Journal of Cellular Biochemistry 92:664–678 (2004)

FAST TRACK

Inactivating Pit-1 Mutations Alter Subnuclear Dynamics Suggesting a Protein Misfolding and Nuclear Stress Response

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Abstract Pit-1, a POU-class nuclear DNA-binding transcription factor, specifies three of the parenchymal cell types in anterior pituitary ontogeny. Using fluorescent fusions and live cell imaging, we have compared the dynamic behavior of wild-type and inactivating Pit-1 point mutations. Fluorescence recovery after photobleaching (FRAP) and real-time extraction data indicate that wild-type Pit-1 has a dynamic mobility profile, with $t_{1/2s} \sim 5-7$ s when expressed from low to high amounts, respectively. Biochemically, Pit-1 is ~50% retained according to direct observation during extraction, indicating a dynamic interaction with nuclear structure. An analysis of transiently expressed Pit-1 carrying two different debilitating mutations reveals that they translocate normally to the nucleus, but exhibit two different levels of mobility, both clearly distinguishable from wild-type Pit-1. At low expression levels, the $t_{1/2s}$ of Pit_{W261C} and Pit_{A158P} are extremely rapid (0.3 and 0.6 s $t_{1/2s}$, respectively). At higher expression levels, unlike wild-type Pit-1, both mutant proteins become immobilized and insoluble, and fractionate completely with the insoluble nuclear matrix. Relative to wild-type, over expression of mutated Pit-1 elicits a nuclear stress response indicated by increased levels of heat shock inducible heat shock protein 70 (Hsp70), and reorganization of heat shock factor-1. The decreased mobility of Pit_{A158P} relative to Pit_{W261C} at low expression levels correlates with its ability to partially activate when expressed at low levels and its ability to bind cognate DNA. At high expression levels, lower Pit_{A158P} activation correlates with its immobilization and insolubility. These data suggest a link between specific rates of intranuclear mobility and Pit-1 transcription function, perhaps to insure sufficient interactions with chromatin, or in the case of non-DNA binding Pit-1, interaction as a repressor (Scully and Rosenfeld [2002]: Science 295:2231–2235). These data imply inactivating mutations can lead to an intranuclear sorting away from transcription related pathways, and at least in part to a misfolded protein pathway. Taken together, caution is suggested when interpreting point (or other) mutational analyses of transactivator function, as new compartmentation, especially in the context of expression levels, may cloud the distinction between defining functional molecular domains and intranuclear processing of misfolded proteins. J. Cell. Biochem. 92: 664–678, 2004.

Key words: chromatin; cell stress; transcription; photobleaching chaperone; ubiquitin; protein folding

Pituitary ontogeny is an important model of neuroendocrine development. Pit-1, a POU-class transcription factor [Andersen and Rosenfeld, 2001], is vital to the differentiation and survival of three separate, but developmentally related, cell types in the anterior pituitary that individually produce growth hormone, prolactin and the β subunit of thyroid stimulating

Grant sponsor: NIH; Grant number: RO1 DK55622; Grant sponsor: National American Heart Association Scientist Development Award (to M.A.M.); Grant number: 9630033N; Grant sponsor: NIH postdoctoral fellowship (to D.L.S.); Grant number: 1F32DK09787; Grant sponsor: NIH-NIA (to Z.D.S.).

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Received 27 October 2003; Accepted 29 October 2003
DOI 10.1002/jcb.20028

hormone [Ingraham et al., 1988; Theill and Karin, 1993; Andersen and Rosenfeld, 1994; Cohen et al., 1996; Treier and Rosenfeld, 1996; Radovick et al., 1998; Schonemann et al., 1998; Dasen and Rosenfeld, 1999; Kioussi et al., 1999].

The mechanism of Pit-1 transcription activation has been investigated using a variety of approaches. First, cell-free [Smith et al., 1995] and ex vivo approaches showed that Pit-1 activation is synergized with itself and with other factors such as Ets-1 [Howard and Maurer, 1995; Bradford et al., 1996, 1997, 2000], and GATA2 [Dasen et al., 1999]. Second, Day [1998] used FRET to demonstrate in vivo interactions with Ets-1. These factors are targets of cell signaling that mediate various physiological and developmental stimuli, and are required for maximal activation in transactivation assays. Third, at the level of chromatin, Pit-1 was shown to interact ex vivo and in vitro with chromatin architectural co-repressors and co-activators [Xu et al., 1998]. In these studies [Xu et al., 1998], changes in domain requirements for histone acetyltransferases and CBP in mediating the effects of various signal transduction pathways to Pit-1 were proposed. Fourth, Pit-1 also influences the transcription machinery in the absence of chromatin. In vitro, cell-free experiments showed that Pit-1 is required for the assembly of an activated RNA polymerase II transcription complex [Sharp, 1995]. Last, Scully et al. [2000], showed that Pit-1 dimers bound to a high affinity site from the prolactin promoter (Prl-1P) are in a different structural configuration from those bound to the proximal high-affinity site in the growth hormone promoter, GH-1. The difference in dimer configuration is due to two base pairs, TT, that extend the Pit-1 DNA contacts on GH-1 relative to Prl-1P. This is biologically significant since it helps explains, in part, why expression of growth hormone is restricted to somatotropes. In addition, the ETS factors do not bind to or activate the GH promoter, suggesting that they also participate in the combinatorial control of lactotrope-specific expression of the prolactin gene [Bradford et al., 1997].

Of significant biological importance is a need to understand Pit-1 function in the context of a nuclear environment, which presents significant challenges for understanding events mediated by transcription factors. At a crude, biochemical level, Pit-1 is equally divided between detergent soluble and insoluble fractions [Mancini et al., 1999]. Spatially, Pit-1 localizes to thousands of discrete foci, which, based on staining intensity in well-fixed cells, co-localize with euchromatin [Mancini et al., 1999]. Transient over expression of Pit-1 carrying mutations that impair its ability to activate transcription shift partitioning to the insoluble fraction concomitant with an abnormal intranuclear localization pattern, including frequent large inclusions [Mancini et al., 1999]. The well-conserved POU-specific domain (PSD) [Andersen and Rosenfeld, 2001] appears necessary to mediate targeting of Pit-1 or a generic fusion to the insoluble fraction [Mancini et al., 1999], though the specific interactions and spatial targeting issues involved remain poorly understood. The functional significance of nuclear solubility and spatial partitioning of Pit-1 is not known. Additionally, with the recent ability to study mobility of fluorescent protein fusions in living cells, is there any relationship between Pit-1 partitioning and its subnuclear mobility?

The mobility of nuclear proteins translationally fused to fluorescent partners is unimpeded by the high concentration of nucleic acids and proteins in the nucleus [Phair and Misteli, 2000]. However, with some nuclear proteins, changes in physiological conditions such as hormone signaling can alter partitioning dynamics in concert with changes in solubility. The decreased mobility of ERα and its coactivator, SRC-1, in response to agonist, correlates positively with repartitioning to the insoluble, nuclear matrix fraction [Stenoien et al., 2001b]. By targeting ER (lac rep-ER) to integrated lac repressor binding sites, Nye et al. [2002] showed decondensation of large scale chromatin structure, which, interestingly, was partially reversed in the presence of agonist. Using a similar approach, Stenoien et al. [2001a] demonstrated that individual proteins present in agonist-dependent, biochemically-defined ER complexes, such as SRC-1 or CBP, are, when examined in vivo, found to be highly dynamic interacting with lacER tethered to the lac operator array. Since ER and Pit-1 are known to synergize on the prolactin enhancer [Day et al., 1990; Simmons et al., 1990; Holloway et al., 1995; Chuang et al., 1997; Fujimoto et al., 1999; Schaufele, 1999], we have begun to further investigate the nuclear behavior of Pit-1.

Here, we report a comparison of fluorescent fusions of wild-type and mutated Pit-1 that reveals a relationship exists between the ability to activate transcription and changes in nuclear mobility. In addition, a novel nuclear "stress" response to over expression of mutated, and presumably misfolded, Pit-1 is also described.

MATERIALS AND METHODS

Wild-Type and Mutant CFP, GFP and YFP Pit-1 Plasmids

PCR primers were designed to add a 5′ *Asp*718 site and 3′ *Bam*HI site to the end of an approximately 370 bp section of pCMV-GFP-Pit-1 (a kind gift from R. Day) that contains a five amino acid spacer 5′ to the Pit 1 start site, creating pCMV-GFP-Pit-1(1–370). The PCR product was digested with *Asp*718 and *Bam*HI and isolated from a 2% NuSieve GTG agarose gel. pEGFP-C1 vector (Clontech, Palo Alto, CA) was digested with *Bsr*GI and *Bam*HI, and electrophoresed in a 0.8% SeaPlaque GTG agarose gel. An in-gel ligation was performed using the insert and vector fragments, resulting in the loss of the *Bsr*GI site. The same PCR product above was used to create a pTRE-XFP Pit-1 clones.

To make full-length, wild-type XFP Pit-1 and introduce point mutations, pEXFP-Pit-1(1–370) was sequentially digested with *Ppu*MI and *Bam*HI, and isolated. Point mutation-containing fragments were prepared from pCEP4-Flag-Pit-1, A158P, W261C, α-1 (E133P/A136P), and α-4 (R183P/A188P) [Mancini et al., 1999] by digesting with *Ppu*MI and *Bam*HI, and in-gel ligations were performed. Mutants obtained from the yeast screen (see below; E174G, N182S, and G147V) were also prepared using the same strategy. Qiagen midi preparation DNA was transfected into HeLa cells to check for correct size by Western blotting, lack of dm66/enhancer-luciferase activity (all negative) and characteristic subnuclear localization patterns compared to pCEP4 versions using anti-Pit1 2C11 antibody [Mancini et al., 1999].

For tet-regulated clones, pTRE-EXFP-Pit-1(1–370) was partially digested first with *Bsa*I, then with *Bam*HI. Point mutation-containing inserts were prepared by digesting with *Bsa*I, then with *Bam*HI. Correct clones were tested as above in HeLa Tet-On cells (Clontech; 1–8 μg/ml doxycycline overnight post transfection). GFP-heat shock transcription factor-1 (HSF-1) was a kind gift of R. Morimoto (Northwestern University), and was subcloned into a YFP vector (Clontech).

Cell Culture and Labeling

HeLa cells were maintained in Opti-MEM I media (Life Technologies, Inc., Gaithersburg, MD) containing 4% FBS (Life Technologies, Inc.). Twenty-four hours before transfection, cells were plated onto poly-D-lysine-coated coverslips in 35-mm wells at a concentration of 10^5 cells per well in media containing charcoal stripped FBS. Transient expression of plasmids was accomplished using with Fugene (Roche, Basel, Switzerland).

Live Microscopy and Fluorescence Recovery After Photobleaching (FRAP)

Cells were grown on 40 mm cover slips in 60 mm plates and transfected with 2.5 μg of each test plasmid, allowed to recover for 4–24 h and then were transferred to a live cell chamber (Bioptechs, Inc., Butler, PA) and maintained in DMEM with 5% stripped FBS at 37°C. This media was re-circulated using a peristaltic pump to which ligand was added. Time-lapse live microscopy was performed using a Deltavision deconvolution microscope (Applied Precision, Issaquah, WA) with images acquired before and at intervals following ligand addition. A Z-series of focal planes were digitally imaged and deconvolved with the DeltaVision constrained iterative algorithm to generate high-resolution images. FRAP was performed using a LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY). A single Z-section was imaged before and at time intervals following the bleach. The bleach was performed using the laser set 488 nm for GFP or 458 nm for CFP at maximum power for 100 iterations (~1 s). For dual FRAP experiments, both were bleached with the same laser setting at 458 nm and simultaneous images corresponding to the CFP and YFP fluorescence were obtained. A minimum of 10 cells were examined at each conditions. Fluorescent intensities of regions of interest were determined using LSM software and data was exported to Excel (Microsoft, Inc., Redmond, WA) for analysis. LSM images were exported as TIF files and final figures were generated using Adobe Photoshop and Illustrator.

Mutagenesis and Selection of PSD Mutants

To facilitate isolation of inactive Pit-1 mutants, we used a system of positive selection in the budding yeast. It is based on the ability of Pit-1 to act as a transcriptional activator when targeted to an exogenous promoter [Ding et al., 1991]. To avoid biasing the screen towards mutations affecting sequence-specific DNA binding alone, we constructed a fusion of Pit-1 to the DNA-binding domain of yeast transcriptional activator GAL4 (amino acids 1–147) and the HA epitope tag. The fusion is expressed in yeast under the control of the ADH promoter from a single-copy (centromere-containing) plasmid vector pAS2CEN (a gift from S.J. Elledge). This plasmid was introduced into a yeast strain containing two reporter genes, *URA3* and *LacZ*, whose expression is dependent upon binding of GAL4 protein, or GAL4 DNA binding domain fused to a transcription activation domain, to the upstream control sequences. Expression of the GAL4–Pit1 fusion in this strain was confirmed by immunoblotting using the 2C11 mAb (not shown). Expression resulted in activation of both *URA3* and *lacZ* genes, as evidenced by the ability of the cells to grow on minimal media without uracil and by β-galactosidase color assay. This system allows positive selection of Pit-1 mutations incapable of transcriptional activation by plating the cells on selective media containing 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing URA3 gene product. Cells expressing wild-type Pit-1 activity cannot grow on this medium.

We performed the mutagenesis by taking advantage of inherent errors of Taq polymerase and homologous recombination in yeast (gap-repair mutagenesis). The plasmid containing the GAL4–Pit-1 fusion was "gapped" with restriction enzymes to remove the PSD (and 40–60 bp of flanking sequence). Independently, this region was PCR-amplified from an intact plasmid, mixed with the gapped vector and used to transform the tester yeast strain. Homologous recombination within the regions of overlap between the gaped plasmid and PCR product results in in vivo reconstitution of complete plasmid encoding the fusion of normal GAL4 to Pit-1 with mutagenized PSD.

Cell Biological Procedures

Cell transfections, transcription assays, preparation of core nuclear matrix, Western blotting with mAB 2C11, and immunofluorescence assays were done as previously described [Mancini et al., 1999; Stenoien et al., 2001b]. Antibody to general heat shock protein 70 (Hsp70) and specifically to the inducible form of Hsp70 was obtained from Stressgen (SPA-812C) and used at 1:500 followed by FITC-conjugated goat anti-rabbit.

RESULTS

Transcription Activation by Fluorescent Fusions of Wild-Type and Mutated Rat Pit-1

In an effort to identify functionally relevant residues in rat Pit-1, a yeast mutagenesis screen was undertaken using degenerate PCR as described in the "Materials and Methods." After mutagenesis and gap repair, we obtained approximately 5,000 transformants. Plating the transformants onto selective media containing 5-FOA identified 40 Pit-1 clones that did not activate URA3 or LacZ transcription. Mutant plasmids were rescued from yeast by transformation into bacteria and re-tested after transformation into the same tester yeast strain. Those transformants that proved transcriptionally negative were assayed for the expression of full-length GAL4–Pit-1 fusion by Western blotting. A majority of these clones expressed truncated proteins. This may be attributed to a selective advantage of the cells expressing truncated Pit-1, since expression of the full-length protein has a deleterious effect on yeast cell growth (M.G.M., I.I.O., M.A.M., unpublished observations).

To examine these and previously identified human and mouse Pit-1 point mutations (Fig. 1a) in living mammalian cells, cDNAs were translationally fused to green fluorescent protein in pEGFP (BD Clontech) vector. The functional competence of the fluorescent fusions (wild-type and mutants), was tested by transient transfection assays (Fig. 1b). In contrast to GFP-Pit-1, which is capable of activating a PRL-Luc reporter, all of the mutations, consistent with the screening results, were inactive in this assay. Note that two mutations identified in this screen, G147V and E174G, were also reported in an earlier, chemical mutagenesis-based yeast screen of Pit-1 DNA-binding domain mutations [Liang et al., 1995], who reported that G147V is competent for DNA-binding in gel shift assay using in vitro transcribed and translated proteins, while E174G is not. We now report that

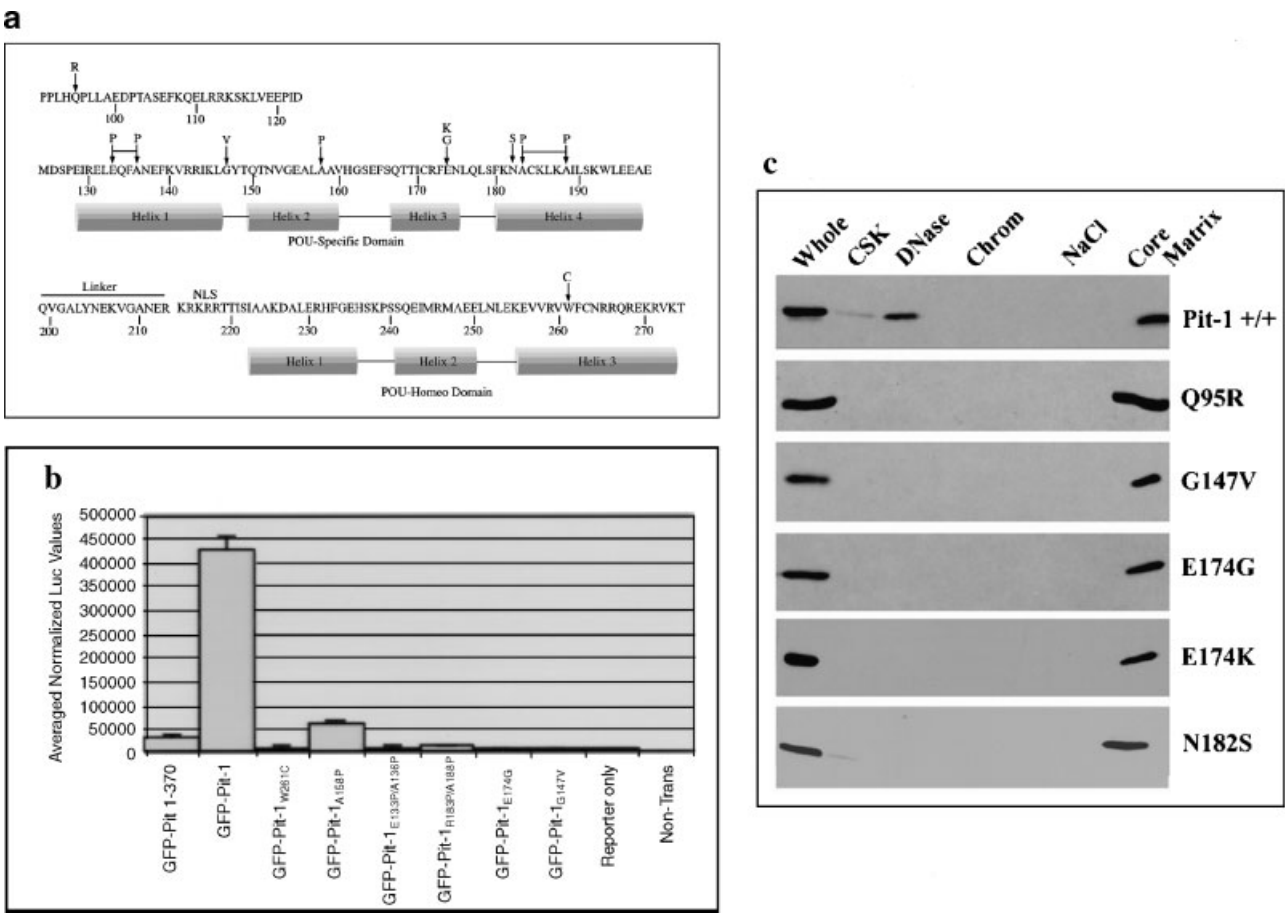

Fig. 1. Analysis of Pit-1 carrying inactivating mutations. **a**: Sequence of Pit-1 POU-specific and homeodomains with relevant mutations. Linker is the flexible linker region separating the POU-SD and POU-HD. NLS is the location of the nuclear localization sequence. A158P and W261C are mutations found in human cases of combined pituitary hormone deficiency (CPHD) [Pfaffle et al., 1992] and the Snell and Jackson dwarf mouse mutation [Li et al., 1990], respectively. **b**: Transcription assays of GFP translationally fused with Pit-1 and mutants. pEGFP-Pit-1 and mutations shown in (a) were transfected along with a luciferase reporter plasmid demonstrate that GFP-Pit-1 is transcriptionally active. The point mutations render Pit-1 inactive with the exception of GFP-Pit-1_{A158P}, which retains partial activity. **c**: Biochemical partitioning of nonfunctional Pit-1. Flag-tagged point mutants (see above) and wild-type rat Pit-1 were transiently transfected into HeLa cells. Core matrix preparations were performed as described in ''Materials and Methods.'' The distribution of epitope-tagged Pit-1 (**top row**) contains a soluble and insoluble component. The solubility partitioning of Pit-1 carrying the indicated point mutations is heavily skewed to the insoluble fraction. PIT-1_{A158P} is a mutation found in patients with CPHD without hypoplasia [Pfaffle et al., 1992].

both of these mutations are incapable of PRL-Luc reporter transactivation in mammalian cells (Fig. 1b). As with previous studies [Mancini et al., 1999], preliminary transient over expression studies, as shown in Figure 1c, reveals Pit-1 protein carrying the mutations in Figure 1a shift into the insoluble nuclear fraction. This issue will be further addressed in experiments below.

It has been reported that the A158P mutation found in patients with combined pituitary hormone deficiency (CPHD) renders Pit-1 inactive in transient co-transfection assays while retaining modified DNA-binding activity in vitro [Pfaffle et al., 1992]. It was also noted that patients in families that were homozygous for the A158P mutation had CPHD without pituitary hypoplasia, while individuals from another family that are compound heterozygous for the A158P allele and a Pit-1 deletion presented with both CPHD and pituitary hypoplasia [Pfaffle et al., 1992]. This suggested that the A158P mutation partially cripples Pit-1, and, perhaps, the increased expression from two A158P alleles allowed Pit-1 to direct embryonic differentiation and lactotrope, somatotrope, and thyrotrope survival [Pfaffle et al., 1992]. The current interpretation of the data on this mutation is that it is recessive and affects the activation function of Pit-1 [Andersen and

Rosenfeld, 2001]. Interestingly, A158P is partially active in transient transfection assays (Fig. 1b). In our experiments, Pit_{A158P} in rat Pit-1 reproducibly demonstrated transcription activity, which appeared to be inversely correlated with expression levels. To test this under regulated conditions, levels of wild-type and Pit_{A158P} were placed under the control of doxycycline. Pit-1-dependent levels of luciferase activity rose steadily with increasing amounts of vector in the presence of doxycycline (8 μg/ml, Fig. 2). Consistent with its impaired transactivation potential, the A158P mutant began its rise at a slightly lower level of activity. In contrast to wild-type Pit-1, the levels of luciferase activity decreased when 250 ng of pTRE-$Pit\text{-}1_{A158P}$ was transfected and stimulated with 8 ng/ml of doxycycline (Fig. 2). Thus, increasing concentrations of this mutant is somehow detrimental to its ability to activate. This does not appear to be due to squelching of general transcription factors since wild-type Pit-1-dependent reporter showed no evidence of decreasing at the same levels of expression (Fig. 2).

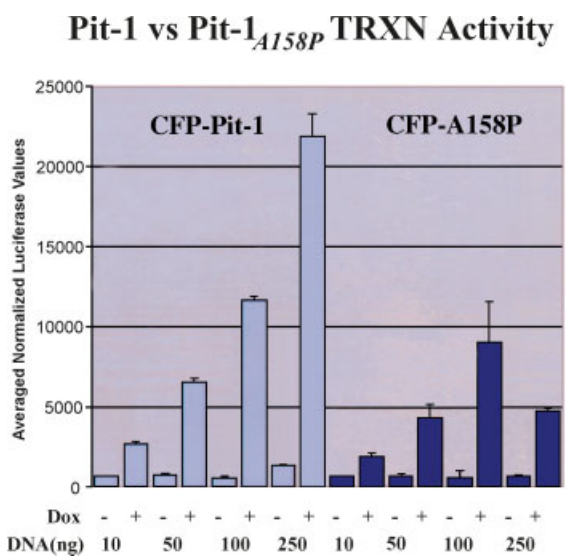

Fig. 2. Higher levels of $Pit\text{-}1_{A158P}$ adversely affect transcription activity. To test the effects of expression levels on CFP-Pit-1 and CFP-$Pit\text{-}1_{A158P}$, cells were transfected with increasing plasmid concentration of doxycycline-regulated Pit-1 mammalian expression plasmids, along with 500 ng of reporter plasmid. Eight microgram per milliliter doxycycline was either present or absent in the media as indicated. CFP-Pit-1 activity increases linearly with increasing DNA concentrations while CFP-$Pit\text{-}1_{A158P}$ activity decreases once a threshold is attained. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cellular Mobility of Wild-Type and Mutated Pit-1

We have previously reported the insolubility and abnormal nuclear morphology of transiently over-expressed flag-tagged-$Pit\text{-}1_{W261C}$ and -$Pit\text{-}1_{A158P}$ mutations using immunofluorescence detection [Mancini et al., 1999]. To examine the nuclear morphology and mobility of Pit-1 in live cells, wild-type and mutated Pit-1 fluorescent fusions were examined microscopically. As a reference point, Figure 3 demonstrates a typical, highly resolved fixed cell pattern of low-level (barely above detection) nuclear distribution of GFP-Pit-1 in transiently transfected HeLa cells. Compared to chromatin densities, Pit-1 is localized to numerous discrete foci scattered throughout the nucleus, which is consistent with that obtained by immunofluorescence. This nuclear distribution is similar to that documented for endogenous and epitope-tagged (Flag) Pit-1, e.g., numerous subnuclear speckles [Mancini et al., 1999]. However, the improved imaging resolution from better optics and restorative iterative deconvolution processing reveals a far more elaborate organization than previously reported. This delicate distribution of Pit-1, not observable in real time microscopy, is due to live cell dynamics (see below).

To compare the mobility of wild-type and mutated Pit-1, FRAP was performed on transiently transfected HeLa cells expressing higher or lower levels of wild-type GFP-Pit-1 (Fig. 4a). Relative higher or lower levels of expression were chosen directly through the eyepiece, with lower expression being just barely visible using a 63× 1.3 N.A. PlanApochromat objective. Following brief, high intensity laser illumination, a clear zone of bleached GFP-Pit-1 is observed. Recovery dynamics of GFP-Pit-1 fluorescence is also comparable at each level of expression, with $t_{1/2s} \sim 5-7$ seconds when expressed from low to high amounts, respectively (note, images from low or high expressers were normalized for presentation). In contrast to wild-type GFP-Pit-1, the FRAP analysis of a low level of Pit-1 carrying the Snell or human mutations, W261C (Fig. 4a) and A158P (Fig. 4b; image data not shown), respectively, revealed a dramatic alteration in nuclear mobility as evidenced by a rapid decline in total nuclear fluorescence over the period of bleach and an recovery average of $t_{1/2s}$ of 0.3 and 0.6 s, re-

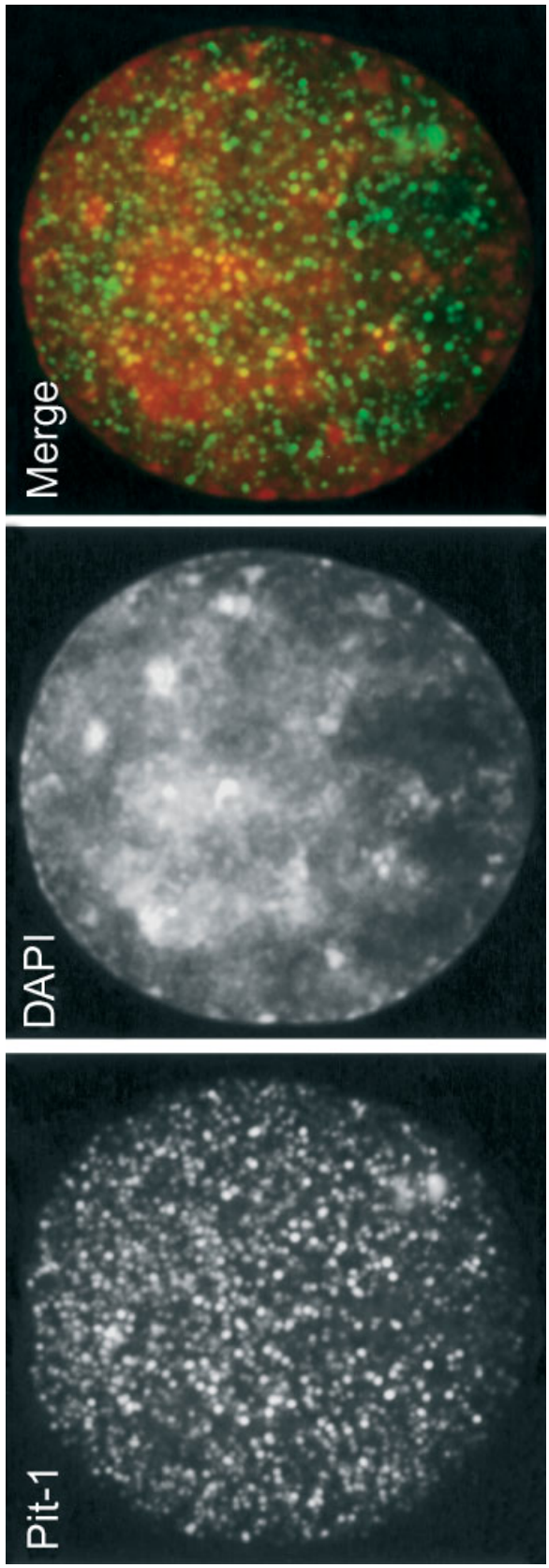

Fig. 3. High resolution, 3D view of tet-regulated GFP-Pit-1 in transiently transfected HeLa using deconvolution microscopy. Chromatin densities are demarcated by labeling with the DNA specific dye, DAPI (**middle**, and red in color figure). GFP-Pit-1 (**left**, and green in color figure) is shown to localize primarily in discrete foci scattered throughout the euchromatin, as was also seen for endogenous Pit-1 [Mancini et al., 1999].

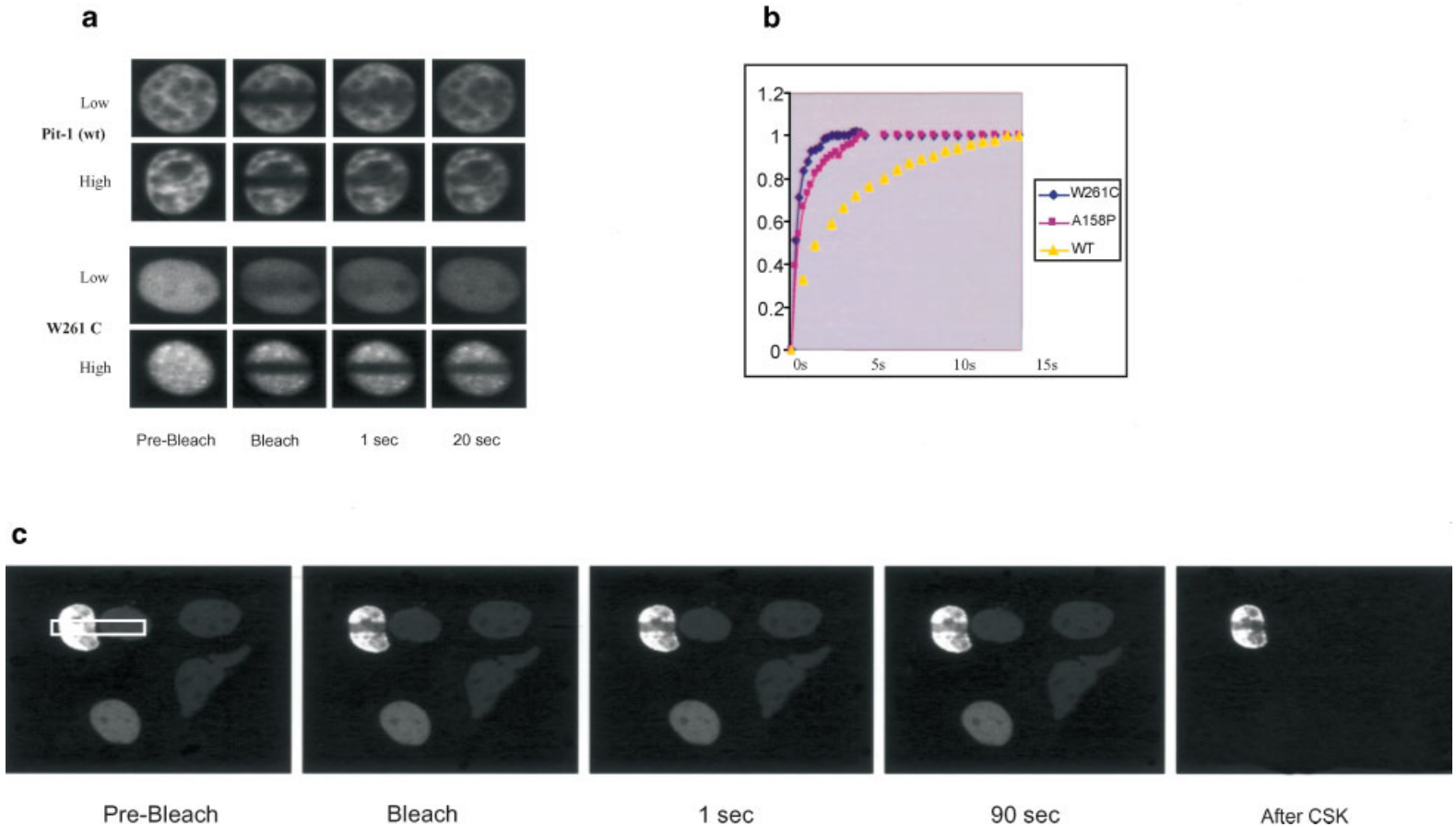

Fig. 4. Fluorescence recovery after photobleaching (FRAP) of GFP-Pit-1 and GFP-Pit-1_{W261C}. **a**: Wild-type Pit-1 exhibits similar recovery dynamics regardless of protein expression levels. As evidenced by the rapid decline in total nuclear fluorescence over the short bleach time, GFP-Pit-1_{W261C} is very mobile at low protein concentrations. In contrast, at high protein levels, GFP-Pit-1_{W261C} becomes completely immobilized. **b**: FRAP recovery curves of GFP-Pit-1 and GFP-Pit-1_{W261C}. FRAP was performed on 10 cells expressing low levels of either GFP-Pit-1 and GFP-Pit-1_{W261C} or GFP-Pit-1_{A158P} and the recovery times were averaged. The fluorescence immediately after the bleach was set to 0 and the fluorescence at complete recovery was set to 1. **c**: Expression level affects GFP-Pit-1_{W261C} mobility and solubility. HeLa cells were transiently transfected with GFP-Pit-1_{W261C}. A box spanning two cells that were obviously expressing different levels was photobleached. The higher expressing cell on the **left** (overexposed to enable visualization of the other cells) showed little recovery after the bleach, and was resistant to detergent extraction. The lower expressing cell on the **right** showed rapid recovery and no resistance to detergent extraction. In the panel, other low expressing cells, which were not bleached, were also completely extracted in detergent. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

spectively. In the recovery curves in Figure 4b, note that there is a distinct difference in the two mutations when expressed at low levels, with the partially active A158P mutant being intermediate between wild-type and the functionally dead W261C mutant.

As demonstrated by a clear bleach zone with little to no recovery after 20 s (Fig. 4a), the W261C mutant routinely presented a different picture of mobility at higher levels of expression (roughly 20–50× higher). These data indicate that this mutated protein, which is defective in DNA-binding, becomes immobilized within the nuclear space when expressed at high levels, and also displays focal accumulations of increased fluorescence. The A158P mutant demonstrated a similar picture of immobilization at higher levels of expression (data not shown). Combined, the data to this point revealed a positive correlation between ability to activate in a transient co-transfection assay (Fig. 1) and intranuclear dynamics (Fig. 4a,b).

In an effort to relate mobility and solubility, real-time extraction experiments [Stenoien et al., 2000] were performed. Figure 4c, presenting data on GFP-Pit-1_{W261C}, shows two adjacent transiently transfected cells, which were photobleached at the same time for direct comparison (boxed area in Fig. 4c). One cell, obviously expressing much less fluorescent protein than the other, demonstrates the same recovery dynamics as the lower expressing cell shown in Figure 4a. Likewise the higher expressing cell also demonstrates the same recovery dynamics as the cell shown in Figure 4a. After detergent extraction of the cells on the coverslip, GFP-Pit-1_{W261C} in the higher expressing cell is resistant, while it is fully extracted in the lower expressing cell. Other lower expressing cells in the field, which were not photobleached (Fig. 4c), showed that GFP-Pit-1_{W261C} is soluble. These observations provide a clear correlation between mobility, solubility, and expression level, and point out that heterotypical expression profiles in a transiently transfected culture, when bulk-processed biochemically, could be misleading if the predominate number transiently over expressing cells were of the immobile, and insoluble variety. Our earlier Western blotting results with over expressing Pit-1 mutants were clearly not reflecting the presumed presence of at least some low expressers [Mancini et al., 1999].

Mutated Pit-1 and Nuclear Stress Response

The next obvious question is: what is responsible for the increased and decreased nuclear mobility of mutated Pit-1 at lower and higher levels of expression, respectively? As mentioned previously, the higher-level expression of Pit-1 mutants presents with an "aggregated" morphology within the nucleus and is insoluble. The morphology and biochemical insolubility is reminiscent of similar presentations by misfolded proteins responsible for neurodegenerative diseases such as Huntington's, spinocerebellar ataxia type 1 (SCA1), and spinal and bulbar muscular atrophy (SBMA, androgen receptor) [Cummings et al., 1998, 1999; Lin et al., 1999; Stenoien et al., 1999; Fernandez-Funez et al., 2000; Krobitsch and Lindquist, 2000; Satyal et al., 2000; Simeoni et al., 2000]. Since these proteins co-localize with proteasomes and chaperones, the latter of which suppresses nuclear aggregation and alters nuclear localization [Cummings et al., 1998; Fernandez-Funez et al., 2000], we tested the possibility of their co-localization with wild-type and mutated Pit-1.

Initially, we ask if a generalized response to exogenous cell stress might affect transcription factor mobility. For this purpose, CFP-Pit-1 and YFP-HSF-1 were co-expressed in HeLa cells. Figure 5 (bottom two panels) shows that a 2 min incubation of the co-expressing cells at 42°C resulted in a reorganization of YFP-HSF-1, typical of HSF-1 [Cotto et al., 1997]. Note that the heat treatment also resulted in a decreased recovery after photobleaching (Fig. 5, compare top two panels with bottom two panels). These data indicate a heat shock itself can reduce mobility of wild-type Pit-1.

Next, we ask if higher expression of Pit-1 mutants demonstrated features similar to heat shock. Figure 6a shows that highly expressed Pit-1_{W261C} co-localizes with Hsp70 [Nollen and Morimoto, 2002]. Indicative of a stress response by the over expression of these mutated proteins, Figure 6b,c demonstrates that high levels of wild-type Pit-1 does not induce the stress-specific form of Hsp70, while the E174G mutant does. Further, a stress response elicited by over expression of mutant Pit-1 proteins is evidenced by an unusual morphological relationship of HSF-1 and Pit-1_{W261C} in nuclear foci. Under these conditions, HSF-1 frequently presents as nuclear accumulations co-localizing with

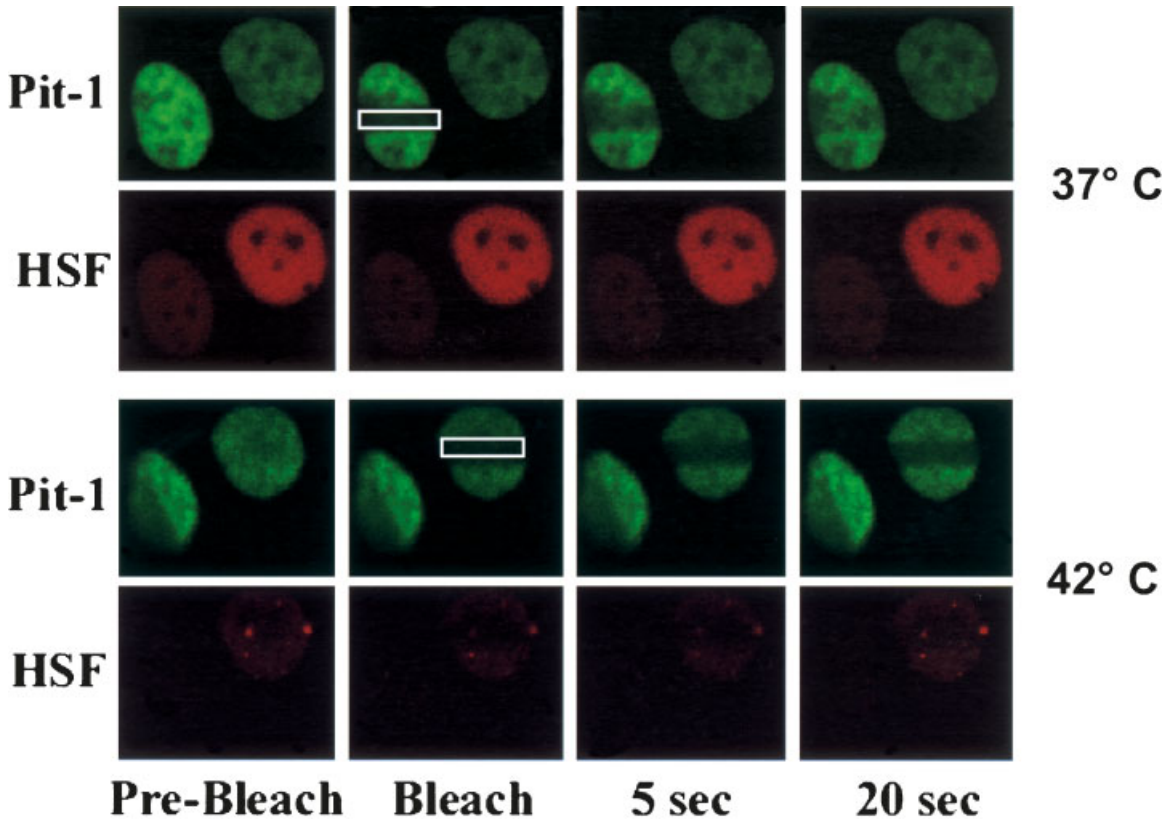

Fig. 5. Exogenous cell stress reduces Pit-1 mobility. Wild-type CFP-Pit-1 (green) and YFP-HSF-1 (red) translation fusions were co-expressed in HeLa cells, which were incubated at 37°C (**top two panels**) or 42°C (**bottom two panels**) for 2 min. Recovery after photobleaching was documented for CFP-Pit-1. Note the reorganization of YFP-HSF-1 as noted previously for HSF-1 [Cotto et al., 1997] and reduced recovery of CFP-Pit-1 subsequent to heat shock. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Pit-1_{W261C} foci (compare Fig. 7a and b). Finally, Western blot analysis comparing lysates from cells over expressing Pit-1 or Pit-1_{W261C} demonstrates slower mobility bands specifically in Pit-1_{W261C} cells consistent with potential ubiquitination (Fig. 7c). Interestingly, the putative ubiquitinated bands are observed in insoluble pellets, consistent with the reduced mobility of Pit-1_{W261C} compared to wild-type. Similar results were observed in the case conditions that immobilize ER, where some ligands or proteasome-inhibition shifted ubiquitinated-like bands of ER specifically to the NM fraction [Stenoien et al., 2001b]. Interestingly, Figure 7c also shows that a significant portion of the bulk isolated Pit-1_{W2561C} pool is soluble and is

Fig. 6. Co-localization with and induction of a heat shock protein 70 (Hsp70) by mutated Pit-1 proteins. **a**: Pit-1_{W261C} nuclear aggregates co-localize with Hsp70. Transiently transfected cells expressing Pit-1_{W261C} were immunostained with antisera specific for Pit-1 (green) and general Hsp70 (red), and counterstained with DAPI for nuclear identification. This image, typical of cells expressing high level of Pit-1_{W261C}, is representative of the significant overlap (yellow) of the immunoreactive proteins. **b** and **c**: High-level expression of a Pit-1 mutant induces expression of inducible Hsp70. HeLa cells were transiently transfected with expression plasmids driving the expression of wild-type (b) or mutant (Pit-1_{E147G}), immunostained and counterstained as in panel (a), but with an antibody to the stress inducible form of Hsp70.

Fig. 7. Over expression of mutant Pit-1 elicits a cell stress response. **a** and **b**: Representative images from HeLa cells co-transfected with either wild-type (a) or Pit-1_{W261C} (b) expression vectors (red), and GFP-HSF-1 in green. In most cases, cells labeled with mAb 2C11 expressing Pit-1_{W261C} exhibit a reorganized HSF-1 profile indicative of cell stress. Note that the ''halo'' of Pit-1_{W261C}, which is a distinct morphology relative to wild-type Pit-1 response to heat shock. **c**: Tet-regulated Pit-1 expression vectors were used to minimize the effects of over expression. Cells were extracted to obtain soluble and insoluble fractions (see ''Materials and Methods''). Immunoblotting using α-Pit-1 demonstrates that Pit-1_{W261C} forms high MW ubiquitin-like ladders, which are only found in the pellet. Note, at lower expression, some soluble mutant Pit-1 is detected.

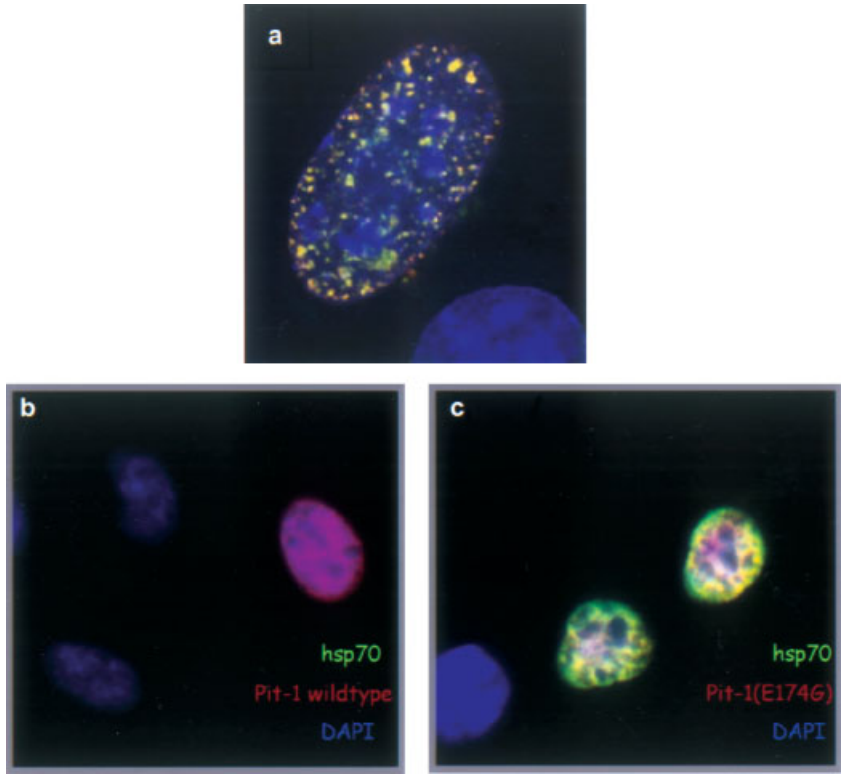
a
b
hsp70
Pit-1 wildtype
DAPI
c
hsp70
Pit-1(E174G)
DAPI

Fig. 6.

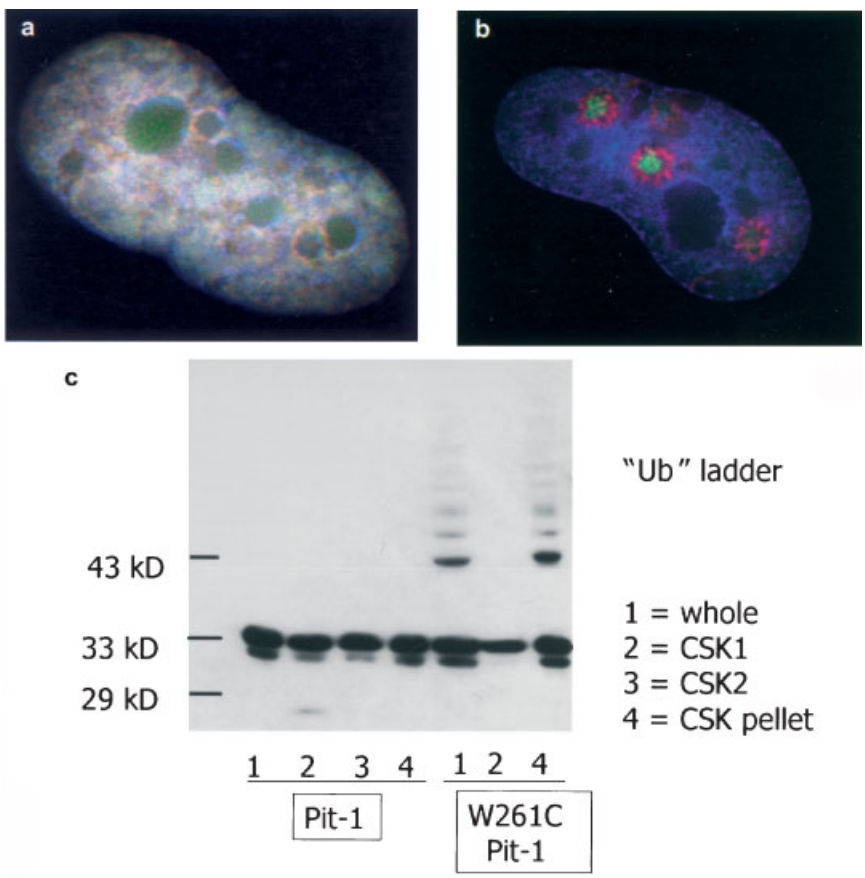
a
b
c
"Ub" ladder
43 kD
33 kD
29 kD
1 = whole
2 = CSK1
3 = CSK2
4 = CSK pellet
1 2 3 4
1 2 4
Pit-1
W261C
Pit-1

Fig. 7.

apparently not ubiquitinated, presumably from lower expressers in the culture.

DISCUSSION

In this report, using a novel yeast screen we have documented additional mutations that inactivate Pit-1 transcription function, two of which had been previously identified. Combined with our functional data here, the evidence suggests that G147V is important for protein–protein interactions, as proposed for the POU-S domain [Andersen and Rosenfeld, 2001]. The E174G mutation, reported in a case of CPHD, is part of a compound heterozygous mutation in human Pit-1, and is reported to have decreased DNA-binding affinity [Brown et al., 1998].

A general theme that emerged in these studies is that decreased nuclear function of Pit-1 correlates strongly and specifically with nuclear (vs. cytoplasmic) insolubility. As with previous mutants examined, the additional mutants also readily translocate from the cytoplasm and are, therefore, not intrinsically insoluble. Only within the nucleus are the mobility, insolubility, and organization affected. However, cause and effect with these mutations must be interpreted with caution. Are they defective because of an inability to interact with DNA and/or other proteins in the transcription machinery (typically demonstrated in an in vitro environment) or are they inactive due to misfolding, aggregation, and insolubility, especially at the routinely over-expressed conditions of transient transfection? We use the term "aggregated" carefully, here, as some misfolded nuclear proteins which appear to be "aggregated" [Kim et al., 2002; Stenoien et al., 2002] can be, in live cell analyses, highly mobile. The Pit-1 mutated proteins that are clearly defective in DNA-binding, such as the mouse dwarf mutation Pit-1_{W261C} [Li et al., 1990] and Pit-1_{E174G} [Brown et al., 1998] may be more clearly assigned to this functional defect. Other types of mutations are more difficult to assign because of their differential partitioning compared to wild-type. This general theme is also likely to extend to other nuclear transcription factors, especially those assayed ex vivo using transient transfection assays.

The mutation in Pit-1 identified in the Dutch families is a case in point. Based on the findings in this study, we reinterpret the genetic data [Pfaffle et al., 1992] to include the possibility that the phenotypes of the Pit-1_{A158P} allele do not solely reflect its inability to activate. In addition, the phenotype may also point toward the propensity of this partially active protein to become insoluble, and ultimately less active (see Fig. 2) at higher levels of expression. Thus, during pituitary development, the levels of Pit-1_{A158P} in homozygous patients are postulated to be low, but high enough for the partially active protein to support differentiation and survival of Pit-1-dependent cell types. Although potentially misfolded, at this level of expression the protein maintenance systems in the nucleus (chaperones and proteasomes) appear operational, perhaps reflected by the intermediate mobility of GFP-Pit-1_{A158P} at low levels of expression (Fig. 4). Later, when there is a physiological demand for higher levels of target hormones (e.g., during growth), the increased levels of Pit-1_{A158P} lead to its becoming immobile and insoluble, perhaps due to overwhelming the chaperone/proteasome maintenance system in the nucleus, resulting in CPHD without hypoplasia. Mice carrying the A158P mutation would be a potential test of this model. Also of interest in this regard is our consistent inability to generate stable clonal lines over expressing Pit-1_{A158P} or other Pit-1 mutants; tet-regulation has made possible some progress in this endeavor, but even slightly leaking expression drastically reduces colony formation (M.G.M., Z.D.S., M.A.M., unpublished observations).

These studies have also uncovered a cellular response to over expression of mutated nuclear transcription factors, which has not been previously appreciated. Increased levels of Pit-1 carrying the W261C mutation clearly lead to higher expression of nuclear levels of inducible Hsp70, with which it can also co-localize. At this point, we can only speculate that the chaperon activity of Hsp70 (and potentially others) is induced to deal with the high levels of misfolded Pit-1_{W261C}. Consistent with induction of inducible Hsp70 and co-localization, we have documented additional cellular signs of stress concomitant with high levels of mutated Pit-1 expression. It was previously shown that HSF-1 nuclear localization is dramatically altered in response to heat shock [Cotto et al., 1997]. We speculate that the intriguing morphological presentation of Pit-1_{W261C} and HSF-1 documented by experiments represented in Figure 7 is a generalized nuclear response to an over abundance of a mutated (misfolded?)

nuclear protein, perhaps similar to a response to heat. If it were a response to over expression alone, it would have been observed with wild-type Pit-1. In this regard, we also note that heat shock in Figure 5 did not elicit an aggregate morphology with wild-type Pit-1. It is interesting that evidence for "ubiquitinated" Pit-1_{W261C} is only evident in the insoluble fraction, suggesting that immobility is somehow associated with ubiquitination and possibly degradation, which is partitioned to this nuclear compartment.

We believe that these manifestations of a nuclear stress response observed for Pit-1 should be cautionary for studies seeking to use transient transfection transcription assays as tests of activator/repressor function. We also wonder: is it possible that these connections possibly manifest themselves in human disease as described above?

In addition to differences in solubility and aggregation, there is a clear difference in the nuclear mobility of mutated and wild-type Pit-1, which is also affected by expression levels. At low levels of expression, Pit-1 carrying a W261C or A158P mutation is extremely mobile. At high levels of expression, their mobility is very low, perhaps immobile (and certainly resistant to extraction, see Fig. 4c). Why is this? Our current speculation is any mutation that disrupts either DNA or protein interactions in Pit-1 promotes increased mobility at low levels of expression, perhaps simply as a result of subtle chaperone interactions, reminiscent of high mobility of unliganded (and chaperone-bound) estrogen receptor [Stenoien et al., 2001b]. At high levels of expression, continued (if not overwhelmed) interactions with chaperones, and ultimately the ubiquitin/proteasome pathway and/or the nuclear matrix drastically impede its mobility, and result in insolubility. Stated differently, the reduced mobility of wild-type Pit-1, relative to its mutated counterparts at low levels of expression, probably reflects the average residence times of all its interactions with DNA and other proteins involved in transcription activation and/or repression. For Pit-1_{A158P}, we observe mobilities inbetween Pit-1_{W261C} and wild-type, probably because Pit-1_{A158P} is capable of binding DNA, at least under in vitro conditions [Pfaffle et al., 1992]. The difference in mobility between Pit-1_{W261C} and Pit-1_{A158P} could be interpreted in this model as the contribution that DNA-binding makes toward the average residence time of interactions within the nucleus. In this model for Pit-1, DNA binding may contribute relatively little toward overall slower mobility of wild-type Pit-1, perhaps due to the rapid "hit and run" nature [McNally et al., 2000] of mostly non-specific interactions with monotonic DNA.

ACKNOWLEDGMENTS

The authors appreciate the technical assistance during the course of these studies by K. Patel, F. Herbert, and Hank Adams, and J. Orengo.

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