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Distinct LIM Domains of Hic-5/ARA55 Are Required for Nuclear Matrix Targeting and Glucocorticoid Receptor Binding and Coactivation

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Abstract Hydrogen peroxide-inducible clone-5 (Hic-5), belongs to the group III LIM domain protein family and contains four carboxyl-terminal LIM domains (LIM1–LIM4). In addition to its role in focal adhesion signaling, Hic-5 acts in the nucleus as a coactivator for some steroid hormone receptors such as the glucocorticoid receptor (GR) and androgen receptor (AR). Based upon its effect on AR transactivation, Hic-5 has also been designated as ARA55. Here, we report mapping studies of Hic-5/ARA55 functional domains and establish that LIM3 and LIM4 are necessary for maximal effects on GR transactivation. However, results from yeast two-hybrid assays demonstrated that these two LIM domains together, while necessary, are not sufficient to interact with the tau2 transactivation domain of GR. LIM4 also functions as a nuclear matrix targeting sequence (NMTS) for Hic-5/ARA55, as it is both necessary and sufficient to target a heterologous protein to the nuclear matrix. Thus, as suggested from previous analysis of LIM domain-containing proteins, separate but highly related LIM domains serve distinct functions. J. Cell. Biochem. 92: 810–819, 2004.

Key words: Hic-5/ARA55; LIM domain proteins; GR

LIM domains were initially discovered and characterized within three homeodomain proteins (Linl-1, Isl-1, and Mec-3) [Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990]. They are unique cysteine-rich motifs that define a double zinc finger approximately 60 amino acids in length and are thought to be important for protein–protein interactions [Schmeichel and Beckerle, 1994]. LIM domains have been found in more than 300 proteins that are localized within various subcellular compartments and have been grouped in three broad classes [Dawid et al., 1998]. Group I LIM domain proteins include LIM homeodomain proteins, LMO proteins, and LIM kinase proteins. Group II LIM domain proteins are cytoplasmic proteins composed mainly of LIM domains. Finally, the group III LIM domain proteins contain variable numbers of LIM domains located at their C-terminus and are found predominantly at focal adhesions. Focal adhesions form inside cells opposing points of cell attachment. They are composed of various proteins, some of which appear to participate in a number of signal transduction pathways acting either as direct mediators of protein phosphorylation [Turner, 2000] or as scaffolds [Matsuya et al., 1998] that attract components of distinct signaling pathways.

Members of the group III LIM domain proteins include paxillin, zyxin, hydrogen peroxide-inducible clone-5 (Hic-5), thyroid interacting protein partner (Trip6), and LIM containing lipoma preferred partner (LPP). Despite their predominant localization within focal adhesions, group III LIM domain proteins

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can also been found in the nucleus. In some cases, specific nuclear export sequences have been identified within group III proteins that function to limit their nuclear residency [Nix and Beckerle, 1997; Petit et al., 2000; Wang and Gilmore, 2001]. Transactivation domains have also been identified in Hic-5, Trip6, and LPP suggesting that group III LIM domain proteins may play a direct role in transcriptional regulation. Transcriptional activation is a well-established property of group I LIM domain proteins including LMO proteins such as ACT, FHL3, and FHL2, which contain little more than four and a half LIM domains (FHL) [Fimia et al., 2000; Muller et al., 2000].

Hic-5, a group III LIM domain protein, contains four carboxyl-terminal LIM domains and is involved in cellular senescence [Shibanuma et al., 1997]. Others and we have found that Hic-5 is a coactivator for certain hormone receptors such as the glucocorticoid receptor (GR) and androgen receptor (AR) [Fujimoto et al., 1999; Yang et al., 2000]. In fact, Hic-5 is also known as ARA55, based upon its effects on AR transactivation [Fujimoto et al., 1999]. Using biochemical fractionations and indirect immunofluorescence assays, we had previously shown that a fraction of transfected as well as endogenous Hic-5/ARA55 is bound to the nuclear matrix [Yang et al., 2000]. This targeting of Hic-5/ARA55 may be related to its interaction with a nuclear matrix targeting sequence (NMTS) located within the tau2 transactivation domain of GR. The C-terminal region of Hic-5/ARA55, which contains seven zinc fingers arranged in four LIM domains (LIM1–LIM4), was required for its interactions with both the nuclear matrix as well as the GR tau2 transactivation domain [Yang et al., 2000].

In this article, we focus on detailed mapping of functional domains of Hic-5/ARA55 that influence GR transactivation and nuclear matrix targeting. In transient transfection assays we found that LIM3 and LIM4 are necessary for the maximal effect of Hic-5/ARA55 on GR transactivation. However, results from yeast two-hybrid assays demonstrated that these two LIM domains together, while necessary, are not sufficient to interact with the tau2 domain of GR. In contrast, LIM4 alone is able to target a heterologous protein to the nuclear matrix, and therefore acts as a functional NMTS for Hic-5/ARA55. Thus, as suggested from previous analysis of LIM domain containing proteins, separate but highly related LIM domains serve distinct functions and in some cases may work cooperatively [Nishiya et al., 1999; Fimia et al., 2000; Jia et al., 2001].

MATERIALS AND METHODS

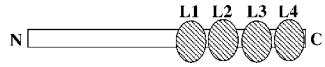
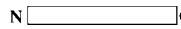
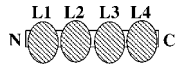
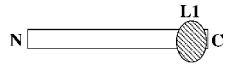
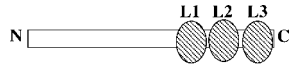
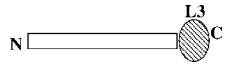
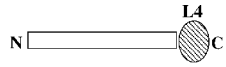
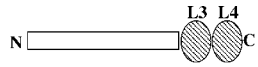
Cell Culture

Cos-1 cells, monkey kidney fibroblasts, were maintained in Dulbecco's modified Essential medium (DMEM, Invitrogen Corporation, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Norcross, GA).

Plasmids

Mammalian expression vectors for full length Hic-5/ARA55 and its deletion mutants (depicted in Table I) were constructed by inserting into pSG5.HA [Chen et al., 1999] the following PCR-amplified *Eco*RI/XhoI DNA fragments; full length Hic-5/ARA55 (amino acids [aa] 1–444), N-terminus of Hic-5/ARA55 (aa 1–200), C-terminus of Hic-5/ARA55 (aa 201–444), N-terminus + LIM1 (aa 1–269), N-terminus + LIM1 and LIM2 (aa 1–325), N-terminus + LIM1, LIM2, and LIM3 (aa 1–385). Mammalian expression vectors for Hic-5/ARA55 N-terminus + LIM3, or LIM4, or LIM3, and LIM4 were constructed by inserting into pSG5.HA that contained the N-terminus of Hic-5 PCR-amplified *Eco*RI-BglII cDNA fragments encoding aa 326–385, aa 386–444, or 326–444, respectively. Mammalian expression vectors for GAL4 DNA-binding domain (DBD) fusion proteins with specific LIM domains of Hic-5/ARA55 (depicted in Table II) were constructed by inserting PCR-amplified *Eco*RI-BamHI DNA fragments encoding LIM1 (aa 211–269) or LIM4 (aa 383–444) into the PM2 vector [Sadowski et al., 1992]. The mammalian expression vector for rat GR used was p6RGR [Godowski et al., 1987]. The mouse GR DBD tau2 expression plasmid, pC7mGR(395–562), and the MMTV-Luciferase reporter (i.e., MMTV Luc) were described previously [Milhon et al., 1997]. Yeast expression vectors for Gal4 activation domain (AD) fusion proteins with Hic-5/ARA55 and its deletions mutants (see Table I) were constructed by inserting into the pGAD424 vector (Clontech Laboratories, Inc., Palo Alto, CA) PCR-amplified *Eco*RI-SalI DNA fragments encoding full length Hic-5/ARA55 (aa 1–444), the N-terminus of Hic-5/ARA55 (aa 1–200), N-terminus + LIM1 (aa 1–269), N-terminus + LIM1

TABLE I. Recombinant Hic-5/ARA55 Plasmids

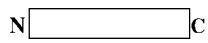
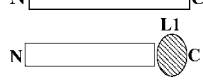
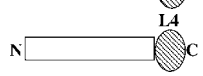
Hic-5/ARA55 deletions	
FL (1–444)	
N-terminal (1–200)	
C-terminal (201–444)	
N + LIM1 (1–269)	
N + LIM1, LIM2 (1–325)	
N + LIM1, LIM2, LIM3 (1–385)	
N + LIM3 (1–200/326–385)	
N + LIM4 (1–200/386–444)	
N + LIM3, LIM4 (1–200/326–444)	

and LIM2 (aa 1–325), N-terminus + LIM1, LIM2, and LIM3 (aa 1–385). The Gal4AD fusion proteins with specific LIM domains were constructed by inserting PCR-amplified *Eco*RI-BglII DNA fragments encoding LIM3 (aa 326–385), LIM4 (aa 386–444), or LIM3 and LIM4 (aa 326–444) into the pGAD424 vector (Clontech Laboratories, Inc.). Yeast expression vectors for GAL4DBD fusion proteins were constructed by inserting PCR-amplified SmaI-SalI DNA fragments containing the following coding regions into pGBT9 (Clontech Laboratories, Inc.): GR tau2 (mouse GR aa 513–562) and GR DBD (mouse GR aa 395–562) as described previously [Yang et al., 2000]. The identity of all constructs was confirmed by DNA sequence analysis. Protein expression of all mammalian expression vectors was confirmed by Western blot analysis.

Transfections for Transactivation Assay Experiments

Cos-1 cells were passed at a density of 1×10^5 cells/well in 6-well plates 1 day before transfection. For the transactivation assays each well was transfected with 0.5 μg of MMTV-Luc, 10 ng of mDBD tau2 plasmid or 0.5 ng of rGR plasmid, and 1 μg of empty vector, Hic-5 full length or deletion expression plasmid. Cells were transfected in triplicate using 20 μl of Superfect reagent (Qiagen, Inc., Valencia, CA) for each of three wells and allowing for a 3 h incubation of cells with transfection mix. The transfection mixture was removed and cells refed with fresh DMEM with 10% FBS. Where indicated dexamethasone, a synthetic glucocorticoid, was added to a final concentration of 10^{-6} M. Cells were lysed using the Cell Culture Lysis Reagent

TABLE II. Recombinant Gal4DBD-Hic-5/ARA55 Plasmids

Gal4DBD derivatives:	
Gal4DBD (1–147)	
Gal4DBD + LIM1 (1–147/211–269)	
Gal4DBD + LIM4 (1–147/383–444)	

(Promega Corp. Madison, WI) 24 h following transfection and luciferase assays performed as described previously [Yang et al., 2000]. The relative luciferase units were normalized to protein concentration. All experiments were repeated three or more times.

Transfections for Nuclear Matrix Preparations

Cos-1 cells were transfected, using the calcium phosphate method, with 3 μg of DNA/60 mm plate. Two plates were used to prepare total cell extract and two plates used for each biochemical fractionation. GAL4DBD-LIM1 and GAL4DBD-LIM4 DNA were transfected with the Superfect reagent (Qiagen, Inc.). Cells were harvested 2 days after transfection, collected in 1× PBS-TE (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5) and pelleted at 6,000 rpm for 4 min. Pellets were then either resuspended in 1× SDS (2× buffer: 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) gel-loading buffer for total cell extract or subject to biochemical fractionations for nuclear matrix preparations.

Nuclear Matrix Preparation and Subcellular Fractionation

Pelleted cells were resuspended in ice-cold CK buffer (10 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl riboside complex, 0.5% Triton X-100, 1.2 mM PMSF, and protease inhibitors) for 5 min on ice and then centrifuged at 6,000 rpm for 3 min. The supernatant was collected and SDS gel-loading buffer was added to a final concentration of 1×. The pellet was then resuspended in DNAse I buffer (same as CK buffer except for 50 mM NaCl and DNAse I, 300 U/ml), and allowed to incubate at 25°C for 30 min. Ammonium sulfate was then added to a final concentration of 0.25 M for 5 min. After centrifugation at 14,000 rpm for 3 min, the supernatant was removed and the nuclear matrix pellet was resuspended in 1× SDS gel-loading buffer.

Western Blots

Western Blot analysis was used to detect transiently expressed HA-tagged Hic-5/ARA55 and its deletion mutants in various subcellular fractions [Tang et al., 1998]. In each case, total expression of Hic-5 or deletion mutant was compared to fractions obtained from equivalent amounts of cells with the use of a monoclonal anti-HA antibody (Roche Applied Science, Indianapolis, IN). Total expression of GAL4DBD-Hic-5/ARA55 LIM1 and GAL4DBD-Hic-5/ARA55 LIM4 was compared to fractions obtained from equivalent amounts of cells with the use of a GAL4DBD antibody (Clontech Laboratories, Inc.). The same blots were probed with a Lamin B antibody (sc-6216, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to provide an internal control for nuclear matrix recovery. Following primary antibody incubation, blots were treated with appropriate HRP-conjugated secondary antibodies (Bio-Rad Laboratories, Richmond, CA) and then subject to a chemiluminescence detection (New England Nuclear, Boston, MA).

Yeast Two-Hybrid Assays

The SFY526 (Clontech Laboratories, Inc.) strain of yeast was transformed using the Lithium method and transformants selected following growth on appropriate selection plates (i.e., minimal media and amino acid mixture lacking leucine and tryptophan, Clontech Laboratories, Inc.). Colonies were picked and allowed to grow in a patch and from this patch a 5 ml culture of selective media was inoculated overnight. Yeast cells were grown and lysed according to procedures outlined by Clontech Laboratories, Inc., to measure beta-galactosidase activity. The substrate used for these assays was *o*-nitrophenyl beta-D-galactopyranoside (ONPG) (Sigma Chemical Co., St. Louis, MO).

RESULTS

Functional Domains of Hic-5/ARA55

The GR tau2 interacting region of Hic-5/ARA55 had previously been mapped to its C-terminal LIM domain-containing region using a yeast two-hybrid assay, while the N-terminal region possesses an intrinsic transactivation domain [Yang et al., 2000]. To further investigate which domains of Hic-5/ARA55 are important for its potentiation of GR tau2 transactivation several deletion mutants (see Table I) were tested in transiently transfected cells. Cos-1 cells were co-transfected with a luciferase reporter gene linked to a glucocorticoid responsive promoter, a GR DBD tau2

expression plasmid [Yang et al., 2000], and plasmids directing the expression of wild type and deletion mutants of Hic-5/ARA55. As shown in Figure 1, deletion of LIM4 from Hic-5/ARA55 reduced its ability to potentiate GR tau2 directed transactivation. The LIM4 C-terminal deletion of Hic-5/ARA55 still maintained approximately 50% of wild-type coactivation activity. However, deletion of both LIM3 and LIM4 from Hic-5/ARA55 eliminated its ability to act as a coactivator for GR tau2 (Fig. 1). The coactivator activity of the Hic-5/ARA55 deletion mutant that included its N-terminal domain and LIM1 and LIM2 was indistinguishable from that of empty vector alone (Fig. 1). When the

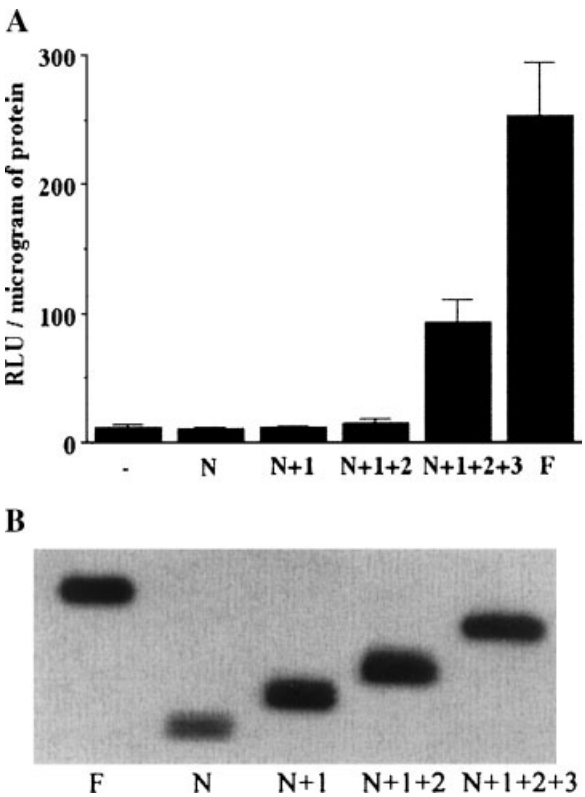


Fig. 1. Mapping of hydrogen peroxide-inducible clone-5 (Hic-5)/ARA55 LIM domain that acts as a coactivator for the glucocorticoid receptor (GR) tau2 transactivation domain. **A**: Cos-1 cells were transfected with 0.5 μg of MMTV-luciferase reporter plasmid, 10 ng of GR DBD tau2 plasmid, and 1 μg of the indicated plasmid encoding Hic-5/ARA55 or its deletion mutants (see ''Materials and Methods'' and Table I for description). Results from replacing the Hic-5/ARA55 plasmid with empty vector (−) are shown in the first column and results for full length (F) Hic-5/ARA55, in the last column. Activity is given in relative luciferase units (RLU) per microgram of protein as the mean ± standard deviation of three separate experiments performed in triplicate. **B**: Western blot analysis of the HA-tagged Hic-5/ARA55 and deletion mutants expressed in representative samples measured in A.

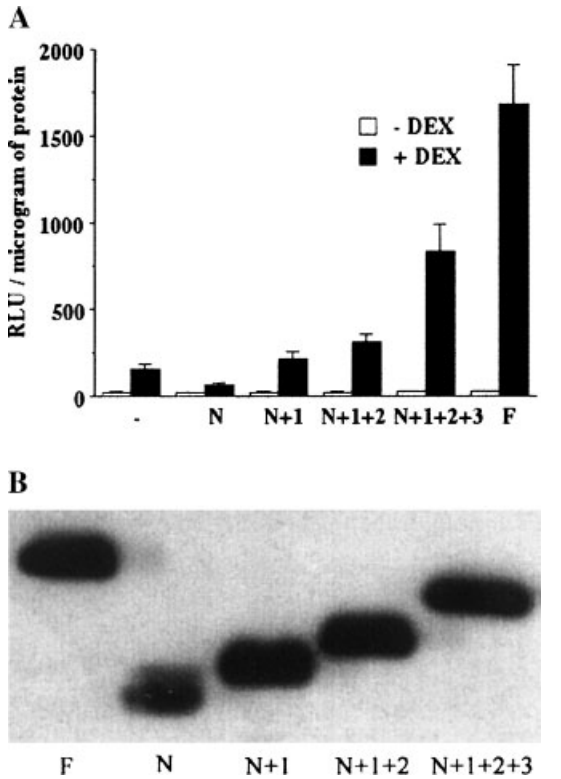


Fig. 2. Mapping of Hic-5/ARA55 LIM domain that acts as a coativator for GR. **A**: Cos-1 cells were transfected with 0.5 μg of MMTV-luciferase reporter plasmid, 0.5 ng of GR plasmid, and 1 μg of the indicated plasmid encoding Hic-5/ARA55 or its deletion mutants (see ''Materials and Methods'' and Table I for description). Results from replacing the Hic-5/ARA55 plasmid with empty vector (−) are shown in the first column and results for full length (F) Hic-5/ARA55, in the last column. Where indicated 10^{-6} M dexamethasone was added 24 h prior to harvesting. Activity is given in RLU per microgram of protein as the mean ± standard error of five separate experiments done in triplicate. **B**: Western blot analysis of the HA-tagged Hic-5/ARA55 and deletion mutants expressed in representative samples measured in A.

Hic-5/ARA55 deletion mutants were tested in the context of full length GR (Fig. 2) analogous results were obtained. While we have yet to obtain tau2 mutants in the context of full length GR that do not affect hormone binding [Milhon et al., 1997], these results suggest that Hic-5/ARA55 effects on the tau2 transactivation domain may be responsible for its action on full length GR.

In order to test whether Hic-5/ARA55 mutants with reduced effects on GR transactivation maintained GR tau2 binding, we performed a yeast two-hybrid assay. For these assays, a yeast strain was used that possessed an integrated beta gal reporter gene controlled by a GAL4UAS element linked to a minimal

promoter. A plasmid encoding Gal4DBD fused to GR tau2 was co-transformed with plasmids encoding deletion mutants of Hic-5/ARA55 (see Table I) fused to the GAL4AD. The extent of LIM domain/GR tau2 interaction was inferred from the results of beta gal assays using yeast cell extracts. The Gal4DBD/ GR DBD chimera and a GAL4AD expression vector were used as negative controls (Fig. 3). As shown in Figure 3, deletion of LIM4 did not significantly affect the interaction between Hic-5/ARA55 and GR tau2. A Hic-5/ARA55 C-terminal deletion that lacks LIM3 and LIM4 was unable to interact with GR tau2 in yeast. The results of the yeast two-hybrid assay are therefore consistent with the transactivation assays and suggest that LIM3, and perhaps LIM4 to some extent, are required for GR transactivation based upon their role in directing Hic-5/ARA55 binding to GR. Interestingly, LIM3 alone, LIM4 alone, or LIM3 and LIM4 together could not bind to GR tau2 when tested in a yeast two-hybrid assay (Fig. 4). Transient transfection assays testing the effects of the N-terminus of Hic-5/ARA55 linked to either LIM3, LIM4, or LIM3 + LIM4 to enhance transcriptional activation by GR DBD tau2 also showed no coactivator activity (Fig. 5). Thus, analogous to many other LIM domain proteins tested [Nishiya et al., 1999; Fimia et al., 2000; Jia et al., 2001], functional interactions between individual LIM domains (i.e., LIM3 and LIM4 of Hic-5/ARA55) and their partner proteins (i.e., GR) may require the stabilizing influences of closely linked LIM domains or other features of the LIM domain containing protein.

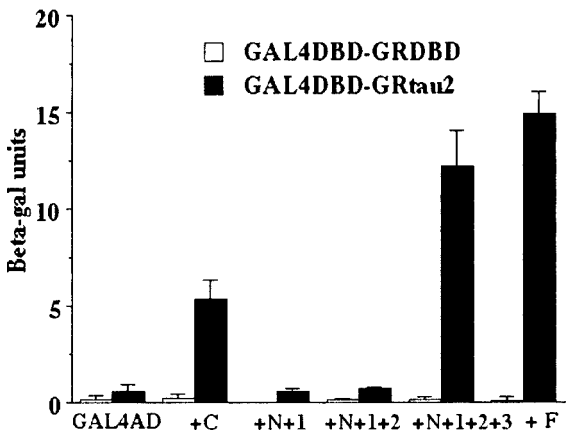


Fig. 3. Mapping of Hic-5/ARA55 LIM domain that binds to GR tau2 in yeast. Yeast strain SFY526 was transformed with plasmids encoding the indicated GAL4AD constructs (see "Materials and Methods" and Table I for description) and either GAL4DBD-GR DBD or GAL4DBD-GR tau2. Activity is given in beta-gal units as the mean ± standard deviation of triplicate determinations.

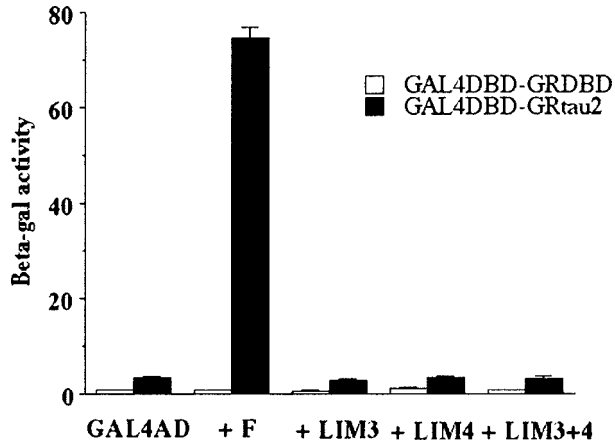


Fig. 4. Analysis of individual Hic-5/ARA55 LIM domain interactions with GR tau2 in yeast. Yeast strain SFY526 was transformed with the indicated GAL4AD constructs (see "Materials and Methods" and Table I for description) and either GAL4DBD-GR DBD or GAL4DBD-GR tau2. The interaction of GAL4DBDtau2 and full length Hic-5/ARA55, was used as a positive control in this experiment. Activity is given in beta-gal units as the mean ± standard deviation of triplicate determinations.

Nuclear Matrix Targeting of Hic-5/ARA55

Biochemical fractionations have been used to establish the nuclear matrix binding of transfected as well as endogenous Hic-5/ARA55 [Yang et al., 2000]. Furthermore, the C-terminal LIM domains were sufficient to direct Hic-5/ARA55 to the nuclear matrix (Fig. 6A, lane 9) while the N-terminal region showed no detectable nuclear matrix binding [Fig. 6A and see ref. Yang et al., 2000]. To further define the NMTS of Hic-5/ARA55, nuclear matrix binding of Hic-5/ARA55 deletion mutants (see Table I) was assessed in transiently transfected Cos-1 cells. Using established fractionation procedures we observed a considerable decrease in nuclear matrix binding of Hic-5/ARA55 when LIM4 was deleted. As shown in Figure 6A and summarized in Table III, Hic-5/ARA55 deletion mutants which have the N-terminal region and LIM1 (lane 12) or LIM1 + 2 (lane 15) or LIM1 + 2 + 3 (lane 18) exhibit very low levels of nuclear matrix binding compared to full length Hic-5/ARA55 (lane 3). Therefore, it appears that the NMTS of Hic-5/ARA55 may be contained within LIM4. In order to test directly this notion, the nuclear matrix targeting activity of individual LIM domains (i.e., LIM1 and LIM4) was tested

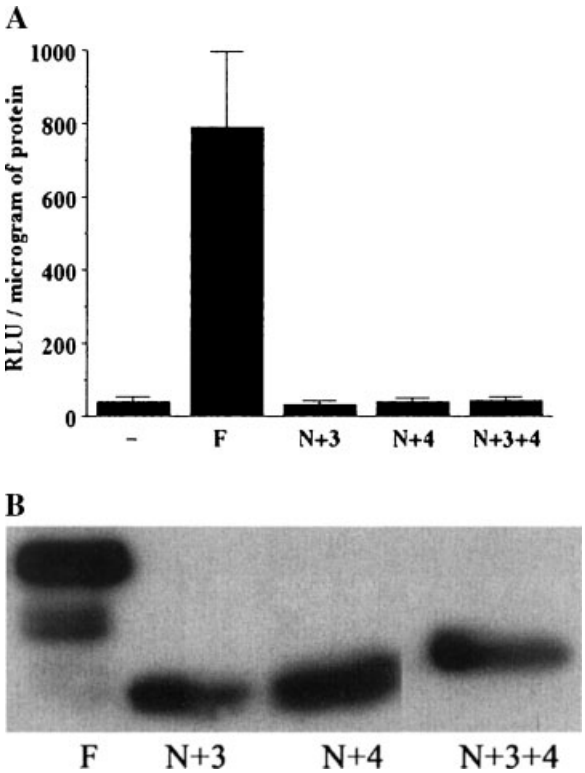


Fig. 5. Analysis of individual Hic-5/ARA55 LIM domain function as a coactivator for the GR tau2 transactivation domain. **A**: Cos-1 cells were transfected with 0.5 μg of MMTV-luciferase reporter plasmid, 10 ng of GR DBD tau2 plasmid, and 1 μg of the indicated plasmid encoding Hic-5/ARA55 or its mutant. Results from replacing the Hic-5/ARA55 plasmid with empty vector (−) are shown in the first column and results for full length (F) Hic-5/ARA55, in the second column. Activity is given in RLU per microgram of protein as the mean ± standard deviation of three separate experiments performed in triplicate. **B**: Western blot analysis of the HA-tagged Hic-5/ARA55 and deletion mutants expressed in representative samples measured in A.

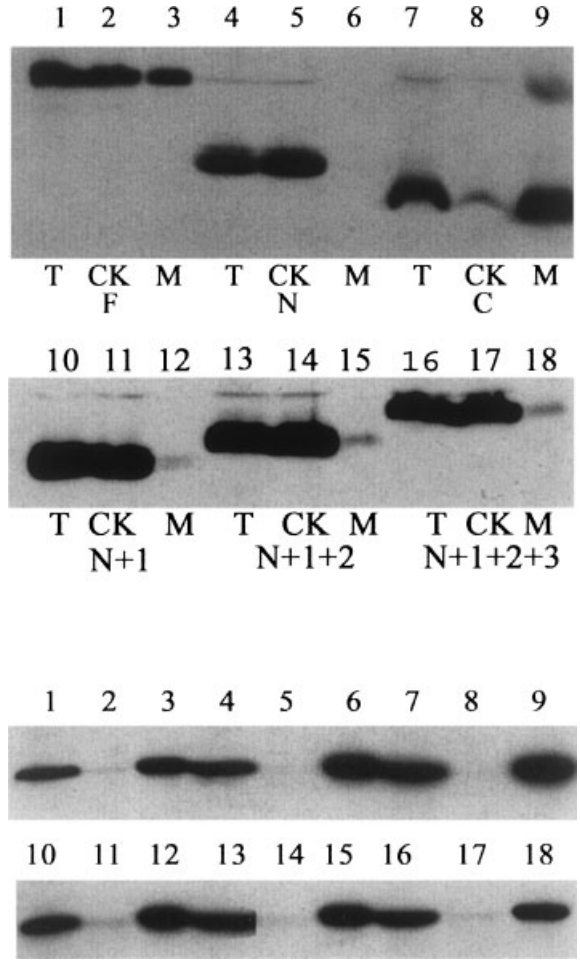


Fig. 6. Mapping of Hic-5/ARA55 LIM domain targeting to the nuclear matrix. Cos-1 cells were transfected with 2 μg of an expression vector for full length (F) Hic-5/ARA55 (aa 1–444) and the following deletion mutants: the N-terminal region (aa 1–200), the C-terminal region (aa 201–444), the N-terminal region with LIM1 (aa 1–269), the N-terminal region with LIM1 and LIM2 (aa 1–325), and the N-terminal region with LIM1, LIM2, and LIM3 (aa 1–385) (see ''Materials and Methods'' and Table I for description). Proteins present in whole cell extracts (**lanes 1**, **4**, **7**, **10**, **13**, and **16**), CK buffer-extracted supernatants (**lanes 2**, **5**, **8**, **11**, **14**, and **17**), or nuclear matrix pellets (**lanes 3**, **6**, **9**, **12**, **15**, and **18**) were separated by SDS–PAGE and subjected to Western blot analysis to detect HA-tagged Hic-5/ARA55 or its deletion mutants (**A**) or the nuclear matrix protein lamin B (**B**).

when linked to the GAL4DBD. GAL4DBD chimeras have been used to map NMTSs for other proteins given the inability of GAL4DBD to interact with the nuclear matrix in transfected mammalian cell lines [Zeng et al., 1997; Tang et al., 1998]. A GAL4DBD chimera containing either LIM4 of Hic-5/ARA55 or LIM1 (see Table II), as a negative control, were assessed for nuclear matrix binding in transfected Cos-1 cells. As shown in Figure 7 and summarized in Table III, the GAL4DBD/LIM4 chimera showed strong nuclear matrix binding (lane 9) while the GAL4DBD/LIM1 chimera (lane 6) and the GAL4DBD (lane 3) exhibited no nuclear matrix binding above background. Panels 6B and 7B show Western blot analysis of the nuclear matrix protein lamin B, and provide a control of nuclear matrix recovery. Thus LIM4 alone of Hic-5/ARA55 is necessary and sufficient for nuclear matrix binding.

DISCUSSION

Hic-5/ARA55 is a LIM-domain containing focal adhesion protein and member of the paxillin family. Like many members of this family it has several LIM domains, which are unique cysteine-rich motifs that define a double zinc finger that are thought to be important for

TABLE III. Summary of Hic-5/ARA55 Nuclear Matrix Binding

Hic-5/ARA55 mutant	Nuclear matrix binding
Full length (1–444)	++++
N-terminal (1–200)	–
C-terminal (201–444)	++++
N + LIM1 (1–269)	+
N + LIM1, LIM2 (1–325)	+
N + LIM1, LIM2, LIM3 (1–385)	+
Gal4DBD-Hic-5/ARA55 derivative	Nuclear matrix binding
Gal4DBD (1–147)	–
Gal4DBD + LIM1 (1–147/211–269)	+
Gal4DBD + LIM4 (1/147/383–444)	++++

protein–protein interactions [Schmeichel and Beckerle, 1994]. The LIM domains of Hic-5/ARA55 are responsible for its association with focal adhesions [Thomas et al., 1999], the nuclear matrix [Yang et al., 2000], the protein tyrosine phosphatase (PTP)-PEST [Nishiya et al., 1999], and possess Zn^{2+}-dependent DNA-binding activity [Nishiya et al., 1998]. Although Hic-5/ARA55 contains four LIM domains, they are not functionally equivalent. LIM3 is required for PTP–PEST interaction [Nishiya et al., 1999], while LIM4 alone, or LIM1 and LIM2 together possess Zn^{2+}-dependent DNA-binding activity [Nishiya et al., 1998], and LIM4 binds hsp27 [Jia et al., 2001]. The C-terminal region containing the four LIM domains binds to the mouse GR tau2 sequence [Yang et al., 2000], and the studies reported here uncover novel functions of two LIM domains of Hic-5/ARA55.

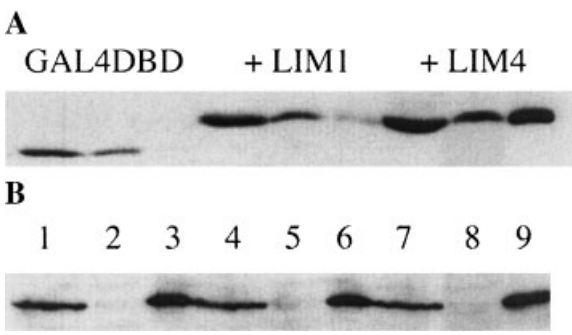


Fig. 7. Nuclear matrix targeting of individual Hic-5/ARA55 LIM domain. Cos-1 cells were transfected with 2 μg of GAL4DBD and fusions of GAL4DBD containing either Hic-5/ARA55 LIM1 or LIM4 (see ''Materials and Methods'' and Table II for description). Proteins present in whole cell extracts (**lanes 1**, **4**, **7**), CK buffer-extracted supernatants (**lanes 2**, **5**, and **8**), or nuclear matrix pellets (**lanes 3**, **6**, and **9**) were separated by SDS–PAGE and subjected to Western blot analysis to detect GAL4DBD (**A**) or the nuclear matrix protein lamin B (**B**).

Transactivation assays performed with the Hic-5/ARA55 deletion mutants suggest that LIM3 and LIM4 are important for the ability of Hic-5/ARA55 to function as a coactivator for GR. These results are likely to reflect a requirement for LIM3 and LIM4 for GR interactions with Hic-5/ARA55, as revealed with a yeast two-hybrid assay. However, the individual LIM3 or LIM4 domains of Hic-5/ARA55 were unable to interact with tau2 in yeast cells and do not function as a coactivator in transfected mammalian cell lines. Analogous results have been obtained in previous studies that mapped distinct functions of individual Hic-5/ARA55 LIM domains. For example, while the interaction between Hic-5/ARA55 and PTP–PEST required LIM3, this domain alone could not bind directly to PTP–PEST in vitro [Nishiya et al., 1999]. In an analysis of the FHL coactivator protein, ACT [Fimia et al., 2000], it was shown that specific arrangements of the LIM domains are essential for its transactivation properties. However, in that study the ability of ACT to interact with itself could be attributed to a single LIM domain [Fimia et al., 2000]. In our study, we were also able to identify a single LIM domain responsible for one interaction, as LIM4 alone acts as a NMTS for Hic-5/ARA55 (Fig. 7). Thus, some individual LIM domains may be sufficient for binding to specific partners, while other LIM domains may require stabilization with other protein domains to maintain functionally relevant interactions with other partners.

The LIM4 domain of Hic-5/ARA55 is the first LIM domain that has been shown to be a NMTS. There is no consensus NMTS since these signals presumably target proteins to different sites on the matrix and to unique acceptor proteins. NMTSs range greatly from small peptide sequences to large protein segments. The only other LIM domain protein that has been shown to target to the nuclear matrix is Lhx3, a LIM homeodomain transcription factor. However, unlike Hic-5/ARA55, the NMTS of Lhx3 maps to its homeodomain and not its LIM domain [Parker et al., 2000]. Proteins bind to the nuclear matrix by interaction with an acceptor protein on the matrix or matrix-attachment regions (MAR) or scaffold attachment regions (SAR) DNA. The LIM4 of Hic-5/ARA55 is capable of binding DNA that resembles MARs/SARs. However, DNA binding can not be the only way in which Hic-5 binds to the matrix

since two other LIM domains of Hic-5/ARA55 are also known to bind this type of DNA [Nishiya et al., 1998]. Perhaps, Hic-5/ARA55 is targeted to the nuclear matrix by binding to an acceptor protein on the matrix. Hic-5/ARA55 could then act as an scaffold or acceptor protein for other proteins that are targeted to the matrix such as GR. Hic-5 also acts as a scaffold or acceptor protein at the focal adhesions by binding proteins like focal adhesion kinase (FAK), cell adhesion kinase (CAK), and PTP–PEST [Fujita et al., 1998; Matsuya et al., 1998; Nishiya et al., 1999]. The deletion of LIM4 Hic-5/ARA55 alone reduces its coactivator function for GR approximately 50%. Therefore, in our transient transfections the nuclear matrix targeting of Hic-5/ARA55 by LIM4 contributes to but is not essential for its coactivation activity. Perhaps, matrix binding plays a role in coactivation of GR responsive genes in "native" chromatin, as opposed to the transiently transfected reporter genes used in this study.

Considerable attention has been focused on focal adhesion proteins in the paxillin family given their role in cell adhesion and migration [Nishiya et al., 2001]. Curiously many members of the paxillin family of group III LIM domain protein are found not only at the focal adhesions but also in the nucleus. For zyxin, Trip6, and LPP specific nuclear export sequences have been identified that function to limit their retention within the nucleus [Nix and Beckerle, 1997; Petit et al., 2000; Wang and Gilmore, 2001]. Transactivation domains have also been identified in LIM proteins such as Trip6 and LPP. It is unclear what mechanisms operate to regulate the presumed trafficking of group III LIM domain proteins between focal adhesions and the nucleus. Other LIM domain protein coactivators have been identified in the LMO subclass FHL such as ACT, FHL2, and FHL3 [Fimia et al., 2000; Muller et al., 2000]. FHL2 is a novel tissue-specific coactivator of the AR [Muller et al., 2000]. In contrast to Hic-5/ARA55, deletion mutants of FHL2 that were used to map its interaction with AR, showed that all LIM domains contribute to its association with AR.

Recently, the Ajuba LIM domain protein was found to shuttle between focal adhesions and the nucleus in response to differentiation-inducing signals [Kanungo et al., 2000]. Perhaps as shown for the c-Abl protein [Lewis et al., 1996], shuttling of group III LIM domain proteins between focal adhesions and the nucleus may also be mediated by specific signals that emanate from the extracellular matrix (ECM). Hormone regulated transcription of some target genes (beta-casein, MMTV) in mammary epithelial cells has been observed to be potentiated upon plating of cells on particular ECMs [Myers et al., 1998]. While the mechanisms responsible for this ECM enhancement of GR transactivation are not known, it may be time to consider the possibility of a direct link between focal adhesions, which serve as the initial entry point of ECM-directed signals, and the nucleus. Group III LIM domain proteins provide an attractive candidate for proteins that could impact ECM-directed signaling by indirect means via the recruitment of multiple signaling intermediates, and direct means through acting as transcriptional coactivators.

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