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Phorbol esters potentiate the induction of class I HLA expression by interferon α

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ABSTRACT **We have studied the effect of phorbol esters on the induction of class I histocompatibility antigen (HLA) expression by interferons (IFNs) in the T-cell line MOLT-4 and in the MOLT-4 mutant YHHH. Addition of IFN-α to phorbol 12,13-dibutyrate-pretreated MOLT-4 cells causes a >20-fold increase in the expression of class I HLA, as compared to a 4- to 7-fold IFN-α-induced increase in control cells. Pretreatment with phorbol 12,13-dibutyrate does not alter the class I HLA response to IFN-γ or the responses of other IFN-induced genes. This effect of phorbol 12,13-dibutyrate reproduces in MOLT-4 cells the phenotype of the mutant YHHH, which also displays a selective enhanced class I HLA response to IFN-α. Pretreatment of YHHH with phorbol 12,13-dibutyrate does not affect any of the responses induced by IFN. These findings suggest the existence of a phorbol ester-sensitive factor, inducible in MOLT-4 and constitutively expressed or modified in YHHH, which operates in the pathway of induction of class I HLA by IFN-α but not in the pathway used by IFN-γ.**

Interferons (IFNs) are regulatory proteins that bind to specific cell-surface receptors (1); induce gene expression (2); and elicit antiviral, antiproliferative, and immunoregulatory responses (3, 4). One of the most generalized IFN responses (reviewed in ref. 5) is the increase in the expression of major histocompatibility complex class I antigens (HLA-A, -B, -C in humans and H-2-K, -D in mice). In human cells, both type I and type II IFNs, acting through different specific receptors (6, 7), enhance class I HLA expression (8–11), mostly accounted for by HLA-B (12, 13).

In the case of IFN-α, transcriptional activation of class I HLA genes takes place shortly after IFN binding (14), and class I HLA mRNA and antigens may accumulate for several days (10, 15). Activation of transcription does not require prior protein synthesis (14) and at the DNA level it is controlled by defined regulatory flanking sequences (14, 16). Apart from these findings, the molecular events that couple IFN-α receptor occupancy with the induction of class I HLA expression remain obscure.

The analysis of spontaneous mutants with an altered class I HLA response to IFN may help in the elucidation of these events. In our laboratory, we have derived a series of such mutants from the T-cell line MOLT-4 (12). This line, originated from a human lymphoblastic leukemia (17), expresses very low constitutive levels of class I HLA, but after prolonged exposure to IFN-α it increases class I HLA expression significantly with a corresponding increase in the level of class I HLA mRNA (10). The YHHH mutant was derived from MOLT-4 by selecting cells with the highest class I HLA expression at progressively lower concentrations of IFN-α. YHHH produces 1 order of magnitude more cell-surface class I HLA in response to IFN-α than does MOLT-4 and shows induction at 100 times lower concentrations of IFN-α (12). Apart from the striking differences in the induction of class I HLA expression by IFN-α, the phenotypes of MOLT-4 and YHHH are identical for the responses to IFN-γ and for the induction of other IFN-sensitive genes (see below). We now report that pretreatment of MOLT-4 cells with phorbol esters, which activate protein kinase C (18, 19), reproduces the phenotype of YHHH in the parental line but has no effect on the IFN-α response of the mutant itself. Based on these findings, we propose that a phorbol ester-sensitive factor, constitutively expressed or modified in YHHH, regulates specifically the pathway of induction of class I HLA expression by IFN-α without interfering with the regulation of other IFN-sensitive genes or with the pathway used by IFN-γ.

MATERIALS AND METHODS

Materials. Recombinant human IFN-α was a generous gift from M. Brunda (Hoffmann–La Roche). Recombinant human IFN-γ was kindly provided by Biogen. Phorbol esters were purchased from Sigma (Poole, Dorset, England). The monoclonal antibody YTH/76.3 was purified from the ascitic fluid of tumor-bearing mice and conjugated to fluorescein isothiocyanate (12, 20, 21). The HLA-B probe was made from the M13 cDNA clone M4117 (13); the probe was generated by Klenow polymerase extension from the M13 universal primer followed by restriction with *Hin*dIII. The 6-16 probe (SC1.2/E1-850) was labeled by random priming (22); this probe, which recognizes the product of the gene described by Kelly *et al.* (23), was made from an independent cDNA clone (T. Evans, A. Bradbury, and C.M., unpublished data).

Cell Cultures. Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum under 5% CO_2/95% air at 37°C. Experiments were started with cells in exponential growth at a density of 0.3×10^6 cells per ml. YHHH is a derivative of MOLT-4 (12).

Cytofluorimetric Analysis. Class I HLA expression was measured with fluorescein isothiocyanate-conjugated monoclonal antibody YTH/76.3, using the fluorescence-activated cell sorter (FACS II, Becton Dickinson), as described (24). Mean fluorescence intensities were calculated as $\Sigma N_i C_i / \Sigma N_i$ (N_i = number of cells in channel C_i). Autofluorescence of unstained cells was subtracted.

mRNA Analysis. Purification of poly(A)$^+$ RNA and dot blot hybridization were performed as described (24).

RESULTS

Treatment of MOLT-4 cells with IFN-α produces a marked increase in the expression of class I HLA antigens, particularly of HLA-B, as recognized by the monoclonal antibody YTH/76.3 (12). Fig. 1*A* shows that treatment of MOLT-4 cells with phorbol 12,13-dibutyrate (PBt_2) for 24–48 hr also generated an increase, albeit lower, in the expression of class I HLA antigens. Addition of PBt_2 together with IFN-α had a

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Abbreviations: IFN, interferon; PBt_2, phorbol 12,13-dibutyrate.

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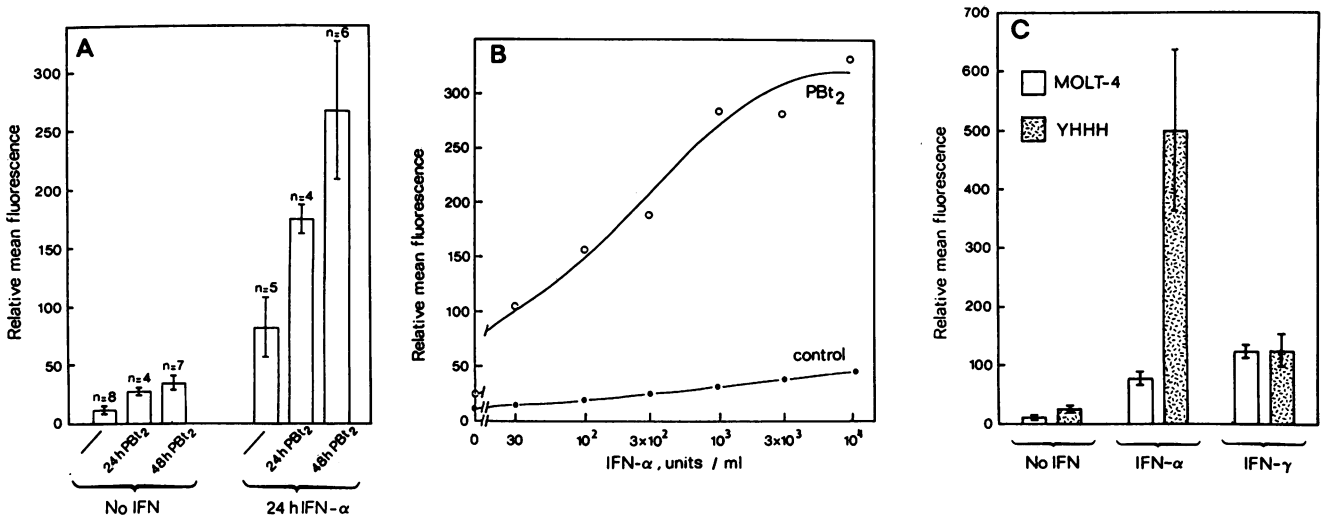


FIG. 1. (*A*) Synergistic effect of PBt_2 and IFN-α on class I HLA expression in MOLT-4 cells. (*B*) Effect of PBt_2 pretreatment on the dose–response of MOLT-4 cells to IFN-α. (*C*) Comparison of the effects of IFN-α and IFN-γ on class I HLA expression in MOLT-4 and YHHH cells. In *A*, MOLT-4 cells were treated for 24 hr with or without recombinant human IFN-α (4000 units/ml); where indicated, 200 nM PBt_2 was added at the time of the initiation of the IFN-α treatment (24 hr PBt_2) or 24 hr before (48 hr PBt_2); results are means ± SD of *n* separate experiments. In *B*, MOLT-4 cells were preincubated with or without PBt_2 for 24 hr and then treated with different concentrations of IFN-α for another 24 hr without changing the medium. In *C*, MOLT-4 cells were treated for 24 hr with IFN-α (4000 units/ml) or IFN-γ (1000 units/ml); results are mean values ± SD of four independent experiments.

synergistic effect. The combined effect of PBt_2 and IFN-α was even larger (23-fold on average) when PBt_2 was added to the cells 24 hr prior to rather than together with IFN-α (Fig. 1*A*). The effect of PBt_2 was dose dependent; half-maximal effect was observed at 5–10 nM PBt_2 and maximal effect was at 100–200 nM (data not shown).

Effect of PBt_2 on the Sensitivity of MOLT-4 and YHHH Cells to IFN-α and IFN-γ. Fig. 1*B* shows that pretreatment of MOLT-4 cells with PBt_2 also affected the dose–response to IFN-α. In the presence of low concentrations of IFN-α, class I HLA expression was barely doubled in control cells, but it increased at least 10-fold in PBt_2-treated cells, a level that could not be reached in control cells even with 100 times higher IFN-α concentrations. Thus, the potentiating effect of PBt_2 in MOLT-4 approaches the effect of the mutations that gave rise to the phenotype of YHHH, a variant of MOLT-4 with increased class I HLA response to low concentrations of IFN-α (12).

The effect of PBt_2 was also studied in YHHH. As in MOLT-4, there was a small increase in the basal level of class I HLA (Fig. 2 *A* and *B*). However, in YHHH, unlike in MOLT-4, PBt_2 did not potentiate the induction of class I HLA (Fig. 2 *C* and *D*), but rather caused a 15–20% inhibition. Thus, pretreatment with PBt_2 increases class I HLA expression induced by IFN-α in MOLT-4 cells but not in the mutant YHHH.

IFN-γ induced the expression of class I HLA antigens in MOLT-4 and YHHH cells to the same extent (Fig. 1*C*). In other words, the YHHH mutations do not affect the pathway of induction used by IFN-γ. Similarly, the marked effect of PBt_2 observed in MOLT-4 with IFN-α (Fig. 2*C*) was not observed when the cells were stimulated by IFN-γ (Fig. 2*E*).

Neither the YHHH Mutations nor PBt_2 Induce Other IFN-α-Sensitive Genes. Dot blot hybridization analysis of poly(A)$^+$ RNA purified from MOLT-4 and YHHH cells showed that after treatment with IFN-α, the level of HLA-B mRNA increased severalfold more in YHHH than in MOLT-4, but the level of mRNA for 6-16, another gene strongly induced by IFN-α in MOLT-4 cells, increased to the same extent in both cell lines (Fig. 3). Hybridization with probes that recognize the mRNAs for β_2-microglobulin and 2′–5′-oligoadenylate synthetase also showed similar levels of induction of these transcripts in both cell lines (data not shown). Thus, the mutations that gave rise to YHHH do not affect the induction of other IFN-α-sensitive genes. Similarly, the effect of PBt_2 on MOLT-4 is also not general to other IFN-α-sensitive genes, since it does not affect the induction levels of 6-16 (Fig. 3).

Role of Protein Kinase C in the Effect of PBt_2. Phorbol and phorbol ester derivatives vary in their ability to bind and activate protein kinase C (18, 26), and, as shown in Table 1, this correlates with the potentiation of the induction of class I HLA expression by IFN-α. This suggests that the effect of phorbol esters on the induction of class I HLA is mediated via protein kinase C. Prolonged incubation with phorbol esters has been shown to down-regulate protein kinase C in several cell types (27–29), but this does not seem to be the case in MOLT-4 and YHHH; total protein kinase C activity in extracts from these cells is the same and was not affected substantially by PBt_2 and/or IFN-α treatment (data not shown).

DISCUSSION

The results presented here demonstrate that previously and newly disclosed alterations of the MOLT-4 phenotype characteristic of the high IFN-α responder mutant YHHH can be reproduced in the parental line MOLT-4 by pretreatment with those phorbol esters that activate protein kinase C. These conclusions are supported by the following findings: First, the mutant YHHH has an increased sensitivity to IFN-α for its effect on class I HLA expression (12), and this property was reproduced in MOLT-4 cells by the PBt_2 pretreatment, as shown in Fig. 1*B*. Second, while YHHH cells and PBt_2-pretreated MOLT-4 cells (Fig. 1*A*) show a strikingly enhanced class I HLA response to IFN-α, they both show a normal response to IFN-γ (Figs. 1*C* and 2*E*), and they are not altered in the response of other IFN-α inducible genes (Fig. 3). Third, YHHH cells are not affected by pretreatment with PBt_2, this being an indication that the change induced in MOLT-4 cells by phorbol ester pretreatment is constitutive in YHHH. The difference in the IFN-α and IFN-γ responses are in line with previous findings (11, 30), suggesting that the pathways of class I HLA induction by type I and type II IFNs are independent.

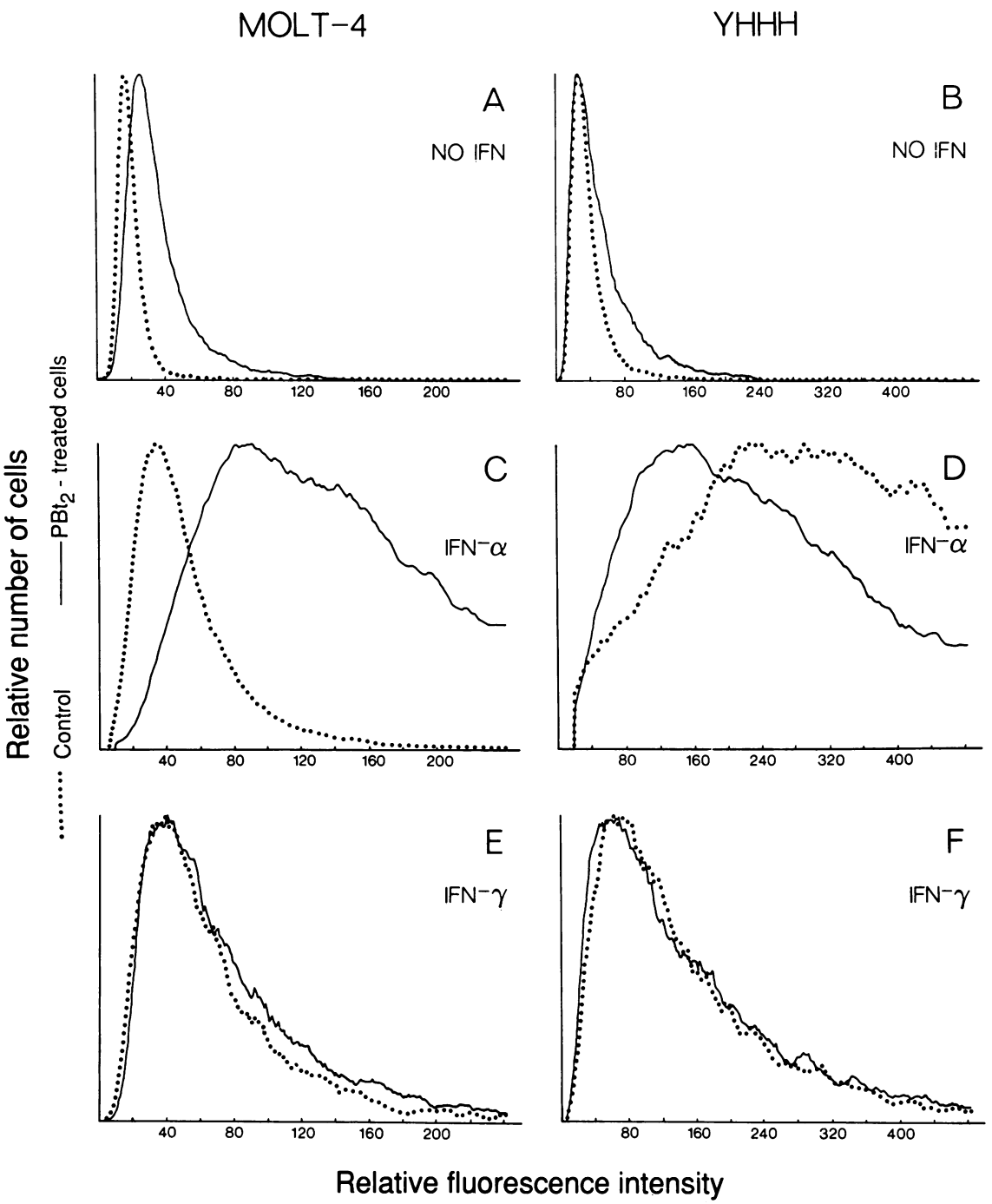


FIG. 2. Cytofluorimetric profiles of MOLT-4 and YHHH cells showing the effect of PBt_2 pretreatment on the induction of class I HLA expression by IFN-α and IFN-γ. Cells were pretreated for 24 hr with or without 200 nM PBt_2 followed by a further 24-hr incubation with or without IFNs. Medium was not changed between treatments. As indicated, MOLT-4 received IFN-α (4000 units/ml) or IFN-γ (1000 units/ml). YHHH cells received IFN-α or IFN-γ (1000 units/ml).

An IFN-induced transient increase of the levels of diacylglycerol has been observed in some cell types (31). It could be argued that YHHH is a mutant with a constitutive high level of diacylglycerol. This phenotype would be mimicked by treatment with phorbol esters. This, however, is unlikely, because the effect we described is not observed with other IFN-induced genes and because such a transient effect is difficult to reconcile with the kinetics of induction of class I HLA expression (10, 15) and with the increased effect of PBt_2 with time (see Fig. 1*A*). We have also measured the number and affinity of IFN-α receptors in MOLT-4 and YHHH and found no significant differences that might explain the phenotype of YHHH (J.D.E. and T. Evans, unpublished results). A recent paper indicates a 2-aminopurine-sensitive step involved in the induction of expression of some genes by IFN (32). Unlike our case, this effect is common to both IFN-α and -γ and affects the induction of gene 6-16.

The effect described in this paper is in some respects reminiscent of the induction of other genes by phorbol esters (33). In particular, metallothionein II is also induced by IFN-α (14, 15) and by phorbol esters (33, 34), but it is not yet known in this instance if the two agents act synergistically. Such effects are most probably mediated by changes in the concentration or activity of factors that regulate gene transcription. For instance, it has been shown that the activities of transcriptional factors AP-1 (33), AP-2 (35), and NFκB (36) are activated by phorbol esters. Interestingly, these purified factors have been shown to bind to sites upstream of H-2

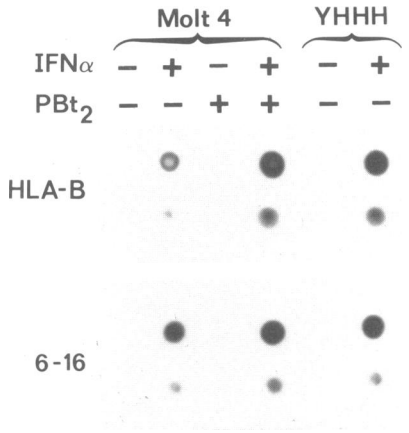
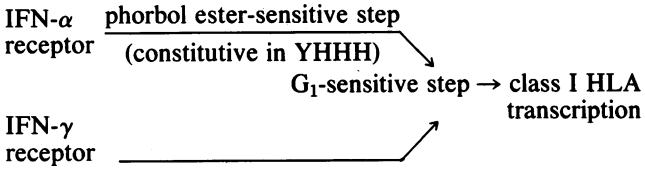


FIG. 3. Effect of PBt_2 on the levels of HLA-B and 6-16 mRNAs induced by IFN-α. MOLT-4 cells were pretreated for 24 hr with or without 200 nM PBt_2 followed by a further 24-hr incubation with or without IFN-α (1000 units/ml). YHHH were treated for 24 hr with or without IFN-α (1000 units/ml). On the first row, each dot contains 2 μg of RNA. On the second row, samples were diluted 1:3. Hybridization with a CD1a probe [cDNA clone FCB6 (25)] was used as a control and showed the same RNA levels in all the samples.

class I genes (35, 37, 38). Class I HLA genes also have recognition motifs for AP-2 and NFκB but do not appear to have conserved the AP-1 site. It is conceivable, therefore, that the phorbol ester effect on class I HLA induction is mediated through one or more of these factors. However, the effect we have described is unique in that the phorbol esters are not activating the genes directly, but are potentiating the activity of another agent, in this case IFN-α.

We have previously reported the superinduction of class I HLA expression by IFNs in G_1/S-arrested cells (24). That effect did not discriminate between IFN-α and IFN-γ and was identical in MOLT-4 and YHHH; therefore, it differs from the effect reported here with phorbol esters. Each effect appears to be operating at a different step of the pathway(s) transducing the IFN signals. The possible relationship between the two steps is schematically represented as follows:



The results presented here may help to dissect the complex intracellular events that modulate the induction of class I HLA expression. We suggest that following treatment of MOLT-4 cells with PBt_2, a putative factor involved in the induction of class I HLA expression by IFN-α is produced or activated. This factor is constitutive in the mutant YHHH. This is why PBt_2 fails to potentiate the induction of class I HLA by IFN-α in YHHH. The putative factor defines a rate-limiting step in the pathway that transduces the IFN-α signal to the class I HLA transcriptional machinery. At this step, the pathway used by IFN-γ differs, since neither the effect of PBt_2 in MOLT-4 nor the high responder phenotype of YHHH is manifested with IFN-γ.

Table 1. Effectiveness of phorbol esters to potentiate the induction of class I HLA expression

Pretreatment	Protein kinase C activation*	Relative mean fluorescence	
		No IFN	IFN-α
None		14	108
Phorbol	−	16	101
4α-Phorbol 12,13-didecanoate	−	16	106
Phorbol 12-myristate 13-acetate	+	29	194
Phorbol 12,13-didecanoate	+	28	270
PBt_2	+	40	346

MOLT-4 cells were pretreated with or without various phorbol derivatives (200 nM) followed by a further 24-hr incubation with or without IFN-α (4000 units/ml). Medium was not changed between treatments. Class I HLA expression was measured as described in Fig. 1.
*From Castagna *et al.* (18).

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