time. Phosphorylation of paxillin (Ser178) or total paxillin protein was visualized by Western blotting using a phospho-specific antibody or an antibody against total paxillin, respectively. Equal protein loading was confirmed by stripping the membrane and reprobing with a β -actin antibody. The protein level was normalized with β -actin protein level and quantified by comparison with non-treated control.

Figure 2.

Si-paxillin inhibits cell proliferation. *A*, schematic diagram for the construction of the siRNA paxillin expression vector. *B*, DNA sequence of the two synthetic oligonucleotide primers. *C*, JB6 Cl41 cells were co-transfected with pcDNA3.1neo and pU6pro mock control or siRNA paxillin and selected with 400 μ g/ml G418. The si-mock and si-paxillin stably transfected cells were disrupted with cell lysis buffer. Total cell lysates were used to analyze paxillin protein levels by Western blotting using a paxillin mAb and β -actin was used to confirm equal protein loading. *D*, the si-mock or si-paxillin stably transfected cells were seeded (3×10^3 /well) in 6-well plates in 5% FBS/MEM. After incubation for the indicated times, cell number was determined using a microscope. *Asterisk indicates a significantly higher rate of growth ($p <$

0.01) of si-mock stably transfected cells compared to si-paxillin stably transfected cells. Data are represented as means $\pm$ S.D. of 3 independent experiments.

Figure 3.

Tumor promoter-induced cell transformation is inhibited in si-paxillin stably transfected cells. Si-mock or si-paxillin stably transfected cells were used to assess colony formation in an anchorage-independent cell transformation assay (soft agar assay) in the presence of EGF or TPA. Cells (8×10^3 /ml) were exposed to EGF (0, 0.1, or 1 ng/ml) or TPA (0, 10, or 20 ng/ml) in 1 ml of 0.33% basal medium Eagle agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO₂ incubator for 10 days (EGF, A and B) or 18 days (TPA, C and D), and the colonies were scored as described by Colburn *et al.* (25). *Asterisk indicates a significantly ($p < 0.01$) greater number of colonies formed by si-mock stably transfected cells compared to si-

paxillin stably transfected cells. Data are represented as means $\pm$ S.D. of 3 independent experiments.

Figure 4.

Si-paxillin inhibits EGF- or TPA-induced AP-1 activity and DNA binding activity. Si-mock or si-paxillin stably transfected cells (80% confluence) were transiently transfected with 2 μ g of AP-1 luciferase reporter plasmid together with 0.1 μ g of a pRL-SV-40 plasmid. At 24 hours after transfection, cells were starved in 0.1% FBS/MEM for another 24 h and then were or were not exposed to either 10 or 50 ng/ml EGF (A) or 5 or 20 ng/ml TPA (B) for an additional 12 hours. The firefly luciferase activity was determined in cell lysates and normalized against *Renilla* luciferase activity. Asterisks (** $p < 0.01$; * $p < 0.05$) indicate significantly greater EGF- or TPA-induced AP-1 activity in si-mock stably transfected cells compared to si-paxillin stably transfected cells. Data are represented as means $\pm$ S.D. of 3 independent experiments.

C, Si-mock or si-paxillin stably transfected cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 24 h. Cells were then treated or not treated with 5 ng/ml EGF, 10 ng/ml TPA, or 4 kJ/m² UVB and harvested after 12 h. After treatment, nuclear proteins (3 µg) were prepared from si-mock or si-paxillin stably transfected cells. Sequence specific AP-1 DNA binding activity was determined by EMSA using a ³²P-labeled oligonucleotide containing the AP-1 binding site as described in "Materials and Methods". To determine the binding specificity, a 5-fold excess of unlabeled AP-1 oligonucleotide as the Specific Competitor (*lane 3*) or unlabeled NF-κB oligonucleotides (*lane 2*) as the Nonspecific Competitor was incubated in similar individual reactions. The Negative Control (*lane 1*) contains no protein and the Control (*lanes 4 and 8*) contains nuclear proteins from untreated cells.

Figure 5.

Si-paxillin inhibits EGF- or TPA- induced phosphorylation and protein level of c-Jun. Si-mock or si-paxillin stably transfected cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 24 h. Cells were then treated or not treated with 1 ng/ml EGF (A) or 10 ng/ml TPA (B) for 15 min. After treatment, the cells were harvested and subjected to Western blotting to detect phosphorylation of various MAP kinases and phosphorylation and protein levels of c-Jun. Equal protein loading was confirmed by stripping the membrane and reprobing with a β -actin antibody.

Figure 6.

Si-paxillin is involved in c-Jun protein down-regulation. **A**, si-mock or si-paxillin stably transfected cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for 24 h. Cells were then treated or not treated with 5 ng/ml EGF, 10 ng/ml TPA, or 4 kJ/m² UVB and harvested after 12 h. Nuclear proteins from the preparations above were used to analyze EGF-, TPA, or UVB-induced c-Jun protein levels by Western blotting. **B**, si-mock or si-paxillin stably transfected cells (80% confluence) were starved by replacing the medium with 0.1% FBS/MEM and culturing for the indicated time. The cells were harvested and subjected to Western blotting to detect c-Jun protein levels. **C**, si-mock or si-paxillin stably transfected cells (80% confluence) were treated with cycloheximide (10 μg/ml), an inhibitor of protein synthesis, and lactacystin (10 μM), an inhibitor of the proteasome degradation pathway, or leupeptin (100 μg/ml), an inhibitor of the lysosomal degradation pathway for 6 h under serum-deprived conditions.