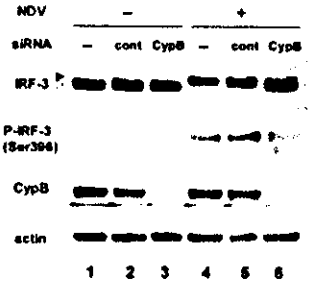

FIG. 2. **Cyclophilin B knockdown inhibits virus-induced IRF-3 phosphorylation.** HT1080 cells were mock-transfected (*lanes 1* and *4*) or transfected with control siRNA (*lanes 2* and *5*) or CypB siRNA (*lanes 3* and *6*), respectively, followed by mock infection (*lanes 1–3*) or infection with NDV for 12 h (*lanes 4–6*). Whole cell extracts were prepared and subjected to immunoblotting using the anti-IRF-3 (C-20) antibody (*first panel*), the anti-IRF-3 Ser(P)-396 antibody (*second panel*), the anti-CypB antibody (*third panel*), or the anti-actin antibody (*fourth panel*). The *solid* and *empty arrowheads* indicate the phosphorylated IRF-3 and the unphosphorylated IRF-3, respectively. The data are representative of three independent experiments.

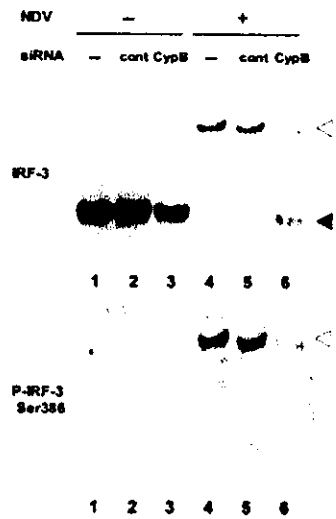

FIG. 3. **Cyclophilin B knockdown inhibits virus-induced IRF-3 dimerization.** Whole cell extracts were prepared as described in Fig. 2 and were analyzed by native PAGE followed by immunoblotting with the anti-IRF-3 (C-20) antibody (*upper panel*) and the anti-IRF-3 Ser(P)-386 antibody (*lower panel*). The *solid* and *empty arrowheads* indicate the monomer and the dimer of IRF-3, respectively. The data are representative of three independent experiments.

not precipitate the endogenous IRF-3 with CypB, even 10 min after the infection. We also tried to characterize the binding domains of IRF-3 with CypB by using mutant IRF-3 expression plasmids. Unfortunately, the IRF-3 expression was inhibited when the CypB plasmid was introduced simultaneously. This is consistent with previous reports that CypB may participate in inducing the degradation of exogenously introduced DNA (18, 19).

Cyclophilin B Is Required for Virus-induced Phosphorylation of IRF-3—To examine the physiological roles of CypB in the IRF-3 function, we performed RNA interference experiments to knock down CypB *in vivo* and then examined the biochemical activities of IRF-3 after NDV infection. As shown in Fig. 2, the expression of CypB was almost completely suppressed by the specific siRNA interference (*lanes 3* and *6*, *third panel*). NDV infection in the mock- or control siRNA-transfected cells resulted in a mobility shift of IRF-3, which reflected the C-terminal phosphorylation of IRF-3 (Fig. 2, *lanes 4* and *5*, *first panel*) (9, 20). However, IRF-3 showed an intermediate mobility in CypB siRNA-transfected/NDV-infected cells (Fig. 2, *lane 6*, *first panel*). When the same blot was reprobed with an antibody that specifically recognized a phosphorylated form of IRF-3 (21), we found that the amounts of phosphorylated IRF-3 (phosphorylated Ser-396) were reduced in the CypB siRNA-transfected cells, as compared with the mock- or control siRNA-transfected cells (Fig. 2, compare *lanes 6* with *lanes 4* and *5*, *second panel*). These results strongly suggest that the knockdown of CypB resulted in the defect in IRF-3 phosphorylation by virus infection.

The Cyclophilin B Knockdown Inhibited Virus-induced IRF-3 Dimerization—It is known that the phosphorylation of IRF-3 is prerequisite for the dimerization of IRF-3 (7, 20). We thus examined the virus-induced dimerization of IRF-3 in siRNA-transfected cells. To do this, we used the native PAGE assay that sensitively detects the difference between the monomer and dimer forms of IRF-3 (22). IRF-3 existed as a monomer in the mock-, control siRNA-, and CypB siRNA-transfected cells in the absence of NDV infection (Fig. 3, *lanes 1–3*). Upon viral infection, the monomer signals were reduced, but newly formed dimer signals were detected in the mock- and control siRNA-transfected cells (Fig. 3, *lanes 4* and *5*). However, significant amounts of the IRF-3 monomer still remained in the CypB siRNA-transfected/NDV-infected cells (Fig. 3, *lane 6*). These results clearly indicated that the specific knockdown of CypB by RNA interference inhibited the IRF-3 dimer formation induced by viral infection. The importance of CypB was again demonstrated in the phosphorylation of Ser-386, which is the critical residue in IRF-3 activation (23). CypB siRNA-transfected/NDV-infected cells failed to phosphorylate Ser-386 (Fig. 3, *panel 2*, *lane 6*).

The Cyclophilin B Knockdown Inhibited IRF-3 Binding to the ISRE and Association with CBP—Phosphorylated IRF-3 undergoes homodimerization (6, 7) and associates with the coactivators CBP/p300 (6, 9). The holocomplex has the ability to specifically recognize the target DNA sequence, called the ISRE (5, 8, 9). We examined the effects of CypB on IRF-3 in terms of the DNA binding to the ISRE and the association with the CBP/p300 coactivator, using electromobility shift assay. In the absence of NDV infection, no DNA-protein complex was observed in the mock-, control siRNA-, and CypB siRNA-transfected cells (Fig. 4, *lanes 1*, *6*, and *11*). NDV infection induced the formation of a DNA-protein complex bound to the ISRE of the ISG15 gene in the mock- and control siRNA-transfected cell extracts (Fig. 4, *lanes 2* and *7*). The addition of specific antibodies against IRF-3 to the binding reactions reduced the amount of the complex band and the induction of supershifted bands, indicating that the complex contained IRF-3 (Fig. 4, *lanes 3* and *8*). The addition of specific antibodies against CBP to the binding reactions reduced the amount of the complex but did not induce the formation of supershifted bands, indicating that complex formation was partly blocked by the antibody (Fig. 4, *lanes 4* and *9*). This is consistent with the previous result that CBP/p300 is involved in the holocomplex of IRF-3 (6–9). However, the knockdown of CypB severely impaired the DNA binding activity of IRF-3 (Fig. 4, *lane 12*). Thus, the inhibition of CypB also resulted in reduced holocomplex formation, which is required for the target gene activation by IRF-3. A chromatin immunoprecipitation analysis showed the *in vivo* binding of IRF-3 to the ISG15 promoter in NDV-infected cells (Fig. 4*B*, *lane 8*) but not in uninfected cells (Fig. 4*B*, *lane 2*). When NDV-infected cells were pretreated with CypB siRNA, the binding was significantly reduced, and no band was detected after 30 PCR cycles (Fig. 4*B*, *lane 12*), although a band was visible after 40 cycles (data not shown). The control siRNA could not reduce the binding (Fig. 4*B*, *lane 10*).

The Cyclophilin B Knockdown Reduced IFN-β Production by Newcastle Disease Virus Infection—Finally, we examined the

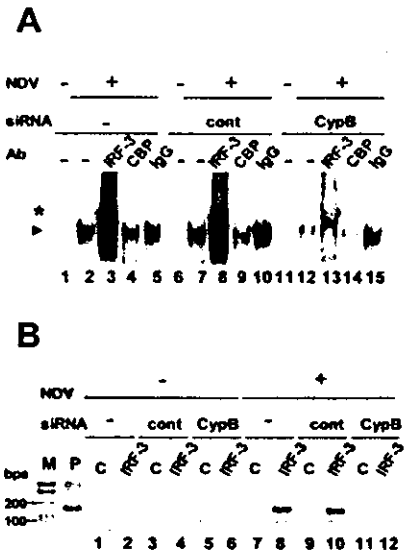

FIG. 4. **Cyclophilin B knockdown inhibits IRF-3 binding to the ISRE and association with CBP.** *A*, whole cell extracts were prepared as described in Fig. 2, and were subjected to electromobility shift assay, using the ISRE of the ISG15 gene as a probe, in the absence (*lanes 1, 2, 6, 7, 11,* and *12*) or presence of the anti-IRF-3 (C-20) antibody (*lanes 3, 8,* and *13*), the anti-CBP antibody (*Ab*) (*lane 4, 9,* and *14*), or control (*cont*) IgG (*lanes 5, 10,* and *15*). The *arrowhead* indicates the IRF-3-containing complex. The *asterisk* indicates the bands supershifted by the anti-IRF-3 antibody. *B*, chromatin immunoprecipitation assay to assess the amounts of IRF-3 bound to ISG15 promoter. *M*, marker; *P*, positive control of genomic DNA. Whole cell extracts prepared as described in Fig. 2 were subjected to the assay, using the promoter region of the ISG15 gene as primers. Each extract was immunoprecipitated with the control IgG (*C*) (*lanes 1, 3, 5, 7, 9,* and *11*) or the anti-IRF-3 (C-20) antibody (*lanes 2, 4, 6, 8, 10,* and *12*). The data are representative of three independent experiments.

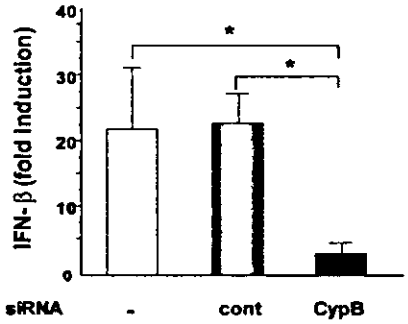

FIG. 5. **Cyclophilin B knockdown reduces IFN-β production by Newcastle Disease Virus infection.** HT1080 cells were mock-transfected, or transfected with control siRNA or CypB siRNA. After 24 h, the cells were infected with NDV for 12 h. The supernatants before and after NDV infection were analyzed by an IFN-β enzyme-linked immunosorbent assay. The results shown are the averages of three independent experiments, with S.E. *bars*. The *asterisk* indicates a p value < 0.05.

effect of CypB on the regulation of IFN-β production. The amount of IFN-β in the culture medium was assayed by enzyme-linked immunosorbent assay, before and after NDV infection. The virus infection induced a more than 25-fold activation of IFN-β in the mock- and the control siRNA-transfected cells. The CypB-siRNA treatment caused a severe defect in the viral-dependent activation of IFN-β. (Fig. 5). These results indicate that CypB is required for the efficient activation of IFN-β production upon viral infection.

DISCUSSION

We have described a novel interaction between IRF-3 and CypB. The present *in vitro* analysis suggests that autoinhibition domain of IRF-3 and the catalytic domain bearing the peptidyl-prolyl isomerase activity of CypB are required for the interaction.

CypB, a member of the cyclophilins, possesses a *cis-trans* peptidyl-prolyl isomerase activity (15). Via their PPIase activity, cyclophilins facilitate protein folding and have been shown to contribute to the maturation and trafficking of several proteins (24). Furthermore, cyclophilins regulate signal transduction cascades, as revealed by their modulation of transforming growth factor-β signaling and the transactivation of c-myb and IRF-4 (25, 26). Among the cyclophilins, CypB is distinguished from the others by the presence of an endoplasmic reticulum-directed signal sequence (15). However, CypB is found not only in the endoplasmic reticulum but also in the extracellular space and the nucleus (15). CypB has been reported to interact with prolactin. The proximal action of prolactin is mediated by its cell surface receptor. PRL activity, however, is also associated with the internalization and translocation of this hormone into the nucleus. To retrotransport it to the nucleus, and to potentiate prolactin-induced proliferation, the interaction with CypB with its PPIase activity is essential (27, 28). Similar to the interaction with prolactin, the PPIase domain of CypB is required for IRF-3 binding in a GST pull-down assay. We also found that retaining either one of the autoinhibition domains of IRF-3 is required for the binding. Previously, Mamane *et al.* (29) reported the interaction between IRF-4, a member of the IRFs, and FKBP52, another member of the immunophilins. They demonstrated that IRF-4 would not co-immunoprecipitate with FKBP52 unless the C-terminal autoinhibition domain of IRF-4 was removed. This observation raises the possibility that the interactions of immunophilins with IRF family proteins are sensitive to the conformations or the ternary structures of IRFs.

As demonstrated by our RNA interference analysis, the specific knockdown of CypB *in vivo* resulted in the inhibition of virus-induced IRF-3 activation. This was confirmed at multiple steps, including phosphorylation, dimerization, DNA binding, coactivator binding, and IFN-β-induction. If the phosphorylation of IRF-3 is a prerequisite for the following events after the virus-induced activation of IRF-3, then it is likely that CypB is involved in the phosphorylation reaction of IRF-3. The early involvement of CypB is supported by the notion that the *in vivo* association of CypB with IRF-3 was only detected 10 min after the infection but not after 30 min. At present, it has been reported that the C-terminal phosphorylation of IRF-3 is mediated by IκB kinase-ε and TANK-binding kinase 1 (10, 11). The mutagenesis of IRF-3 revealed key residues for virus-induced activation. Substitutions of the serine residues at 385 or 386 to alanine, glutamic acid, or aspartic acid made the molecule unresponsive to stimuli (7, 21). Substitutions of other serine/threonine residues, present at positions 396, 398, 402, 404, and 405, to aspartic acid made IRF-3 constitutively active (20). Recently, Servant *et al.* (21) identified Ser-396 and Ser-398 as the minimal phosphorylation sites critical for activation, based on their observation that S396D and S396D/S398D are constitutively active. More recently, Mori *et al.* (23) identified Ser-386 as the target of IRF-3, using an antibody that specifically detects the phosphorylation of Ser-386. As protein kinases are often associated with, in addition to their regulators, molecular chaperones that sometimes need to exert their specificity for the substrates, it is interesting to speculate that CypB associates with the IRF-3 kinases, IκB kinase-ε and TANK-binding kinase 1, in a similar manner. Although it will be important in the future to determine whether it is the CypB binding, catalytic activity, or both that is responsible for its effect on IRF-3, our results indicate that CypB plays a significant role in modulating IFN-β gene expression via its interaction with IRF-3.

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