Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11831–11835, December 1994
Cell Biology

Nuclear accumulation of interferon γ

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Communicated by Elwood V. Jensen, June 7, 1994

ABSTRACT **Examination of the interferon γ (IFN-γ) amino acid sequence revealed two conserved basic amino acid clusters similar to the prototype nuclear localization signal. We followed the fate of cell surface receptor-bound IFN-γ in murine leukemia L1210 cells. A time- and temperature-dependent accumulation of murine IFN-γ in the cell nucleus could be demonstrated by autoradiography and indirect immunofluorescence after the rapid isolation of nuclei. Human IFN-γ was also internalized and translocated to the nucleus of murine L1210 cells transfected with and expressing the human IFN-γ receptor, but it appeared to be retained by the nucleus only transiently. IFN-γ molecules chemically crosslinked to their cell surface receptor remain capable of being translocated to the nucleus even as part of a receptor–ligand complex. Thus, the bipartite nuclear localization signal sequence appears to be functional and suggests that nuclear targeting could participate in IFN-γ signal transduction.**

Murine and human interferon γ (MuIFN-γ, HuIFN-γ) elicit similar cellular responses after binding to their species-specific cell surface receptors and subsequent internalization. Among these effects are antiproliferative and antiviral activity, modulation of surface antigens, and immunoregulation (1–3). Whether internalization of IFN-γ is required for biological activity is still unclear. It was previously shown that binding to its receptor is not sufficient for HuIFN-γ to elicit a physiologic response in murine cells (4, 5). Nevertheless, biological activity was observed after either microinjection of HuIFN-γ (6) or the direct intracellular production of HuIFN-γ in murine cells (7). Earlier work suggested that interferon would bind to nuclear membrane receptors (8, 9). More recently, our understanding of post-cell-membrane receptor signaling pathways has increased and nuclear targeting of hormones and growth factors has been demonstrated (10). Analysis of nuclear protein structure has uncovered a consensus sequence of basic amino acids required for efficient transport from the cytosol to the nucleus. This amino acid sequence, the prototype of which is the basic sequence found in the large tumor antigen of simian virus 40 or nucleoplasmin (reviewed in ref. 10), is termed the nuclear localization signal (NLS). Close examination of murine and human IFN-γ sequences revealed two conserved stretches of basic amino acids similar to the NLS (11). Furthermore, crystallographic analysis of HuIFN-γ indicated that these stretches of basic amino acids are exposed on the surface of the protein (12). Antibodies directed against the first basic epitope, Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Gln-Lys [residues 84–94 of HuIFN-γ) (12), abolish biological activity without inhibiting binding to its receptor (11). Recent analysis of HuIFN-γ analogs showed that the second (C-terminal) basic cluster (residues 128–131 of HuIFN-γ) is equally essential for a biological response and that partial or complete removal of this cluster results in drastic loss of activity (13). Thus, we asked whether the conserved clusters of basic amino acids function as NLSs *in vivo*—that is, whether IFN-γ is targeted into the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture. L1210 murine leukemia cells and transfected L1210 cells [clone 2/10-14, containing the entire gene for the HuIFN-γ receptor (4)] were cultured as previously described (3).

Binding Assays. ^{125}I-labeled IFN-γ (^{125}I-IFN-γ) binding assays and acid elutions were performed on 2–5 × 10^7 cells as previously described (2, 3).

Isolation of Nuclei. Suspensions of 2–5 × 10^7 cells in 1 ml of culture medium were laid over 2 ml of 20% Ficoll-Paque (Pharmacia) in lysis buffer [PBS/10 mM $MgCl_2$/10 mM $CaCl_2$/0.1% dimethyl sulfoxide/2% (vol/vol) Nonidet P-40/1.6% (vol/vol) Triton X-100, pH 7.4] all Sigma, supplemented with 5% fetal bovine serum (FBS) and centrifuged at 200 × *g* for 8 min at 4°C. After aspiration of supernatant, pellets containing nuclei were resuspended in lysis buffer without FBS. At this point preparations were examined for cytoplasmic lactate dehydrogenase (LDH) as an indicator of contaminating cytoplasm and were found to be LDH negative.

Autoradiography. Cells or nuclei were incubated at saturating (≥3 nM) concentrations of ^{125}I-MuIFN-γ or ^{125}I-HuIFN-γ at 4°C or 37°C for 2 h. Cells and nuclei were subsequently washed in PBS, pH 7.4, containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$, and allowed to adhere to polylysine-coated glass chamber slides (Nunc) for 15 min. After removal of supernatant, cells were fixed with paraformaldehyde (4% in PBS) for 3 min, rinsed copiously with 5% FBS in PBS, and dried. Slides were coated with NTB-2 photographic emulsion (Kodak), developed at 48 h intervals, and counter-stained with Giemsa.

Indirect Immunofluorescence. Cells fixed as above were permeabilized (0.1% Nonidet P-40, 10 min at 4°C) and incubated with a rabbit anti-recombinant MuIFN-γ polyclonal antiserum raised in our laboratory at a dilution of 1:100. The secondary antibody, a fluorescein isothiocyanate (FITC)-goat anti-rabbit antibody (Sigma), was used at a 1:50 dilution. DAPI (4′,6-diamidino-2-phenylindole) solutions for staining nuclei were adjusted to 10 ng/ml to give similar light intensity as specific signals. Prior to microscopy (Olympus IMT2), cells were embedded in 50% glycerol in PBS, pH 8, containing *p*-phenylenediamine at 1 mg/ml to reduce photobleaching (14). Photographs are pseudocolor representations of digitized images acquired after intensification (equal gain for all pictures) and background subtraction as described (15).

Crosslinking of IFN-γ to Its Receptor. Cells were treated with saturating concentrations (≥3 nM) of murine or human ^{125}I-IFN-γ (2 h, 4°C), washed twice with PBS (pH 7.4,

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Abbreviations: FITC, fluorescein isothiocyanate; Hu, human; IFN-γ, interferon γ; LDH, lactate dehydrogenase; Mu, murine; NLS, nuclear localization signal.

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supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$), and crosslinked with disuccinimidyl suberate (DSS) as described (2, 3). After 15 min cells were pelleted and resuspended in 1 ml of medium. Nuclei were immediately prepared for the first time point (0.25 h) together with noncrosslinked controls. All other samples were cultured for 2, 4, 8, or 20 h at 37°C before nuclei were isolated. After two washes in lysis buffer, nuclear pellets were frozen at −20°C until SDS/PAGE (16) and autoradiography (3).

RESULTS

The presence of two potential nuclear targeting sequences within the IFN-γ molecule prompted us to investigate the possible nuclear translocation of both murine and human IFN-γ in parental L1210 murine leukemia cells and transfected L1210 cells containing the complete HuIFN-γ receptor gene (4). In an initial time course study, cells were allowed to bind ^{125}I-MuIFN-γ at 4°C for 2 h. By taking advantage of a protocol for the rapid isolation of nuclei, we detected nuclear accumulation of externally bound ^{125}I-MuIFN-γ at the earliest time after a shift from 4°C (binding) to 37°C (internalization). To ensure that only internalized ^{125}I-MuIFN-γ was measured in isolated nuclei, cells were acid washed (2) to elute all externally bound ^{125}I-IFN-γ prior to nuclear isolation. Fig. 1*A* shows the time-dependent internalization of ^{125}I-MuIFN-γ in L1210 cells (*Inset*) and the parallel accumulation of ^{125}I-MuIFN-γ in the nucleus. Maximal uptake by the cells (60–90 min) preceded the plateau reached in the nucleus. The autoradiogram in Fig. 1*B* shows ^{125}I-MuIFN-γ in lysates of cells (lane 1). ^{125}I-MuIFN-γ accumulated in nuclear extracts (lanes 3–15) for at least 4 h. A 50-fold excess of unlabeled ligand competes with ^{125}I-MuIFN-γ for binding to cells at 4°C (lane 2) and translocation into nuclei at 37°C (lane 16). The excess unlabeled ligand reduces but does not eliminate the signal. Contamination of the nuclear fraction with ^{125}I-MuIFN-γ seems unlikely, as nuclei prepared under similar conditions without acid elution at 4°C showed insignificant labeling (see Fig. 2 *A* and *B*, C→N). Similar experiments employing ^{125}I-HuIFN-γ resulted in faster nuclear accumulation with subsequent loss (data not shown, compare Fig. 4).

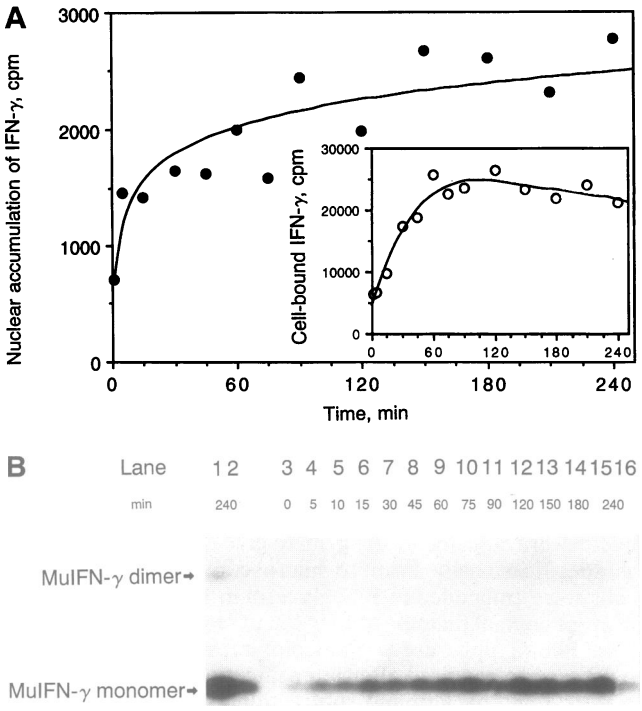

FIG. 1. (*A*) Kinetics of ^{125}I-MuIFN-γ accumulation in the nuclei of L1210 cells (●) at 37°C as compared to specifically internalized ^{125}I-MuIFN-γ (○, *Inset*). (*B*) Autoradiograph of an SDS/polyacrylamide gel containing cell extracts prepared at 4°C (lanes 1 and 2) and nuclear extracts (lanes 3–16) prepared at different times after a temperature shift to 37°C. Specific binding at 4°C was ≈50,000 cpm per 2 × 10^6 cells per tube. L1210 cells in lane 2 and lane 16 were incubated in the presence of a 50-fold excess of unlabeled MuIFN-γ. Control cells maintained at 4°C were not acid-eluted (lanes 1 and 2). All other cells were shifted to 37°C and acid-eluted prior to nuclear isolation (lanes 3–16).

In a different approach, we followed ^{125}I-labeled murine and human IFN-γs through cells by autoradiography, varying the temperature and comparing cells with isolated nuclei. We investigated the binding (4°C) and internalization (37°C) of ^{125}I-IFN-γs on whole cells, to ensure a receptor-mediated uptake of IFN-γ, or on isolated nuclei, circumventing receptor-dependent steps. Fig. 2 shows four panels each of which is subdivided into six subpanels for murine and human IFN-γ at 4°C and 37°C. The right side of each panel shows cells or nuclei after receptor saturation with the indicated ^{125}I-IFN-γ, while the left side of each panel shows control cells or nuclei that received, in addition to the labeled IFN-γ, a 50-fold excess of the respective unlabeled IFN-γ. The 50-fold excess of unlabeled IFN-γ gives a comparable specific signal reduction for both murine and human IFN-γ. Fig. 2 *A* and *B* represents the results at 4°C, a temperature that prevents ligand internalization. Specific labeling can be found peripherally and on the cells with both murine (*A*) and human (*B*) ^{125}I-IFN-γ. Microscopic inspection suggests a surface rather than cytoplasmic localization (Fig. 2*B*, ^{125}I-HuIFN-γ-treated cells). Nuclei, prepared after murine or human ^{125}I-IFN-γ had bound to the cell surface receptor at 4°C, are poorly labeled compared with their controls (central subpanel), which is in agreement with the inability of receptors to internalize with bound ligand at this temperature. In contrast, nuclei incubated with murine or human ^{125}I-IFN-γ at 4°C were able to specifically associate with either IFN-γ (Fig. 2 *A* and *B*, *Bottom Right*) in contrast to controls which show random grains (Fig. 2 *A* and *B*, *Bottom Left*).

Fig. 2 *C* and *D* represents similar experiments but performed at 37°C. At this temperature, receptor-bound ^{125}I-IFN-γ is internalized and may be translocated to the nucleus. Fig. 2*C* shows pronounced specific labeling of L1210 cells (*Top Right*) and nuclei that received ^{125}I-MuIFN-γ via its receptor (*Middle Right*) as compared with the respective controls (*Top Left* and *Middle Left*, respectively). Treatment of isolated nuclei (*Bottom Right*) with ^{125}I-MuIFN-γ permitted direct nuclear labeling. The same is true for ^{125}I-HuIFN-γ (Fig. 2*D*, *Bottom Right*). Moreover, transfected L1210 cells showed substantial nuclear labeling at 37°C (Fig. 2*D*, *Top Right*), but not at the surface, in agreement with the suggested mechanism of receptor-mediated ligand internalization followed by nuclear accumulation. Nuclei, isolated after ^{125}I-HuIFN-γ had bound to the cells, were labeled heavily compared to controls (Fig. 2*D*, *Middle Right*, and *Left*, respectively). Nuclei were isolated in the presence of detergents and tested for the presence of LDH, a cytoplasmic marker. The absence of LDH suggests that no cytoplasmic or membrane-derived proteins associated unspecifically with radioactive ligand.

To confirm that IFN-γ accumulates in the nucleus and to further exclude any possible contamination of nuclei with IFN-γ during isolation, a nondisruptive approach was chosen to follow MuIFN-γ in L1210 cells. Cells were treated with MuIFN-γ incubated at 4°C or 37°C for 2 h, washed, and fixed. To detect MuIFN-γ, polyclonal anti-rMuIFN-γ antibody was used for indirect immunofluorescence after amplification with a FITC-conjugated secondary antibody. Fig. 3 shows that a specific fluorescence signal can be found mainly on the surface of cells at 4°C (*Middle Left*) but the signal colocalized with nuclei at 37°C (*Bottom Left*). Shown in the top row for comparison is one of several controls, cells at 4°C that were

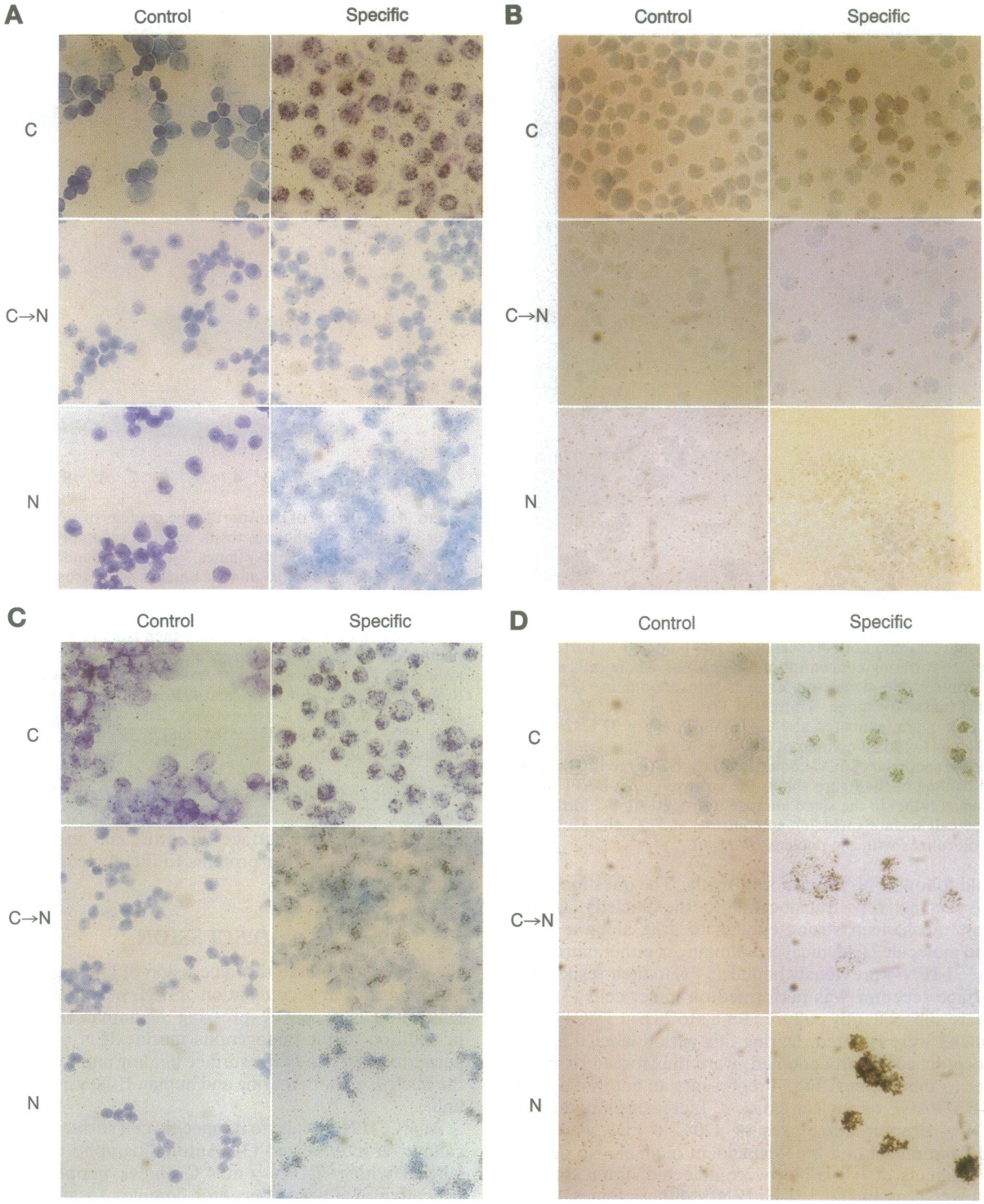

FIG. 2. Autoradiography of L1210 cells after binding of ^{125}I-MuIFN-γ at 4°C (*A*) and 37°C (*C*) and of transfected L1210 cells expressing the HuIFN-γ receptor after binding ^{125}I-HuIFN-γ at 4°C (*B*) and 37°C (*D*). Controls were treated with a 50-fold excess of the appropriate unlabeled IFN-γ to give a comparable (between murine and human IFN-γ) reduction of the signal without eliminating it. Binding was performed at the receptor-saturating concentration (≥3 nM) on either whole cells (C, C→N) or isolated nuclei (N). Shown are cells (C), nuclei prepared after receptor-mediated IFN-γ binding (4°C) and internalization (37°C) (C→N), and nuclei directly exposed to ^{125}I-IFN-γ (N) at the respective temperatures.

not treated with MuIFN-γ. These results show that the temperature-dependent nuclear accumulation of IFN-γ does occur in intact cells and suggest directional transport as the mechanism rather than diffusion.

Neither autoradiography nor indirect immunofluorescence can conclusively discriminate between nuclear-membrane-associated IFN-γ or intranuclear IFN-γ. Furthermore, a small protein like IFN-γ (MuIFN-γ, 16 kDa; HuIFN-γ, 17 kDa) might, even as dimer (12, 17), diffuse across the nuclear membrane rather than being guided by a NLS (18–21). Inhibitors of the translocation step could not be employed since they interfered with the ability of the cells to internalize IFN-γ (data not shown). However, because L1210 cells do not rapidly degrade the two IFN-γs (refs. 2 and 3 and Fig. 4),

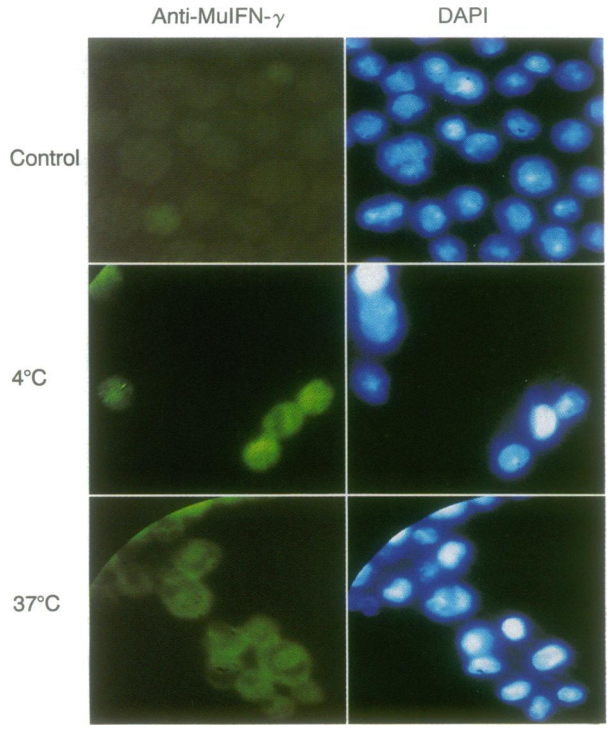

FIG. 3. Nuclear localization of MuIFN-γ in L1210 cells visualized by indirect immunofluorescence. IFN-γ binding was performed at receptor saturating concentration (≥3 nM) on whole cells except for the control. No signal was evident with preimmune serum or secondary antibody alone. Untreated (*Top Row*) and MuIFN-γ-treated cells were incubated at 4°C (*Middle Row*) or 37°C (*Bottom Row*). The left vertical column of the figure shows the pseudocolor signal of the secondary FITC-conjugated antibody (green). The right vertical column of the figure shows the corresponding DAPI signal of the nuclei (blue) in the same microscopic field. At 4°C MuIFN-γ labels the cell surface (*Middle*). In contrast, at 37°C, the MuIFN-γ signal colocalizes with the nucleus (*Bottom*).

we could follow IFN-γ transport directly. The question was: If IFN-γ is actively translocated to the nucleus, is the transport mechanism strong enough to drag along a large attached molecule? Chemical crosslinking of either murine or human ^{125}I-IFN-γ previously bound at 4°C to their respective cell surface receptor was performed on intact cells (3, 21). Cells were subsequently cultured for up to 20 h at 37°C before their nuclei were isolated. During this period labeled ligand and receptor, covalently coupled, were thus forced to move together to the nucleus. Fig. 4 represents an autoradiogram after SDS/PAGE of nuclear extracts. Increasing amounts of ^{125}I-IFN-γ–receptor complexes of 120–130 kDa were extracted from nuclei in a time-dependent manner. No degradation products of ^{125}I-IFN-γ were detected. Interestingly, while fewer crosslinked ^{125}I-HuIFN-γ dimers (lanes 8–12) (17) were present after 2 h, ^{125}I-MuIFN-γ (lanes 2–6) persisted as surface-derived, crosslinked dimer in the nuclei. Also, only ^{125}I-MuIFN-γ showed an additional faint band of an apparent molecular mass of 37–40 kDa which was consistently observed but which did not increase with time, arguing against it being a degradation product.

Although ligand internalization is receptor-mediated, an intracellular association of IFN-γ with its receptor might not be necessary since a significant amount of free ligand (monomer and crosslinked dimer) was detected in nuclear extracts within 15 min. This agrees with the kinetics described in Fig. 1 and suggests that the nuclear accumulation process is due to a signal indigenous to IFN-γ and not the receptor. Moreover, this experiment demonstrates that IFN-γ does not simply diffuse into the nucleus, but must be actively transported due to the high molecular mass of the IFN-γ–receptor complex. This observation implies that IFN-γ, once internalized through the surface receptor, may even serve as a carrier to deliver other proteins to the nucleus under physiological conditions.

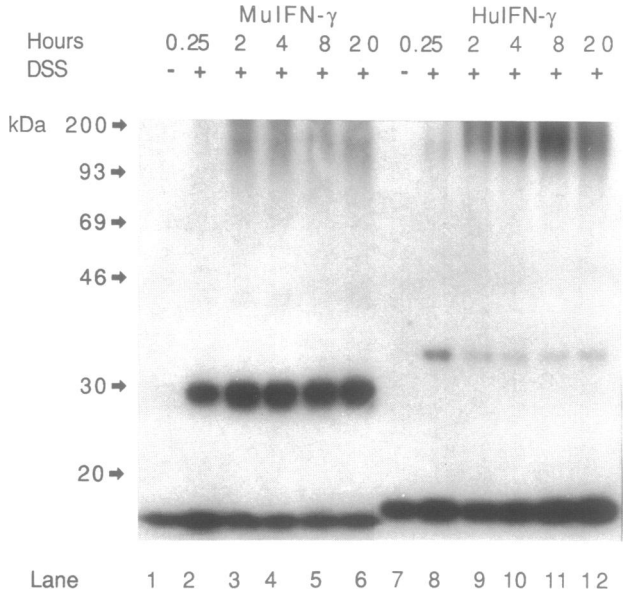

FIG. 4. Kinetics of transport to the nucleus of ^{125}I-labeled ligand after chemical crosslinking to its specific receptor. ^{125}I-MuIFN-γ (lanes 1–6) or ^{125}I-HuIFN-γ (lanes 7–12) was bound to their respective cell surface receptors at 4°C. Ligands were crosslinked to their receptors in all samples except for the controls (lanes 1 and 7). The covalent IFN-γ–receptor complex was allowed to internalize at 37°C for the indicated times. Nuclei were then isolated and their components were resolved by SDS/gel electrophoresis followed by autoradiography. Specifically incorporated ^{125}I-MuIFN-γ corresponded to 661, 1951, 5765, 4356, 5075, and 4024 cpm at time points 0.25 (−/+ DSS), 2, 4, 8, and 20 h, respectively. The values for ^{125}I-HuIFN-γ were 959, 1980, 6308, 5443, 5568, and 5733 cpm at the same time points, respectively.

DISCUSSION

We demonstrate here the succession of binding, internalization, and nuclear accumulation of IFN-γ. Transfected murine leukemic L1210 cells, expressing the human IFN-γ receptor in addition to the endogenous murine IFN-γ receptor (4), permitted us to study the surface binding and translocation to the nucleus of both murine and human IFN-γ in the same cell line.

At 4°C, IFN-γ binds to its specific cell surface receptor but cannot be internalized (3). Autoradiography of intact cells labeled with ^{125}I-IFN-γ at 4°C shows peripheral labeling indicative of a ligand–receptor complex located at the cell surface. Indirect immunofluorescence yields similar results. Ligand–receptor complex internalization can be triggered by raising the temperature. At 37°C the ^{125}I-IFN-γ signal becomes localized internally while the cell surface signal diminishes in a time-dependent process. Acid elution of cell-surface-bound ligand progressively removes less ^{125}I-MuIFN-γ with time as the ligand accumulates in nuclei at 37°C (Fig. 1). Similarly, immunofluorescence on intact cells displays a loss of surface label concomitant with intense colocalization of label with the nucleus. This pattern is retained in isolated nuclei at 37°C and in cells that are disrupted at 37°C after binding ligand, but the pattern does not hold at 4°C (Fig. 2).

The data in Fig. 1 suggest that only about 10% of ^{125}I-MuIFN-γ appears to enter the nucleus under these experi-

mental conditions, which seems to underestimate the real percentage. Autoradiography (Fig. 2 *C* and *D*), immunofluorescence (Fig. 3), and chemical crosslinking (Fig. 4), which might increase retention in and prevent leakage from the nucleus, indicate that a much higher proportion of MuIFN-γ enters the nucleus. However, ^{125}I-HuIFN-γ bound to its receptor in a heterologous cell background behaves differently. Although HuIFN-γ translocates to the nucleus even faster than the homologous murine ligand, it is not as stably retained (Fig. 4). It is possible that expression of HuIFN-γ receptors alone (chromosome 6) is insufficient to elicit a biological response. Only the additional presence of human chromosome 21 led to a biological response to HuIFN-γ, even in heterologous cells (22–25). It is thus tempting to propose that nuclear localization of HuIFN-γ in the murine L1210 cells is transient due to the lack of a factor(s) from human chromosome 21 that is involved in nuclear retention.

Our results suggest that the putative bipartite NLS sequence present in the murine and human IFN-γ molecule is functional *in vivo* and that nuclear targeting of IFN-γ could be involved in the induction of a cellular response. Furthermore, our observations are in line with previous findings suggesting an intracellular role for IFN-γ to be biologically active (6, 7). A change in the first MuIFN-γ NLS sequence Lys-Ala-Lys-Lys (residues 86–89) to Val-Leu-Ser-Leu rendered the protein biologically inactive (our unpublished data). Also, cleavage of the C-terminal NLS sequence has been shown to inactivate HuIFN-γ (13). Together these findings allow us to hypothesize that at least some of the effects of IFN-γ on gene expression require translocation of the intact cytokine to the nucleus.

Biologically effective nuclear accumulation probably involves other species-specific factors, the absence of which could explain the lack of activity in murine cells of HuIFN-γ. However, our data permit us to propose a direct role of IFN-γ in the cascade of events involved in signal transduction. Other cytokines, such as platelet-derived growth factor (26) and basic fibroblast growth factor (27), have been shown to contain functional NLSs.

Only smaller molecules (<30–50 kDa) diffuse freely across the nuclear membrane, hence the need for a NLS and an active transporter for larger molecules (10, 18–21). The presence of a NLS and the fact that translocation into the nucleus is saturable imply the existence of a cytoplasmic acceptor protein. A protein, previously described as having a molecular mass of 60–70 kDa (28), mediates translocation to the nucleus, a temperature-dependent process that consumes ATP and depends on the concentration of acceptor protein (19, 20, 29). Early reports about the presence of IFN-γ receptors on nuclear membranes (8, 9) might be attributable to NLS-recognition proteins (28, 30). IFN-γ dimers might reach a critical size which necessitates an active mechanism to cross the nuclear envelope. It is possible, therefore, that IFN-γ might serve as a vehicle to deliver other protein(s) to the nucleus. Alternatively, IFN-γ, once inside the nucleus, might interact with nuclear components involved in the regulation of gene expression. Although IFN-γ exhibits a high affinity for polynucleotides (31), no specific DNA binding site is known.

Despite the need to be bound to a specific receptor to be internalized (3), the data in Fig. 4 indicate that the majority of IFN-γ enters the nucleus without its receptor. The chemical crosslinking of IFN-γ to its receptor generates a covalent complex which travels through the cell in a time-dependent manner. This experiment demonstrates that nuclear accumulation of ligand occurs earlier than the accumulation of the ligand–receptor complex, which can be explained in part by the low efficiency of crosslinking. The importance of these data lies in the fact that even under this nonphysiological condition, the nuclear translocation of IFN-γ can drive the IFN-γ receptor (≈90 kDa) into the nucleus. Whether IFN-γ dissociates from the receptor under physiological conditions prior to entering the nucleus is unclear. Several laboratories showed recently that activation by IFN-γ and IFN-α results in the phosphorylation of latent cytoplasmic transcription factors that function in different combinations at distinct DNA binding sites (32–34). Although it remains to be demonstrated whether IFN-γ associates with nuclear components, the present data suggest that nuclear translocation of IFN-γ may be one of the intracellular events that lead to the biological effects mediated by this cytokine.

We thank G. R. Adolf and D. Lando for recombinant murine and human IFN-γ, respectively. We are grateful to M. Aguet for the L1210 clone 2/10-14. Also, we thank Maïté Coppey for her expert assistance with the immunofluorescence and J. Sancéau and G. Merlin for helpful advice. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Association pour la Recherche Médicale (ARC). T.B. was supported by a fellowship (poste vert) from INSERM and an Yvette Mayent Fellowship at the Institut Curie.

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