A CRM1-dependent Nuclear Export Pathway Is Involved in the Regulation of IRF-5 Subcellular Localization*

Received for publication, July 12, 2004, and in revised form, November 11, 2004
Published, JBC Papers in Press, November 19, 2004, DOI 10.1074/jbc.M408452200

Rongtuan Lin‡‡‡, Long Yang‡, Meztli Arguello‡‡**, Claudia Penafuerte§§, and John Hiscott‡‡‡‡

From the ‡‡ Terry Fox Molecular Oncology Group, Lady Davis Institute for Medical Research, and the Departments of §Microbiology and Immunology and ¶¶ Medicine, McGill University, Montreal, Quebec H3T 1E2, Canada

Interferon regulatory factors (IRFs) are involved in gene regulation in many biological processes including the antiviral, growth regulatory, and immune modulatory functions of the interferon system. Several studies have demonstrated that IRF-3, IRF-5, and IRF-7 specifically contribute to the innate antiviral response to virus infection. It has been reported that virus-specific phosphorylation leads to IRF-5 nuclear localization and up-regulation of interferon, cytokine, and chemokine gene expression. Two nuclear localization signals have been identified in IRF-5, both of which are sufficient for nuclear translocation and retention in virus-infected cells. In the present study, we demonstrate that a CRM1-dependent nuclear export pathway is involved in the regulation of IRF-5 subcellular localization. IRF-5 possesses a functional nuclear export signal (NES) that controls dynamic shuttling between the cytoplasm and the nucleus. The NES element is dominant in unstimulated cells and results in the predominant cytoplasmic localization of IRF-5. Mutation of two leucine residues in the NES motif to alanine, or three adjacent Ser/Thr residues to the phosphomimetic Asp, results in constitutively nuclear IRF-5 and suggests that phosphorylation of adjacent Ser/Thr residues may contribute to IRF-5 nuclear accumulation in virus-induced cells. IKK-related kinases TBK1 and IKKα have been shown to phosphorylate and activate IRF-3 and IRF-7, leading to the production of type 1 interferons and the development of a cellular antiviral state. We examined the phosphorylation and activation of IRF-5 by TBK1 and IKKα kinases. Although IRF-5 is phosphorylated by IKKα and TBK1 in co-transfected cells, the phosphorylation of IRF-5 did not lead to IRF-5 nuclear localization or activation.

The success of the innate host defense to viral and bacterial infections is dependent on the ability of the cell to detect the presence of the invading pathogen. In response to the recognition of components specific to viruses and bacteria, the host cell activates several signal transduction cascades that produce protein messengers in the form of cytokines and chemokines that impede viral/bacterial replication and spread through innate and adaptive immune mechanisms (1, 2). Type I interferons secreted by virus-infected cells activate the innate immune machinery, modulate adaptive immune responses, promote apoptosis of infected cells, and induce an antiviral program in uninfected cells (3, 4).

Molecular regulation of IFN1 gene expression is tightly regulated by extra- and intracellular signals generated during primary infection, culminating in the activation of NF-κB, AP-1, and interferon regulatory factor (IRF) transcription factors that trigger an immediate early IFN response characterized by the release of IFNβ and IFNα1 (5–7). Once produced, secreted IFN acts in a paracrine fashion to induce gene expression in neighboring cells through engagement of cell surface IFN receptors. Activation of the JAK-STAT signaling pathway leads to the formation of STAT1/2 heterodimers, which in conjunction with IRF-9 (or interferon-stimulated gene factor 3) bind to interferon-stimulated-response elements found in hundreds of IFN-induced genes including 2’-5’ oligoadenylate synthase, Mx, double-stranded RNA-activated kinase, and major histocompatibility complex class I, resulting in the induction of proteins that impair viral gene expression and replication (1, 8). In addition, IFNs have other pleiotropic effects in the host, with important roles in apoptosis, growth inhibition, and development of protective immune responses via increased expression of major histocompatibility complex class I proteins (9) and other components of adaptive immunity. IFNs thus link the innate immune responses to adaptive immunity (5, 10).

Biochemical, molecular biological, and gene knock-out studies have demonstrated that the members of the interferon regulatory factor family play important roles in pathogenesis, cytokine signaling, hematopoietic differentiation, regulation of cell cycle, and apoptosis (reviewed in Refs. 3, 7, and 11–14). Among the members of the IRF family, IRF-3 and IRF-7 play essential roles in the virus-induced type I IFN gene expression (15–22). Phosphorylation of the C-terminal serine of both IRF-3 and IRF-7 is essential for nuclear localization and transactivation, and both proteins play complementary rather than redundant roles in the regulation of IFN and chemokine gene expression (15, 16, 18, 19, 22–27). The net result is the generation of the antiviral activity of IFNs including induction of apoptosis, inhibition of cell growth, and immune response modulation. Recently, the IKK-related kinases, IKKα (28) and

1 The abbreviations used are: IFN, interferon; NES, nuclear export signal; IRF, interferon regulatory factors; NLS, nuclear localization sequence; IKK, IκB kinase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; LMB, leptomycin B; GFF, green fluorescent protein; EGFP, enhanced GFP; CBP, CREB-binding protein; CREB, CAMP-response element-binding protein; aa, amino acids; TBK1, TANK-binding kinase 1; CRM1, chromosome region maintenance 1; NDV, Newcastle disease virus.
TBK1 (29–31), were shown to be essential signaling components required for IRF-3 and IRF-7 phosphorylation (32, 33).

IRF-5 is an additional direct transducer of virus-mediated signaling that plays a role in the expression of multiple cytokine/chemokines (34–36). The sequence of human IRF-5 cDNA was first published in the NCBI GenBank™ by T. Mak’s group (accession number U51127), and the transcript encoded 504 amino acids (isoform a). Three IRF-5 transcript variants have been reported: IRF-5 transcript variant 2 spliced out 48 nucleotides corresponding to the 5’ portion of exon 6 and contained an additional 30 nucleotides (encoded 498 aa, isoform b, accession number NM_032643); IRF-5 transcript variant 3 and 4 spliced out 48 nucleotides corresponding to the 5’ portion of exon 6 encoding an identical protein of 488 aa (accession numbers AY504946 and AY504947). Mori et al. (37) also identified two transcript variants: transcript b spliced out 48 nucleotides corresponding to the 5’ portion of exon 6, and transcript c spliced out 34 nucleotides corresponding to exon 5. Using cell line overexpression of the 488 aa (encoded by variant 3 or 4) IRF-5, Barnes et al. (34) demonstrated that IRF-5 was specifically activated by NDV but not by Sendai virus and that IRF-5 induction also up-regulates expression of IFNA genes. Furthermore, overexpression of IRF-5 induced multiple cytokines and chemokines in infected BJAB cells (35).

IRF-5 can act as both an activator and a repressor of IFN gene expression depending on the IRF-interacting partner: IRF-5 cooperates with IRF-3 in the stimulation of IFNA gene transcription and suppresses IRF-7-mediated IFN gene expression (36). As a direct p53 target gene product, IRF-5 also inhibits the growth of tumor cells both in vitro and in vivo (37, 38). IRF-5-mediated growth inhibition is associated with a p53-independent G2-M cell cycle arrest and with the stimulation of multiple cell cycle regulatory and proapoptotic genes including Bak, caspase 8, Bax, and p21 (38).

Nucleocytoplasmic trafficking of protein and RNA molecules plays an important role in eukaryotic cell function (39). A related family of shuttling transport factors, importins and exportins, recognizes nuclear localization sequence (NLS)-containing or nuclear export sequence (NES)-containing proteins and coordinates trafficking between the nucleus and the cytoplasm. CRM1 (exportin 1) has been identified as an export receptor that recognizes NES sequences directly and is responsible for the export of NES-containing proteins (reviewed in Refs. 39 and 40). The pharmacological compound leptomycin B (LMB) directly interacts with CRM1 and blocks NES-mediated protein export (41).

Like IRF-3 and IRF-7, IRF-5 is localized to the cytoplasm in unstimulated cells and accumulates in the nucleus following virus infection (15, 16, 22, 23, 25, 27, 34). IRF-5 contains a functional NES (15, 16, 42) and a functional NLS (42). Two NLSs have been identified in the N- and C-terminal regions of IRF-5, and both of these NLS elements are necessary for virus-induced nuclear translocation (35, 36). In this report, we demonstrate that in addition to the two NLS elements, IRF-5 possesses a functional NES that controls dynamic shuttling between the cytoplasm and the nucleus. In unstimulated cells, both NLS and NES are active, but the NES element is dominant, resulting in the predominant cytoplasmic localization of IRF-5. Although IRF-5 is phosphorylated by IKKe and TBK1 in co-transfected cells, phosphorylation of IRF-5 did not promote cytoplasmic to nuclear translocation, stimulation of IRF-5 transactivation potential, or induction of IFN promoters.

MATERIALS AND METHODS

Plasmid Constructions and Mutagenesis—Plasmids encoding IKKe, TBK1, RANTES/pGL3, IFNB/pGL3, IFNA14/pGL3, and pRLTK were described previously (26, 32). Human IRF-5a cDNA was amplified by reverse transcription-PCR from the Namalwa B-cell line, and human IRF-5b cDNA was amplified by reverse transcription-PCR from the MC-CAR B-cell line. Both cDNAs were cloned into pcDNA3.1Zeo (FLAG-IRF-5a and FLAG-IRF-5b) and pEGFP-C1 (GFPA-IRF-5a and GFPA-IRF-5b). DNA sequencing was performed for confirmation of mutations. The IRF-5 point mutants including 1) A157/159 (NES), 2) D156/158/160/160 (3D), 3) A156/158/160 (3A), 4) D437/441/443/446 (4D), and 5) D156/158/160/160/D437/441/443/446 (7D) were generated by overlap PCR-mediated mutagenesis. The IRF-5 deletion mutants including 1) 1–450, 2) 1–400, 3) 1–250, 4) 1–200, 5) 1–150, and 6) 161–504 were generated by PCR. The CRM1 cDNA was obtained from Dr. Grosveld at St. Jude Children’s Hospital.

Cell Culture, Transfections, and Luciferase Assays—Transfections for luciferase assay were carried out in either human embryonic kidney 293 cells grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, glutamine, and antibiotics or BJAB B-cell line grown in RPMI 1640 (Wisent) supplemented with 10%, heat-inactivated fetal bovine serum. Subconfluent 293 cells were transfected with 50 ng of pRLTK reporter (Renilla luciferase for internal control), 100 ng of pGL3 reporter (firefly luciferase, experimental reporter), and 200 ng of expression plasmids by calcium phosphate co-precipitation method. Electroporation was performed at 850 microfarads and 250 V for BJAB cells. Luciferase assays were performed following a transfection of 2.5 μg of the reporter gene, 2.5 μg of the Renilla internal control (pRLTK), and 5 μg of expression plasmids into 10 × 10⁶ BJAB cells. The reporter plasmids were: RANTES pGL3, IFNB pGL3, and IFNA14 pGL3 reporter genes; the transfection procedures were described previously (43). At 24 h after transfections, the reporter gene activities were measured by dual-luciferase reporter assay, according to the manufacturer’s instructions (Promega).

Subcellular Localization of GFP-IRF-5—To analyze the subcellular localization of GFP-IRF-5 proteins, wild type or mutated forms of GFP-IRF-5 expressing plasmids (2 μg) were transiently transfected into subcon-
fluorescent COS-7 or Hela cells by Lipofectamine (Invitrogen). At 24 h after transfection, cells were left untreated or treated with 10 ng/ml LMB (a gift from Dr. Minoru Yoshida) for 60 min or the indicated times. GFP fluorescence was analyzed in living cells, as indicated in the legend for Fig. 1.

Western Blot Analysis—For Western blotting, 2.0 μg of pEGFP-C1, GFP-IRK, or GFP-TBK with FLAG-IRF-3 or FLAG-IRF-5 was co-transfected into HEK293 cells. At 24 h after transfection, whole cell extracts (20 μg) were prepared and subjected to SDS-PAGE in a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to Hybond transfer membrane (Amersham Biosciences) in a buffer containing 30 mM Tris, 200 mM glycine, and 20% methanol for 1 h. The membrane was blocked by incubation in phosphate-buffered saline containing 5% dried milk for 1 h and then probed with anti-FLAG, anti-GFP, or anti-Myc antibody (1:1000–1:3000). Immunocomplexes were detected by using ECL (15).

RESULTS

Cytoplasmic Localization of IRF-5 Is Sensitive to Treatment with LMB—Two NLSs have been identified in N- and C-terminal regions of IRF-5, and both NLSs are necessary for virus-induced nuclear translocation (35, 36). However, in unstimulated cells, IRF-5 resides in the cytoplasm (34), and only following virus infection does IRF-5 accumulate in the nucleus (34). To determine whether a CRM1-dependent nuclear export pathway is involved in IRF-5 subcellular localization, isoforms a and b of IRF-5 were linked to GFP, transfected into COS-7 cells, and examined for LMB-induced changes in subcellular localization (Fig. 1). Fluorescence microscopy analysis of these two GFP fusion proteins revealed that both IRF-5 isoforms localized exclusively to the cytoplasm in untreated cells; upon LMB treatment, both fusion proteins accumulated in the nucleus within 15 min (Fig. 1B). Since LMB is a CRM1-specific inhibitor (41, 44–46), this initial observation suggested that
the subcellular localization of IRF-5 may be regulated by CRM1.

Localization of the IRF-5 NES Element—To investigate the sequences of IRF-5 that may exhibit nuclear export activity, the subcellular localization of a series of deletion mutants encompassing amino acids 1–450, 1–400, 1–200, 1–250, 1–150, and 161–504 of isoform a fused to GFP was examined (Fig. 2 A). As shown in Fig. 2B, mutants 1–450, 1–400, 1–250, and 1–200 were predominantly cytoplasmic in untreated cells, and following LMB treatment, these IRF-5 forms accumulated in the nucleus (Fig. 2B), indicating that the putative NES element was localized to the N-terminal 200 aa. The IRF-5 peptide of aa 1–150 (deleted of the C-terminal residues 151–504) was constitutively localized to the nucleus (Fig. 2B). IRF-5 with a deletion of N-terminal residues 1–160 (aa 161–504) was also predominantly nuclear in untreated and LMB-treated cells (Fig. 2B). Western blotting analysis with anti-GFP antibody indicated that all deletion mutants were correctly expressed (data not shown). These results therefore localize a putative NES element to an N-terminal region of IRF-5 between aa 150 and 160; NES activity is LMB-sensitive, and its association with the CRM1-dependent export machinery may determine the cytoplasmic localization of IRF-5 in unstimulated cells.

Mutational Analysis of the IRF-5 NES—Sequence inspection revealed that residues 150–160 contain a stretch of hydrophobic amino acids resembling the NES consensus (LXXXLXXLXL) (Fig. 3A). Since CRM1 is known to recognize leucine-rich or other hydrophobic motifs (47), residues 150–160 may constitute a functional NES. To test this possibility, point mutations within the putative NES motif in which two leucine residues were replaced with alanine (L157A/L159A, IRF-5-NES) (Fig. 3A) were generated. Replacement of these two residues within the context of full-length IRF-5 isoform a leads to nuclear accumulation of the mutated IRF-5 protein (Fig. 3B), indicating that these two leucine residues are important for nuclear export of IRF-5. Interestingly, these two leucine residues are flanked by two serine residues and one threonine residue. To determine whether these Ser/Thr residues are involved in the regulation of IRF-5 subcellular localization, the three residues were substituted with the phosphomimetic Asp or Ala in GFP-IRF-5 (Fig. 3A) and examined for subcellular localization. As shown in Fig. 3B, the mutation of these residues to phosphomimetic Asp (D156/158/160, IRF-5–3D) resulted in constitutively nuclear IRF-5, whereas the alanine substitution (A156/158/160, IRF-5–3A) did not change the subcellular localization. Western blotting analysis with anti-GFP antibody indicated that all point mutants were correctly expressed (data not shown). This result suggested that the phosphorylation of adjacent Ser/Thr residues may contribute to IRF-5 nuclear accumulation in virus-induced cells.

The putative NES peptide and the A157/159 peptide (mutNES) were fused to the C-terminal end of GFP (Fig. 4A), and as shown in Fig. 4B, the NES peptide but not mutNES peptide was capable of directing GFP to the cytoplasm; furthermore, GFP-NES export from the nucleus was sensitive to leptomycin (Fig. 4B), thus demonstrating that the IRF-5 NES is sufficient to direct GFP to the cytoplasm. Taken together, these data indicate that residues 150–160 of IRF-5 function as an authentic NES that is responsible for the cytoplasmic localization of IRF-5 in unstimulated cells.

Physical Interaction between IRF-5 and CRM1—To examine the possibility that IRF-5 functionally associated with the CRM1 in vivo, interactions between IRF-5 and CRM1 were investigated by co-immunoprecipitation using 293 cells co-transfected with CRM1 and wild type or mutated forms of IRF-5 expression plasmids (Fig. 5). After immunoprecipitation of Myc-tagged CRM1 from cell extracts with anti-Myc antibody, immunoblot analysis revealed that FLAG-tagged wild type
IRF-5 co-precipitated with Myc-tagged CRM1 (Fig. 5, lane 2) but not with preimmune serum (data not shown) or with anti-Myc antibody in the absence of Myc-tagged CRM1 (Fig. 5, lane 1). Interaction required functional NES since the IRF-5 3D and IRF-5 NES mutants did not immunoprecipitate with Myc tag CRM1 (Fig. 5, lane 3, 4, and 5); also, co-transfected FLAG-tagged IRF-7 did not co-precipitate with the Myc-tagged CRM1 (Fig. 5A, lane 6).

Transactivation of IFNA and IFNB Promoters by IRF-5—Next, the capacity of IRF-5 to regulate gene expression was analyzed by transient transfection in human 293 HEK cells and BJAB cells using the IFNA14 and IFNB promoters in reporter gene assays. Expression of wild type IRF-5 did not enhance IFNA14 promoter activity and only slightly increased IFNB promoter activity (Fig. 6, A and B). Co-transfection of IRF-5–3D, the constitutively nuclear IRF-5, minimally induced IFNA14 and IFNB promoter activity between 3- and 4-fold (Fig. 6A and B), indicating that nuclear accumulation alone is not sufficient for full activation of IRF-5. It has been reported that IRF-5 protein contains potential serine phosphorylation sites in the C-terminal region between aa 471 and 486 and that IRF-5 protein contains potential serine phosphorylation sites in the C-terminal region between aa 471 and 486 and that phosphorylation of C-terminal Ser residues may contribute to IRF-5 transactivation.

Phosphorylation of IRF-5 by IKKε and TBK1—C-terminal phosphorylation of IRF-5 and IRF-7 promotes cytoplasmic to nuclear translocation, DNA binding, association with CBP/p300 histone acetyltransferases, and transactivation of target genes such as interferons and chemokines (15, 16, 18–24). Recently, the IKK-related kinases, IKKε and TBK1, were shown to be essential signaling components required for direct C-terminal phosphorylation and activation of IRF-3 and IRF-7 transcription factors (32, 33). Likewise, virus infection has been shown to induce IRF-5 phosphorylation, nuclear translocation, and transactivation (34–36). To determine whether
IRF-5 Possesses an NES Element

293 cells were transfected with a pRLTK control plasmid, a luciferase reporter plasmid containing the IFNA1, IFNA2, IFNA4, IFNA7, or IFNA14 promoter and expression plasmids encoding IRF-5, IRF-7, IKKα, or TBK1 as indicated. Luciferase activity was measured at 24 h after transfection. Relative luciferase activity was measured as fold activation (relative to the basal level of reporter gene in the presence of pcDNA3 vector after normalization with co-transfected RLU activity); the values represent the average of three experiments done in duplicate with variability between 10 and 25%.

| IFNA1 | IFNA2 | IFNA4 | IFNA7 | IFNA14 |
|-------|-------|-------|-------|--------|
| pCMV2 + GFP 0.7 1.0 1.0 1.0 1.0 | pCMV2 + IKKα 1.2 1.3 2.4 2.3 2.3 | pCMV2 + TBK1 1.2 1.3 2.3 2.2 2.3 | IRF-5 + GFP 1.2 1.3 2.2 2.1 2.2 | IRF-5 + IKKα 1.2 1.3 2.2 2.1 2.2 | IRF-5 + TBK1 1.2 1.3 2.2 2.1 2.2 |
| IRF-5 - IFNB 1.2 1.3 2.2 2.1 2.2 | IRF-7 + GFP 1.2 1.3 2.2 2.1 2.2 | IRF-7 + IKKα 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + IFNA14 1.2 1.3 2.2 2.1 2.2 | IRF-7 + IRF-5 (D) 1.2 1.3 2.2 2.1 2.2 |
| IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 | IRF-7 + TBK1 1.2 1.3 2.2 2.1 2.2 |

**Fig. 8.** Activation of IFNB and IFNA14 reporter gene activation by active form of IRF-3, IRF-5, and IRF-7. 293 HEK cells were transfected with a pRLTK control plasmid, a luciferase reporter plasmid containing the IFNA1 (A) or IFNA14 (B) promoter, and the expression plasmids encoding wild type and mutated forms of IRF-3, IRF-5, and IRF-7 as indicated. At 24 h after transfection, cells were infected with Sendai virus (SV) or left uninfected as indicated. Luciferase activity was analyzed at 24 h after transfection by the dual-luciferase reporter assay as described by the manufacturer (Promega). Relative luciferase activity was measured as fold activation (relative to the basal level of reporter gene in the presence of pcDNA3 vector after normalization with co-transfected RLU activity); values are mean ± S.D. for three experiments.

IRF-5 is phosphorylated by IKKe and TBK1, IRF-3- and IRF-5-expressing plasmids were transiently co-transfected with IKKe- or TBK1-expressing plasmids in 293 HEK cells. Both IKKe and TBK1 induced slower migrating forms of IRF-3 and IRF-5 (Fig. 7A, lanes 2, 3, 5, and 6). Co-expression of IKKe or TBK1 also stimulated IRF-5 dimerization measured by co-immunoprecipitation (Fig. 7B, lanes 2 and 3). However, IKKe or TBK1 did not induce nuclear translocation of IRF-5 (Fig. 7C). Thus, although IKKe or TBK1 co-expression was sufficient to induce IRF-5 phosphorylation and dimerization, IKKe- or TBK1-induced phosphorylation and dimerization of IRF-5 did not stimulate IRF-5 nuclear translocation.

IKKe and TBK1 Phosphorylation Does Not Activate IRF-5 Transcriptional Activity—Reporter gene analyses were performed with luciferase expression driven by the IFNA1, IFNA2, IFNA4, IFNA7, and IFNA14 promoters to determine the ability of IKKe and TBK1 to stimulate IRF-5-dependent gene expression. As shown in Table I, row 4, IRF-5 expression alone did not increase IFNA promoter activity. In contrast, expression of IRF-7 alone stimulated IFNA promoter activities between 5- and 10-fold (Table I, row 7). Expression of IKKe or TBK1 alone activated IFNA1 promoter 5- and 10-fold, respectively, but only minimally induced IFNA2, IFNA4, IFNA7, and IFNA14 promoter activities between 1.2- and 3.4-fold (Table I, rows 2 and 3). Co-expression of IRF-7 together with IKKe or TBK1 strongly activated these IFNA promoters (900- to over 9000-fold induction, Table I, rows 8 and 9). However, co-expression of IRF-5 with IKKe or TBK1 did not increase IFNA promoter activities (Table I, rows 5 and 6), thus indicating that IRF-5 is not activated by IKKe- or TBK1-dependent phosphorylation and dimerization.

IRF-5 Only Weakly Stimulates Type I IFN Promoter Activities—It was reported that IRF-5 was able to enhance the transcription from IFNB, IFNA1, IFNA2, and IFNA14 promoter in virus-infected cells (34, 35). To assess the role of IRF-3, IRF-5, and IRF-7 in the activation of type I IFN gene expression, wild type and constitutively active forms of IRF-3, IRF-5, and IRF-7 were co-transfected with the luciferase reporter gene driven by the IFNB and IFNA14 promoters. As shown in Fig. 8A, IFNB promoter was strongly activated by both IRF-3 and IRF-7. The constitutively active form of IFNB promoter (34, 35) activated the IFNA14 luciferase reporter gene 250-fold, the constitutively active form of IFNB promoter 250-fold, whereas phosphomimetic forms of IFNB (IFNB-7(4D)) and IFNB (IFNB-7(5D)) only resulted in 7–8-fold stimulation of IFNB promoter activity. The IFNA14 promoter was activated 1000-fold by the constitutively active form of IFNB promoter (IFNA14-7(4D)) (Fig. 8B) but was only weakly stimulated by phosphomimetic forms of IFNB and IRF-5 (between 4- and 6-fold, Fig. 8B). Similar results were obtained with IFNA1, IFNA2, IFNA4, and IFNA7 promoters (data not shown). These results demonstrate that IRF-5 is a weak activator of type I IFN gene expression.

**DISCUSSION**

The results of the present study demonstrate that in addition to the two functional NLSs in the N- and C-terminal regions, IRF-5 possesses an authentic NES that is necessary for cytoplasmic retention in unstimulated cells. Following treatment with leptomycin B, a known CRM1-specific inhibitor (34, 44–46), IRF-5 rapidly accumulated in the nucleus (Fig. 1B), indicating that IRF-5 is subject to active nuclear export. Deletion
and point mutational analyses revealed that a hydrophobic motif (LQRMLPSLSLT) located between residues 150–160 of IRF-5 functions as an NES element. Alanine substitution of two critical leucine residues, as indicated in bold letters (LQRMLPSASAT) resulted in the nuclear accumulation of the mutated protein in unstimulated cells. Furthermore, the addition of a single copy of the wild type but not mutated NES of IRF-5 downstream of GFP confers the property of LMB-sensitive cytoplasmic localization to the fusion protein. Our findings suggest that IRF-5, like the IRF-3 protein, actively shuttles between the nuclear and cytoplasmic compartments in unstimulated cells.

The IRF-5 protein possesses two traditional monopartite NLS elements that are solely responsible for nuclear localization of IRF-5 protein in virus-infected cells (35). Mutation of these two NLS elements led to the cytoplasmic localization of the resulting protein in both infected and uninfected cells. It has been suggested that the 3’ NLS is exposed and responsible for the transactivation activity of IRF-5 in uninfected cells, whereas the 5’ NLS is masked either by an intramolecular interaction or by association with another protein. Phosphorylation of serine residues in the C-terminal region of IRF-5 results in the exposure of the 5’ NLS and accumulation of IRF-5 in the nucleus (35). Both NLSs and NES in IRF-5 are constitutively active, but nuclear export is dominant in unstimulated cells (Fig. 1).

As shown in Fig. 3, the NES motif of IRF-5 contains two serine residues and one threonine residue. The substitution of these three residues to phosphomimetic Asp (35) was variant 3. Like IRF-3 and IRF-7, IRF-5 is selectively phosphorylated and activated in NDV- and vesicular stomatitis virus-infected cells but not in Sendai virus-infected cells (34–36). Recent studies have identified IKKe/TBK1 as components of the virus-activated kinase responsible for IRF-3 and IRF-7 phosphorylation in response to virus infection (32, 33). Although co-expression of IKKe or TBK1 resulted in the phosphorylation (Fig. 7A) and dimerization (Fig. 7B) of IRF-5, IKKe/TBK1 did not induce IRF-5 nuclear accumulation (Fig. 7C) or stimulate IRF-5 transactivation activity (Table I). This result suggests either that a distinct signaling pathway is involved in the activation of IRF-5, or alternatively, that IRF-5 simply is not an activator of IFN gene expression. This possibility has been suggested by recent experiments indicating that heterodimerization of IRF-5 with IRF-3 or IRF-7 confers transactivation capacity (36).

Based on recent observations that IRF-5 is a p53-targeted gene (37), the primary physiological role of IRF-5 may be to regulate gene expression during cell growth or as a response to DNA damage, as recently reported (38).

Acknowledgments—We thank Drs. Minoru Yoshida and G. Grosveld for reagents used in this study and members of the Molecular Oncology Group, Lady Davis Institute for helpful discussions.

REFERENCES
1. Samuel, C. E. (2001) Clin. Microbiol. Rev. 14, 778–809
2. Akira, S., Takeda, K., and Kaisho, T. (2001) Nat. Immunol. 2, 675–680
3. Taniguchi, T., Ogasawara, K., Takaoka, A., and Tanaka, N. (2001) Annu. Rev. Immunol. 19, 623–655
4. Katze, M. G., He, Y., and Gale, M., Jr. (2002) Nat. Rev. Immunol. 2, 675–687
5. Biron, C. A. (2001) Immunity 14, 661–664
6. Levy, D. E., and Garcia-Sastre, A. (2001) Cytokine Growth Factor Rev. 12, 141–156
7. Levy, D. E., Marié, I., Smith, E., Prakash, A., Yoneyama, M., Suhara, W., Fujita, T., Barnes, B., Lubyova, B., Pitia, P. M., Taniguichi, T., Ogasawara, K., Takaoka, A., Tanaka, N., Taki, S., and Sato, M. (2002) J. Interferon Cytokine Res. 22, 87–93
8. Sen, G. C. (2001) Annu. Rev. Microbiol. 55, 255–281
9. Stark, G. R., Kerr, I. J., Williams, B. R. G., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
10. Biron, C. A. (1997) Curr. Opin. Immunol. 9, 24–34
11. Servant, M. J., Grandvaux, N., and Hiscott, J. (2002) Biochem. Pharmacol. 64, 985–992
12. Servant, M. J., ten Oever, B., and Lin, R. (2002) J. Interferon Cytokine Res. 22, 49–58
13. Hiscott, J., Pitia, P., Genin, P., Nguyen, H., Heyboubre, C., Manane, Y., Algarte, M., and Lin, R. (1999) J. Interferon Cytokine Res. 19, 1–13
14. Manane, Y., Heyboubre, C., Genin, P., Algarte, M., Servant, M. J., LePage, C., DeLuca, C., Kwon, H., Lin, R., and Hiscott, J. (1999) Gene (Amst.) 237, 1–14
15. Lin, R., Heyboubre, C., Pitia, P. M., and Hiscott, J. (1998) Mol. Cell. Biol. 18, 2986–2996
16. Yoneyama, M., Suhara, W., Fukuhara, Y., Fukada, M., Nishida, E., and Fujita, T. (1999) EMBO J. 17, 1087–1095
17. Au, W. C., Moore, P. A., LaFleur, D. W., Tombal, B., and Pitha, P. M. (1998) J. Biol. Chem. 273, 29210–29217
18. Lin, R., Mamane, Y., and Hiscott, J. (1999) Mol. Cell. Biol. 19, 2465–2474
19. Lin, R., Heyboubre, C., Genin, P., Pitia, P. M., and Hiscott, J. (1999) Mol. Cell. Biol. 19, 959–966
20. Marié, I., Durbin, J. E., and Levy, D. E. (1998) EMBO J. 17, 6660–6669
21. Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998) FEBS Lett. 425, 112–116
22. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000) Immunity 13, 539–548
23. Wathelet, M. G., Lin, C. H., Parakh, B. S., Ronco, L. V., Howley, P. M., and Maniatis, T. (1998) Mol. Cell. Biol. 18, 507–518
24. Weaver, B. K., Kumar, K. P., and Reich, N. C. (1998) Mol. Cell. Biol. 18, 561–571
25. Choo, P. L., Kuo, G., Vlahos, R., Cao, Y., Berger, K., Overby, R. L., Drozda, A., Brown, C., Varmus, H. E., and Rice, C. M. (1989) Nature 340, 425–429
26. Levy, D. E., and Garcia-Sastre, A. (2001) Cytokine Growth Factor Rev. 12, 141–156
27. Levy, D. E., Marié, I., Smith, E., Prakash, A., Yoneyama, M., Suhara, W., Fujita, T., Barnes, B., Lubyova, B., Pitia, P. M., Taniguichi, T., Ogasawara, K., Takaoka, A., Tanaka, N., Taki, S., and Sato, M. (2002) J. Interferon Cytokine Res. 22, 87–93
28. Sen, G. C. (2001) Annu. Rev. Microbiol. 55, 255–281
29. Stark, G. R., Kerr, I. J., Williams, B. R. G., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
30. Biron, C. A. (1997) Curr. Opin. Immunol. 9, 24–34
25. Lin, R., Genin, P., Mamane, Y., and Hiscott, J. (2000) Mol. Cell. Biol. 20, 6342–6353
26. Lin, R., Mamane, Y., and Hiscott, J. (2000) J. Biol. Chem. 275, 34320–34327
27. Marie, I., Smith, E., Prakash, A., and Levy, D. E. (2000) Mol. Cell. Biol. 20, 8803–8814
28. Peters, R. T., Liao, S. M., and Maniatis, T. (2000) Mol. Cell 5, 513–522
29. Pomerantz, J. L., and Baltimore, D. (1999) EMBO J. 18, 6694–6704
30. Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motoyama, N., Ikeda, K., Karin, M., and Nakanishi, M. (2000) Nature 404, 778–782
31. Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Hie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) EMBO J. 19, 4976–4985
32. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscott, J. (2003) Nat. Immunol. 4, 491–496
33. Barnes, B. J., Moore, P. A., and Pitha, P. M. (2001) J. Biol. Chem. 12, 12
34. Barnes, B. J., Kellum, M. J., Field, A. E., and Pitha, P. M. (2002) Mol. Cell. Biol. 22, 5721–5740
35. Barnes, B. J., Field, A. E., and Pitha-Rowe, P. M. (2003) J. Biol. Chem. 278, 16630–16641
36. Mori, T., Anazawa, Y., Hizumi, M., Fukuda, S., Nakamura, Y., and Arakawa, H. (2002) Oncogene 21, 2914–2918
37. Barnes, B. J., Kellum, M. J., Pinder, E. A., Frisancho, J. A., and Pitha, P. M. (2003) Cancer Res. 63, 6424–6431
38. Weiss, K. (1998) Trends Biochem. Sci. 23, 185–189
39. Ulman, K., Powers, M. A., and Forbes, D. J. (1997) Cell 90, 967–970
40. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) Cell 90, 1051–1060
41. Kumar, K. P., McBride, K. M., Weaver, B. K., Dingwall, C., and Reich, N. C. (2000) Mol. Cell. Biol. 20, 4159–4168
42. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1999) Mol. Cell. Biol. 16, 1401–1409
43. Kudo, N., Matsumori, N., Tsoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9112–9117
44. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) Science 278, 143–144
45. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) Cell 90, 1041–1050
46. Nakielny, S., and Dreyfuss, G. (1999) Cell 99, 677–680
47. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998) FEBS Lett. 441, 106–119
A CRM1-dependent Nuclear Export Pathway Is Involved in the Regulation of IRF-5
Subcellular Localization
Rongtuan Lin, Long Yang, Meztli Arguello, Claudia Penafuerte and John Hiscott

J. Biol. Chem. 2005, 280:3088-3095.
doi: 10.1074/jbc.M408452200 originally published online November 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408452200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 48 references, 21 of which can be accessed free at
http://www.jbc.org/content/280/4/3088.full.html#ref-list-1