Monocyte Cells and Cancer Cells Express Novel Paxillin Isoforms with Different Binding Properties to Focal Adhesion Proteins*

Yuichi Mazaki‡, Shigeru Hashimoto§, and Hisataka Sabe‡,**

From the Institute for Virus Research, Kyoto University, Kyoto 606, Japan, [Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Kyoto 619-02, Japan and §Center for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan.

The versatility of integrin functions is mediated by engagement of a number of proteins that assemble with integrins. Among them, paxillin is one of the important molecules interacting with a variety of signaling molecules and cytoskeletal building blocks. We report here that paxillin is not a single molecule with a unique physiological property. We identified two human paxillin isoforms, β and γ. These isoforms have distinct amino acid insertions; each consists of a distinct exon, at the same site of previously reported paxillin (paxillin α). Several proteins were co-precipitated with paxillin, and we found that β bound to focal adhesion kinase but weakly to vinculin, and γ bound to vinculin but only weakly to focal adhesion kinase, although both bound equally to talin. No additional proteins were found to bind to β and γ over those binding to α. Unlike the α isoform, β and γ mRNAs were not detected in normal tissues, but several cancer cells expressed both α and β proteins simultaneously. All three isoform proteins were expressed in promonocytic cells with ratios comparable with each other, and the expression patterns were altered during differentiation of floating promonocytic cells into adherent macrophage-like cells. Therefore, each isoform of paxillin exhibits distinct expression and different biochemical as well as physiological properties and thereby appears to act as a distinct module involved in different functions of integrins.

Integrin αβ heterodimers mediate cell adhesion to extracellular matrixes (ECMs)1 and to other cells. Integrin-mediated cell adhesion plays a critical role in directing cell motility, growth, differentiation, survival, and invasion through intracellular regulation of a variety of signals, such as protein tyrosine phosphorylation, calcium ion mobilization, inositol lipid metabolism, and proton efflux (for reviews, see Refs. 1–7). Integrins themselves have no intrinsic enzymatic activity; integrins appear to function by interacting with other proteins possessing cytoskeletal as well as catalytic signaling properties, primarily with their cytoplasmic domains (for reviews, see Refs. 4 and 7–9). A number of proteins have been identified as able to associate with the cytoplasmic domains of integrins or are localized to focal adhesion plaques where integrins interact with the ECM to connect the extracellular environment with intracellular effectors, resulting in the organization of the actin-containing cytoskeleton (for reviews, see Refs. 7 and 10–12) (13–15). Conversely, intracellular events also affect integrin conformation and affinity to the ECM (for reviews, see Refs. 1 and 16).

It has been demonstrated that triggering of an integrin fibronectin receptor response involves hierarchies of protein interactions that assemble with the integrin (13, 14). Moreover, integrin signaling pathways vary with integrin isoforms even within a single cell type (17). Thus, to achieve a high diversity of integrin-mediated signals, different cytoskeletal building blocks and signal transducers may be engaged to each distinct integrin and focal assembly. Based on our current knowledge of the biochemical properties of protein-protein interactions among focal adhesion proteins, several possible architectures of complexes formed at focal adhesions or connecting integrins to actin stress fibers can be drawn. In this regard, α-actinin, talin, vinculin, and tensin have been shown to bind directly to actin; and α-actinin, talin, and focal adhesion kinase (Fak) (15) bind directly to the cytoplasmic domain of integrin β1 (for review, see Ref. 7). Paxillin is also associated with the cytoplasmic peptide of integrin β1 (15; also see below). These proteins may interact with each other to connect integrin and actin. For example, vinculin binds to talin, tensin, and α-actinin (for review, see Ref. 7); Fak binds to paxillin (18–20), which binds to vinculin (18, 21; also see below). Different combinations of these actin- and integrin-binding proteins may then form different architectures of focal assembly frameworks. Protein modification such as tyrosine (and perhaps serine and threonine) phosphorylation, which mediates protein binding to the src homology 2 (SH2) domains could also participate in the alteration of the organization of the focal adhesion assembly (for review, see Ref. 7). Moreover, a variety of alternative splicing products exist not only for integrins and ECM proteins but also for focal adhesion proteins, such as vinculin (22–25) and Fak-related nonkinase (26). Paxillin was originally identified as a substrate for the v-Src tyrosine kinase (27) and shown to be highly localized at focal adhesion plaques (21). CDNA cloning of human and chicken paxillin has revealed their primary structure (18, 28). At its COOH terminus, paxillin contains four repeats of the LIM domain, which may mediate protein-protein interactions, as in the case of zyxin (29). Proteins interacting with these LIM domains are unknown. Besides putative LIM-mediated binding, paxillin has been shown to bind to vinculin (18, 21, 30),

* This work was supported by the Japan Science and Technology Corp., Precursory Research for Embryonic Science and Technology, and Grants-in-aid 07272220 and 08264216 from the Ministry of Education, Science, and Sports of Japan (to H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Minase Research Institute, Ono Pharmaceutical Co., Ltd., 3-1-1 Sakurai Shimamoto, Mishimagun, Osaka 618, Japan.

‡ To whom correspondence should be addressed: Institute for Virus Research, Kyoto University, Kawanabe Shogoin, Sakyoiku, Kyoto 606, Japan. Tel.: 81-75-751-4026; Fax: 81-75-751-5766.

1 The abbreviations used are: ECM, extracellular matrix; Fak, focal adhesion kinase; GST, glutathione S-transferase; SH, src homology; PAG, polycrylamide gel electrophoresis; FCS, fetal calf serum; TPA, 12-O-tetradecanoylphorbol acetate; PCR, polymerase chain reaction; Ab, antibody; kb, kilobase.
talin (28), and Fak (18-20). In addition, paxillin binds to the SH3 domain of c-Src (31). Paxillin can also be recovered within a complex of proteins binding to the cytoplasmic domain peptide of integrin β1 (15). Although it is not clear whether this binding is direct, Fak is not essential for this binding (15). Moreover, tyrosine phosphorylation of paxillin is accompanied by cell adhesion to the ECM and disappears when the cell adhesions are not maintained (32). This cell adhesion-dependent tyrosine phosphorylation of paxillin creates binding sites for the SH2 domains of COOH-terminal Src kinase (33) and v-Crk, an adapter molecule that consists only of SH2 and SH3 domains (34-36). Inhibition of cell adhesion-mediated protein tyrosine phosphorylation has been shown to affect the formation of focal adhesions and cell cycle progression to S phase (32). Therefore, binding of paxillin to cytoskeletal building blocks, which may result in connection of integrin with actin, and interaction of paxillin with a variety of signaling molecules highlight the important role of paxillin in focal adhesions. Paxillin and its modifications, such as tyrosine phosphorylation, appear to be crucial in regulating focal adhesion assembly, as well as integrin-mediated signal transduction.

When we analyzed paxillin on two-dimensional SDS-PAGE, we detected at least eight different spots cross-reacting with the anti-paxillin antibody. Several seemed to be generated by tyrosine and serine phosphorylation (27, 33, 35). However, it is also possible that paxillin consists of several isoforms of primary protein structure. Thus, we were sensitive to this possibility when we isolated paxillin cDNAs. Moreover, we chose U937 human promonocyte cells as a source of mRNA for potential isolation of paxillin isoforms, the expression of which might be restricted to floating cells or change during the differentiation of the cells into macrophage-like cells, accompanied with altered cell adhesion (37). Here, we report two different isoforms of paxillin, β and γ, generated by insertion of distinct exons. These two novel isoforms, along with the isoform previously reported (isoform α; Ref. 28), were expressed simultaneously in monocyte cells. Several cancer cells expressed both α and β isoforms, but not the γ isoform. Biochemical analysis revealed that these isoforms exhibit different binding properties to several proteins, including Fak and vinculin. Moreover, during the maturation of monocyte cells, the protein level of the γ isoform was increased severalfold. The pattern of the expression of the β isoform was also changed during this process. Thus each isoform of paxillin appears to have distinct physiological functions, possibly through formation and maintenance of different architectures and signaling complexes of focal adhesions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—U937, Jurkat, and HPB-ALL cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% FCS; HeLa and BOCS23 cells were in Dulbecco's modified Eagle's medium with 10% FCS; NIH3T3 cells were in Dulbecco's modified Eagle's medium with 5% FCS; and K562 cells were in Ham's F-12 medium with 10% FCS. For the differentiation of U937 cells α-interferon treated cells were seeded at 3 × 10^6 cells/ml with 1.6 × 10^-7 M 12-O-tetradecanoylphorbol acetate (TPA) for 3 days (37). The differentiation was assessed by the appearance of about 50% of cells with an adherent macrophage-like phenotype as described (37), and those cells undifferentiated and still floating after the TPA treatment were washed off before harvesting the cells. For radiolabeling, HeLa cells were incubated in the presence of 0.1 mM [3H]leucine (80 Ci/mmol, Moravek Biochemicals) in Dulbecco's modified Eagle's medium (without l-leucine, Life Technologies, Inc.) containing 10% dialyzed FCS for 7 h, after being starved for l-leucine by incubating in Dulbecco's modified Eagle's medium (without l-leucine) with 10% dialyzed FCS for 30 min.

**Isolation of the cDNAs**—Standard methods (38) were used for DNA and RNA manipulations, unless otherwise stated. Polyadenylated RNA was prepared from cultured U937 cells according to the manufacturer's instructions (QuickPrep mRNA purification kit, Pharmacia Biotech Inc.), and cDNA was synthesized from poly(A)+ mRNA templates using random primers and reverse transcriptase (Pharmacia). PCR amplifications of human paxillin cDNA fragments were performed in a combination of oligonucleotides (primer 1-24, 5'-CCGATCTCATGGAAGGATCCTGACGCCCCTG-3'; primer 922-942, 5'-CAGCTTGTCAGAACTGAGTGTCGACC-3'; and a combination of primer 844-865, 5'-GGAAGAGACCTGGGAACCTGCAGC-3'; and primer 1648-1674, 5'-GGCTTGGACCCCTGAGAG-3') and (primer 922-942, 5'-CAGCTTGTCAGAACTGAGTGTCGACC-3'; and primer 1648-1674, 5'-GGCTTGGACCCCTGAGAG-3'). Nucleotide numbers in this article correspond to the sequence of human paxillin isoform a, with nucleotide A at the first ATG codon as 1. After digestion of the former PCR products with BamHI and SphiI (located at nucleotide 905 of isoform α) and the latter with SphiI and EcoRI, these DNA fragments were ligated with BamHI-EcoRI-cleaved pGEX-2T vector (Pharmacia) using T4 DNA ligase (Takara Shuzo). The resulting plasmids were transformed into Escherichia coli DH5α, and each clone was isolated and subjected to DNA sequencing analysis.

**Isolation of a Genomic DNA Fragment of Paxillin—High molecular weight DNA isolated from human placental tissue was subjected to PCR amplification using oligonucleotides of primer 742-768 (5'-GTTCACCTCCACCAAGACAGGACGCG-3') and primer 889-915 (5'-CCTCGACGCCCTGCTG-3') and primer 922-942 (5'-CCCTGCCTGTCCTGAGACC-3') to isolate a genomic DNA fragment encompassing paxillin β- and γ-specific exons. A DNA fragment produced was isolated by separating on 0.7% agarose gel electrophoresis and then ligated into pT7Blue(R) vector bearing single 3'-T overhangs at its EcoRV site (Novagen). After transformation of the resulting plasmid into E. coli NovaBlue clones (Novagen), a single clone was isolated and subjected to restriction enzyme mapping and sequencing analysis.

For PCR amplification of β- and γ-specific exons from this genomic DNA fragment, combinations of oligonucleotides of primer β-3 (5'-ATCAGAGCAGATGAAAGACGAGCGC-3') and primer β-3' (5'-CCCTGCCTGTCCTGAGACC-3'), specific for isoform β, and primer γ-5' (5'-GGCTTGGACCCCTGAGAG-3') and primer γ-3' (5'-CTGTAGACACGGGACTG-3'), specific for isoform γ, were used (see Fig. 1D).

**Exogenous Expression of Paxillin Isoforms**—For the exogenous expression of paxillin in mammalian cells, pBabePuro vectors (39) bearing paxillin cDNAs were constructed as follows. BglI-EcoRI fragments of each paxillin isoform were isolated from the pGEX constructs, ligated with a synthetic double strand DNA fragment containing the 5'-end coding Kazak sequences (underlined) of paxillin (28): 5'-GATCTCTGGCCTAGTACGATCCTGGCAGCCCTG-3' and 3'-GGCCGGCTACCTGTCGTAGGTCCGG-5'. The ligated fragments were then ligated into pBabePuro vector cleaved with BamHI and EcoRI. After isolation of single clones with each isoform, constructs were confirmed by sequencing analysis.

The resulting plasmid DNAs were transfected into BOSC 23 cells (40) by the calcium phosphate precipitation method, and 48 h after transfection recombinant viruses bearing pBabePuro and paxillins were collected. NIH3T3 cells were then infected with these viruses. After culturing the infected cells for 1 week in the presence of 2 μg/ml puromycin (Sigma), cells were harvested and analyzed for expression of paxillin by immunoblotting as described above.

**GST Fusion Proteins**—pGEX-2T vectors containing cDNA sequences encoding the human paxillin isoforms were expressed in E. coli as glutathione S-transferase (GST) fusion proteins by isopropyl-β-D-thiogalactopyranoside induction. Bacterial lysates were incubated with glutathione-Sepharose beads (Pharmacia) for 2 h at 4 °C and then washed extensively with 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1% aprotinin, and 2 μg/ml leupeptin), as described previously (33, 35).

**Protein Binding Assay**—Cell lysates were prepared at 4 °C by solubilizing cells with 1% Nonidet P-40 buffer and clarifying for 10 min at 10,000 × g as described previously (33). Each 500 μg of lysates was incubated with 5 μg of the GST fusion proteins bound to glutathione-Sepharose beads at 4 °C for 2 h. Beads were then washed four times, or six times for radiolabeled cell lysates, with 1% Nonidet P-40 buffer, resuspended in Laemmli SDS sample buffer, and incubated for 5 min at 100 °C.

Proteins recovered with the beads were separated by 8% SDS-PAGE and transferred to membrane filters (Immobilin P, Millipore). After blocking with Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 5% bovine serum albumin (Sigma), filter membranes were probed with 2 H. Sabe and H. Hanafusa, submitted for publication.
with appropriate antibodies as described previously (33, 35). The antibodies retained on filter membranes were then detected by a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and visualized by an enzyme-linked chemiluminescence method according to the manufacturer's instructions (Amersham Corp.). The same filter was subjected to immunoblotting analysis sequentially with different antibodies, according to the manufacturer's instructions (Amersham Corp.). The same filter bodies retained on filter membranes were then detected by a peroxida-

te of γ.'  

Antibodies Against Paxillin—Anti-paxillin antibodies were generated by immunizing rabbits with a keyhole limpet hemocyanin-conjugated synthetic peptide of Lys-Glu-Lys-Pro-Lys-Arg-Aas-Gly-Gly-Arg-Gly-Leu-Glu-Asp-Val-Arg-Pro-Ser-Val-Cys, which corresponds to amino acids 199–217 of the isoform α sequence. The COOH-terminal Cys residue is for conjugation with keyhole limpet hemocyanin. Antibodies raised (Ab 199–217) were purified using the peptide used for the immunization. For generation of antibodies specific for paxillin isoforms β and γ, rabbits were immunized with synthetic peptides of Ile-Gln-Asp-Leu-Glu-Gln-Arg-Ala-Asp-Gly-Gly-Ars-Cys-Trp-Ala and Ala-Gly-Trp-Pro-Arg-Gly-Gly-Arg-Ser-Pro-Gly-Gly-Glu-Gly, each conjugated with a multiple antigenic peptide (41) for antibodies specific for the β isoform, or with synthetic peptides of Leu-Val-Ser-Ile-Ser-Ser-Val-Ser-Glu-Gly-Glu-Glu-Glu-Val-Pro-Arg-His-Pro-Cys-Ala and Ala-Ars-Arg-Pro-Gly-Gly-Gly-Arg-Ser-Pro-Gly-Gly-Glu-Gly, each conjugated with multiple antigenic peptide for the γ isoform. For immunoprecipitation of paxillin, a monoclonal antibody against paxillin (Transduction Laboratories) was used, coupled with protein G-Sepharose (Pharmacia). Mouse IgG1 protein used as a negative control was purchased from Sigma.

RESULTS

Isolation of Human Paxillin Isoform cDNAs—Among four paxillin cDNA clones we isolated using mRNA prepared from U937 cells, two cDNA clones showed the same sequence as the human paxillin reported previously (Ref. 28 and Fig. 1). However, the other two cDNA clones appeared to have additional sequences, as assessed by restriction enzyme mapping analysis. Sequence analysis revealed that each of these longer cDNAs contains distinct sequences, both of which were inserted at the same site (just after amino acid Lys777, i.e. between nucleotides 831 and 832) of the reported paxillin se-
sequences (Fig. 1B). The inserted sequences are shown in Fig. 1D. Both of them were linked in frame to both the 3′- and 5′-ends of the flanking sequences of human paxillin. Except for these inserted sequences, both cDNAs showed the same sequence as that previously reported for human paxillin (data not shown). We hereafter call the reported paxillin isoform α and our longer isoforms β and γ, respectively. Isoform β contains a 34-amino acid insert, and γ contains a 48-amino acid insert (Fig. 1, B and D).

**Analysis of Genomic DNA Encompassing the β- and γ-Specific Exons**—To examine how these isoforms are generated, we isolated a human genomic DNA fragment containing these inserted sequences. We have amplified high molecular weight human genomic DNA using a combination of two oligonucleotides, each corresponding to nucleotides 742–768 and 889–915 of human paxillin α (Fig. 1B, primers 742–768 and 889–915). This amplification generated a single DNA band of 6.4 kb in length (Fig. 1C). PCR amplification of high molecular weight genomic DNA with β and γ exon-specific primers (Fig. 1B, combinations of β-5′- and β-3′-, and γ-5′- and γ-3′), which were synthesized based on the inserted sequences of β and γ isoforms, also generated a single band each, of the expected size (Fig. 1C). The 6.4-kb DNA fragment was isolated from the gel and cloned directly into the pT7Blue(R) vector. PCR amplification of this cloned DNA fragment using combinations of oligonucleotides specific for isoforms β and γ generated precise sizes of DNA fragments, each corresponding to the inserted sequences of isoforms β and γ, respectively (data not shown). We then sequenced the DNA fragment using β- and γ-specific oligonucleotides, as well as the two oligonucleotides used for the generation of the DNA fragment. As shown in Fig. 1D, just after the sequences corresponding to the codon for Lys277 of paxillin α began sequences unrelated to the paxillin cDNAs. An exon encoding the inserted sequence of isoform β was found near the 3′-end of the genomic DNA fragment. Downstream of the 3′-end of this exon are again unrelated sequences of 142 nucleotides in length. After this region, another exon encoding the inserted sequence of isoform γ began. Interestingly, this exon was directly connected to the coding region of Phe276 and thereafter of paxillin α sequences (Fig. 1, B and D).

**Exogenous Expression of Paxillin Isoforms in Fibroblasts**—To verify the protein products of these cDNAs, we expressed these cDNAs in fibroblasts. We used a virus infection system with BOSC 23 virus-packaging cells and the pBabe vector, which uses the murine retrovirus long terminal repeat as a promoter. As shown in Fig. 2, each isoform cDNA produced a protein of the expected size in NIH3T3 cells that reacted with polyclonal anti-paxillin antibodies, which were raised against amino acids Lys199–Val217 of isoform α (Ab 199–217). Diffuse bands in each isoform may represent tyrosine and serine phosphorylation of paxillin, as was shown with the α isoform of paxillin (27, 35). With the expression of these isoforms of paxillin in NIH3T3 cells, no significant morphological change or anchorage-independent growth of cells was observed (data not shown).

**Isoforms β and γ Show Distinct Binding Properties to Focal Adhesion Proteins**—To examine possible functional differences in integrin-mediated cell adhesions or focal adhesion assemblies, we then assessed the protein binding properties of the paxillin isoforms. We expressed GST fusion proteins of each isoform in E. coli. Cellular proteins that co-precipitated with these recombinant proteins coupled to glutathione beads were then analyzed. Isoform α has been shown to bind to Fak, talin,
and vinculin (18–21, 28), and these proteins were indeed co-precipitated with our GST fusion protein of paxillin α (Fig. 3). It should be noted that there were large differences in the relative affinities of the GST-paxillin α protein toward each of these proteins; with our conditions in the presence of an excess amount of the fusion protein, more than 50% of Fak protein in the cell lysates appeared to be recovered, whereas only less than several percent of proteins were recovered in the case of vinculin and talin (Fig. 3). With fusion proteins of β and γ isoforms, amounts of these focal adhesion proteins co-precipitated were different from those with the α isoform (Fig. 3). Compared with the binding of isoform α, isoform β binds to Fak and talin similarly but exhibits only marginal binding to vinculin; isoform γ binds to vinculin and talin similarly but only weakly binds to Fak. Essentially the same results were obtained with cell lysates prepared from HeLa and U937 cells (Fig. 3).

We also examined paxillin-binding proteins using radiolabeled cell lysates. As shown in Fig. 4, at least eight different protein bands were detected as co-precipitating with the three isoforms over those seen with negative control GST protein. Among these isoforms, γ exhibits much decreased binding toward cellular proteins. Judging from the molecular sizes, protein band E may correspond to Fak, and band B may be talin. Visualization of vinculin, which bound only weakly, as assessed by the above experiments, may be hindered by band E. In addition to these proteins, six bands were detected: A, C, D, F, G, and H, all of which bound to isoform α. Compared with isoform α, isoform β bound to bands A, C, D, G, and H similarly but not to band F; isoform γ bound to band A similarly, weakly to band H, and with marginal levels to bands C, D, E, and G. No additional protein bands were seen with isoforms β and γ compared with those observed with isoform α. Again, with radiolabeled cell lysates prepared from U937 cells, essentially the same patterns of protein binding were obtained as with HeLa cell lysates (data not shown).

Expression of Paxillin α, β, and γ mRNAs—Expression of α mRNA has been studied in normal human tissues, as well as several cultured cells (28). Consistent with the previous report, our analysis showed that paxillin, as detected by a DNA probe encompassing a 1–1072-nucleotide sequence of the α isoform, is expressed ubiquitously in most normal tissues (Fig. 5). Among cancer cells examined using the same DNA probe, high levels of paxillin mRNA were detected in HeLa S3 epithelial carcinoma cells, K562 chronic myelogenous leukemia cells, SW480 colorectal adenocarcinoma cells, A549 lung carcinoma cells, and G361 melanoma cells; marginal levels of expression were detected in HL-60 promyelocytic leukemia cells and Raji Burkitt’s lymphoma cells, and paxillin expression was not detected in HL-60 promyelocytic leukemia cells (Fig. 5A). On the other hand, with DNA probes each specific for β and γ isoforms, expression of β and γ mRNAs were not detected in any of the normal tissues we have examined (Fig. 5B; data not shown). Expression of β mRNA, however, was clearly detected in SW480 cells, with low levels in HeLa, K562, and A549 cells. β mRNA was not detected in HL-60, Molt 4, Raji, and G361 cells. γ mRNA was not detected in any of above cell lines in the same mRNA blot filters (data not shown). mRNAs each specific for α, β, and γ isoforms, on the other hand, were clearly detected in U937 cells using a reverse transcription-PCR amplification method (data not shown).

Expression of α, β, and γ Proteins—We have generated three types of polyclonal antibodies; one was Ab 199–217, which recognizes all three isoforms, and the other two were types of polyclonal antibodies each against β and γ isoforms. The specificity and sensitivity of these antibodies were assessed by the use of GST fusion proteins of each paxillin isoform (Fig. 6). To detect isoform expression, paxillin was first immunoprecipitated with the anti-paxillin monoclonal antibody and then blot-
Changes in the Levels and the Patterns of Isoform Expressions during Differentiation of Monocytic Cells—To explore further the possible physiological differences of these paxillin isoforms, we examined the isoform expression during monocyte maturation. U937 cells were treated with TPA for 3 days to differentiate the cells into macrophage-like cells. Changes in the expression of integrins as well as changes in the binding and spreading of cells toward different ECMs have been documented in this process (42, 43). As shown in Fig. 6A, expression of the γ isoform was increased severalfold after the differentiation, whereas the isoform consisted of two protein bands in either stage of the cells. With the β isoform, one additional protein band appeared, and the slow-migrating fractions of the isoform were increased markedly, which may represent increased phosphorylation of β isoform after the differentiation and adherence of U937 cells.

DISCUSSION

We characterized two novel isoforms of human paxillin generated by exon insertion. These novel isoforms exhibited different expression, and the patterns of the expression were altered during monocyte maturation. Moreover, biochemical analysis using recombinant proteins revealed differences in binding to several proteins, including focal adhesion proteins.

Sequence analysis of the novel paxillin cDNAs revealed that both the β and γ isoforms have distinct insertions just after Lys277 of human paxillin α. Both of these insertions are followed by Phe278 and the remainder of the isoform sequence. Analysis of the genomic structure revealed that there is indeed an exon breakpoint just after the codon for Lys277. After this codon begins an intron, and the exon encoding the sequence inserted in isoform β lies about 6 kb 3′ of this junction. This β-specific exon is followed by a short intron and then by an exon encoding the sequence inserted in isoform γ. The γ-specific exon connects directly to the sequence encoding Phe278. All of these introns have consensus 5′-GT and 3′-AG splice sites. The γ-specific exon ends with AG, followed by the TTC codon for serine (44, 45). There may be additional exons, other than β- and γ-specific exons, within this 6.4-kb DNA fragment. So far, however, we have not detected other transcripts by reverse transcription-PCR amplification of cellular mRNAs using the 5′- and 3′-flanking sequences (primers 742–768 and 889–915) of the 6.4-kb DNA fragments as primers.

We showed that each GST fusion form of paxillin isoforms co-precipitates focal adhesion proteins with a different affinity. Our experiments using radiolabeled cell lysates suggested that no additional proteins are bound to the β and γ isoforms over those binding to paxillin isoform α, and the γ isoform exhibits much decreased affinity toward cellular proteins. The β and γ isoforms bind to talin with affinities comparable to that of isoform α. However, binding to Fak and vinculin is altered greatly; β binds to Fak strongly but quite weakly to vinculin, whereas γ binds to vinculin but only weakly to Fak. Moreover, several proteins, such as Fak, vinculin, and bands D, F, and G in Fig. 4, appear to bind selectively to either β or γ isoforms. Deletion analysis of paxillin α has suggested that amino acids 56–100 are responsible for Fak and vinculin binding, and amino acids 100–277 bind to talin (18, 28). Our β and γ isoforms contained inserts between Lys277 and Phe278 of isoform α. This kind of intronless exon-exon junction is found in other genes, such as caldesmon (44, 45). There may be additional exons, other than β- and γ-specific exons, within this 6.4-kb DNA fragment. So far, however, we have not detected other transcripts by reverse transcription-PCR amplification of cellular mRNAs using the 5′- and 3′-flanking sequences (primers 742–768 and 889–915) of the 6.4-kb DNA fragments as primers.
from this cell line, each paxillin isoform showed essentially the same patterns of binding to vinculin and talin as in Fak-positive cell lysates (data not shown). Thus, Fak is not essential for the binding of paxillin isoforms to vinculin and talin.

The architecture of the focal adhesion assembly may not be unique. It has not yet been established which focal adhesion proteins are used with which integrins or focal adhesions. The different and selective binding of γ isoforms to Fak and vinculin, for example, may involve localization of vinculin to certain types of focal adhesions independent of Fak. Indeed, the fact that focal adhesions are present in Fak-deficient fibroblasts indicates that Fak is not essential for certain types of adhesions (46). We also showed that the binding of the β isoform to vinculin is weak compared with those of isoforms α and γ. However, it should be noted that the amounts of vinculin as well as talin that bound to paxillin were quite low, compared with Fak binding to paxillin (see Fig. 3). Thus, it should be studied further whether the weak interaction of paxillin with vinculin and talin, detected in vitro, could have certain physiological relevance.

Northern blotting analysis showed that mRNAs for both the β and γ isoforms were undetectable in normal tissues, whereas paxillin mRNA was clearly detected in most of normal tissues using a DNA probe encompassing nucleotides 1–1072 of the α isoform. This result indicates that isoform α is indeed expressed in normal tissues, as reported previously (28). Since DNA probes specific for the β and γ exons were relatively short (102 and 144 nucleotides in length, respectively), it may have been difficult to detect any low levels of the β and γ mRNAs. However, a DNA probe of similar length prepared from isoform α cDNA using primers 742–768 and 889–915 (see Fig. 1B) did detect paxillin mRNA expression clearly in normal tissues as well as several cancer cells (data not shown).

Although the β isoform mRNA is not detected in normal tissues, it is expressed in several cancer cells, such as SW480, HeLa, K562, and K549 cells. These β mRNA expression results recall the similar results of the increased levels of Fak mRNA observed only in metastatic and invasive cancers with no detectable expression in normal adult tissues or most benign tumors (47). Although the cancer cells expressing the β isoform are highly tumorigenic in vivo, SW480 cells, in which the β isoform was detected at the highest level among cancer cells examined, do not exhibit the highest metastatic and invasive activity in vitro (for example, see Refs. 48 and 49). Thus, the correlation between the expression of the paxillin isoforms and tumorigenesis, metastasis, and invasiveness, processes in which integrins are deeply involved (50–52; for review, see Ref. 53), is interesting but should be studied in more detail.

Expression of the γ isoform is much restricted. The γ isoform was not detected in any of samples we examined, except in U937 promonocytic cells. Since other cells with hematopoietic lineages, such as Jurkat, HPB-ALL, Raji, HL-60, Molt 4, and K562, were negative in its expression, expression of the γ isoform seems not to be specific for every hematopoietic lineage but more restricted for certain differentiation stages, including that of monocyte maturation.

All three isoform proteins were expressed in U937 cells at levels comparable with each other. We found that the protein level of the γ isoform was markedly increased when U937 cells were differentiated into adherent monocyte- and macrophage-like cells by TPA. Modification of the β isoform was also accompanied by this process. In undifferentiated U937 cells, the amount of the γ isoform was smallest among the three isoforms. On the other hand, the amount of the γ isoform appeared to become higher than that of the β isoform after the differentiation and adherence of cells onto culture dishes. Hu-

man monocyte precursor cells express several families of integrins, and patterns of their expression are greatly altered during the monocyte differentiation that accompanies the changes in the binding properties of the cell toward different ECMs (54, 55). These properties are important in the homing and positioning of mononuclear cells at the sites of inflammation. Indeed, in U937 cells, TPA treatment, which mimics most of the effects of tumor growth factor β1, enhances the expression of integrins α2 and αv, with a concomitant decrease in integrin α4 expression (42, 43). Although the expression of integrin types varies greatly during the monocyte maturation, altered expression of integrins per se does not seem to be enough to account for the changes of cell adherence and motility (56). It is, thus, quite important to examine how much the changes in the expression of paxillin isoforms contribute to this process. Also, it would be quite interesting to see whether each isoform of paxillin assembles selectively with different types of integrins in monocyte cells.

In conclusion, these paxillin isoform clones are useful tools with which to explore possible differences in the components, regulatory mechanisms, and signaling cascades of different types of focal adhesions within a single cell as well as among different types of integrins and cells. Although paxillin expression in brains of day 10 or day 12 chicken embryos, as well as in the human brain, is quite low (21, 28, 57), we have detected high levels of paxillin mRNA in neural tubes during the early stages (stages 15–20, 50–72 h) of chicken embryogenesis, when neural crest cells actively migrate along their ECMs. Although the β and γ isoforms were undetectable in normal tissues, they may have been expressed at embryonic stages, at which cell movements and differentiation actively take place for morphogenesis. More precise analysis of the differences in the expression and localization of each paxillin isoform will contribute to clarifying our understanding of the roles of paxillin and focal adhesions during embryogenesis, cell adhesion, movement and invasion, and cancer metastasis.

Acknowledgments—We are grateful to Goro Eguchi for support and encouragement throughout this work, Tetsuya Taga for U937 cells, Warren S. Pear and David Baltimore for BOSC 23 cells, Hartmut Land for pBabe vector, Heidi Greulich and Tomohoro Kurosaki for critical reading of the manuscript, and Manami Hiraishi for technical assistance.

REFERENCES
1. Hynes, R. O. (1992) Cell 69, 11–25
2. Schwartz, M. A. (1992) Trends Cell Biol. 2, 304–308
3. Juliano, R. L., and Haskill, S. (1995) J. Cell Biol. 129, 577–585
4. Sastry, S. K., and Horwitz, A. F. (1995) Curr. Opin. Cell Biol. 5, 819–831
5. Shattil, S. J., Ginsberg, M. H., and Brugge, J. S. (1994) Curr. Opin. Cell Biol. 6, 695–704
6. Schwartz, M. A., and Ingber, D. E. (1994) Mol. Biol. Cell 5, 389–393
7. Clark, E. A., and Brugge, J. S. (1995) Science 268, 233–239
8. Damsky, C. H., and Werb, Z. (1992) Curr. Opin. Cell Biol. 4, 772–781
9. Pavalko, F. M., and Otey, C. A. (1994) Proc. Soc. Exp. Biol. Med. 202, 282–293
10. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) Annu. Rev. Cell Biol. 4, 487–525
11. Turner, C. E., and Burridge, K. (1991) Curr. Opin. Cell Biol. 3, 849–853
12. Schaller, M. D., and Parsons, J. T. (1994) Curr. Opin. Cell Biol. 6, 705–710
13. Miyamoto, S., Akiyama, S. K., and Yamada, K. M. (1995) Science 267, 883–885
14. Miyamoto, S., Teramoto, H., Coss, O. A., Gutkind, J. S., Barbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) J. Cell Biol. 131, 791–805
15. Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, J. T. (1995) J. Cell Biol. 130, 1181–1187
16. Ginsberg, M. H., Du, X., and Plow, E. F. (1992) Curr. Opin. Cell Biol. 4, 766–771
17. Zhang, Z., Vuori, K., Reed, J. C., and Ruoslahti, E. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 6161–6165
18. Turner, C. E., and Miller, J. T. (1994) J. Cell Sci. 107, 1583–1592
19. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1995) Mol. Biol. Cell 6, 637–647
20. Tashihana, K., Sato, T., D’Avirro, N., and Morimoto, C. (1995) J. Exp. Med. 182, 1089–1100
21. Turner, C. E., Glenney, J. R., and Burridge, K. (1990) J. Cell Biol. 111, 849–853

Y. Mazaki and H. Sabe, unpublished data.
1059–1068

22. Glukhova, M. A., Kabakov, A. E., Frid, M. G., Ornatsky, O. I., Belkin, A. M., Mukhin, D. N., Orekhov, A. N., Koteliantsy, V. E., and Smirnov, V. N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9542–9546

23. Belkin, A. M., and Koteliantsy, V. E. (1987) FEBS Lett. 220, 291–294

24. Belkin, A. M., Ornatsky, O. I., Kabakov, A. E., Glukhova, M. A., and Koteliantsy, V. E. (1988) J. Biol. Chem. 263, 6631–6635

25. Moiseyeva, E. P., Weller, P. A., Zhidkova, N. I., Corben, E. B., Patel, B., Jasinska, I., Koteliantsy, V. E., and Critchley, D. R. (1993) J. Biol. Chem. 268, 4318–4325

26. Schaller, M. D., Bergman, C. A., and Parsons, J. T. (1993) Mol. Cell Biol. 13, 785–791

27. Glenney, J. R., and Zokas, L. (1989) J. Cell Biol. 108, 2401–2408

28. Salgia, R., Lin, J-L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Patel, B., Jasinska, I., Koteliansky, V. E., and Critchley, D. R. (1993) J. Biol. Chem. 270, 5039–5047

29. Wood, C. K., Turner, C. E., Jackson, P., and Critchley, D. R. (1994) J. Cell Sci. 107, 709–717

30. Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., and Seidel-Dugan, C. (1993) J. Biol. Chem. 269, 14946–14956

31. Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovic, L., Herlyn, M., and Buck, C. A. (1990) Cancer Res. 50, 6757–6764

32. Chan, B. M. C., Matsuura, M., Takada, Y., Zetter, B. R., and Hemler, M. E. (1991) Science 251, 1600–1602

33. Felding-Haberman, B., Mueller, B. M., Romerdahl, C. A., and Cheresh, D. A. (1992) J. Clin. Invest. 89, 2018–2022

34. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

35. Akiyama, S. K., Olden, K., and Yamada, K. M. (1995) Cancer Metastasis Rev. 14, 173–189

36. Van de Water, L., Aronson, D. A., and Braman, V. (1988) Cancer Res. 48, 5730–5737

37. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989) J. Cell Biol. 110, 1321–1330

38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

39. Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., and Seidel-Dugan, C. (1993) J. Biol. Chem. 269, 14956–14963

40. Turner, C. E., Schaller, M. D., and Parsons, J. T. (1993) J. Cell Sci. 106, 637–645

41. Posnett, D. N., McGrath, H., and Tam, J. P. (1988) J. Biol. Chem. 263, 1719–1725

42. Ferreira, O., Garcia-Pardo, A., and Bianco, C. (1990) J. Exp. Med. 171, 351–356

43. Bauvois, B., Rouillard, D., Sanceau, J., and Wietzerbin, J. (1992) J. Immunol. 148, 3912–3919

44. Hayashi, K., Yano, H., Hashida, T., Takeuchi, R., Takeda, O., Asada, K., Takahashi, E., Kato, I., and Sobue, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 12122–12126

45. Glenney, J. R., and Zokas, L. (1989) J. Cell Biol. 108, 2401–2408

46. Salgia, R., Lin, J-L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Patel, B., Jasinska, I., Koteliansky, V. E., and Critchley, D. R. (1993) J. Biol. Chem. 269, 4318–4325

47. Schaller, M. D., Bergman, C. A., and Parsons, J. T. (1993) Mol. Cell Biol. 13, 785–791

48. Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., and Seidel-Dugan, C. (1993) J. Biol. Chem. 268, 14956–14963

49. Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovic, L., Herlyn, M., and Buck, C. A. (1990) Cancer Res. 50, 6757–6764

50. Chan, B. M. C., Matsuura, M., Takada, Y., Zetter, B. R., and Hemler, M. E. (1991) Science 251, 1600–1602

51. Felding-Haberman, B., Mueller, B. M., Romerdahl, C. A., and Cheresh, D. A. (1992) J. Clin. Invest. 89, 2018–2022

52. Akiyama, S. K., Olden, K., and Yamada, K. M. (1995) Cancer Metastasis Rev. 14, 173–189

53. Van de Water, L., Aronson, D. A., and Braman, V. (1988) Cancer Res. 48, 5730–5737

54. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989) J. Cell Biol. 110, 1321–1330

55. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

56. Schaller, M. D., and Parsons, J. T. (1995) Mol. Cell Biol. 15, 2635–2645

57. Gierleski, B., O. A., Pattengale, P. K., Janssens, M. W., and Nilsson, K. (1981) Nature 292, 848–850

58. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.