Paxillin Isoforms in Mouse

LACK OF THE γ ISOFORM AND DEVELOPMENTALLY SPECIFIC β ISOFORM EXPRESSION

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Paxillin, a focal adhesion protein, exists as multiple isoforms in humans (α, β, and γ). To understand more about the physiological role of each isoform, we have employed the mouse system. We found that although the α and β isoforms are present in the mouse, the γ isoform is not. The α isoform protein was detected clearly in most adult tissues, whereas the β isoform protein was almost undetectable except in spleen, testis, thymus, and lung. On the other hand, mRNAs of both isoforms were detectable in all tissues we examined. High levels of the β isoform protein was detected in peritoneal exudate macrophage cells in adult mouse as well as in cultured fibroblasts, together with the α isoform. The α isoform was expressed at a constant level throughout the embryonic stages we examined, whereas the β isoform protein was detected at the mid-stages of development and increased to levels almost equal to those of the α isoform during the late stages of embryogenesis. Therefore, unlike the α isoform, expression of the β isoform protein is restricted in adult tissues. Moreover, we showed that α and β isoforms were colocalized within the same focal adhesion plaques, and cytoplasmic pools of both isoforms exist in the perinuclear area, colocalized with the Golgi apparatus.

Paxillin was originally identified as a substrate for the v-Src tyrosine kinase (1) and shown to be highly localized at focal adhesions in fibroblasts (2). A number of results indicate the importance of paxillin in the focal adhesion assembly and the integrin-mediated signal transduction. Paxillin can interact directly with several focal adhesion proteins including vinculin, talin, and integrin β1 (2–5). Paxillin also binds directly to signal transducers of protein tyrosine kinases such as focal adhesion kinase (5, 6) and c-Src (7), and is tyrosine phosphorylated upon cell to substratum adhesion (8), thus creating the binding sites for the Src homology 2 domains of Csk and Crk (9–11). Inhibition of this tyrosine phosphorylation blocks the formation of the focal adhesions and cell cycle progression into S-phase (8). We have also shown that paxillin is hyperphosphorylated on serine and down-regulated during mitosis where the cell adherence to the extracellular matrices is greatly reduced (12). Moreover, papilloma E6 protein binds to paxillin, and this binding correlates with disruption of the actin cytoskeletal architecture in the virus-infected cells (13, 14).

The paxillin cDNA originally isolated from fibroblasts was the α isoform of chicken (3) and human (4), and we identified two more human isoforms, β and γ (15). The β and γ isoforms of paxillin are generated by the insertion of specific exons into the same site as the α isoform. We have shown that mRNA of the α isoform is expressed in most normal human tissues, whereas mRNA of the β and γ isoforms are almost undetectable. On the other hand, human cancer cells and monoblast cells cultured in vitro have been shown to express these isoforms simultaneously. Notably, expression of the γ isoform is up-regulated during the differentiation of floating monoblast cells into adherent monocyte/macrophage-like cells. Moreover, each isoform exhibits different binding properties to focal adhesion proteins, suggesting distinct physiological roles for each isoform.

Because one purpose of analyzing paxillin isoforms is to delineate and understand their possible roles in cancer metastasis, we began with analysis of human material (15). Here, as an extension of our previous study, we analyzed paxillin in the murine system. We first isolated the murine genomic DNA fragments corresponding to the human β- and γ-specific exons. Our analysis revealed that rodents do not bear an exon that is specific to the γ isoform, although both the α and β isoforms are present. We present evidence that the γ-specific exon in humans evolved from ancestral genomic sequences that are also present in rodents. We also examined the expression of paxillin protein isoforms in adult and embryonic tissues and their subcellular localization. The possible biological relevance of the difference in the γ isoform between human and mouse is also discussed.

EXPERIMENTAL PROCEDURES

Tissues and Cells—129/Sv mice and BALB/c mice were obtained from National Institute for Genetics (Shizuoka, Japan). Peritoneal exudate cells were isolated from BALB/c mice 4 days after injecting Brewer’s thioglycolate medium. Hepatocellular carcinomas were developed in transgenic mice carrying the SV40 T antigen under the control of the albumin promoter (16). Embryos were obtained from BALB/c mice. NIH 3T3 cells were cultured with Dulbecco’s modified Eagle’s medium with 5% fetal calf serum.

Isolation of Mouse Genomic Clones Encapsulating the β-Specific Exon of Paxillin—Standard methods (17) were used for DNA and RNA manipulations, unless otherwise stated. A dASH genomic library of the 129/Sv mouse was screened using human paxillin DNA fragments corresponding to the β- and γ-specific exons (15) as probes. After hybridization in a solution containing 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA at 65 °C for 16 h, filters were washed for 10 min at room temperature with 2× SSC and 0.1% SDS, and washed three times with 0.5× SSC and 0.1% SDS at 55 °C for 20
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| min. Positive phase clones were then isolated, and their inserted genomic DNA fragments were cut out using EcoRI or XhoI and ligated into pBluescript vector (Stratagene, La Jolla, CA). After transformation of the resulting plasmid into Escherichia coli JM109, each clone was isolated and subjected to restriction enzyme mapping and sequencing analysis.

**Isolation of a Genomic DNA Fragment of Paxillin from Different Mice Strains**—High molecular weight DNA isolated from tails of 129/Sv mice, BALB/c mice, Mus musculus musculus, and Mus spretus (the latter two were a gift from K. Kitada) were subjected to PCR amplification using oligonucleotides 5′-GAGCTCTAGGGGCTACTCTGAGATGTTTTCTTCTTCACTGATCTTTGCTCTGGCCATGAA-3′. Each two resulting plasmid DNA was confirmed by sequencing.

**Construction and Expression of Tagged Paxillin cDNAs**—Each paxillin isoform cDNA in pBabePuro vector (15) was fused with a mutant form of green fluorescent protein (EGFP) or a myc sequence as follows: for EGFP-paxillin α construction, BamHI-EcoRI fragments of paxillin α cDNA were isolated from the pGEX/paxillin α plasmid DNA (15) and ligated into PstI-XhoI vector cleaved with BglII and EcoRI. The ligated fragments were then isolated from this pEGFP-C1 construct, blunted, and ligated into pBabePuro vector cleaved with SmaI. For myc-paxillin β construction, BglII-EcoRI fragments of paxillin β cDNA were isolated from the pGEX/paxillin β (15), ligated with a synthetic double strand DNA fragment containing the 5′-end coding region of paxillin, Kozak sequence and the myc sequence: 5′-GATCTCCGGCCACTGGAGCAGAAGTCAGCAGACCTGAGCGCCCTG3′-3′-GGGCCGGTTACCTCGTCTTCGACTAGAGGCTC-3′, which encodes 4.7-kilobase pairs downstream of the exon sequence 5′-AACAAGCAGAAGTCAGCAGAGCC-3′. The ligated fragments were then ligated into pBabePuro vector cleaved with BamHI and EcoRI. Each resulting plasmid DNA was confirmed by sequencing.

**Protein Detection**—Cell extracts from whole embryos or tissues isolated from insoluble materials by centrifuging at 10,000 g for 10 min at 4 °C, lysates were precleared with protein G-Sepharose (Amersham Pharmacia Biotech). Each 3 mg of protein recovered was then washed with PBS, the cells were soaked in PBS containing 2% BSA for 30 min followed by incubation with primary antibodies in PBS containing 2% BSA at 37 °C for 1 h. The cells were then washed with PBS and incubated with rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory, Inc.) in PBS containing 2% BSA at 37 °C for 1 h. After being washed with PBS, the cells were washed with PBS and slides were mounted with PBS containing 50% glycerol and 0.1% p-phenylenediamine (Acris Organics, NJ). Photographs were taken using MRC1024 confocal laser-scanning microscope (Bio-Rad). Antibodies used for the immunostaining analysis were against the myc-tag epitope (9E10; Babco, Richmond, CA), vinculin (VIN11–5; Sigma), GRP78 (19) (Santa Cruz Biotechnology, Santa Cruz, CA), and Golgi 58-kidetal protein (20) (clone 58K-9; Sigma). EGFP-tagged paxillin isoforms were detected by their autofluorescence after cells were incubated for 6–8 h at 30 °C and then fixed as above. F-actin was visualized with BODIPY 581/591 phalloidin (Molecular Probes, Eugene, OR).

**RESULTS**

**Absence of the γ-Specific Exon in Mouse Genome**—The β and γ isoforms of human paxillin are generated by insertion of specific exons into Lys-277 and Phe-278 of the α isoform (15). Using human paxillin cDNAs encompassing β- and γ-specific sequences as probes, we isolated five clones from a murine 129/Sv genomic library (Fig. 1A). After the subcloning of each genomic DNA fragment into pBluescript, restriction enzyme mapping and partial nucleotide-sequencing analysis (data not shown) revealed 14.4 kilobase pairs of paxillin genomic sequence. We then focused on the region encompassing the exon encoding Lys-277 to the exon encoding Phe-278 of the α isoform (Fig. 1B). Sequencing analysis revealed that the β-specific exon, with 85% homology to that of the human β-specific exon, was found about 4.7-kilobase pairs downstream of the exon encoding Lys-277. Of note, the position of the mouse β exon relative to 5′- and 3′-flanking exons is similar to that of the human β exon (Fig. 1B). The deduced amino acid sequences encoded by the human and mouse β exons exhibited 71% homology.

The γ-specific human exon lies 142 nucleotides downstream of the β exon, and connects directly to the 3′-flanking exon encoding Phe-278 of the α isoform (15). The nucleotide sequence of the β exon and its 3′-flanking exon in the 129/Sv mouse showed some 62% homology with the corresponding
region in the human genome (Figs. 1B and 2). However, an exon corresponding to the human γ exon was not found in this region. The same nucleotide sequences were obtained from all five original phage clones. We then PCR-amplified the sequences corresponding to this region from high molecular weight DNAs isolated from 129/Sv mouse, BALB/c mouse, M. spretus-specific exon. Exons are enclosed in boxes, and their amino acid sequences deduced from nucleotide sequences are also shown. The consensus sequences of the exon-intron junction are indicated by an underline. Genomic structure encompassing the human paxillin β and γ isoform-specific exons (15) is shown above for comparison. B, BamHI; H, HindIII; P, PstI; S, SphI.

FIG. 1. Mouse genomic structure and nucleotide sequence encompassing the paxillin β-specific exon. A, mouse genomic DNA fragments (clones of Pax 1, 2, 3, 8, and 9) isolated from a DASH genomic library of 129/Sv mouse using human paxillin cDNA fragments corresponding to the β- and γ-specific exons as probes. Relative positions of other exons of mouse paxillin genome are shown above. B, nucleotide sequence surrounding the mouse paxillin β-specific exon. Exons are enclosed in boxes, and their amino acid sequences deduced from nucleotide sequences are also shown. The consensus sequences of the exon-intron junction are indicated by an underline. Genomic structure encompassing the human paxillin β and γ isoform-specific exons (15) is shown above for comparison. B, BamHI; H, HindIII; P, PstI; S, SphI.

which was made against amino acids 199–217 of the human α isoform and thereby recognizes all three human isoforms (15). Amino acids 199–217 of human paxillin are 84.2% identical (with 100% similarity) to the corresponding amino acids of murine paxillin (data not shown), and this polyclonal antibody recognized mouse and human paxillin equally well. As shown in Fig. 3A, all the tissues examined expressed paxillin as detected by Ab 199–217. The highest level of paxillin expression was observed in spleen. On the other hand, probing the filters with polyclonal anti-β antibody revealed that only marginal levels of the β isoform were expressed in most of these tissues. The polyclonal anti-β antibody was originally made against human β-specific amino acid sequence (15) but was equally reactive with the mouse β isoform and the human β isoform, as assessed with glutathione S-transferase fusion proteins of the human and mouse β isoforms (data not shown). Moreover, the same amount of recombinant α and β paxillin were included in each gel as internal standards, and the immunoblots were probed first with the anti-β, then Ab 199–217. Therefore, the paxillin bands detected with Ab 199–217 in Fig. 3A appear to represent primarily paxillin α. The expression of the α isoform mRNA can be detected by RT-PCR independently of that of the β isoform, and we have confirmed the α isoform mRNA expression in these tissues (Fig. 4A).

Using human RNA blot filters purchased from a commercial source, we have shown previously that the mRNAs of the β and the γ isoforms were almost undetectable in normal human tissues (15). However, in mice, significant levels of the β protein expression were detected in spleen, testis, thymus, and lung, although the levels of the β isoform in these tissues were still much lower than those of the α isoform, except in lung. Similar levels of β isoform expression were also detected in mouse bone marrow cells (data not shown). Moreover, the transcripts specific to the mouse paxillin β isoform were clearly detected in all the tissues examined, although the levels were again much lower than those of the α isoform in most tissues (Fig. 4B).

We also examined β isoform expression in mouse hepatocellular carcinoma tissues (16). The paxillin protein expression was similar to that observed in normal liver with an undetectable level of the β isoform (Fig. 3A).

High Levels of the α and β Isoforms Are Expressed During Embryogenesis—We then examined embryos. Immunoblotting of all embryonic protein samples revealed two distinct bands reacting with Ab 199–217. Probing the same filters with the anti-β antibody indicated that the upper bands in each preparation correspond to the β isoform (Fig. 3B). The lower bands, thus, appeared to correspond to the α isoform. Moreover, it should be noted that a fraction of paxillin in embryonic tissues is known to be tyrosine phosphorylated (21), causing smearing of the bands when separated on SDS-polyacrylamide gel electrophoresis, as seen in Fig. 3B. Again, the expression of the α isoform and the β isoform genes was confirmed by RT-PCR methods (data not shown), and the same amount of recombinant proteins was included on each gel as an internal standard. Judging from the lower bands (Fig. 3B), the level of isoform α per μg of embryo cell extract remained essentially constant throughout different stages of embryonic development that we examined (9.5–17.5 days post coitus (dpc)). Expression of the β isoform protein was clearly detected in 9.5- and 11.5-dpc embryos, whereas the levels of the β isoform were two to three times lower than those of the α isoform. In 15.5- and 17.5-dpc embryos, however, the β isoform expression was almost equal to that of the α isoform.

The α and β Isoforms Are Expressed in Peritoneal Exudate Cells Isolated from Adult Mice as Well as Cultured Fibro-

region in the human genome (Figs. 1B and 2). However, an exon corresponding to the human γ exon was not found in this region. The same nucleotide sequences were obtained from all five original phage clones. We then PCR-amplified the sequences corresponding to this region from high molecular weight DNAs isolated from 129/Sv mouse, BALB/c mouse, M. musculus molossinus and M. spretus. Genomic paxillin sequences from these different strains or sub-species of rodents also showed homology to each other and did not contain an exon corresponding to the human γ exon (Fig. 2). Moreover, we have sequenced the entire region of the 129/Sv mouse genome from the exon encoding Lys-277 to the exon encoding Phe-278 of the α isoform shown in Fig. 1B and did not find the γ exon (data not shown). Therefore, we conclude that the mouse genome does not encode the γ isoform of paxillin.

β Isoform Expression Is Restricted in Adult Mouse Tissue Whereas the α Isoform Is Expressed Ubiquitously—We then examined protein expression of the α and β isoforms in normal adult tissues of mice. We used polyclonal antibody Ab 199–217.
We have reported that the human monoblast cell line U937 expresses all three paxillin isoforms (15). The same was true with another human monoblast cell line, THP-1.\(^2\) Paxillin isoform expression in mouse monocyte/macrophage cells was therefore examined. We isolated peritoneal exudate cells/macrophages from adult mice treated with thioglycolate. As shown in Fig. 3C, these cells expressed the \(a\) and \(b\) isoforms at similar levels. Smearings of the bands was primarily because of phosphorylation (data not shown). We also examined isoform expression in cultured NIH 3T3 fibroblasts and found that the cells expressed both isoforms simultaneously. Unlike the peritoneal exudate cells/macrophages, however, the level of expression of the \(b\) isoform was significantly lower than that of the \(a\) isoform (Fig. 3C). The same was true with Swiss 3T3 and 3Y1 fibroblasts (data not shown).

**Colocalization of the \(a\) and \(b\) Isoforms in Focal Adhesions, the Cell Periphery and Perinuclear Regions in Fibroblasts**—We next examined the subcellular localization of the each isoform in NIH3T3 fibroblasts. Upon solubilization of fibroblast cells, paxillin was recovered partly in the soluble fraction and partly in a particulate fraction (data not shown). We made epitope-tagged constructs of each paxillin isoform; the \(a\) and \(b\) isoforms with an EGFP-tag, and the \(g\) isoform with a myc-tag. As shown in Fig. 5 a–c, no significant difference in subcellular localization was observed, and a significant fraction of each isoform was indeed localized to the focal adhesions. Both isoforms were also detected in certain regions of the cell periphery. In addition to these regions, we noticed that immunostaining of endogenous paxillin with the anti-paxillin antibody gave rise to a remarkable staining of the perinuclear regions of membranous organelle structures in fibroblasts (data not shown). Because this kind of immunostaining often gives a nonspecific signal around perinuclear regions, we were not confident of the localization of paxillin in these regions. However, the localization of paxillin at the perinuclear regions was also seen with EGFP-tagged paxillin \(a\) (Fig. 5 a), with myc-tagged paxillin \(b\) (Fig. 5 b), with myc-tagged paxillin \(a\), and with EGFP-tagged paxillin \(b\) (data not shown). Neither fluorescence from EGFP per se nor the staining of mock-transfected cells with an anti-myc antibody gave rise to staining around these perinuclear regions (data not shown). We thus conclude that a significant fraction of paxillin is localized to the perinuclear regions in adhered fibroblasts. A similar localization was observed with Swiss 3T3 and 3Y1 fibroblasts (data not shown).

Colocalization of paxillin with vinculin and F-actin was also examined with epitope-tagged constructs of paxillin \(a\) (Fig. 5, d–i) and paxillin \(b\) (data not shown). Endogenous vinculin was colocalized with paxillin \(a\) and \(b\) at focal contacts and the cell periphery (Fig. 5, d–f). It is noteworthy that a minor fraction of vinculin was also seen at the perinuclear regions (Fig. 5e). However, unlike paxillin, the relative amount of vinculin in these perinuclear regions was less than that observed in focal contacts (Fig. 5f). The pattern of F-actin localization appeared to be similar to that of paxillin, but only a small population of the two proteins were colocalized (Fig. 5, g–i). Paxillin appears to localize next to the front edge of F-actin at each focal adhesion.

To examine to which regions paxillin was localized within the perinuclear area, we visualized the submembranous structures of the endoplasmic reticulum and the Golgi apparatus. Paxillin was not colocalized with the GRP78 protein used as an endoplasmic reticulum marker, although the overall pattern of GRP78 expression overlapped with that of the perinuclear localization of paxillin (Fig. 5, j–l). Colocalization of the Golgi 58-kilodalton protein with paxillin was observed (Fig. 5, m–o). Subpopulation of paxillin also seemed to be localized to the trans-Golgi network, which is characterized by the condensed localization of the 58-kilodalton protein (Fig. 5, m–o).

**DISCUSSION**

We have shown in this paper that the mouse genome encodes the \(a\) and \(b\) isoforms of paxillin but not the \(g\) isoform. Mouse genomic sequences adjacent to the 3′-end of the \(b\) exons showed significant sequence homology to the human \(g\) exon, yet comprise introns in rodents. Therefore, the \(g\)-specific exon may have evolved from ancestral sequences also present in rodents. Our previous analysis with human system showed that the mRNA of the \(a\) isoform of paxillin is expressed in most normal tissues, whereas isoform \(b\) and \(g\) messages are not (15). Our

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\(^2\) Y. Mazaki and H. Sabe, unpublished results.
analysis of the murine system confirmed that the α isoform protein is indeed expressed in all normal adult tissues we examined, whereas the β isoform protein is expressed only at marginal levels or is almost undetectable. However, it should be noted that several tissues such as spleen, testis, thymus, and lung express detectable levels of the β isoform protein. A subset of cells in spleen, testis, and thymus are not static but motile. In lung, a number of leukocytes invade and are activated; and we showed that high levels of the β isoform protein are expressed in all normal adult mouse tissues and hepatocellular carcinomas. Each normal tissue was prepared from 5–6-week-old 129/Sv mice. Hepatocellular carcinomas (Liver cancer) were from transgenic mice carrying the SV40 T antigen (16). B, expression of paxillin isoforms in mouse embryos. Embryos of different stages (indicated as dpc) were prepared from BALB/c mice. C, expression of paxillin isoforms in mouse cells. Peritoneal exudate macrophage cells were prepared from 5–6-week-old 129/Sv mice by a thioglycolate treatment. NIH 3T3 cells were cultured with Dulbecco’s modified Eagle’s medium with 5% fetal calf serum.

We showed that both isoforms are indeed localized to focal adhesion plaques. Among focal contacts in a single cell, there is no selective localization of either isoform. Focal adhesion proteins in general are believed to have relatively large cytoplasmic pools. Our analysis of confocal images indicated that both isoforms of paxillin in cultured fibroblasts are not present throughout the cytosol but rather are primarily associated with perinuclear submembranous structures that colocalized with the Golgi apparatus. Vinculin, another focal adhesion protein, was similarly localized, although the relative amount of its perinuclear localization was much less than that of paxillin.
Several small GTP-binding proteins including Rho and Rac are involved in the regulation of focal adhesions and the dynamic regions of the cell periphery (24, 25), and it is noteworthy that the myosin Myr4 protein bearing Rho-GTPase-activating activity, for example, localizes to the Golgi apparatus (26). It is well documented that integrins are transported to the cell surface and are internalized continuously (27) via coated pits (28). Paxillin may not be essential for the translocation of integrins to the plasma membrane (29). However, it is not yet well established how and where intracytoplasmic proteins are transported to and assemble with the cytoplasmic domains of integrins. Orchestration of protein assembly among different focal contacts in a single cell may also be necessary for cell motility. Paxillin itself is a soluble protein and has no Golgi retention signal, and thus identification of the protein(s) that anchors paxillin to the perinuclear submembranous structures or the Golgi apparatus may provide clues for further understanding the regulation of focal assemblies, as well as cell motility.

It is surprising that rodents do not have the γ isoform of paxillin. The γ isoform of paxillin is expressed in human monoblast cells, and its expression is enhanced severalfold during maturation of the cells into macrophage-like cells (15), implying the γ isoform in integrin avidity, cell motility, and trans-invasion. Moreover, we recently found that primary preparations of keratinocytes, fibroblasts, and vein endothelial cells when cultured in vitro express relatively high levels of the γ isoform protein. Unlike the other two isoforms, the γ isoform only weakly interacts with focal adhesion kinase (15). The γ isoform-specific exon, on the other hand, contains a proline-rich sequence that can bind to certain Src homology 3 domains in vitro. Thus, the γ isoform appears to participate in transmitting an integrin-mediated signal, distinct from signals transmitted by the α and β isoforms. There may be a number of differences between human and mouse with regards to cell adhesions and signalings. For example, CD58 (LFA-3) (30), which is expressed in several types of human cells including monocytes, keratinocytes, fibroblasts, and vein endothelial cells, and acts as a counter receptor for CD2-mediating cell adhesions between killer T cells and their target cells (31–33), appears to be absent in rodents. In rodents, CD48, which is also present in humans, seems to involve the function of the human CD58 (34–36). Elucidation of the role of the γ isoform may unveil differences in cell adhesion mechanisms and its signaling between human and mouse. This may also contribute to our evaluation of rodents as animal model systems for human diseases such as cancer, arteriosclerosis, and asthma and to the development of medical strategies that target the cells involved in these diseases.
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