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Involvement of FAK and PTP-PEST in the Regulation of Redox-Sensitive Nuclear-Cytoplasmic Shuttling of a LIM Protein, Hic-5

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ABSTRACT

The LIM protein Hic-5 is a focal adhesion protein shuttling in and out of the nucleus through the redox-sensitive nuclear export signal, and unlike other focal adhesion proteins including paxillin, the protein most homologous to Hic-5, it accumulates in the nucleus under oxidative conditions and participates in the transcription of c-*fos* and $p21^{Cip1}$ genes. Here, we examined the roles of the interacting partners of Hic-5, focal adhesion kinase (FAK) and protein tyrosine phosphatase PEST (PTP-PEST), in the nuclear translocation of Hic-5 and found that they were inhibitory. Interestingly, the interaction of Hic-5 with FAK was regulated by specific cysteines near the binding site and decreased in cells under oxidative conditions. Its interaction with PTP-PEST was also sensitive to the oxidant. These results suggest that the nuclear-cytoplasmic shuttling of Hic-5 is regulated by its interacting partners at focal adhesions or in the cytoplasm in a redox-sensitive manner, coordinating its role at focal adhesions with that in the nucleus, depending on the redox state of cells. Cytochalasin D or a phorbol ester also induced nuclear accumulation of Hic-5, which was inhibited by scavengers of reactive oxygen species (ROS), suggesting that besides oxidants, endogenously produced ROS induced the nuclear accumulation of Hic-5. *Antioxid. Redox Signal.* 7, 335–347.

INTRODUCTION

Hic-5 is a LIM protein of the paxillin family (35) that contains four zinc finger motifs classified as LIM domains (6) in its C-terminus and three or four LD motifs (5) in its N-terminus, both of which are known as protein–protein interacting interfaces (2). As reviewed by Turner (41), this family includes paxillin, Hic-5, pax B, and leupaxin and is mainly localized at focal adhesions as one of the components constituting the signaling and structural complex that mediates a physical link between the extracellular matrix and intracellular actin cytoskeleton through integrins and coordinates cell adhesion status with various cellular processes, such as migration, growth, survival, and differentiation (9, 31).

Hic-5 is known to interact with multiple signaling and structural proteins, such as focal adhesion kinase (FAK) (8), PYK2/Cakβ (19), protein tyrosine phosphatase PEST (PTP-PEST) (24), vinculin (4, 11), Arf-Gap family protein GIT1 (26), Csk (39), and PKL (42) and is supposed to function as an adaptor protein at focal adhesions for integrin-signal transduction. These interacting factors are common to paxillin, although paxillin also binds to Crk through its SH2 domain, which is absent from the Hic-5 protein (39).

Recent findings indicate that some LIM proteins, such as zyxin, lipoma-preferred partner (LPP), and thyroid receptor interacting protein-6 (Trip6), shuttle between focal adhesions and the nucleus in a Crm1/exportin-dependent manner (for reviews, see 1, 44). Our recent study added Hic-5 to the list (37). Paxillin was also found to have a nuclear export signal (NES)-like sequence and shown to traffic in and out of the nucleus (45). In the nucleus, these proteins were presumed to participate in transcriptional control or other nuclear events through their association with the transcriptional machinery or nuclear matrix (for reviews, see 1, 3). For example, Trip6

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was reported to interact with thyroid hormone receptor as a potential coactivator for transcription, and LPP is also likely involved in transcriptional activity, although their target genes in the nucleus are unidentified (28, 43). Similarly, Hic-5 was proposed to have a role in the nucleus as a coactivator of transcription through our series of work. We found that the c-*fos* and p21 genes were potential targets of coactivator activity and that Hic-5 presumably provided a scaffold for the transcriptional activity at Sp1 sites on which to assemble the complex, including Sp1, Smad3, and p300 (13, 23, 36, 38). In addition, Yang *et al.* reported that Hic-5 interacted with the transactivation domain of the glucocorticoid receptor and worked as a coactivator for this transcription factor in association with the nuclear matrix (46). The focal adhesion protein, Cas-interacting zinc finger protein, also has the ability to translocate to the nucleus where it regulates the expression of matrix metalloproteinases (22). Thus, evidence has accumulated indicating that a class of adhesion proteins primarily localized at focal adhesions shuttles to the nucleus and there engages in activities distinct from those at adhesion sites, such as transcriptional regulation. However, the relationship between the functions at these two locations, including the potential role to relay information from adhesion sites to the nucleus and the mechanisms regulating the shuttling, are still poorly understood.

Among the shuttling proteins, Hic-5 is characterized by its nuclear accumulation under oxidative conditions. In most other cases, no signals or regulation has been defined that could induce the bulk redistribution of the proteins from focal adhesions to the nucleus, except FHL2, which belongs to the LIM-only protein family and translocates to the nucleus in response to Rho signals (21). Our detailed analysis clarified that the nuclear import of Hic-5 was directed by the four LIM domains of the C-terminus cooperating as an unconventional nuclear localization signal, and the export, on the other hand, was regulated by a novel type of Crm1/exportin-dependent NES consisting of a leucine-rich Rev-type stretch of one of the LD motifs, LD3, together with the two nearby cysteines in the N-terminus. Importantly, the NES showed redox sensitivity and was inactivated under oxidative conditions, thereby localizing Hic-5 in the nucleus under the conditions. The redox sensitivity of NES was regarded as the critical basis for Hic-5 nuclear accumulation and its function in the nucleus to up-regulate the c-*fos* gene in response to the oxidative conditions in cells, and the two cysteines of NES were identified as responsible for the sensitivity (37). Overall, Hic-5 appeared to be a shuttling protein specialized to deal with changes in the redox state in cells, coordinating cell adhesion status with gene expression.

In yeast, the proteins Yap1/Pap1 share some of the properties of Hic-5. They are AP-1-like yeast transcription factors that are localized in cytoplasm in nonstress conditions, but translocate into the nucleus under oxidative conditions and regulate gene transcription for the stress response (15, 40). Like Hic-5, they have redox-sensitive NESs containing two or three cysteine residues besides a leucine-rich stretch whose inactivation results in their nuclear accumulation under oxidative conditions (14). The amino acid sequences of the NESs of Yap1/Pap1 showed a limited similarity to those of Hic-5, whereas a difference was noticed in the precise disposition of cysteine residues and leucine-rich stretch in the NES module (37). Additionally, it should be pointed out that the core of the NES of Hic-5, the LD3 motif, was also assigned as an interface crucial to interact with FAK (25), whereas such a functional overlap has not been reported for the NES of Yap1/Pap1. This fact implied that FAK had some effect on the NES function of Hic-5 through binding to the motif.

In the present study, we studied the regulation of the redox-dependent nuclear shuttling of Hic-5, focusing on the roles of its interacting partners, FAK and PTP-PEST.

MATERIALS AND METHODS

Cell culture

Mouse C3H10T1/2 fibroblastic cells, MC3T3 osteoblastic cells, and COS-7 cells were grown in minimum essential medium (MEM) (C3H10T1/2) or Dulbecco's modified MEM (MC3T3 and COS-7) supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37°C in an atmosphere of 5% CO_2 in air.

Antibodies and chemicals

Antibodies to tag were obtained as follows: Anti-Flag polyclonal and monoclonal (M2) antibodies from Sigma (St. Louis, MO, U.S.A.), anti-hemagglutinin (HA) monoclonal antibody (12CA5) from Boehringer Mannheim (Indianapolis, IN, U.S.A.), and anti-myc monoclonal antibody (9E10) from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Anti-Hic-5 and anti-FAK monoclonal antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.), and anti-vinculin monoclonal antibody was from Sigma.

Leptomycin B (LMB), cytochalasin D (CD), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Diethyl maleate (DiM) was from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). Colcemid was from Life Technologies, Inc. (Rockville, MD, U.S.A.).

Plasmids

Expression vectors for wild-type Hic-5 used in this study were pCG-LD1m*hic-5* (HA tag) (25), Flag-m*hic-5* (37), and FlagLD1m*hic-5* generated by inserting the PCR-amplified full-length cDNA of LD1m*hic-5* into the cloning vector of pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) including the Flag tag. PCR amplification was carried out with *pfu* grade polymerase, and the amplified insert was verified by DNA sequencing. Flag-mCfl/NSm*hic-5* was constructed enzymatically by excising the mCfl/NSm*hic-5* fragment from the pCG version (37) and inserting it into pcDNA3 containing the Flag tag.

FlagPax was engineered by inserting the PCR-amplified full-length paxillinα cDNA into pcDNA3. Myc-tagged wild-type (pSRα-FAK) and kinase-defective FAK [FAK_{KD} (K454R)] and HA-tagged PTP-PEST (HA-PTP-PEST) together with their mutants [active center-destroyed (C231S) or Pro-2 domain-deleted] were as described previously (24, 25).

Immunocytochemistry

The expression plasmids were introduced into cells using the conventional calcium phosphate precipitation method. Immunocytochemistry was performed as described previously (11). Fluorescein isothiocyanate-conjugated anti-mouse IgG and tetramethylrhodamine B isothiocyanate-conjugated anti-rabbit IgG from Dako (Copenhagen, Denmark) were used as the secondary antibodies. Fluorescence microscopy was carried out using an Axioskope microscope (Carl Zeiss) equipped with a high-speed cooled digital CCD camera fluorescence imaging system (ARGUS/HiSCA).

Immunoprecipitation and immunoblot analysis

The expression plasmids were introduced into cells with TransIT-LT1 reagent purchased from PanVera (Madison, WI, U.S.A.). After being incubated in complete medium for 24 h, the cells were processed for immunoprecipitation. After a wash with phosphate-buffered saline, an aliquot of cells was lysed in buffer containing 0.5% sodium dodecyl sulfate, 150 m*M* NaCl, and 50 m*M* Tris (pH 6.8) and analyzed by immunoblotting as the lysate fraction, or lysed in immunoprecipitation buffer [0.5% NP-40, 150 m*M* NaCl, 50 m*M* Tris (pH 6.8), 10 m*M* NaF, 10 m*M* sodium pyrophosphate, 0.4 m*M* sodium orthovanadate, and protease inhibitor mixture (Wako Pure Chemical Industries, Ltd., Osaka, Japan)]. After preclearing of the lysate (~200 μg of total protein per sample) with normal IgG (Dako) immobilized on protein A-Sepharose for 1 h at 4°C, immunoprecipitation was performed with antibodies immobilized on protein A-Sepharose with gentle agitation for 2 h at 4°C. After immunoprecipitation, the resins were washed with the immunoprecipitation buffer supplemented with 1% bovine serum albumin three times, followed by phosphate-buffered saline three times.

The bound proteins were eluted by boiling in sodium dodecyl sulfate sample buffer for immunoblotting (11). The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG antibodies from Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.), and bound antibodies were visualized with the enhanced chemiluminescence detection system.

RESULTS

The nuclear accumulation of Hic-5 protein induced by hydrogen peroxide (H_2O_2) was inhibited by FAK and PTP-PEST

The nuclear-cytoplasmic shuttling of proteins is potentially regulated by their interacting molecules, and Hic-5 was shown to bind to multiple signaling molecules, including FAK and PTP-PEST. We here examined the effects of FAK and PTP-PEST on the nuclear accumulation of Hic-5 under oxidative conditions. C3H10T1/2 cells were transfected with the expression vector for FAK (myc tag) or PTP-PEST (HA tag) and exposed to 1.5 m*M* H_2O_2 for 30 min; then the localization of endogenous Hic-5 along with that of exogenously expressed FAK and PTP-PEST was visualized with the antibody to Hic-5 and to the tags, respectively. In the cells overexpressing FAK or PTP-PEST, the nuclear accumulation of Hic-5 was almost completely inhibited, whereas Hic-5 accumulated in most surrounding cells that did not express FAK or PTP-PEST at detectable levels, as seen in Fig. 1, suggesting a regulatory role for FAK and PTP-PEST in the Hic-5 nuclear accumulation.

FAK and PTP-PEST were localized in the cytoplasm under the conditions inducing nuclear accumulation of Hic-5 and inhibited the nuclear translocation of Hic-5

Currently, we have only a limited amount of knowledge on the regulation of the subcellular localization of FAK and PTP-PEST, particularly in relation to that of Hic-5. The results in Fig. 1 indicated that, in contrast to Hic-5, FAK and PTP-PEST did not change their localization remarkably on treatment with H_2O_2, being distributed mostly in the cytoplasm. Their cytoplasmic localization was also unchanged by the treatment with DiM and LMB, which efficiently induced the nuclear accumulation of Hic-5 (Fig. 2). Because LMB is an inhibitor for Crm1/exportin-dependent NES, the unresponsiveness to LMB suggested that FAK and PTP-PEST did not harbor NES to shuttle and, in addition, that they did not accompany Hic-5 into the nucleus during its nuclear-cytoplasmic shuttling.

Moreover, FAK or PTP-PEST coexpressed with Hic-5 in the same cells remarkably inhibited the nuclear translocation of Hic-5 induced by LMB as below (Fig. 3). In the cells expressing Hic-5 exogenously together with FAK, the Hic-5 stayed in the cytoplasm in the presence of LMB (Fig. 3A, an arrow), whereas Hic-5 accumulated in the nucleus in cells in which the expression of FAK was undetectable (Fig. 3A, arrowheads). Similarly, the coexpression of PTP-PEST inhibited the nuclear accumulation of Hic-5 by LMB (Fig. 3B). The results, quantified by counting the nuclear localized Hic-5 among the cells expressing Hic-5 under the cotransfection with FAK or PTP-PEST, demonstrated the inhibitory effects of FAK and PTP-PEST on the nuclear translocation of Hic-5 in a more convincing way (Fig. 3C). The inhibitory effect of FAK in Fig. 3C appeared to be underestimated, because the ratio of the nuclear localized Hic-5 was as low as 0.12 when it was evaluated only among the cells expressing a high level of FAK. The results also showed that these inhibitory effects were retained in the kinase or phosphatase activity-negative forms of FAK and PTP-PEST, FAK(KD) or PTP-PEST (C231S), suggesting that the effect was achieved by their interaction with Hic-5 itself and unrelated to their enzymatic activities. On the other hand, the effect of PTP-PEST (delta Pro2) whose Pro2 domain was deleted was attenuated compared with the wild type, consistent with the fact that this domain was needed to interact with Hic-5 (24). These results indicated that Hic-5-interacting proteins, FAK and PTP-PEST, present in the cytoplasm, did not translocate into the nucleus with Hic-5, but rather inhibited its nuclear translocation by binding to it.

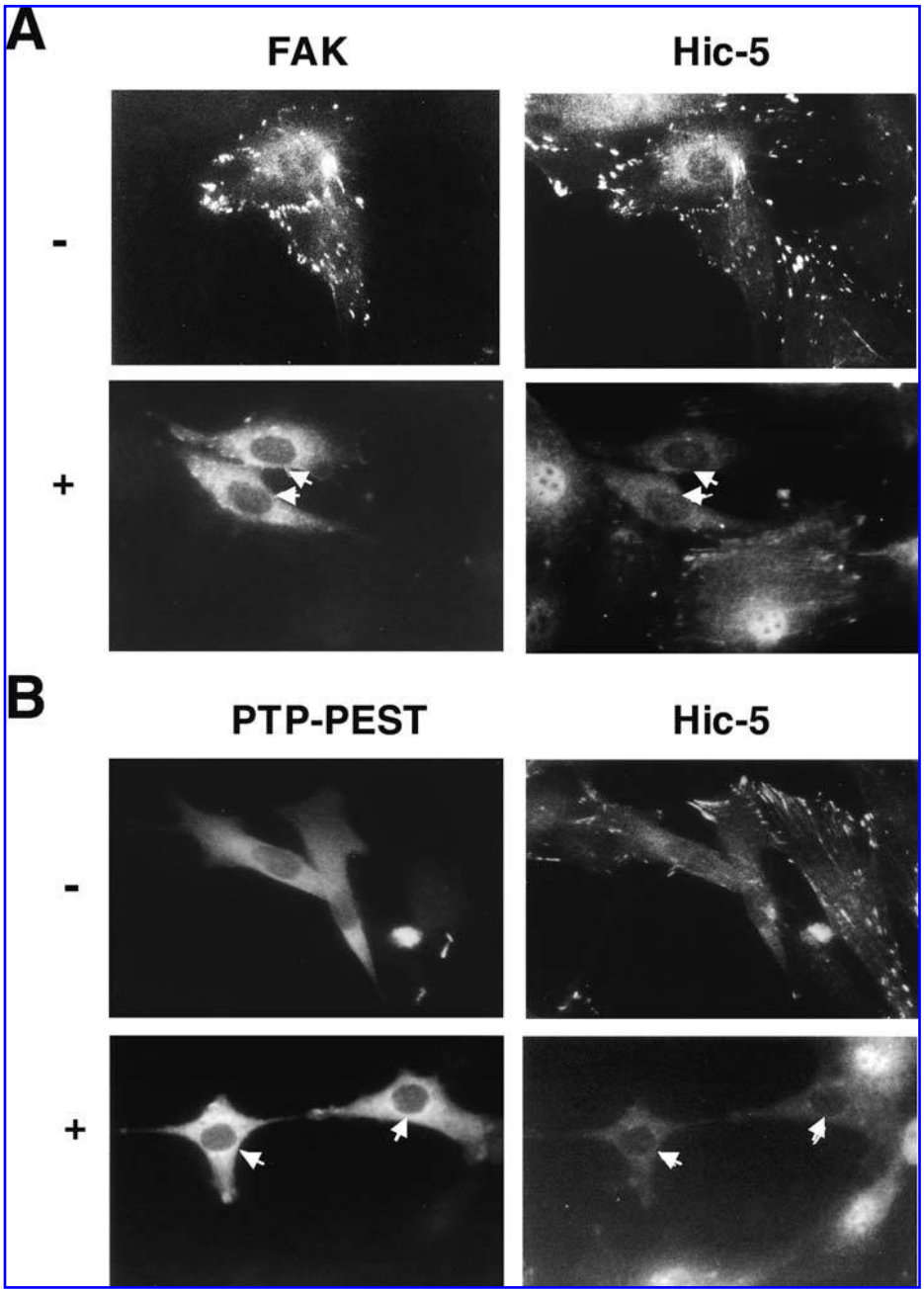

FIG. 1. Overexpression of FAK and PTP-PEST inhibited the nuclear accumulation of Hic-5 induced by H_2O_2. The expression vectors for FAK [pSRα-FAK (myc-tag)] (**A**) or PTP-PEST (HA-PTP-PEST) (**B**) were introduced into C3H10T1/2 cells by the conventional calcium phosphate method. After 24 h, the cells were treated (+) or untreated (−) with 1.5 m*M* H_2O_2 for 60 min and immunostained simultaneously with the antibodies to the tags [myc (FAK); 9E10, HA (PTP-PEST); 12CA5] and to Hic-5 as described in Materials and Methods. The arrows indicate the cells expressing exogenously introduced FAK or PTP-PEST.

Oxidant-sensitive interactions of Hic-5 with FAK and PTP-PEST

Considering the roles of FAK and PTP-PEST as negative regulators in the cytoplasm and focal adhesions for the nuclear translocation of Hic-5 as described above, it seems necessary for Hic-5 to be released from FAK and PTP-PEST upon translocation to the nucleus. To test this possibility, we examined the interaction of Hic-5 with FAK and PTP-PEST under the condition localizing most Hic-5 in the nucleus. The expression vector for Hic-5 (Flag-tagged) was cotransfected with that for FAK or PTP-PEST (HA-tagged) in COS-7 cells, and after 24 h, the cells were lysed following treatment with 2 m*M* DiM for 45 min. Then the Hic-5 protein was immunopre-

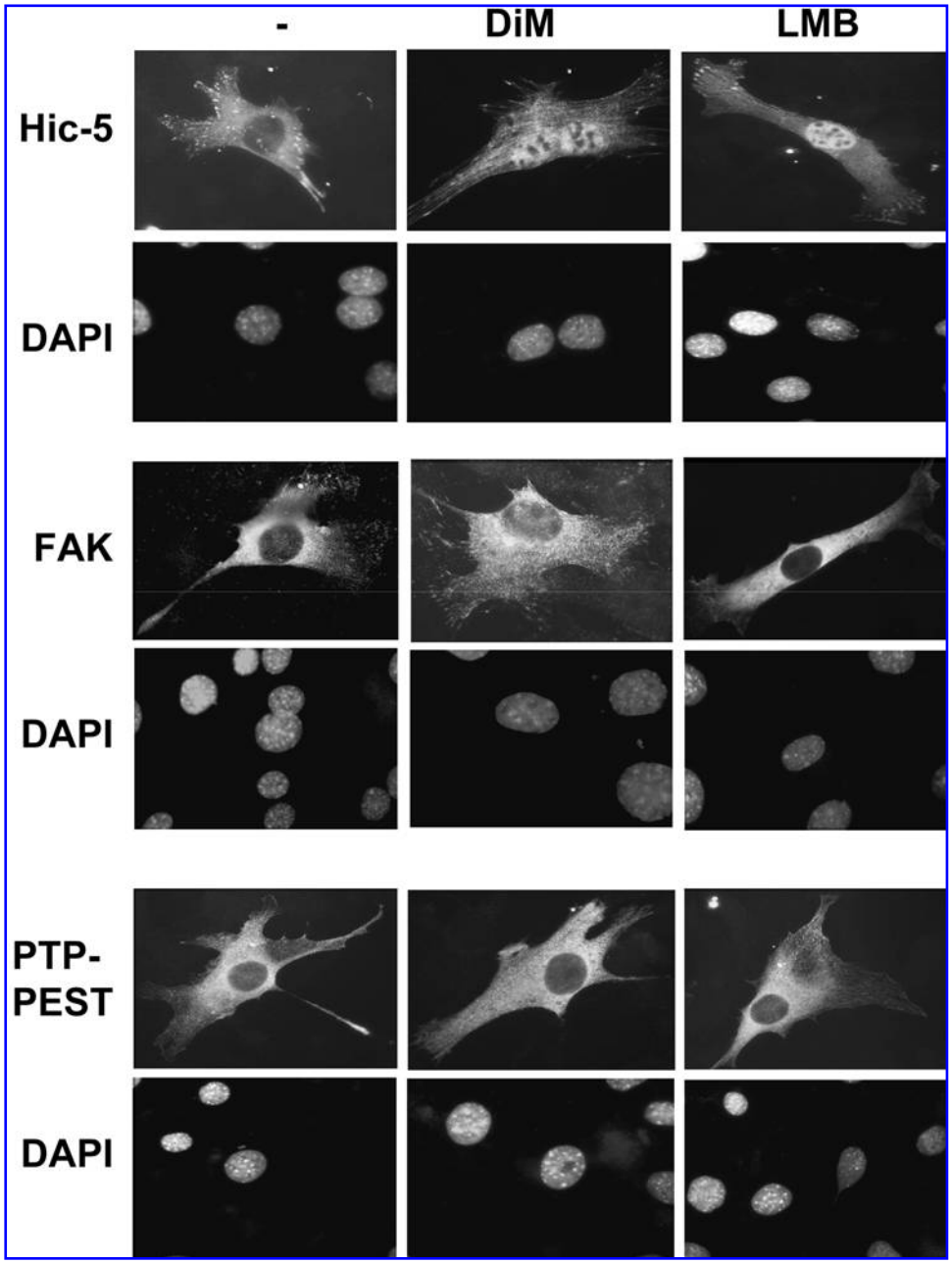

FIG. 2. Subcellular localization of Hic-5, FAK, and PTP-PEST in the absence or presence of DiM or LMB. The expression vectors for Hic-5 [pCG-LD1m*hic-5* (HA-tag)], FAK [pSRα-FAK (myc-tag)], and PTP-PEST (HA-PTP-PEST) were introduced into C3H10T1/2 cells with the conventional calcium phosphate method. After 24 h, the cells were treated or untreated with 2 m*M* DiM for 45 min or with 10 ng/ml LMB for 5 h, and processed for immunostaining using the antibodies to the tags as above. The nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI).

cipitated from the cell lysate with the antibody to the Flag tag, and FAK or PTP-PEST in the immunocomplex was detected by immunoblotting using the antibody to FAK or that to HA tag, respectively. Figure 4A indicates that the treatment with DiM resulted in a considerable decrease in the interaction of FAK with Hic-5. H_2O_2 also decreased the interaction above a dose of 0.5 m*M*, consistent with the induction of the nuclear accumulation of Hic-5 (37) (Fig. 4C). Interestingly, the interaction of FAK with paxillin was marginally affected by the same treatment (Fig. 4B). In contrast to the interaction with FAK, the interaction with PTP-PEST was reduced in both Hic-5 and paxillin by the oxidant. Recently, we found that Hic-5 was able to self-associate (unpublished observations). This self-association of Hic-5 was almost unaffected by the treatment, excluding an overall deleterious effect of the oxidant on Hic-5 protein (Fig. 4A).

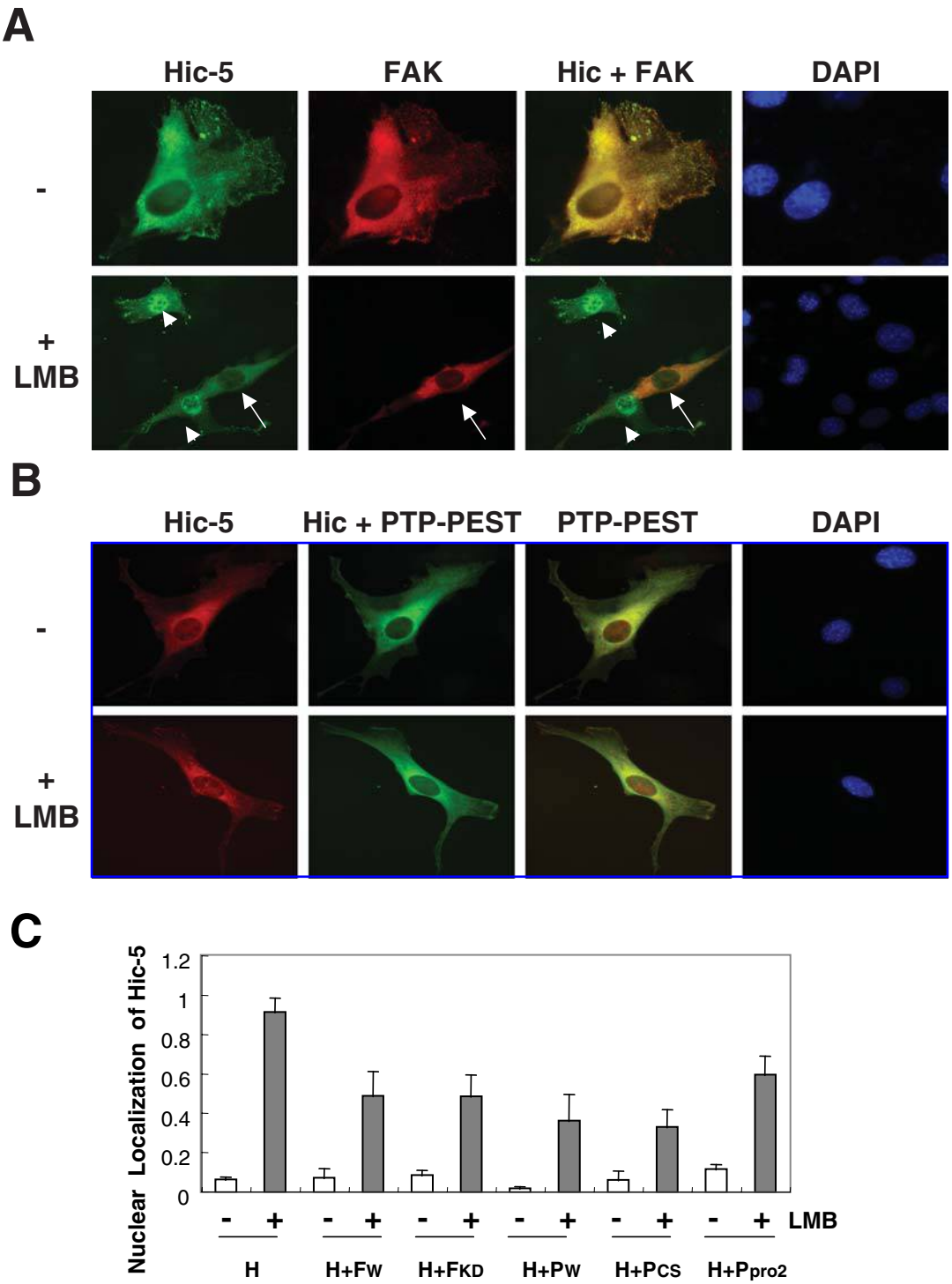

FIG. 3. Inhibition of the nuclear translocation of Hic-5 by FAK and PTP-PEST. The expression plasmid for Hic-5 (FlagLD1m*hic-5*) was cotransfected into C3H10T1/2 cells with that for FAK (pSRα-FAK) (**A**) or with that for PTP-PEST (HA-PTP-PEST) (**B**), treated or untreated with LMB (10 ng/ml, 5 h), and then immunostained simultaneously for Hic-5 (Flag tag) and FAK (myc tag) or PTP-PEST (HA tag) using the antibodies to the tags (Flag; anti-Flag polyclonal, myc; 9E10, HA; 12CA5) as above. The nuclei were labeled with DAPI. (**C**) Quantitative analysis of the inhibitory effects of FAK and PTP-PEST on the LMB-induced nuclear localization of Hic-5 observed in A and B. The nuclear localization was scored microscopically, and the ratio was graphed (means ± SD derived from a series of experiments repeated more than three times; in each of them, >50 cells expressing Hic-5 were scored). H, Hic-5; FW, wild type of FAK; FKD, kinase-defective FAK (K454R); PW, wild type of PTP-PEST; PCS, phosphatase activity-defective PTP-PEST (C231S); Ppro2, PTP-PEST with Pro2 domain deleted. The expression vectors for enzymatically defective FAK or PTP-PEST were as described previously (24, 25).

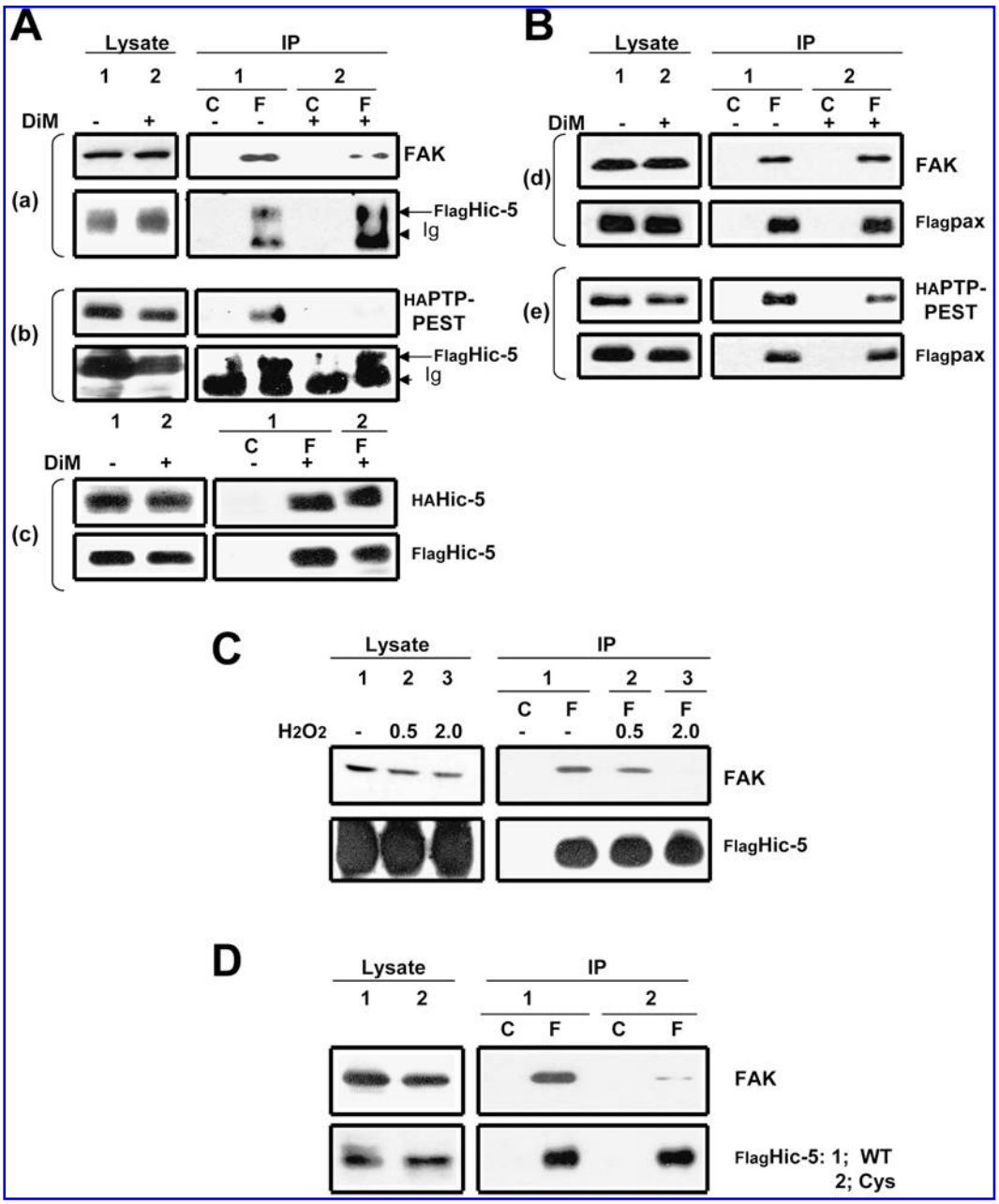

FIG. 4. Oxidant-sensitive interaction of Hic-5 with FAK and PTP-PEST. (**A**) Immunoprecipitation (IP) analysis of the interaction of Hic-5 with FAK or PTP-PEST under oxidative stress. The expression vectors for Hic-5 [(pCG-LD1m*hic-5* (HA tag) or FlagLD1m*hic-5*], FAK (pSRα-FAK), and PTP-PEST (HA-PTP-PEST) were cotransfected into COS-7 cells in the following combinations with Trans-IT-LT1. (**a**) Flag-Hic-5 + FAK, (**b**) Flag-Hic-5 + HA-PTP-PEST, (**c**) HA-Hic-5 + Flag-Hic-5. At 24 h after transfection, the cells were treated with (**2**) or without (**1**) 2 m*M* DiM for 45 min, lysed, and immunoprecipitated with the polyclonal antibody to Flag tag (F) or control IgG (C). Then the immunocomplex was analyzed by immunoblotting as described (11). The antibodies used for immunoblotting were monoclonal antibodies to Flag tag (M2), FAK, and HA tag (12CA5). (**B**) Immunoprecipitation analysis of the interaction of paxillin with FAK or PTP-PEST under oxidative stress. The expression vectors for paxillin (FlagPax), FAK, and PTP-PEST were cotransfected into COS-7 cells in the following combinations and analyzed as in A. (**d**) Flag-pax + FAK, (**e**) Flag-pax + HA-PTP-PEST. (**C**) Immunoprecipitation analysis of the interaction of Hic-5 with FAK on H_2O_2 treatment. The expression vectors for Hic-5 (FlagLD1m*hic-5*) and FAK were cotransfected into COS-7 cells. At 24 h after transfection, the cells were untreated (**1**) or treated with 0.5 (**2**) or 2.0 (**3**) m*M* H_2O_2 for 30 min, lysed, and immunoprecipitated with the polyclonal antibody to Flag tag (F) or control IgG (C). Then the immunocomplex was analyzed by immunoblotting with the monoclonal antibodies to FAK and to the Flag tag (M2). (**D**) Immunoprecipitation analysis of the interaction of wild type and Cys mutant of Hic-5 with FAK. The expression vector for wild-type Hic-5 (Flag-m*hic-5*) (**1**) or Cys mutant Hic-5 (Flag-mCfl/NSm*hic-5*) (**2**) was cotransfected with that for FAK into COS-7 cells with Trans-IT-LT1 and analyzed as in C.

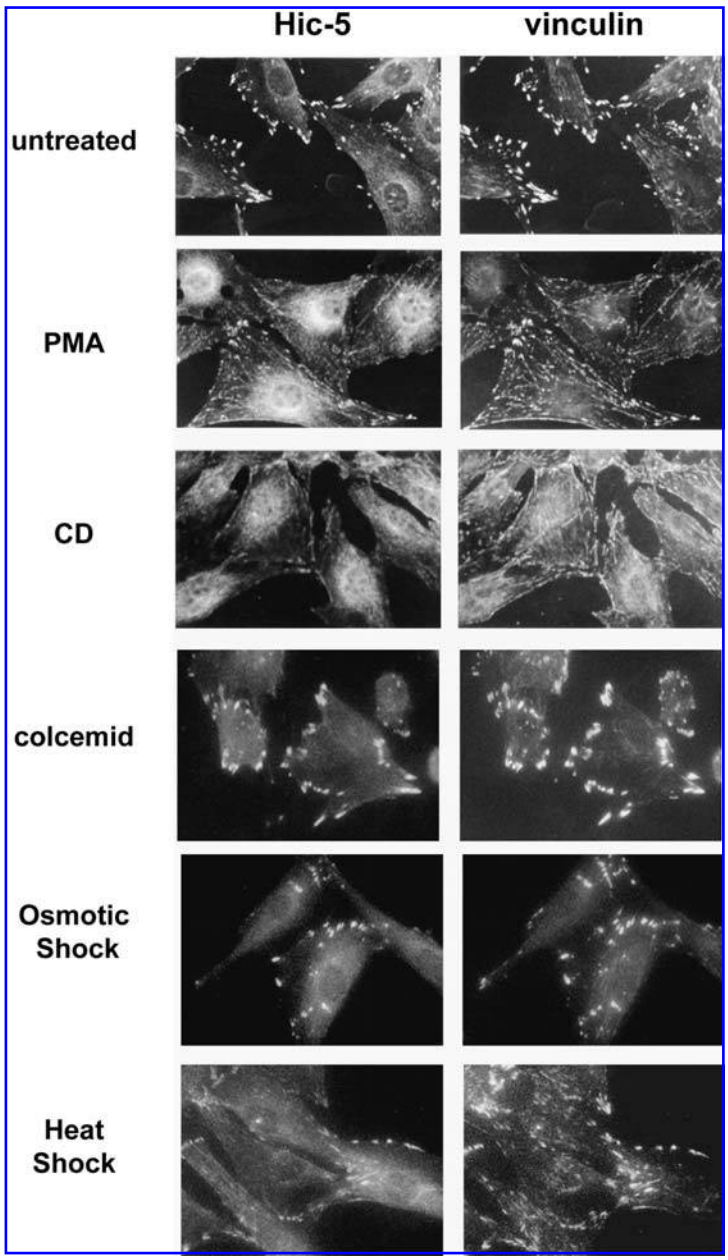

FIG. 5. The nuclear accumulation of Hic-5 induced by CD and PMA, but not by other stresses and a microtubular disruptant. MC3T3 cells were treated with 0.125 μ*M* PMA (30 min), 0.5 μ*M* CD (30 min), 0.5 μg/ml colcemid (30 min), osmotic shock (0.3 *M* sorbitol, 30 min), and heat shock (42°C, 60 min). The localization of endogenous Hic-5 and vinculin was examined by immunocytochemistry with the antibodies to Hic-5 and vinculin.

For the binding of Hic-5 and paxillin to FAK, the LD3 of Hic-5 and LD4 of paxillin, which corresponds to the LD3 of Hic-5, in their N-terminus were shown to play an important role (4, 25), and between the two LD motifs, the amino acid composition was almost identical with one exception out of eight amino acids. Thus, the redox sensitivity of the interaction with FAK that was observed in Hic-5, but not in paxillin, was striking and suggested that another motif other than LD3 that was responsible for the sensitivity was involved in the interaction of Hic-5 with FAK. Most likely, it was the two cysteines found around LD2 of Hic-5. The cysteines were only present in the N-terminus of Hic-5 and substituted with asparagine and serine in the corresponding sites of paxillin. To prove the involvement of the cysteines in the interaction of Hic-5 with FAK, we compared the interaction between the wild and mutant forms of Hic-5 in which the two cysteines were substituted as in paxillin and found that the mutations resulted in a marked decrease in the interaction in the absence of the oxidant (Fig. 4D).

From these results, it is most likely that the interaction of Hic-5 with FAK, which was governed by its LD3 and the cysteines, held Hic-5 at focal adhesions or in the cytoplasm under normal conditions, thereby suppressing its nuclear translocation, and that the oxidants interfered with the interaction through the cysteines of Hic-5, thereby facilitating the nuclear translocation of Hic-5. Likewise, PTP-PEST exhibited an inhibitory effect on the nuclear translocation of Hic-5, suggesting its contribution to the cytoplasmic retention of

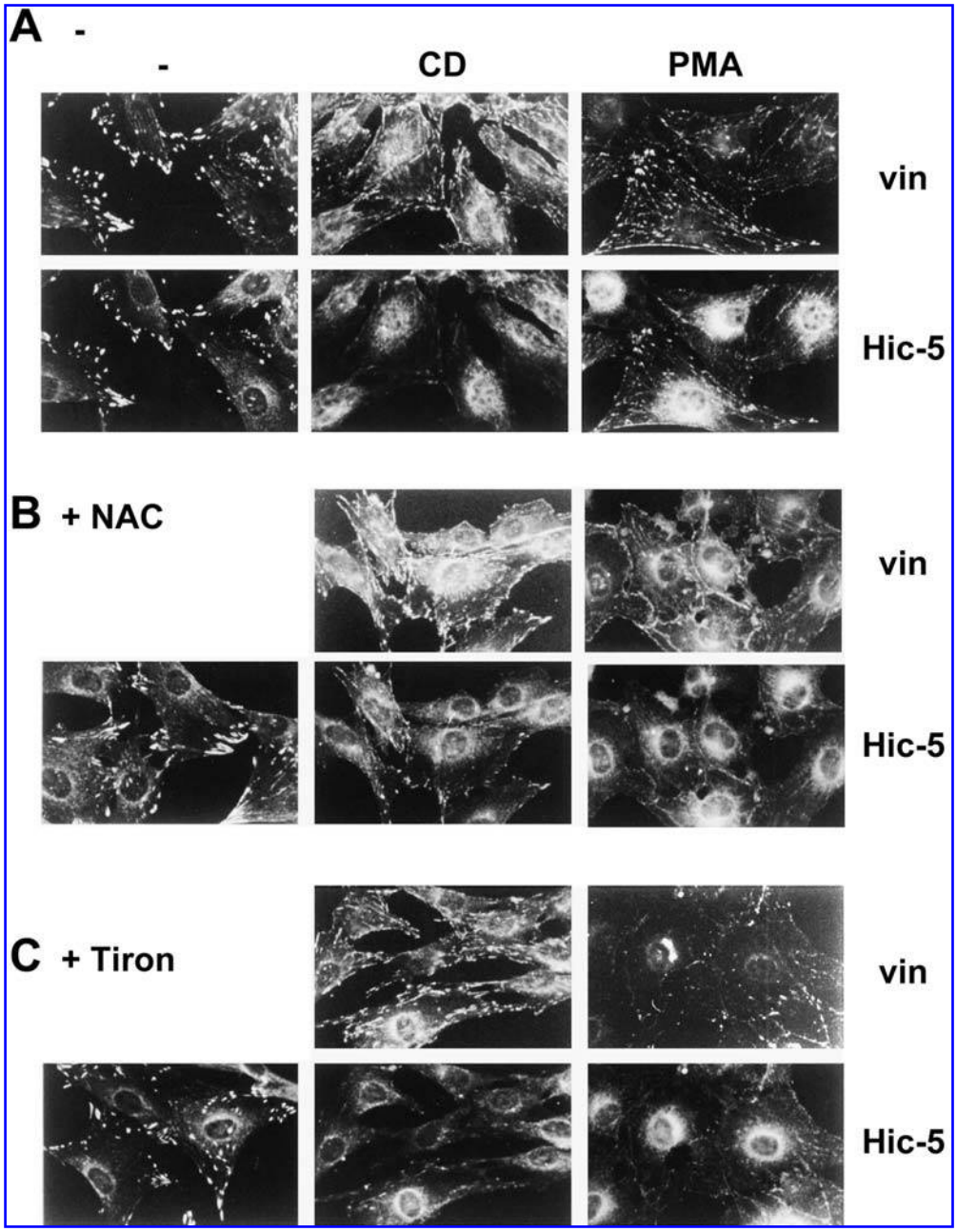

FIG. 6. CD- and PMA-induced nuclear localization of Hic-5 was inhibited by ROS scavengers. MC3T3 cells were pretreated with 10 m*M* NAC (**B**), 10 m*M* Tiron (**C**), or vehicle (**A**) for 40 min and then treated with 0.5 m*M* CD for 90 min and 0.1 μ*M* PMA for 60 min. The localization of endogenous Hic-5 and vinculin was examined as in Fig. 5.

Hic-5 under normal conditions and, thus, Hic-5 must be released from PTP-PEST prior to its nuclear translocation. In fact, the interaction was weakened by the oxidants. However, it was less likely that the release of Hic-5 from PTP-PEST was a determinant of the nuclear translocation of Hic-5, because paxillin was also released from the interaction to some extent, but hardly localized in the nucleus under oxidative conditions. With regard to other interacting molecules, such as vinculin, GIT1, Csk, and PKL, the redox sensitivity of their interaction with Hic-5 and their effects on its nuclear translocation are as yet undetermined.

CD- and PMA-induced nuclear accumulation of Hic-5 was mediated by endogenously produced reactive oxygen species (ROS)

We found that CD, an inhibitor for actin polymerization, and PMA induced the nuclear localization of Hic-5, although their effects were less pronounced than that of H_2O_2 (Fig. 5). Colcemid, a microtubule-disrupting drug, which evoked a cell-shape change as drastic as CD and PMA, did not affect the localization of Hic-5 at focal adhesions. Unlike the oxidative stress caused by H_2O_2, neither heat nor osmotic stress

significantly affected the localization of Hic-5. These observations suggested that Hic-5 was localized in the nucleus not merely as a consequence of stress or a change of cell shape, but with specificity and some biological significance.

An increase in the ROS concentration in cells is an event commonly caused by treatment with H_2O_2, CD, and PMA (17, 32, 33). In addition to CD, other agents disrupting the actin cytoskeleton were also recently reported to stimulate cells to release ROS, including H_2O_2 (12). Thus, we here addressed the possibility that ROS produced endogenously in the cells by CD and PMA induced the nuclear localization of Hic-5 using antioxidants such as *N*-acetyl-L-cysteine (NAC) and Tiron. The cells were pretreated with 10 m*M* NAC (Fig. 6B), 10 m*M* Tiron (Fig. 6C), or vehicle (Fig. 6A) for 40 min and then treated with 0.5 μ*M* CD for 90 min, 0.1 μ*M* PMA for 60 min, or vehicle (−). The localization of endogenous Hic-5 and vinculin was examined by indirect immunofluorescence labeling with the antibodies to Hic-5 and vinculin. The nuclear localization of Hic-5 induced by CD and PMA was inhibited by pretreatment of the cells with the antioxidants (Fig. 6).

DISCUSSION

In the present study, we analyzed the molecular mechanism regulating the nuclear-cytoplasmic shuttling of Hic-5, focusing on the roles of its interacting molecules, FAK and PTP-PEST. Our observations can be summarized as follows: FAK and PTP-PEST consistently distributed in the cytoplasm and/or at focal adhesions negatively affected the Hic-5 translocation to the nucleus. Of interest, the interactions of Hic-5 with these molecules were redox-sensitive such that the oxidants weakened the interactions and, thus, promoted the nuclear translocation of Hic-5. We also found that specific cysteines were involved in the interaction with FAK, which possibly make the interaction responsive to the redox state in cells. Together with previous findings, we concluded that the nuclear localization of Hic-5 was negatively regulated by the interactions with FAK and PTP-PEST in addition to the function of NES, all of which were repressed under oxidative conditions, leading to the nuclear accumulation of Hic-5.

Roles of the two cysteines and LD3 motif in the interaction with FAK and in the NES function

At a molecular level, the roles of the two cysteine residues, which are not present in paxillin, are noteworthy in the redox-sensitive regulation of the nuclear localization of Hic-5. Our previous work identified them as a component of the NES of Hic-5 whose role was to sensitize the NES function to the redox state of cells, although the exact mode of their action was unknown. In the present study, they were suggested to regulate the redox-sensitive interaction of Hic-5 with FAK. Thus, it appears that the cysteines were involved in two separate functions of Hic-5: one is the NES function and the other is the interaction with FAK. Alternatively, it is possible that they were primarily involved in the interaction with FAK. In this case, they were considered to promote the cytoplasmic retention of Hic-5 through the interaction, and as a consequence, to complement NES activity under normal conditions. Both possibilities seemed to be compatible with the observations thus far obtained. As mentioned above, besides the cysteines, LD3 also appeared to carry out dual functions, NES and the interaction with FAK. Thus, we currently speculate that LD3 and the cysteines together constitute a machinery operating as redox-sensitive NES closely coupled to the interaction with FAK in order to guarantee tight regulation of Hic-5 localization at focal adhesions and the nucleus, depending on the cellular redox state, and eventually enable Hic-5 to regulate stress response coordinately with other cellular activities, particularly those associated with adhesive states as discussed below. In the case of Yap1, the cysteines form an intramolecular disulfide bond under oxidative conditions and, thus, regulate NES (16). To our knowledge, there is no evidence indicating the involvement of NESs of the yeast proteins in the interaction with other proteins or other functions in cells. Further study of the precise roles of the cysteines and LD3 would prove how the two apparently distinct functions of NES and of the interface for the interaction with FAK are interconnected to each other and, it is hoped, provide a unique example of redox-sensitive regulation of Crm1-dependent NES, involving a signaling molecule downstream of integrin.

FAK as a key molecule regulating Hic-5 subcellular localization and its functions in cells

To localize Hic-5 in the right place at the right time is important for the integrity of cellular behavior, considering its distinct functions at focal adhesions and the nucleus, and much has to be solved for a comprehensive understanding of the biological functions of Hic-5. For example, how are the functions as an adaptor at focal adhesions and as a transcriptional coactivator in the nucleus related to each other, how is the incorporation into the signaling complex at focal adhesions compromised by the machinery for translocation to the nucleus, and what is the biological significance of the functions of Hic-5 and its nuclear-cytoplasmic shuttling that were sensitive to the redox state of cells?

In the present study, we showed the importance of FAK as a regulator of the nuclear-cytoplasmic shuttling of Hic-5, and our previous study assigned FAK as a critical molecule for Hic-5-mediated integrin signaling at focal adhesions (25). Together, these findings suggested that, under normal conditions, Hic-5 mostly engaged in the regulation of integrin signaling through interaction with FAK at focal adhesions, and in response to the oxidative state, being released from FAK in the signaling complex at focal adhesions, Hic-5 accumulated in the nucleus and in turn participated in the transcriptional regulation. The change cancels the negative regulation of paxillin-FAK signaling by Hic-5 at focal adhesions and upregulates gene transcription, including c-*fos* and $p21^{Cip1}$. As a consequence, cells are considered to acquire more motile capacity through paxillin-FAK signaling along with adaptation to oxidative condition through the gene expression. In summary, FAK could be regarded as a molecule directing the localization of Hic-5 and, thus, its function in a redox-dependent manner.

Similar to the interaction with FAK, that with PTP-PEST was also redox-sensitive. The domain of Hic-5 necessary for the interaction was LIM3, one of the four LIM domains con-

taining conserved cysteines and histidines crucial for the formation of the zinc finger structure (24). Although the molecular mechanism underlying the redox sensitivity of the interaction of Hic-5 with PTP-PEST remained unsolved, cysteines and histidine residues of LIM3 might be specifically sensitive to the redox state of the cells, thereby undergoing a conformational change and regulating the interaction with PTP-PEST in a redox-sensitive manner. LIM3 was also critical for the localization of Hic-5 at focal adhesions (24). However, it was unlikely that PTP-PEST was involved in the localization of Hic-5 to focal adhesions, because the majority of PTP-PEST was apparently distributed in the cytoplasm, and Hic-5 retained its capacity to localize at focal adhesions in PTP-PEST knockout cells (unpublished observations). Thus, PTP-PEST is expected to play a role in regulation of the nuclear translocation of Hic-5 mainly in the cytoplasm, whereas FAK is involved at focal adhesions and in the cytoplasm**.**

Cytoskeletal change, ROS production, and Hic-5 nuclear localization

The treatments with CD and PMA induced the nuclear localization of Hic-5 (Fig. 5). The results of Fig. 6 suggested that ROS was the most likely inductive signal of the nuclear localization of Hic-5 induced by CD and PMA. Intriguingly, Kheradmand *et al*. (12) demonstrated that ROS were released upon actin reorganization and served as a mediator coupling the cell-shape change to gene expression in the nucleus. Hic-5 might be one of the effectors of ROS in such a signaling pathway.

As inferred from the distribution of vinculin, a focal adhesion marker, whose localization at focal adhesions weakened after the treatments, the actin cytoskeleton was affected so that the focal adhesion structures collapsed. However, it is unlikely that the nuclear localization of Hic-5 is only the consequence of the release from focal adhesions. For example, the Hic-5 protein mutated in LIM domains, LIM3, lost the ability to be localized at focal adhesions (24). Nevertheless, it was not until the cells were exposed to H_2O_2 that the mutated protein accumulated in the nucleus (unpublished observations). The mechanisms underlying the nuclear accumulation of Hic-5 by H_2O_2 are as discussed above.

Besides CD and PMA, numerous cellular stimuli increase ROS production. These stimuli include growth factors such as serum (33), tumor necrosis factor-α (30), interleukin-1 (20), transforming growth factor-β (27, 34), platelet-derived growth factor (29), lipopolysaccharide (7), and phorbol esters (18). Recently, ROS have emerged as important molecules in the physiological response of vascular cells, including growth, migration, and production and modification of the extracellular matrix (10). Interestingly, Hic-5 was recently found to be highly expressed in vascular and visceral smooth muscle cells (47). Thus, Hic-5 may play an important role in these cells as a mediator of ROS signaling in response to the above-listed stimuli. An understanding of the roles of Hic-5 *in vivo* in tissues containing smooth muscle would give us an idea of the biological significance of ROS in these tissues, as well as the mechanisms underlying the physiological and pathophysiological responses of vascular systems to ROS, and of therapeutic targets of vascular diseases.

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ABBREVIATIONS

CD, cytochalasin D; DAPI, 4,6-diamidino-2-phenylindole; DiM, diethyl maleate; FAK, focal adhesion kinase; HA, hemagglutinin; H_2O_2, hydrogen peroxide; LMB, leptomycin B; LPP, lipoma-preferred partner; MEM, minimum essential medium; NAC, *N*-acetyl-L-cysteine; NES, nuclear export signal; PMA, phorbol 12-myristate 13-acetate; PTP-PEST, protein tyrosine phosphatase PEST; ROS, reactive oxygen species; Trip6, thyroid receptor interacting protein-6.

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