Partner-regulated interaction of IFN regulatory factor 8 with chromatin visualized in live macrophages

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IFN regulatory factor (IRF) 8 is a transcription factor that directs macrophage differentiation. By fluorescence recovery after photobleaching, we visualized the movement of IRF8-GFP in differentiating macrophages. Recovery data fitted to mathematical models revealed two binding states for IRF8. The majority of IRF8 was highly mobile and transiently interacted with chromatin, whereas a small fraction of IRF8 bound to chromatin more stably. IRF8 mutants that did not stimulate macrophage differentiation showed a faster recovery, revealing little interaction with chromatin. A macrophage activation signal by IFN- γ /LPS led to a global slowdown of IRF8 movement, leading to increased chromatin binding. In fibroblasts where IRF8 has no known function, WT IRF8 moved as fast as the mutants, indicating that IRF8 does not interact with chromatin in these cells. However, upon introduction of IRF8 binding partners, PU.1 and/or IRF1, the mobility of IRF8 was markedly reduced, producing a more stably bound component. Together, IRF8–chromatin interaction is dynamic in live macrophages and influenced by partner proteins and immunological stimuli.

real-time mobility | transcription factor | fluorescence recovery after photobleaching

Gene expression in immune cells is controlled by binding of transcription factors to chromatin targets. A classic view is that active transcription factors stably bind to the chromatinized promoter to drive transcription, a view mostly derived from biochemical studies *in vitro*. With the advent of live cell technologies, the views on the behavior of transcription factors are changing. Studies by fluorescence recovery after photobleaching (FRAP) of many nuclear proteins show that they are highly mobile and only transiently interact with chromatin (1, 2). Proteins showing rapid mobility include general transcription factors, chromatin modifiers, and DNA replication factors (3–9). Some DNA-specific transcription factors, such as nuclear hormone receptors, are also highly mobile in live nuclei (10–12). In addition, Stat1 (signal transducer and activator of transcription 1), a transcription factor that regulates IFN responses, is shown to be mobile before and after translocation into the nucleus (13), although E2F and retinoblastoma protein appear to be more stationary (14). In contrast, core histones, stable components of chromatin, are essentially immobile, showing little recovery after photobleaching (15). A consensus emerging from these studies is that FRAP recovery primarily reflects interactions of nuclear proteins with chromatin and the surrounding genomic DNA (hereafter referred to as chromatin) (16). Nevertheless, photobleaching technologies are still in their early phase of application, and mechanisms regulating FRAP mobility are not fully understood. FRAP mobility might reflect an intrinsic chromatin-binding property of a protein. However, the mobility may be influenced by other factors, such as soluble molecular constituents and structural components of the nucleus. In addition, most transcription factors associate with partner proteins and are assembled into macromolecular complexes, but the effects of

protein–protein interactions on fluorescence recovery have not been extensively studied. Moreover, many FRAP studies so far reported are qualitative, lacking quantitative insight into the chromatin-binding events. More recent efforts to fit FRAP data to mathematical models begin to provide a clearer knowledge on the property of FRAP mobility (16, 17).

IRF8, a member of the IFN regulatory factor (IRF) family, is a key factor that guides the development of macrophages (18). We previously established an *in vitro* model system where *IRF8*^{−/−} myeloid progenitor cells called Tot2 differentiate into macrophages upon IRF8 introduction (19). Concomitant with this differentiation, Tot2 cells become responsive to IFN- γ /LPS, a macrophage-activating signal that triggers expression of genes that are important for innate immunity. Without IRF8, Tot2 cells remain undifferentiated and grow continuously. IRF8 interacts with partner proteins, PU.1 and IRF1, to regulate target gene expression (20–22). By interacting with PU.1, IRF8 can bind to the EICE (Ets/IRF composite element) *in vitro*; interaction with IRF1 allows IRF8 to bind to the IFN-stimulated response element. Both interactions require the C-terminal IRF association domain (IAD) and the DNA-binding domain (DBD) of IRF8 (20, 23). PU.1 also binds to IRF4, a factor structurally similar to IRF8 (24–26). IRF8, IRF1, and PU.1 are assembled together on some promoters in activated macrophages (18, 27–31).

The present study describes a dynamic behavior of IRF8 as it functions in Tot2 macrophages. We show that the majority of IRF8 is moving very fast while transiently and repeatedly binding to the chromatinized genome in macrophages. There was a small fraction of IRF8 that stayed longer on chromatin, the observation supported by mathematical modeling. This “stop-and-go” type of movement was markedly altered when cells were stimulated by a macrophage activation signal. In addition, we show that an interaction with partner proteins, PU.1 and IRF1, critically affects the mobility of IRF8, thereby providing a mechanism that regulates IRF8–chromatin interactions. To our knowledge, this report is the first to describe a real-time movement of a transcription factor in functioning immune cells.

Materials and Methods

DNA Constructs. Full-length mouse IRF8 cDNA was cloned into pEGFP-N3 vector (Clontech) after TAA deletion at the XhoI/BamHI site. The insert was then recombined into the retroviral MSCV (murine stem cell virus) vector through the XhoI/NotI site (19). The K79E and R289E mutants (19, 32) were inserted into the MSCV vector as above. Full-length murine PU.1 cDNA was cloned into MSCV through the EcoRI site. MSCV-IRF8–

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Abbreviations: IRF, IFN regulatory factor; FRAP, fluorescence recovery after photobleaching; EICE, Ets/IRF composite element; IAD, IRF association domain; DBD, DNA-binding domain; MSCV, murine stem cell virus; BiFC, bifluorescence complementation; YFP, yellow fluorescent protein; BFP, blue fluorescent protein.

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CD8t was generated by cloning IRF8 cDNA into a region upstream of the internal ribosome entry site element in the MSCV-CD8t vector (33).

Cells and Transduction. The derivation and maintenance of Tot2 cells are described in ref. 19. Macrophage differentiation was induced by transduction of Tot2 cells with WT-IRF8 vector as described in ref. 19. To induce macrophage differentiation in Tot2 expressing the mutants (K79E-GFP or R289E-GFP), cells were retransduced with the MSCV-IRF8-CD8t vector and selected with the magnetic cell sorting system by using anti-CD8 antibody. NIH 3T3 cells were maintained in DMEM (Cellgro, Mediatech, Washington, DC) with 10% FBS and antibiotics. Cells were transduced with indicated retrovirus by spinoculation ($1,300 \times g$ at 32°C for 1 h) with $4 \mu\text{g/ml}$ polybrene and selected with $2 \mu\text{g/ml}$ puromycin 48 h after spinoculation. Tot2 cells were stimulated with IFN- γ at 200 units/ml and LPS from *Escherichia coli* (Sigma) at 200 ng/ml for 4 h.

FRAP. Cells plated on a chambered coverslip in 1% methylcellulose (Methocult, Stem Cell Technologies, Vancouver) were kept at 37°C by using an air stream stage incubator (ASI 400, Nevtek, Burnsville, VA). Live-cell imaging was performed on a Zeiss 510 confocal microscope by using the 488-nm line of an Ar laser with a $\times 100$, 1.3-numerical aperture oil immersion objective. Photobleaching was carried out on a small circular area (0.5- μm radius, 25 pixels) in the nucleus at the maximum laser power. Thirty prebleach images were acquired before a bleach pulse of 115 ms. Fluorescence recovery was monitored at low laser intensity (0.2% of a 45-mW laser) at 45-ms intervals for 16 s. FRAP experiments were performed on at least 15 independent cells, and data were averaged to generate a single FRAP curve. FRAP data were analyzed to fit to the recently developed mathematical binding models (17).

Bifluorescence Complementation (BiFC). To construct plasmids for BiFC (34), we first prepared the YNN, YNC, and YCC vectors from EYFP-C1 and E-YFP-N1 (Clontech). The YNN vector contained the N-terminal half of enhanced yellow fluorescent protein (YFP) (YN, amino acids 1–154) followed by a multicloning site (MCS); the YNC vector contained a MCS followed by YN with a stop codon. The YCC contained a MCS followed by the C-terminal half of enhanced YFP (YC, amino acids 155–238) with a stop codon. IRF8-YC, IRF8K79E-YC, and IRF8R289E-YC were generated by inserting IRF8 or its mutant cDNAs into the YCC vector after removing their stop codons. PU.1-YN was constructed by inserting PU.1 cDNA into YNN. IRF1-YN was constructed by inserting IRF1 cDNA into YNC after deletion of the IRF1 stop codon. YN, YC, and other cDNAs were prepared either by PCR using Pfu polymerase or restriction enzyme digestions. Cells were transfected with 0.25 μg of YFP constructs and 0.5 μg of pEBFP (Clontech) for 24 h. Cells were allowed to stand at 30°C for 1 h, and YFP signals were viewed on a confocal microscope.

Results

IRF8-GFP Stimulates Macrophage Differentiation in Tot2 Myeloid Progenitor Cells. With the aim of studying how IRF8 moves in the nucleus and interacts with chromatin and chromatinized targets in differentiating macrophages, WT IRF8 and two mutants, K79E and R289E, were labeled with GFP (Fig. 1A) and introduced into *IRF8*^{-/-} Tot2 cells. Each mutant carries a single amino acid substitution either in the DBD or the IAD. Unlabeled WT-IRF8, but not the mutants, induces macrophage differentiation in Tot2 cells (19, 32). Upon introduction, WT-IRF8-GFP uniformly distributed in the nucleus (with the exception of nucleoli), with little GFP signal in the cytoplasm (Fig. 1B). Cells underwent macrophage differentiation in a manner

Fig. 1. Induction of macrophage differentiation by IRF8-GFP. (A) IRF8-GFP constructs tested in FRAP. WT and mutant IRF8 were labeled with GFP at the C terminus in the MSCV vector. The DBD and IAD are marked. K79E and R289E carry a point mutation in the indicated position. (B) General morphology of Tot2 cells and distribution of IRF8-GFP, K79E-GFP, and R289E-GFP was examined 6 days after transduction. (Top) Wright–Giemsa staining. (Middle) GFP distribution. (Bottom) GFP with DAPI (DNA) staining. (C) Expression of macrophage-specific *c-fms* and *scavenger receptor (SR)* mRNA was examined by semiquantitative RT-PCR after transduction with indicated vectors.

identical to that observed with the unlabeled counterpart: Within a few days, Tot2 cells became adherent and increased cytoplasmic areas with altered nuclear architecture (Fig. 1B). Macrophage differentiation was further confirmed by the expression of macrophage-specific genes, *c-fms* and *scavenger receptor* (Fig. 1C), and gaining of the responsiveness to IFN- γ /LPS to induce IL-12p40 and inducible NO synthase after IRF8 transfer (19) (not shown). The levels of IRF8 expressed in Tot2 cells were regarded as physiological, because they were below the levels of endogenous IRF8 in a macrophage cell line, RAW264 cells (19). The K79E-GFP and R289E-GFP, in contrast, distributed in both the cytoplasm and the nucleus and did not induce macrophage differentiation. In our rough estimation, WT-IRF8 and the mutants were expressed at similar levels in the nucleus (Table 1, which is published as supporting information on the PNAS web site). These data validated the use of GFP-labeled IRF8 for further study.

FRAP Analysis Reveals Two Distinct Binding Components for IRF8. A small circular area within the nucleus was briefly photobleached, and the recovery of fluorescent signal was measured every 45 ms for 16 s. Fig. 24 depicts kinetics of fluorescence recovery observed with WT-IRF8 and the mutants. Most WT-IRF8 recovered within 6 s after photobleaching, indicating that the great majority of IRF8 moved very rapidly in the nucleus. Although rapid, this recovery was significantly slower than free GFP alone (<1s; not shown), revealing binding events for IRF8. Importantly, fluorescence recovery plateaued at $\approx 90\%$, indicating the existence of a small pool of IRF8 that moved more slowly. To test whether fluorescence recovery is a function of IRF8-GFP expression levels, we performed FRAP assays with two groups of cells, one expressing IRF8-GFP at low levels and the other expressing IRF8-GFP at higher levels (about twice, according to GFP signals). The recovery patterns were virtually identical in the two groups (Fig. 7, which is published as supporting information on the PNAS web site). Thus, the variation of IRF8-GFP levels in Tot2 cells did not affect the FRAP mobility pattern,

which is expected of the nuclear environment where chromatin is in large excess relative to a transcription factor (17).

To gain quantitative information on IRF8 mobility, the recovery data were fitted to the mathematically derived FRAP models that account for diffusion and binding events (17). IRF8 recovery curves were best fit to the two-binding-state model, consistent with the interpretation that IRF8 mobility is composed of two fractions: 85% of IRF8 molecules were weakly and transiently bound to chromatin, whereas 11% were more stably bound to chromatin. The average time of binding for the fast-moving component was estimated to be <0.1 s, whereas that of the latter was >25 s. The remaining 4% of IRF8 were of freely diffusing species (Fig. 2B; for details, see Table 2, which is published as supporting information on the PNAS web site). It is of note that when FRAP measurements were extended beyond 40 s, IRF8 recovered almost fully, indicating that, unlike core histones, even the more stably bound IRF8 is mobile but exchanges more slowly than the faster moving counterpart (Fig. 8, which is published as supporting information on the PNAS web site). As seen in Fig. 2A, recovery of the mutants was faster than that of WT-IRF8 and reached 100% within 4 s after bleaching. These data are consistent with the minimal interaction of the mutants with chromatin. Analysis of these data with the above models revealed that, in contrast to WT-IRF8, only a single binding state predominated in these mutants (Table 2). We noted that the two mutants showed a slight difference in the recovery profiles, in that R289E recovered slightly more slowly than K79E, and data fit also to the models. Mobility of a protein is influenced by various factors, such as soluble macromolecules and other structural components in the nucleus (35). The faster recovery of IRF8 mutants compared with WT-IRF8 (Fig. 2A) may be attributed to a possible difference in their environment rather than an inherent difference in mobility. Tot2 cells expressing the mutants were in the progenitor state, different from macrophages where WT-IRF8 was expressed. It was therefore important to study the mobility of the mutants and WT-IRF8 in the same cellular environment. To this end, Tot2 cells were first transduced with the mutants and then retransduced with WT-IRF8, which allowed cells expressing the mutant to differentiate into macrophages. If the mobility is a reflection of the environment in which the mutants were placed, they should move as

slowly as WT-IRF8 in differentiating macrophages. In FRAP analyses in Fig. 2 *C* and *D*, both mutants recovered equally fast in undifferentiated and differentiating cells. These results indicate that IRF8 fluorescence recovery is not a measure of cellular environment but signifies its intrinsic mobility. To investigate whether the mobility of IRF8 changes during Tot2 differentiation, FRAP analyses were performed on each day for 6 days after IRF8 transduction. Although differentiation was not synchronous, cells on days 2–3 tended to show early to intermediate morphology, whereas those on days 4–6 tended to display a morphology of more advanced macrophage differentiation (19). FRAP profiles tested on different days were virtually identical for both WT and mutant IRF8, indicating that the global pattern of IRF8–chromatin interaction does not change during differentiation (data not shown). FRAP experiments were performed on day 4 hereafter.

A Macrophage Activation Signal Globally Alters IRF8 Mobility. A combination of IFN- γ and LPS constitutes a macrophage activation signal that triggers large changes in gene expression in macrophages (36). To study whether macrophage activation affects IRF8 mobility, FRAP analysis was performed with cells stimulated with IFN- γ and LPS for 4 h. After stimulation, WT-IRF8 appeared to redistribute within the nucleus: GFP signals were seen in small speckles, rather than in uniform distribution as observed before treatment (Fig. 3A). The mutants, however, were distributed uniformly in both the cytoplasm and nucleus before and after treatment. FRAP analysis revealed a significant delay in WT-IRF8 recovery after IFN- γ /LPS, as evidenced by a right shift in the recovery curve (Fig. 3A). Furthermore, the recovery reached a plateau at $\approx 75\%$, lower than that of untreated cells (90%). In contrast, recovery of the IRF8 mutants was unaffected by IFN- γ /LPS (Fig. 3B and C). Thus, IFN- γ /LPS lowered the overall mobility of WT-IRF8 (but not mutants) and additionally increased a stably bound component. The alteration of IRF8 mobility likely represents genome-wide changes in IRF8–chromatin interactions.

IRF8 Moves Faster in Fibroblasts. To gain insight into a mechanism by which the mobility of IRF8 is regulated, we performed FRAP analysis in NIH 3T3 cells where endogenous IRF8 is not

Fig. 3. Alteration of IRF8 mobility after IFN- γ /LPS stimulation. FRAP analysis was performed with Tot2 cells expressing WT-IRF8 (A) or mutants (B and C) treated with IFN- γ /LPS for 4 h. (Right) Distribution of IRF8 or the mutants in untreated or IFN- γ /LPS-treated cells.

expressed. As shown in Fig. 4B, WT-IRF8 distributed both in the nucleus and cytoplasm in these cells, although the nucleus showed higher fluorescence intensity than the cytoplasm. The mutants distributed in the nucleus and cytoplasm with similar intensity. As expected, transduction of IRF8 constructs did not alter morphology and growth properties of NIH 3T3 cells during the period we tested. In FRAP analysis, WT-IRF8 recovered with surprising rapidity, reaching virtually 100% in 5–6 s, a recovery clearly faster than in Tot2 cells (Fig. 4A). The FRAP curve of WT-IRF8 was superimposable with those of the two mutants, which also recovered very rapidly. Model fitting indicated that IRF8 had only a single weak binding state as a predominant form, consistent with little interaction of IRF8 with chromatin in NIH 3T3 cells.

IRF8 Interacts with PU.1 and IRF1 in Live NIH 3T3 Cells. Among other possibilities, we postulated that the mobility of IRF8 depends

Fig. 4. FRAP analysis in NIH 3T3 cells. (A) FRAP analysis was performed with NIH 3T3 cells transduced with WT-IRF8 and the indicated mutants. (B) Distribution of IRF8-GFP in NIH 3T3 cells viewed 4 days after transduction.

Fig. 5. Visualization of IRF8-partner interactions in live NIH 3T3 cells. (A) Cells were transfected PU.1-YN plus IRF8-YC, K79E-YC, or R289E-YC along with pEBFP. YFP complementation was detected 24 h after transfection. Reconstituted YFP signals are shown in Upper. YFP and BFP signals are merged in Lower. (B) Cells were transfected with IRF1-YN plus IRF8-YN or mutants, and BiFC experiments were performed as above. (C) Efficiency of BiFC. Transfection efficiency (blue bars) was estimated by counting BFP⁺ cells. BiFC efficiency was estimated by counting YFP⁺ cells (yellow bars). More than 500 cells on several independent fields were counted.

on its interaction with partner proteins. In this scenario, IRF8 does not efficiently interact with chromatin in NIH 3T3 cells, because partner proteins are not expressed at sufficient levels. Plausible candidates for partners that might influence IRF8 mobility are PU.1 and IRF1 (20). PU.1, an immune system-specific Ets member, is constitutively expressed in Tot2 cells and forms a complex with IRF8 to bind to the composite element EICE *in vitro* (19), as has been shown for IRF4 (24–26). PU.1 is not expressed in NIH 3T3 cells. IRF1 is expressed in Tot2 cells at a low level and induced by IFN- γ and LPS. IRF8 interacts with IRF1 to bind to the IFN-stimulated response element (ISRE) (20, 23). The K79E and R289E mutants, due to a defective DBD or IAD, respectively, do not bind either to EICE or ISRE *in vitro* (19).

Before testing the effect of the partners on IRF8 mobility, it was necessary to ascertain whether IRF8 and the partners interact with each other in fibroblasts. Although the interaction of IRF8 with these partners was documented *in vitro*, it has not been demonstrated in live cells. To study a real-time interaction of IRF8 with the partners, we used the BiFC assay (34, 37). In this method, YFP is split into the N-terminal and C-terminal halves, and each half is fused to a partner protein. Although the split YFP halves are nonfluorescent, they can complement each other to reconstitute YFP signals, provided that the two proteins interact with each other. The BiFC provides a simple method to visualize a protein–protein interaction in living cells. In Fig. 5A, the N-terminal half of YFP was fused to PU.1 (PU.1-YN), and the C-terminal half of YFP was fused to IRF8 and the mutants (IRF8-YC) and transfected into NIH 3T3 cells, along with pEBFP. The latter vector emits blue fluorescence and was included for transfection efficiency. Cotransfection of PU.1-YN and IRF8-YC produced intense fluorescence signals distributed throughout the nucleus (except nucleoli), indicating an interaction of the two proteins. In contrast, the R289E mutant did not reconstitute fluorescence signals, consistent with the previous *in vitro* results, which showed that the intact IAD is required for an interaction with PU.1. Interestingly, the K79E mutant reconstituted YFP signals in the nucleus, indicating an interaction with PU.1 *in vivo*. This result was somewhat unexpected, because

K79E fails to form a complex with PU.1 on the EICE *in vitro*, consistent with the requirement of the DBD for interaction (19, 26). As expected, no fluorescence signals were detected in cells transfected with PU.1-YN or IRF8-YC alone (data not shown). The combination of IRF1-YN and IRF8-YC also showed complementation between IRF1 and WT-IRF8 and K79E, but not R289E (Fig. 5B), further supporting the idea that IRF8 interacts with this partner through the IAD without requiring the DNA-binding activity *in vivo*. To confirm that transfection efficiency was comparable for all pairs of constructs and to assess the efficiency of complementation, we counted cells with YFP and blue fluorescent protein (BFP) signals. In Fig. 5C, transfection efficiency (BFP⁺ cells) was $\approx 45\%$ for all pairs, of which $\approx 70\%$ of cells generated YFP signals with both WT-IRF8 and K79E, illustrating highly efficient complementation. The transfection efficiency of R289E was similar to that of WT-IRF8 and K79E, verifying that the lack of complementation was not due to low transfection efficiency.

Partner Proteins Enhance Binding of IRF8 to Chromatin in NIH 3T3 Cells and Macrophages. FRAP assays were next performed in NIH 3T3 cells expressing PU.1 or IRF1. Cells were first transduced with GFP-labeled WT-IRF8 or mutants followed by the second transduction with an unlabeled partner. As judged by immunostaining, $>90\%$ of cells were positive for IRF8 and partners, attesting to high transduction efficiency (Fig. 6A and B Right). In FRAP assays in Fig. 6A, PU.1 expression significantly lowered IRF8 mobility, which was most noticeable in an early phase of recovery, although the recovery reached $\approx 100\%$ in later times. In contrast, alteration of FRAP profiles was not observed for the mutants, including K79E, even though this mutant interacted with PU.1 *in vivo* (Fig. 5A). FRAP experiments with IRF1 (Fig. 6B) likewise showed a reduction of WT-IRF8 mobility but not of mutants. Indeed, IRF1 caused a more pronounced slowdown in WT-IRF8 recovery compared with PU.1, and the recovery did not reach 100% in 16 s. These data indicate that PU.1 and IRF1 enhance an interaction of IRF8 with chromatin, provided that IRF8 has an intact DBD, pointing to the role of DNA-binding activity in regulating IRF8 mobility. Furthermore, FRAP analysis was performed in cells expressing both PU.1 and IRF1 (Fig. 6C). In the presence of both partners, IRF8 recovered even more slowly, where the recovery curve was further shifted to the right. The FRAP profiles were indicative of an additive effect of the two partners rather than a competition by them. These data indicate that PU.1 and IRF1 independently and together influence the mobility of IRF8 in NIH 3T3 cells.

Lastly, to ascertain whether a partner protein regulates IRF8 mobility in macrophages, FRAP analysis was performed with *IRF1*^{-/-} and *IRF1*^{+/+} macrophages. We found that IRF8-GFP was more mobile in *IRF1*^{-/-} macrophages than in their *IRF1*^{+/+} counterparts, where fluorescence recovery approached 100% (Fig. 9, which is published as supporting information on the PNAS web site).

Discussion

By FRAP analysis, we found that the majority of IRF8 was highly mobile in macrophage nuclei. In addition, there was a small, less mobile pool of IRF8 in these cells. The mathematical fitting of our data into the recently developed FRAP models validated these observations and showed that the behavior of IRF8 fits the best to a two-binding-state model. Thus, we estimate that $\approx 85\%$ of IRF8 is transiently interacting with chromatin and that 11% is more stably bound. In contrast, the K79E and R289E mutants both exhibited faster mobility than WT-IRF8. Not only did their recovery curve lack a slower, less mobile component, but the fast-recovering species showed a greater mobility than that of WT-IRF8, consistent with a negligible interaction with chromatin. Given that these mutants are transcriptionally defective,

Fig. 6. FRAP analysis after coexpression of IRF1 and PU.1. (A) (Left) FRAP analysis was performed with NIH 3T3 cells expressing WT-IRF8-GFP or mutant GFP alone or coexpressing unlabeled PU.1. (Right) Cells that had been analyzed for FRAP were fixed and stained for DNA (DAPI, Upper) or PU.1 (red) to confirm coexpression of PU.1. (B) FRAP analysis was performed with NIH 3T3 cells expressing WT-IRF8-GFP or mutant GFP alone or coexpressing unlabeled IRF1 and stained for DNA and IRF1 as above. (C) Cells coexpressing WT-IRF8 and one or two partners were tested for FRAP.

mobility of IRF8 appears to reflect transcriptional competence, correlating with the ability of IRF8 to interact with chromatin. The more stably bound WT-IRF8 species seen in FRAP may partly represent IRF8 binding to chromatinized targets in live nuclei. That even the fast-moving species of WT-IRF8 recovered more slowly than the mutants may indicate that this species, too, interacts with targets to some degree, although some of the species may be nonspecifically scanning chromatin (16, 17). It is of note that the slower recovery of WT-IRF8 does not necessarily imply active engagement in transcription. Rather, IRF8 mobility measured in FRAP may point mostly to a steady-state, genome-wide interaction with chromatin. We noted that FRAP profiles in Tot2 cells were very similar during 6 days of macrophage differentiation. Thus, this on-and-off interaction of IRF8 with chromatin is not restricted to certain stages of development and occurs continuously throughout macrophage differentiation. Together, the results indicate that IRF8 binds to chromatin in a highly dynamic fashion following two-phase binding kinetics. Our findings are compatible with the view that many transcription factors are constantly scanning the entire genome by transiently contacting chromatin and forming a global, rapidly reversible network (16).

The recovery profile of WT-IRF8 was markedly altered when Tot2 cells were exposed to IFN- γ and LPS. We observed not only

a delay in the initial phase of WT-IRF8 recovery but also an increase in the less mobile component. These data indicate that macrophage activation increases genome-wide interaction of IRF8 with chromatin. The signal-induced slowdown of IRF8 mobility is reminiscent of a ligand-induced delay in estrogen receptor mobility (11). Similar to IFN- γ /LPS, estrogen triggers a large change in transcription, altering functional activity of the cells. Thus, a signal-induced shift in the nuclear factor mobility likely represents a global modification of the interaction between a transcription factor and chromatin that is linked to changes in gene expression. At present, the mechanism by which IFN- γ /LPS alters IRF8 mobility is not fully evident. An increase in the expression level or posttranslational modification of partner proteins may explain the change. LPS is shown to increase phosphorylation of PU.1, whereas IFN- γ enhances expression of IRF8 and IRF1 (38, 39). Supporting the idea that partner proteins play a role in influencing IRF8 mobility, we found that the timing of delay in FRAP recovery after IFN- γ /LPS addition correlated with that of IRF1 induction (Fig. 10, which is published as supporting information on the PNAS web site).

Unlike the case in macrophages, WT-IRF8 recovered surprisingly rapidly in NIH 3T3 cells, showing almost the same mobility as that of the mutants. Clearly, IRF8 does not efficiently interact with chromatin in the fibroblasts where IRF8 partner proteins are not expressed at a significant level. Visualization of real-time interaction between IRF8 and PU.1/IRF1 by BiFC supported the idea that partner proteins influence IRF8 mobility. In BiFC, WT-IRF8 and the K79E mutant (but not R289E) interacted with both partners, suggesting that DNA-binding activity is not required for an interaction with either partner *in vivo* under these conditions, an observation differing from that seen *in vitro* (24–26). However, the possibility that reconstituted YFP altered

the affinity of K79E in our BiFC experiments cannot be excluded. The subsequent FRAP experiments support the importance of protein–protein interaction for IRF8 mobility in that ectopic expression of PU.1 and IRF1 in NIH 3T3 cells lowered the mobility of IRF8. It is significant that the mobility of K79E was unchanged by the expression of either partner, despite the fact that this mutant avidly interacted with both partners in BiFC. Thus, a protein–protein interaction is not sufficient for interaction of IRF8 with chromatin, and DNA-binding activity is required in addition. Our results are in line with the view that FRAP primarily measures an interaction of a nuclear protein with chromatin (16). When PU.1 and IRF1 were coexpressed, IRF8 recovery displayed a pattern consistent with an additive effect, indicating that the two partners are capable of interacting with IRF8 independently and jointly influencing IRF8 mobility. This observation may be in accord with the reports that IRF8, PU.1, and IRF1 form a ternary complex in some cases (29, 30).

In summary, this study offers a glance at the global behavior of IRF8 in live macrophages. IRF8 is constantly scanning chromatin in the entire nucleus with two distinct binding kinetics. The interaction of IRF8 with chromatin is governed by its interaction with partner proteins and is modulated by an immunological signal. Together, live cell technologies offer a means by which to view dynamic movement of a transcription factor as it acts during development and immune responses in the cell.

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