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# Specific Decrease in the Level of Hic-5, a Focal Adhesion Protein, During Immortalization of Mouse Embryonic Fibroblasts, and Its Association With Focal Adhesion Kinase

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**Abstract** Hic-5 is a paxillin homologue with four LIM domains in its C-terminal region, localized mainly in focal adhesions in normal fibroblasts. Hic-5 is also known to associate with focal adhesion kinase (FAK) or the related CAKβ, and with vinculin. In the present study, we examined changes in Hic-5 and paxillin protein levels in primary mouse embryo fibroblasts (MEF) during mortal and immortal stages. The Hic-5 level was markedly decreased when cells became immortalized, whereas that of paxillin was increased. The vinculin level was not changed significantly. Hic-5 was mainly localized in focal adhesion plaques of mortal MEF but was localized in the nuclear periphery in the immortalized MEF; the number of focal adhesion plaques was decreased in these cells. Mouse Hic-5 contains three LD domains in its N-terminal half, and the first LD domain (LD1) appears to be involved in interaction with FAK. However, this interaction was not essential for recruitment of Hic-5 to focal adhesions, since its subcellular localization was similar in $FAK^{-/-}$ cells. Forced expression of Hic-5 decreased colony forming ability of MEF from $FAK^{+/+}$ mice, but not of $FAK^{-/-}$ cells. These observations suggested the involvement of Hic-5 in determination of cellular proliferative capacity in collaboration with other cytoskeletal components. J. Cell. Biochem. 76:411–419, 2000. 

**Key words:** Hic-5; focal adhesion; paxillin; FAK; immortalization

The hic-5 gene was first isolated a transforming growth factor-β1 (TGF-β)- and hydrogen peroxide-inducible gene from mouse osteoblastic cells by differential hybridization. It was shown to encode a protein with a molecular weight of about 55 kDa with striking similarity to paxillin [Shibanuma et al., 1994]. Its expression was increased during cellular senescence of normal human fibroblast cells and was decreased in immortalized or tumorigenic cell lines [Shibanuma et al., 1994]. Forced expression of hic-5 in immortalized fibroblasts induced a senescence-like phenotype including cessation of growth, enlargement of cells and increased expression of extracellular matrix [Shibanuma et al.,1997], and enhanced differentiation in osteoblastic cells [Shibanuma et al., 1998]. In human peripheral platelets, Hic-5 was shown to be highly expressed, but paxillin was essentially absent [Hagmann et al., 1998]. These observations suggested involvement of Hic-5 in negative regulation of cell growth.

The Hic-5 protein is a new member of the LIM protein family [Schmeichel and Beckerle, 1994; Shibanuma et al., 1997] and is similar to paxillin in its C-terminal four LIM domains. Leupaxin is another member of this family that is specifically expressed in leukocytes [Lipsky et al., 1998]. Paxillin is a vinculin-binding protein that co-localizes with focal adhesion kinase (FAK) and integrins in focal contacts [Turner et al., 1990]. Recent immunocytochemical studies showed that Hic-5 protein was also localized to focal adhesions in the rat fibroblast cell line WFB and associated with CAKβ, a FAK-homologue [Matsuya et al., 1997]. Focal adhesions are specialized sites of cell adhesion to the extracellular matrix [Burridge, 1986] and are composed of integrins (receptors for extracellu-

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lar matrix proteins), cytoskeletal proteins, and signaling molecules such as protein tyrosine kinases and small G proteins [Haynes, 1992; Clark and Brugge, 1995; Burridge et al., 1992; Tapon and Hall, 1997]. Proteins that are localized in focal adhesions are thought to play important roles in regulation of signals elicited by extracellular matrix components.

The N-terminal region of Hic-5 contains Pro-rich and LD domains; mouse Hic-5 has three LD domains, whereas its human homologue has four [Matsuya et al., 1997]. The LD domain has been suggested to be involved in protein-protein interactions [Brown et al., 1996]. Interaction of extracellular matrix and integrins is essential for proliferation, activation, as well as differentiation in many types of cells [Clark and Brugge, 1995]. Changes in these components affect cellular phenotypes, including morphology, growth potential, and malignancy [Schwartz, 1997]. In the present study, we examined changes in localization and levels of Hic-5 during immortalization of normal mouse embryonic cells in vitro and analyzed the interaction with FAK to gain insight into the molecular basis of the functions of Hic-5.

## MATERIALS AND METHODS

### Cell Culture

Primary mouse embryo fibroblasts were prepared from pregnant mouse embryos on day 15 of gestation. Tissues were dispersed with trypsin and cultured in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) in a $CO_2$-incubator. Cells were passaged at a 1:4 split ratio every week ($5 \times 10^5$ cells/100-mm dish) and entered senescence stage after about 40 population doublings, after which spontaneously immortalized cells appeared. Fibroblasts from FAK knockout mice were established as described previously [Ilic et al., 1995]. Colony-forming ability of MEF was measured by transfection with neomycin-resistant pRc/CMV or its Hic-5 expressing vector (S5) by the calcium phosphate-precipitation method as described previously [Shibanuma et al., 1997]. Transfection efficiencies were normalized using luciferase activity obtained by co-transfection with pGL2-luciferase (control) (Promega, Madison, WI). After transfection, cells were cultured in the presence of 800 μg/ml of G418 for 3 weeks, and numbers of colonies were scored. For estimation of colony formation of $FAK^{-/-}$ MEF, pYN3215 [Schaefer et al., 1988] that confers hygromycin resistance was used instead of pRc/CMV, and transformants were selected in medium containing hygromycin (100 μg/ml). The normal human diploid fibroblasts TIG-3 were obtained from the Tokyo Metropolitan Institute of Gerontology. The immortalized cell line SVts8 derived from TIG-3 and cell lines with extended life span (SVts9–3, and SVts9–4) were gifts from Dr. Ide of Hiroshima University [Tsuyama et al., 1991].

### Preparation of Antibody Against Hic-5

The anti-mouse Hic-5 polyclonal antibody (#1024) was raised against the recombinant Hic-5 amino-terminal region (amino acids 2–194) from immunized New Zealand white rabbits. The serum was purified by affinity chromatography on an antigen-conjugated Sepharose 4B column. Antibodies against FAK and paxillin were purchased from Transduction Laboratories, and that against vinculin was obtained from Sigma Chemical Co. (St. Louis, MO).

### Immunostaining and Western Blotting

Cells were rinsed with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde in PBS, permeabilized in 0.2% Triton X-100 in PBS, blocked in 3% bovine serum albumin (BSA) in PBS, and treated with the first antibody at room temperature for 1 h. After washing three times in 0.1% Tween 20 in PBS, cells were treated with the second antibody (FITC-labeled monoclonal anti-mouse IgG, or rhodamin-conjugated anti-rabbit IgG: obtained from Dako). After extensive washing, cells were visualized under a fluorescent microscope. For Western blotting, cells were lysed in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate) on ice. Cell lysates were cleared by centrifugation; supernatants were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, 0.1 M dithiothreitol). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes (Hybond C, Amersham), and the membranes were blocked with BSA. Membranes were incubated with the second antibody (horseradish peroxidase [HRP]-conjugated anti-rabbit IgG or anti-mouse IgG), and were

detected with chemiluminescence using a Renaissance kit (NEN Life Science Products, Boston, MA).

### Construction of N-Terminal Deletions of Hic-5

DNA fragments encoding N-terminal regions of Hic-5 were generated by PCR and inserted into *Eco*RI and *Xho*I sites of pGEX-5X-1 (Pharmacia Biotech, Uppsala, Sweden) inframe to construct GST-N, GST-N1, GST-N2, GST-N3, and GST-N4. These constructs encoded Hic-5 fragments containing amino acids 2–194, 31–194, 66–194, 119–194, and 153–194, respectively. The fusion proteins were expressed by incubation of *Escherichia coli* harboring expression vectors with 1 mM isopropyl-D-thiogalactopyranoside for 3 h at 30. The bacteria were collected by centrifugation, lysed by sonication, and solubilized in PBS containing 1 % Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μ/ml leupeptin. In vitro association of FAK and the deletion mutants of Hic-5 was measured by incubation of MEF extracts and the GST-fusion forms of Hic-5 expressed in *E. coli*, followed by binding to glutathione-Sepharose beads and Western blotting using anti-FAK antibody.

### Determination of Cap Site of Human Hic-5

The 5′-terminus of human Hic-5 mRNA was determined using a 5′-Full RACE kit from Takara Co. (Kyoto). TIG-3 mRNA was used as a template for synthesis of cDNA, and cDNA was concatemerized and polymerase chain reaction (PCR)-amplified using forward and reverse primers (5′-GTACAGCACGGTATGCAAG, and 5′-CATTTGTGGAGCTCACCACC, respectively). Amplified products were sequenced using a kit from Pharmacia.

## RESULTS

### Levels of Hic-5 and Paxillin During Serial Cultivation of MEF

The hic-5 mRNA level is significantly decreased in cells that have the potential to proliferate indefinitely [Shibanuma et al., 1994]; Hic-5 protein is localized to the focal adhesions [Matsuya et al., 1997]. Mouse embryo fibroblasts (MEF) are readily immortalized when cultured in vitro, and Hic-5 protein levels were examined during the immortalization process. MEF were serially passaged and reached a senescence-like stage after about 30–40 population doublings. Spontaneously immortalized cells appeared thereafter. Total cell extracts were prepared from cells at various passages and subjected to SDS-PAGE. Levels of Hic-5 and other components of focal adhesions were determined by Western blotting. The level of Hic-5 was decreased markedly in immortalized cells, as was observed in cultured human cells, whereas the level of its homologue paxillin was significantly increased after immortalization (Fig. 1). Protein levels of focal adhesion kinase (FAK) and vinculin did not change significantly. Independently immortalized MEF showed similar changes in the levels of Hic-5 and paxillin (data not shown). Levels of Hic-5 was also decreased in immortalized human fibroblasts SVts8 (data not shown).

### Localization of Hic-5 in Mortal and Immortal MEF

The subcellular localization of Hic-5 was analyzed during immortalization. Cells in monolayers were stained with antibodies against Hic-5 or vinculin. Specificity of the antibodies was verified by the observation that they mainly gave single bands on Western blotting. As shown in Figure 2, Hic-5 was localized in a distinct dotted pattern in normal MEF during the mortal stage (before 40 population doublings), and was precisely co-localized with vinculin. These structures were thought to be focal adhesion plaques. Cells at later passages (population doublings of about 50) showed flattened morphology, a typical phenotype of senescent fibroblasts, but Hic-5 was localized to adhesion plaques similarly to the observations in young cells. In the case of immortalized cells, however,

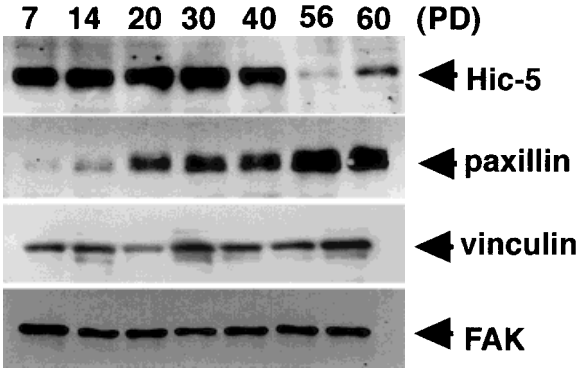


**Fig. 1.** Changes in protein levels in immortalized cells. Cellular extracts of MEF at each population doubling (PD) were subjected to SDS-PAGE, and Western blotting using antibodies against Hic-5, paxillin, vinculin, and FAK.

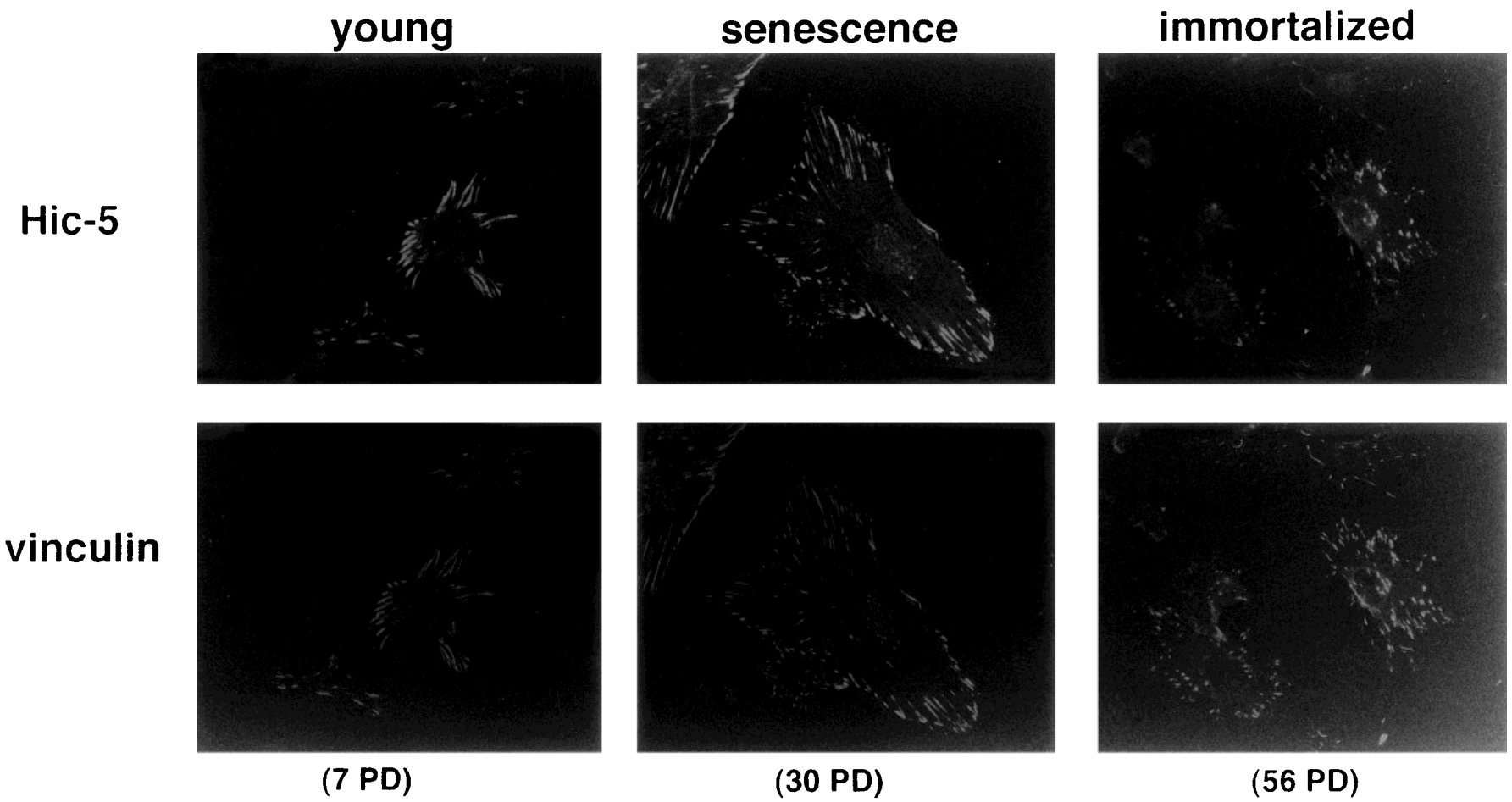


**Fig. 2.** Subcellular localization of Hic-5 and vinculin during immortalization of MEF. MEF at population doublings of 7, 30, and 56 were fixed and treated with polyclonal antibody against Hic-5 or monoclonal anti-vinculin antibody. Cells were then immunostained with FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies.

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  1 :   MSRLGAPKER PPETLTPPPP YGHQPQTGSG ESSGTTGDKD HLYSTVCKPR SPKPVAPVAP

 61 :   PFSSSSGVLG NGLCELDRLL QELNATQFNI TDEIMSQFPS SKMAEGEEKE DQSEDKSSPT
                                 LD1
121 :   VPPSPFPAPS KPSATSATQH LDRLMASLSD FRVQNHLPAS GPPQPPAASP TREGCPSPPG
                                          LD2
181 :   QTSKGSLDTM LGLLQSDLSR RGVPTQAKGL CGSCNKPIAG QVVTALGRAW HPEHFLCSGC
                 LD3
241 :   STTLGGSSFF EKDGAPFCPE CYFERFSPRC GFCNQPIRHK MVTALGTHWH PEHFCCVSCG

301 :   EPFGEEGFHE REGRPYCRRD FLQLFAPRCQ GCQGPILDNY ISALSALWHP DCFVCRECLA

361 :   PFSGGSFFEH EGRPLCENHF HAQRGSLCAT CGLPVTGRCV SALGRRFHPD HFTCTFCLRP

421 :   LTKGSFQERA SKPYCQPCFL KLFG
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**Fig. 3.** Amino acid sequence of mouse Hic-5. LD domains are boxed. Boldface, LIM domains.

staining with anti-Hic-5 in the nuclear periphery seemed to appear in almost all the cells; even staining in the focal adhesion remained. Hic-5 is a paxillin homologue, and paxillin colocalizes with Hic-5 and vinculin. The subcellular distribution of paxillin, however, did not change significantly during passages (data not shown).

## Determination of Hic-5 Domains Responsible for Association With FAK

Comparison of amino acid sequences of mouse and human Hic-5 indicated that they are highly homologous, except in their N-terminal regions. The amino acid sequence of human Hic-5 reported by Matsuya et al. (1997) did not include the first methionine, but we isolated the cap site of human Hic-5 cDNA by a 5′-Full RACE method using mRNA from normal human fibroblasts, and its nucleotide sequence was shown to include methionine (Fig. 3). The predicted amino acids sequence indicated that human Hic-5 contains 17 extra amino acids that are absent in the mouse counterpart that has been isolated at present. Several deletion mutants of mouse Hic-5 were prepared to determine the regions necessary for association with FAK. N-terminal deletions were constructed as GST-fusion proteins, and their association with FAK was examined by in vitro binding experiments (Fig. 4). Deletion of 66 amino acids from the N-terminus of mouse Hic-5 (NΔ1, NΔ2) decreased the binding activity to FAK to about one-half, but further deletion down to amino acid 119 (NΔ3) resulted in complete loss of the binding. Thus, the region from amino acids 66–119 that contains the LD domain 1 (LD1) seems to be most critical for association with FAK, but other domains in the N-terminal re-

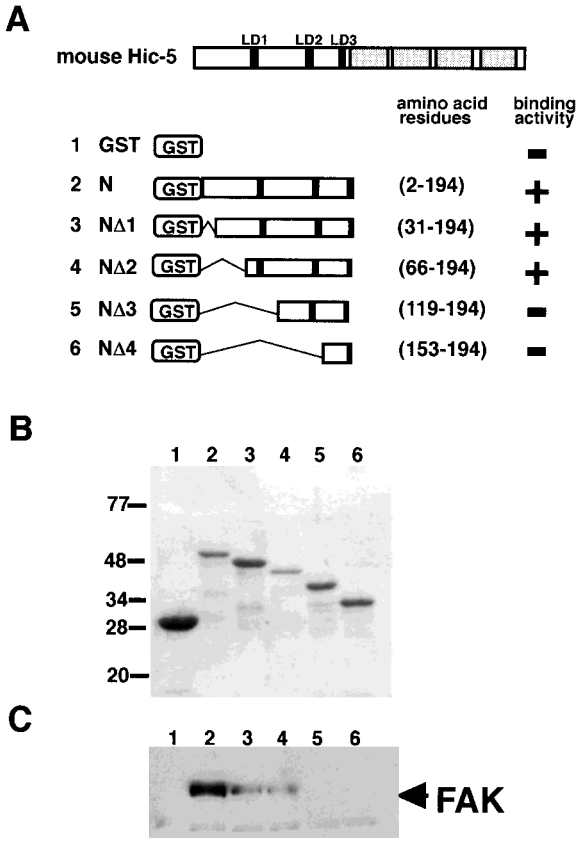


**Fig. 4.** Binding of N-terminal truncated Hic-5 and FAK. **A:** Schematic representation of GST-fusion proteins of N-terminal regions of Hic-5. **B:** Coomassie blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of GST-fusion proteins synthesized in *E. coli*. C, MEF cell extract was incubated with each GST-fusion protein, and mixed with glutathione-Sepharose beads. After washing, proteins bound to the beads were eluted and subjected to SDS-PAGE. Proteins were blotted onto membranes and detected with anti-FAK antibody.

gion may help stabilization of the binding. The LIM domains did not affect the binding to FAK (data not shown).

### Distribution of Hic-5 in FAK$^{-/-}$ Fibroblasts

As Hic-5 binds to FAK, one of the components of focal adhesions, it was interesting to examine the subcellular localization of Hic-5 in MEF from FAK knockout mice. Western blotting indicated that immortalized (population doublings, more than 80) FAK$^{-/-}$ cells contained significantly higher levels of Hic-5 (data not shown), and the results of immunochemical analysis shown in Figure 5 indicated that Hic-5 is localized in the focal adhesions in FAK$^{-/-}$ similarly to the case in FAK$^{+/+}$ cells.

### Effects of Hic-5 on Colony Formation of MEF

We previously reported that forced expression of Hic-5 decreased colony forming ability of fibroblasts [Shibanuma et al., 1997]. This biological effect of Hic-5 was examined using MEF from FAK$^{+/+}$ and FAK$^{-/-}$ mice. FAK$^{+/+}$ cells were transfected with G-418-resistance plasmid (pRc/CMV) or Hic-5 expression vector, and cultured in the presence of G-418. Numbers of G-418-resistant colonies were decreased by the forced expression of full-length Hic-5. As FAK$^{-/-}$ cells were resistant to G-418, hygromycin resistance was used as a selection method. Forced expression of Hic-5 showed essentially no effect on colony formation in FAK$^{-/-}$ MEF (Fig. 6a). Furthermore, N-terminal deletion mutant (NΔ3) that lost an ability to associate with FAK did not decrease the colony-forming ability of FAK$^{+/+}$ MEF. Expression levels of transfected Hic-5 were almost the same both in FAK$^{+/+}$ and FAK$^{-/-}$ cells (Fig. 6b). Thus, the interaction between Hic-5 and FAK is necessary for decrease in colony formation of MEF.

## DISCUSSION

### Levels of Hic-5 and Paxillin in Immortalized MEF

The results of the present study showed that Hic-5 protein levels were decreased significantly in immortalized MEF, but the levels of its homologue, paxillin, were increased. Normal fibroblasts reach cellular senescence after a finite number of cell divisions in culture [Hayflick and Moorhead, 1961]. Some cells escape senescence and are immortalized either spontaneously or by genotoxic agents or tumorigenic viruses [Gallahon et al., 1998]. These events are generally irreversible; the mechanisms un-

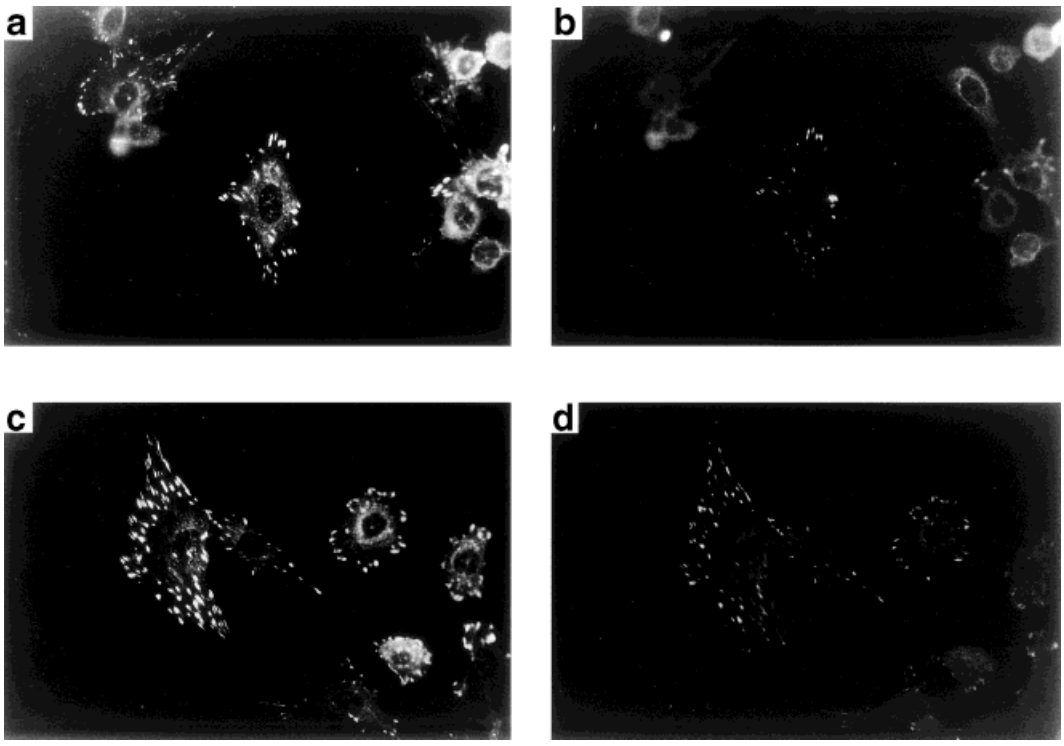


**Fig. 5.** Immunochemical staining of MEF from FAK$^{+/+}$ (**a,b**) and FAK$^{-/-}$ (**c,d**) mice. Cells were fixed and stained with anti-Hic-5 (a,c) and anti-vinculin (b,d) antibodies as described in Fig. 2.

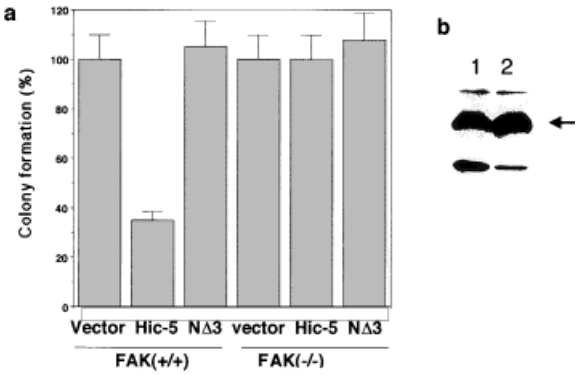


**Fig. 6.** Effects of forced expression of Hic-5 on colony formation of MEF from $FAK^{+/+}$ or $FAK^{-/-}$ mice. **a**, $FAK^{+/+}$ cells were transfected with pRc/CMV, full-length Hic-5, or NΔ3 expression vector, and colonies were scored after selection with G-418. In the case of $FAK^{-/-}$ cells, hygromycin resistance was used as a selection marker, and numbers of hygromycin-resistant colonies were scored. Mean numbers of colonies/dish in the control (three independent experiments) were 105.3 ± 8.9 and 124.2 ± 10.5 in $FAK^{+/+}$ and $FAK^{-/-}$ cells, respectively. **b**, Western blotting of HA-tagged Hic-5 transfected to $FAK^{+/+}$ (**lane 1**) and $FAK^{-/-}$ cells (**lane 2**), stained with anti-HA antibody as described previously [Nishiya et al., 1999]. Arrow, Hic-5.

derlying these phenomena are thought to be different from those that participate in regulation of the cell cycle. The immortalization step has not been well characterized at the molecular level but is generally accompanied by changes in karyotypes, cell morphology [Kuroki and Huh, 1993], and loss of tumor suppressors such as p53 [Harvey and Levine, 1991]. Numerous other alterations in gene expression are induced during cellular senescence and immortalization [Imai et al., 1994; Satoh, et al., 1994; Parchinas et al., 1997], but changes in extracellular matrix-related and cytoskeletal components are most frequently detected [Sottlie et al., 1989; Kumazaki, et al., 1991]. Extracellular matrix proteins bind to their receptor, integrin, and elicit intracellular signals through activation of signaling molecules that associate with focal adhesions. These changes are coupled with alterations in organization of the cytoskeleton and gene expression. Levels of vinculin and vimentin mRNA are decreased in immortalized fibroblasts or in established cell lines such as NIH3T3 or Balb 3T3 and their distribution are also altered [Kaneko et al., 1995]. Expression of the gene encoding the focal adhesion protein Hic-5 is regulated during immortalization, and these changes may affect cellular growth potential through alterations of extracellular matrix-related signals.

## Subcellular Distribution of Hic-5

Hic-5 was localized mainly in focal adhesions in mortal mouse fibroblastic cells, as reported previously [Matsuya et al., 1997; Hagmann et al., 1998], but it was also partly localized to perinuclear regions in immortalized MEF (Fig. 2) along with a marked decrease in the total Hic-5 protein level. Hic-5 and vinculin are colocalized in mortal fibroblasts, but vinculin remained in the focal adhesions in immortalized MEF. The distribution of other focal adhesion components such as FAK and paxillin was not changed significantly (data not shown), but the majority of CAKβ, a FAK homologue [Sasaki et al., 1995], was found in the perinuclear region of the immortalized rat fibroblast cell line WFB [Matsuya et al., 1997]. In mortal and immortal MEF, however, the CAKβ level was below the limit of detection (data not shown).

Focal adhesions are transmembrane bridges of integrin-associated extracellular matrix (ECM) and intracellular signaling molecules such as protein tyrosine kinases and effectors such as small G proteins [Nobes and Hall, 1995; Tapon and Hall, 1997]. Cell-cell or cell-substratum interactions affect cellular functions related to growth and differentiation. Focal adhesions are multiprotein complexes composed of integrins, tyrosine kinases such as FAK, and CAKβ, adaptor proteins such as paxillin, and cytoskeletal components such as talin and vinculin [Clark and Brugge, 1995; Schwartz, 1997].

The formation of focal contact is also regulated by actin-containing filaments and is impaired in tumorigenic cells, possibly through deletions in tumor suppressor genes such as p53 or activation of some types of oncogenes such as c-myc [Tlsty, 1998]. As a result, the normal patterns of expression of genes encoding extracellular matrix proteins, surface receptors, signaling molecules, or nuclear transcription factors are altered. Changes in subcellular localization of Hic-5 may reflect changes in the overall organization of focal adhesions that leads to deregulation of growth potential. The change from cytoplasmic distribution of mortalin, a senescence-inducing protein, to the perinuclear locale is detected during cellular immortalization [Wadhwa et al., 1993]. Determination of subcellular localization of paxillin to sites of focal adhesion has been shown to be mediated by LIM3 [Brown et al., 1996], and LIM3 of Hic-5 is also involved in the determination of its

subcellular localization [Fujita et al., 1998; Nishiya et al., 1999]. Hic-5 associates with FAK through its N-terminal region, and its localization in focal adhesions was not altered in FAK$^{-/-}$ cells. Thus, the association of Hic-5 to FAK may not be involved in targeting of Hic-5 to focal adhesions. We recently found that Hic-5 binds to the protein tyrosine phosphatase PTP-PEST [Nishiya et al., 1999] that is localized to focal adhesions, at least in part [Cote et al., 1998]. Protein tyrosine phosphatase (PTEN) is also localized to the focal adhesions whose components are rich in tyrosine phosphorylation [Tamura et al., 1998]. These interactions and changes in subcellular localization may also play roles in regulation of cell growth through modulating tyrosine phosphorylation level as an interface for tyrosine kinase and phosphatase.

### Involvement of FAK in Inhibition of Colony Formation by Hic-5

Hic-5 associates with several focal adhesion proteins such as FAK, CAKβ, vinculin, and talin. In the present study, however, we focused our attention on FAK, as it is one of the key factors in transduction from integrin signals [Clark and Brugge, 1995]. The N-terminal region of Hic-5, including the LD1 domain, appears to be involved in the interaction with FAK. Hic-5 level was increased in FAK$^{-/-}$ cells, but its subcellular distribution was similar to that in FAK$^{+/+}$ cells (Fig. 5). However, the interaction of these two molecules appears to be involved in inhibition of colony formation induced by Hic-5. Colony formation of FAK$^{+}$ cells was decreased by full-length Hic-5, but that of FAK$^{-/-}$ cells was not affected, and Hic-5 NΔ3 that failed to associate with FAK did not affect colony formation (Fig. 6a). As mentioned above, immortalized FAK$^{-/-}$ cells contained higher levels of Hic-5, and the results of Fig. 6 showing that transfection of Hic-5 did not affect colony formation in FAK$^{-/-}$ cells may be caused by the adaptation of the cell growth to higher levels of Hic-5. Whether this effect is mediated through kinase activity of FAK remains unknown; further studies are required to clarify the requirement of tyrosine kinases in growth suppression by Hic-5.

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