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THE INHIBITORY MECHANISM OF IN VITRO PROTEIN PHOSPHORYLATION BY A NONPROTEIN CHROMOPHORE REMOVED FROM NEOCARZINOSTATIN

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Summary

The inhibitory effect of a nonprotein chromophore removed from neocarzinostatin on protein phosphorylation by nuclear protein kinase in vitro has been studied. Low levels of the chromophore greatly inhibited protein phosphorylation in vitro. This inhibition, however, was not selectively dependent on the indicated kinases and their different phosphate acceptors (histones and non-histone protein). In contrast, the protein component (apoprotein) of neocarzinostatin did not affect the phosphorylation even at a concentration 400-times higher than that of the chromophore. Moreover, apoprotein suppressed the chromophore-induced inhibition of protein phosphorylation in vitro in proportion to the apoprotein concentrations. Kinetic and analytical experiments suggest that the chromophore-induced inhibition of protein phosphorylation seems to be due to the binding of the chromophore to the kinases. In addition, we found that ultraviolet irradiation as well as methanol extraction can release the chromophore from neocarzinostatin, but it exhibits no inhibitory activity of DNA synthesis in growing cells. The fact that the chromophore-induced inhibition of protein phosphorylation in vitro was not sensitive to ultraviolet irradiation, which rapidly inactivated the ability of the chromophore to induce DNA degradation in vitro, suggests that there are different actions involved in the two inhibitions induced by the chromophore which is removed from neocarzinostatin.

Introduction

Neocarzinostatin, an antitumor antibiotic, is a single polypeptide with a molecular weight of 10700 [1,2]. Low levels of neocarzinostatin selectively inhibit DNA synthesis in sensitive bacteria and mammalian cells [3,4] and also induce DNA degradation in vitro and in vivo [4—11]. Recently, we [12—14] and the Goldberg group [15,16] have reported separately that neocarzinostatin possesses a nonprotein chromophore which can be removed from neozarzinostatin by methanol extraction. We have determined a part of the chemical structure of the chromophore, which is a derivative of a naphthalenecarboxylic acid [13]. In addition, we [17] and the Goldberg group [16,18] have shown recently that low levels of the chromophore not only inhibit DNA synthesis but also induce DNA degradation in growing cells. The evidence suggests that the chromophore is responsible for the biological activities of neocarzinostatin, such as the inhibition of DNA synthesis in growing cells and the induction of DNA degradation in vitro and in vivo [15—18].

In this paper we describe the inhibitory effect of the chromophore on in vitro protein phosphorylation by nuclear protein kinases from mouse spleen cells. The chromophore-induced inhibition of protein phosphorylation in vitro seems to be due to the binding of the chromophore to the kinases. However, this inhibition by the chromophore was not selectively dependent on the indicated kinases and their phosphate acceptors (histones and non-histone protein). Moreover, ultraviolet irradiation, which greatly inactivates the ability of the chromophore to inhibit DNA synthesis in growing cells, did not affect the ability of the chromophore to inhibit protein phosphorylation by the kinases in vitro. This finding suggests strongly that there are different biochemical mechanisms involved in the two distinct inhibitions induced by the chromophore.

Materials and Methods

Chemicals

Neocarzinostatin was kindly supplied by Dr. Y. Koyama (Kayaku Antibiotic Res. Corp., Tokyo). [γ-^{32}P]ATP (19.0 Ci/mmol) was obtained from Amersham Corp., calf thymus histones (H1, H2a, H3 and H4) from Boehringer Mannheim GmbH, and Sephadex G-25 from Pharmacia.

Kinase substrates

Calf thymus histones (H1, H2a, H3 and H4) and 13000 dalton non-histone chromatin protein from calf thymus chromatin [19] were used as phosphate acceptors of cyclic AMP-dependent and cyclic AMP-independent protein kinases purified from the nuclei of mouse spleen cells [20].

Nonprotein chromophore and apoprotein preparation from neocarzinostatin

All preparation procedures were carefully carried out in a dark room, because the biological activities of nonprotein chromophore and neocarzinostatin were rapidly inactivated by light and by ultraviolet irradiation [12—15]. Nonprotein chromophore and apoprotein were prepared from neocarzinostatin powder as described in previous papers [14,17].

Nuclear protein kianses

The two distinct protein kinases, cyclic AMP-dependent (M_r 68 000) and cyclic AMP-independent protein kinase (M_r 45 000), were separately purified from the nuclei of mouse spleen cells by using DEAE-cellulose, Sephacryl S-200 gel filtration, phosphocellulose column chromatography and 5—25% (v/v) glycerol density gradient centrifugation (165 000 × *g* for 17 h at 4°C), successively, as reported previously [19,20]. Cyclic AMP-dependent and cyclic AMP-independent protein kinase were partially purified about 950-fold and 820-fold, respectively, with activity yields of 25% and 23%, based on the crude enzyme preparation (0.5 M KCl extracts). These two kinases were stored at −20°C in the presence of 5% glycerol and 2 mM dithiothreitol.

Assay of protein kinase activity

The complete reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 8 mM magnesium acetate, 10 μg bovine serum albumin, 20 μg phosphate acceptor (histone of 13 000 dalton nonhistone chromatin protein), 100 μM [γ-^{32}P]ATP (3000 cpm/pmol) and either 0.5 μg of cyclic AMP-dependent protein kinase or 0.3 μg of cyclic AMP-independent protein kinase. After incubation (10 min at 37°C), the enzyme reaction was arrested by the addition of 0.5 ml of 0.1 M sodium pyrophosphate containing bovine serum albumin (1 mg/ml) and 0.5 ml of 20% trichloroacetic acid. The precipitate formed was filtered through a glass filter paper (Whatman, type GF/F). After drying, the ^{32}P radioactivity contained in the filter was determined in a liquid scintillation spectrometer. The activity of cyclic AMP-dependent protein kinase was assayed in the presence of 1 μM cyclic AMP.

Protein measurements

Protein was determined by the methods of Lowry et al. [21] and of Bradford [22], using bovine serum albumin as standard protein.

Results

Effect of nonprotein chromophore, apoprotein and neocarzinostatin on histone H2a phosphorylation in vitro

The effect of nonprotein chromophore, apoprotein and neocarzinostatin on histone H2a phosphorylation by cyclic AMP-dependent protein kinase was examined. Table I shows that low levels of the chromophore greatly inhibited histone H2a phosphorylation, whereas only 2% of the phosphorylation was inhibited by apoprotein even at a concentration 200-times higher than that of the chromophore. Neocarzinostatin also inhibited kinase activity, but a 30-times higher concentration was required to create an effect similar to that of the chromophore. These results show that the chromophore of neocarzinostatin is responsible for the inhibition of histone H2a phosphorylation by the kinase in vitro, as has been shown in the inhibition of DNA synthesis in growing cells and the induction of DNA degradation in vitro and in vivo by the chromophore [12,13].

TABLE I

EFFECT OF A NONPROTEIN CHROMOPHORE, APOPROTEIN AND NEOCARZINOSTATIN ON HISTONE H2a PHOSPHORYLATION BY CYCLIC AMP-DEPENDENT PROTEIN KINASE IN VITRO

The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 8 mM magnesium acetate, 1 μM cyclic AMP, 10 μg bovine serum albumin, 20 μg histone H2a, 100 μM [γ-^{32}P]ATP (3000 cpm/pmol) and 0.5 μg purified cyclic AMP-dependent protein kinase. The mixture was incubated for 10 min at 37°C in the presence of either the nonprotein chromophore, apoprotein (protein component of neocarzinostatin) and neocarzinostatin at the indicated concentrations.

Addition	Concentration (μg)	Histone H2a phosphorylation	
		cpm	%
None		40 641	100
Nonprotein chromophore	0.2	36 983	91.0
	1.0	15 850	39.0
	5.0	3 042	7.5
Apoprotein	5.0	41 453	102
	25.0	42 060	103
	200.0	39 828	98.0
Neocarzinostatin	1.0	37 706	92.8
	5.0	30 346	74.7
	25.0	23 572	58.0

Substrate and kinase specificity

Since low levels of nonprotein chromophore greatly inhibited histone H2a phosphorylation by cyclic AMP-dependent protein kinase in vitro, we tested whether the inhibitory effect of the chromophore on protein phosphorylation was dependent on either the nuclear kinases or their phosphate acceptors. The obtained results are summarized in Table II. The results show that cyclic AMP-independent protein kinase greatly phosphorylates a 13 000 dalton non-histone chromatin protein as reported previously [19]. The chromophore greatly inhibited the phosphorylation of these two proteins (histone H2a and 13 000 dalton non-histone chromatin protein) by the kinases, but there was no signi-

TABLE II

EFFECT OF A NONPROTEIN CHROMOPHORE ON PROTEIN PHOSPHORYLATION BY TWO DISTINCT PROTEIN KINASES IN VITRO

The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 8 mM magnesium acetate, 10 μg bovine serum albumin, 20 μg phosphate acceptor (histone H2a or 13 000 dalton non-histone chromatin protein), 100 μM [γ-^{32}P]ATP (3000 cpm/pmol) and purified nuclear kinase (0.5 μg cyclic AMP-dependent protein kinase or 0.3 μg cyclic AMP-independent protein kinase). The mixture was incubated for 10 min at 37°C in the presence or absence of the chromophore (2 μg). The activity of cyclic AMP-dependent protein kinase was assayed in the presence of 1 μM cyclic AMP, which had no effect on the inhibitory activity of the chromophore under the assay conditions.

Substrate	Cyclic AMP-dependent protein kinase (pmol)		Cyclic AMP-independent protein kinase (pmol)	
	—Chromophore	+Chromophore	—Chromophore	+Chromophore
Histone H2a	13.2	1.3	9.9	1.0
13 000 dalton non-histone chromatin protein	9.0	0.9	44.1	2.7

ficant difference between the phosphorylation using two distinct kinases and their substrates, including other substrates (histone H1, H3 and H4).

Kinetics of nonprotein chromophore on protein phosphorylation

Since the inhibition of protein phosphorylation induced by the chromophore in vitro is not selectively dependent on the indicated kinases and their substrates, kinetic experiments were carried out to determine the inhibition using histone H2a and cyclic AMP-dependent protein kinase. The obtained results indicated that the apparent K_m and V values for histone H2a of the kinase were $2.35 \cdot 10^{-6}$ M and 0.25 nmol/min per mg protein, whereas those for the kinase in the presence of the chromophore (1 μg) were $2.89 \cdot 10^{-5}$ M and 0.16 nmol/min per mg protein, respectively (Fig. 1A). The apparent K_m and V values for ATP of the kinases were $7.71 \cdot 10^{-7}$ M and 0.51 nm/min per mg protein, whereas those for the kinase in the presence of the chromophore (1 μg) were $1.12 \cdot 10^{-6}$ M and 0.43 nmol/min per mg protein, respectively (Fig. 1B). These results show that the chromophore-induced inhibition of histone H2a phosphorylation by the kinase in vitro was both competitive and noncompetitive inhibition (mixed-type inhibition). A similar result was obtained with 13 000 dalton non-histone chromatin protein and cyclic AMP-independent protein kinase. In addition, in order to determine the binding of the chromophore to cyclic AMP-dependent protein kinase, the kinase was incubated with various concentrations of the chromophore for 10 min at 37°C in the absence of [γ-^{32}P]ATP and Mg^{2+}. The reaction mixture was analyzed by 5—25% glycerol density gradient centrifugation (165 000 × g for 17 h at 4°C). The obtained results showed that the kinase activity was reduced to an extent dependent on the chromophore concentrations. Moreover, these results were reproducible if the reaction mixture was dialyzed against 20 mM Tris-HCl (pH 7.5) containing

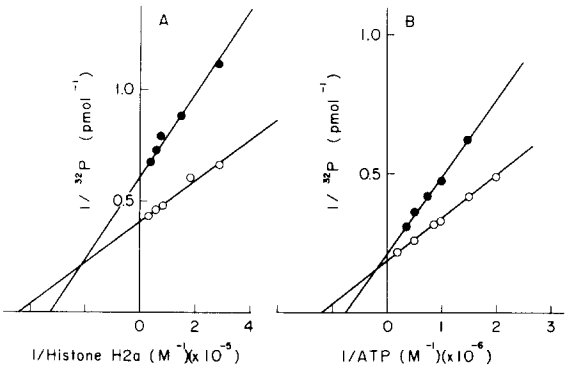

Fig. 1. K_m values of cyclic AMP-dependent protein kinase for histone H2a and ATP. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 8 mM magnesium acetate, 1 μM cyclic AMP, 10 μg bovine serum albumin, 0.5 μg purified AMP-dependent protein kinase, 20 μg histone H2a and 100 μM [γ-^{32}P]ATP (3000 cpm/pmol). The mixture was incubated for 10 min at 37°C in the presence or absence of the chromophore (1 μg). A, K_m values of the kinase for histone H2a in the presence and absence of the chromophore (1 μg). The K_m values were determined at various concentrations of histone H2a (M_r 14 000) in the presence of 100 μM [γ-^{32}P]ATP. B, K_m values of the kinase for ATP in the presence and absence of the chromophore (1 μg). The K_m values were determined at various concentrations of [γ-^{32}P]ATP in the presence of 20 μg histone H2a as a phosphate acceptor of the kinase. These K_m values were averages obtained from four different experiments ($P < 0.001$ using Student's t-test).

50 mM KCl and 2 mM dithiothreitol overnight at 4°C before ultracentrifugation. These results suggest that the binding of the chromophore to the kinase results in the reduction of protein phosphorylation in vitro.

Suppressive effect of apoprotein

The above experiments show that the chromophore greatly inhibits protein phosphorylation by nuclear kinases at one-tenth the concentration of neocarzinostatin and that no effect of apoprotein (protein component of neocarzinostatin) on the phosphorylation is detected at even a concentration 100-times higher than that of the chromophore. This result suggests that apoprotein may suppress the chromophore-induced inhibition of protein phosphorylation in vitro. To test this possibility, the effect of apoprotein on the chromophore-induced inhibition of histone H2a phosphorylation by cyclic AMP-dependent protein kinase was examined by adding various cocnentrations of apoprotein. The results obtained showed that apoprotein suppressed the chromophore-induced inhibition of phosphorylation in proportion to the apoprotein concentrations. A similar effect of apoprotein on the chromophore-induced inhibition of protein phosphorylation was observed when other phosphate acceptors, such as histones (H1, H3 and H4) and 13 000 dalton non-histone chromatin protein, were used instead of histone H2a.

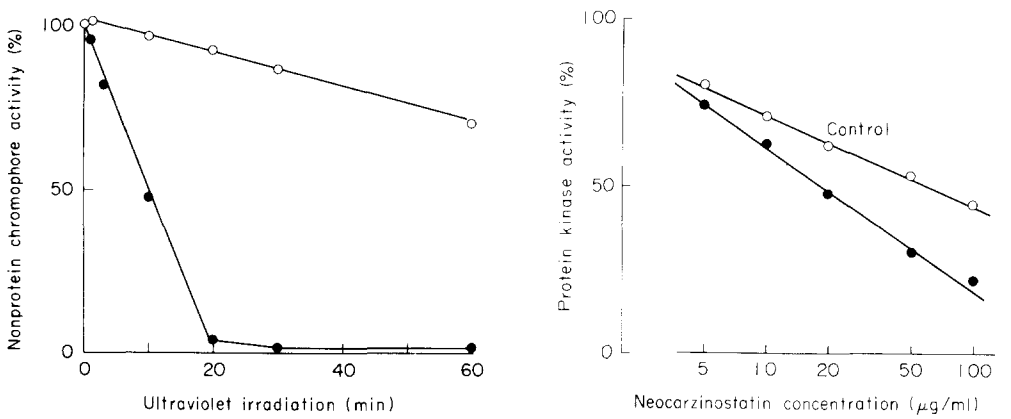

Fig. 2. Effect of ultraviolet irradiation on the chromophore activity. The chromophore prepared from neocarzinostatin by methanol extraction was dissolved in 1.0 ml distilled water (1 mg/ml) and then ultraviolet-irradiated at 3000 $\mu W/cm^2$ per s for the indicated periods at room temperature. The effect of the ultraviolet-irradiated chromophore (2 μg) on histone H2a phosphorylation by cyclic AMP-dependent protein kinase was tested. The effect of the same chromophore (2 μg) on the incorporation of [^{3}H]thymidine (0.5 $\mu Ci/ml$) into the acid-insoluble fraction of mouse L cells was also tested. The mouse L cells ($2 \cdot 10^5$ cells) were previously cultured at 37°C in minimal essential medium containing 10% bovine serum albumin. The cells were further cultured at 37°C for 3 h in the presence or absence of the ultraviolet-irradiated chromophore (2 μg). Protein kinase activity, ○; incorporation of [^{3}H]thymidine into the acid-insoluble fraction, ●.

Fig. 3. The stimulating effect of ultraviolet irradiation on neocarzinostatin-induced inhibition of histone H2a phosphorylation. The indicated concentrations of neocarzinostatin were ultraviolet-irradiated separately for 30 min at 3000 $\mu W/cm^2$ per s in a dark room at room temperature. After that, the inhibitory effects of ultraviolet-treated and untreated neocarzinostatin were examined and then compared. The kinase activity was determined under the same conditions as described in Table I, using histine H2a and purified cyclic AMP-dependent protein kinase. Inhibitory activity of histone H2a phosphorylation in vitro by neocarzinostatin (control), ○; and by ultraviolet-treated neocarzinostatin, ●.

Effect of ultraviolet irradiation on nonprotein chromophore activity

It has been reported that ultraviolet irradiation rapidly inactivates biological activities of neocarzinostatin, i.e., the inhibition of DNA synthesis in growing cells and the induction of DNA degradation in vitro and in vivo [12,17]. The effect of ultraviolet irradiation on the biological activity of nonprotein chromophore was examined. Fig. 2 shows that histone H2a phosphorylation by cyclic AMP-dependent protein kinase in vitro is reduced by about 30% when the chromophore is ultraviolet irradiated at 3000 $\mu W/cm^2$ per s for 60 min at room temperature, whereas the ability of the chromophore to inhibit DNA synthesis in growing cells is completely inactivated by ultraviolet irradiation within 20 min. Moreover, in vitro, no inducing activity of DNA degradation of the ultraviolet-irradiated chromophore was observed. It should be noted that the inhibitory effect of neocarzinostatin on the phosphorylation was enhanced slightly when neocarzinostatin was ultraviolet-irradiated (Fig. 3). This observation suggests the possibility that the chromophore may be released from neocarzinostatin by ultraviolet irradiation, as shown by methanol extraction [12—15].

Release of nonprotein chromophore from neocarzinostatin by ultraviolet irradiation

The above observation that ultraviolet irradiation of neocarzinostatin stimulated the chromophore-induced inhibition of protein phosphorylation significantly suggests the possibility that the chromophore may be released from neocarzinostatin by ultraviolet irradiation. This was tested by the following experiments: neocarzinostatin powder (50 mg) dissolved in 1.0 ml distilled water was irradiated with an ultraviolet-lamp for 30 min at 3000 $\mu W/cm^2$ per s at room temperature in a dark room. The ultraviolet-irradiated neocarzinostatin was passed though a Sephadex G-25 column (1.2 × 34 cm) washed previously with distilled water to separate the substrate(s) released from neocarzinostatin. The column chromatography showed two distinct peaks. The main peak was eluted at void volume fractions and the other minor peak was eluted near V_t fractions (Fig. 4). However, no minor peak was observed in the control run (under the same conditions) of the neocarzinostatin not treated by ultraviolet. High inhibitory activity (about 60%) of histone H2a phosphorylation was detected in the minor peak fractions of ultraviolet-irradiated neocarzinostatin, whereas negligible reduction of the phosphorylation was detected in the minor peak fractions of the control (Fig. 4). The ultraviolet absorption spectrum of the material(s) (nonprotein chromophore) released from neocarzinostatin by ultraviolet irradiation (fraction 34) was similar to that of the chromophore prepared from neocarzinostatin by methanol extraction [13—15]. The purities of both chromophore preparations from neocarzinostatin were about 96%, estimated from nuclear magnetic resonance data. Fig. 5 shows that the ultraviolet absorption spectrum of the chromophore from neocarzinostatin by methanol extraction is slightly different from that of the material released from neocarzinostatin by ultraviolet irradiation between 270 nm and 350 nm (λ_{max} = 340 nm). However, the ultraviolet absorption spectrum of the chromophore released from neocarzinostatin by methanol extraction was shifted to that of the material released from neocarzinostatin by ultraviolet irradiation. Although both the ultraviolet-irradiated chromophore prepared from neocar-

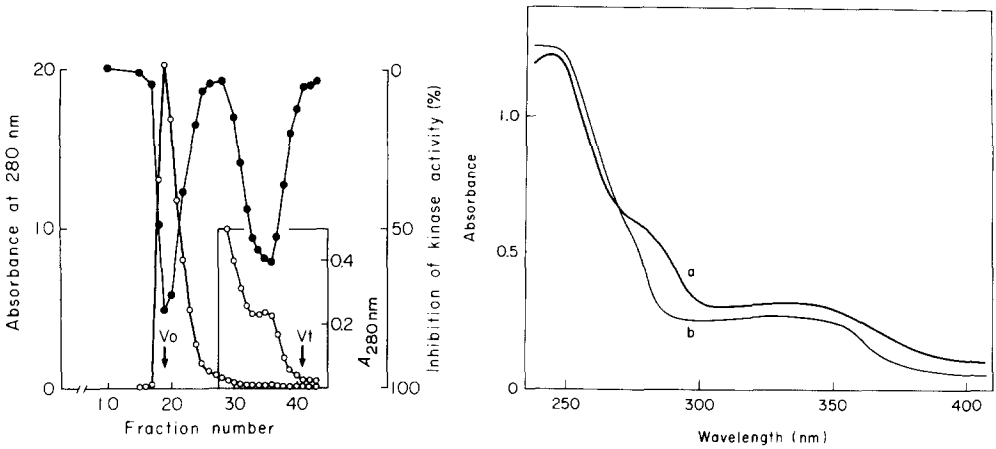

Fig. 4. Nonprotein chromophore released from neocarzinostatin by ultraviolet irradiation. Neocarzinostatin (50 mg) dissolved in 1.0 ml of distilled water was ultraviolet-irradiated for 30 min at 3000 $\mu W/cm^2$ per s in a dark room. The ultraviolet-irradiated neocarzinostatin was passed through a Sephadex G-25 column (1.2 X 34 cm) previously washed with distilled water, and 1.0 ml of the fraction was collected. Absorbance of the fractions was measured at A_{280nm} and the inhibitory effect of the fractions on histone H2a phosphorylation by cyclic AMP-dependent protein kinase was determined. The frame of the figure (between fraction 28 and fraction 45) was extended to 10-times that of the original size. A_{280nm}, ○; inhibition of histone H2a phosphorylation (%), ●.

Fig. 5. Ultraviolet absorption spectrum of the chromophore released from neocarzinostatin by ultraviolet irradiation and by methanol extraction. Two preparation of the chromophores (from neocarzinostatin by ultraviolet irradiation and from neocarzinostatin by methanol extraction) were dissolved separately in distilled water (100 μg/ml). a, nonprotein chromophore from neocarzinostatin by methanol extraction; b, nonprotein chromophore from neocarzinostatin by ultraviolet irradiation.

zinostatin by methanol extraction and the chromophore released from neocarzinostatin by ultraviolet irradiation exhibited no inhibitory activity of DNA synthesis in growing cells and also no inducing activity of DNA degradation in vitro, both, however, inhibited protein phosphorylation by the kinase in vitro. These results suggest strongly that ultraviolet irradiation release nonprotein chromophore with loss of biological activities, such as the inhibition of DNA synthesis in growing cells and the induction of DNA degradation in vitro, and also suggest that ultraviolet irradiation induces some chemical alteration of the chromophore and that this chemical change may involve the inactivation of its biological activity, except for the inhibitory ability of the chromophore to inhibit the protein phosphorylation by the kinase in vitro.

Discussion

Present evidence shows that a nonprotein chromophore from neocarzinostatin inhibits greatly in vitro protein phosphorylation by the binding of the chromophore to the nuclear kinases. This inhibition by the chromophore is not selectively dependent on the indicated kinases and their phosphate acceptors (histones and non-histone protein). Moreover, we found that apoprotein (the protein component of neocarzinostatin) suppresses the chromophore-induced inhibition of protein phosphorylation in proportion to the apoprotein concen-

tration. Since neocarzinostatin is reconstituted from the purified chromophore and apoprotein at optimal ratios [23], the suppressive effect of apoprotein of the chromophore-induced inhibition of protein phosphorylation in vitro seems to be due to the competitive binding of the chromophore between apoprotein and the kinases.

We [12–14] and the Goldberg group [15,16] have reported separately that the chromophore can be removed from neocarzinostatin by methanol extraction, and also showed that the ultraviolet absorption spectrum of neozarzinostatin shifts to that of pre-neocarzinostatin [24], which is physicochemically similar to apoprotein [12,14]. We present here the result that ultraviolet irradiation releases nonprotein chromophore from neocarzinostatin without reducing its inhibitory activity of protein phosphorylation in vitro (Fig. 4). With regard to the ultraviolet irradiation of the chromophore removed from neocarzinostatin by methanol extraction, the following points should be considered: (1) ultraviolet irradiation rapidly inactivates the ability of the chromophore to induce DNA degradation in vitro and to inhibit DNA synthesis in growing cells; (2) the ultraviolet absorption spectrum of the chromophore from neocarzinostatin by methanol extraction has a definite shoulder between 270 nm and 300 nm, whereas no such shoulder is observed after ultraviolet irradiation; and (3) the ultraviolet absorption spectrum of the ultraviolet-irradiated chromophore from neocarzinostatin by methanol extraction is similar to that of the ultraviolet-released chromophore. Such a consideration may reveal the presence of at least two distinct chromophores of neocarzinostatin: one, which is responsible for the induction of DNA degradation in vitro (or inhibition of DNA synthesis in growing cells), is ultraviolet-sensitive and the other, which is responsible for the inhibition of protein phosphorylation in vitro, is ultraviolet-insensitive. The separation of these two chromophores has not yet been accomplished. However, there is another possibility that ultraviolet irradiation induces some chemical alteration of the chromophore and the chemical change may involve the inactivation of the activity of the chromophore to inhibit DNA synthesis in growing cells and to induce DNA degradation in vitro.

The chromophore-induced inhibition of DNA synthesis in growing cells is completely dependent on the ability of the chromophore to induce DNA degradation in vitro, since the two inhibitory activities of the chromophore are inactivated quantitatively by ultraviolet irradiation (unpublished results). This and previous observations suggest that the chromophore-induced DNA degradation results in the inhibition of DNA synthesis in growing cells [12,17]. However, we found here a dissimilarity between the chromophore-induced inhibition of protein phosphorylation in vitro and the chromophore-induced inhibition of DNA synthesis in growing cells (or the chromophore-induced DNA degradation in vitro), because ultraviolet irradiation greatly inactivated the ability of the chromophore to inhibit DNA synthesis in growing cells, but no significant effect was observed in the protein phosphorylation (Fig. 2). This finding strongly supports the possible explanation that the chromophore-induced inhibition of protein phosphorylation in vitro is a different biochemical action from that of the chromophore-induced DNA degradation in vitro.

For the complete understanding of the inhibitory mechanism of the chromophore-induced protein phosphorylation in vitro, the complete chemical struc-

ture of the chromophore and the binding sites of the chromophore on the apoprotein or the kinases are being actively studied.

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