NEOCARZINOSTATIN (NCS) AND MICROTUBULE-ASSOCIATED KINASE

Sir:

Neocarzinostatin (NCS), antitumor antibiotic, is a single polypeptide with a molecular weight of 10,700[1,2]. Recently, it has been shown that NCS possesses a nonprotein chromophore (designated as NPC), which can be removed from NCS by methanol extraction[3~7], and also demonstrated that NPC is responsible for the NCS-induced inhibition of DNA synthesis in growing cells and for the NCS-induced DNA degradation *in vitro* and *in vivo*[5~7]. We have determined a part of the chemical structure of NPC, which is a derivative of naphthalenecarboxylic acid[3]. In addition, we previously showed that NCS inhibits cell spreading, cell surface capping and microtubular paracrystal formation by the binding of NCS to the cell surface receptors on the cell membrane[8,9]. Although the detailed mechanisms of these NCS-induced inhibitions are still unknown, it would seem that these inhibitions may be involved in the impairment of the membrane-microtubule systems[9]. Moreover, recent reports described that microtubular proteins are associated with ATP-dependent protein kinase activity and their phosphorylations and dephosphorylations may play a role in the regulation of the assembly and/or biological function of the proteins[10~12].

On the basis of these findings, the present study was undertaken to test the effect of NCS and NPC on protein phosphorylation by microtubule-associated kinase *in vitro*. We found that NCS and NPC greatly inhibit the protein phosphorylation *in vitro* by the binding of NPC to the kinase. Available data suggest the possibility that the NCS-induced inhibition of cell spreading, cell surface capping and microtubular paracrystal formation may be associated with the inhibition of the kinase activity by NPC at cell surface levels.

We used microtubular protein (Mr=55,000), which was associated with cAMP-dependent protein kinase, purified from rabbit brain cells[11]. The activity of microtubule-associated kinase was assayed in the presence of [γ-^{32}P]ATP and a phosphate acceptor (histone or nonhistone protein), as described in a previous paper.[13] After incubation at 37°C for the appropriate periods, the [^{32}P]radioactivity of the phosphorylated substrate was determined[13]. NPC and protein component of NCS, carrier protein of NPC (designated as PC), were prepared carefully from NCS powder in a dark room as described in a previous report[5].

The activity of microtubule-associated kinase assayed under various conditions are summarized in Table 1, when histone H2a was used as a phosphate acceptor. The optimal conditions of Mg^{2+} and cAMP were found to be 8 mM and 1 μM. cAMP (1 μM) highly enhanced the kinase activity (about five-fold). cAMP and Mg^{2+} could not be substituted by cGMP and Ca^{2+}, respectively. No effect of N-ethylmaleimide (NEM), which is an inhibitor of SH-enzymes, on the kinase activity was detected. NCS and NPC highly inhibited histone H2a phosphorylation, whereas no effect of PC on the phosphorylation was detected at an even 100-times higher concentration than that of NPC. This result show that NPC is responsible for the NCS-induced inhibition of histone H2a phosphorylation by the kinase *in vitro*. However, the NPC-induced inhibition of the phosphorylation was suppressed by the addition of PC. This suppression was in proportion to the PC concentrations (data not shown). In addition, the kinase functioned in the presence of calf thymus histones (H1, H2a, H3 and H4) and 13,000 dalton nonhistone chromatin protein (13,000 NHCP), which is a specific phosphate acceptor of nuclear cAMP-independent protein kinase from mouse spleen cells[13]. However, human immunoglobulin, phosvitin, actin, myosin and bovine serum albumin did not act as phosphate acceptors for the kinase. We used histone H2a for the study since the protein served as the most effective phosphate acceptor for the kinase under the conditions. The obtained results show that the substrate requirements and the optimal conditions of the kinase activity *in vitro* are similar to those of cAMP-dependent protein kinase from rabbit muscle and from the nuclei of mammalian cells[14].

It has been reported that the cell cycle traverse is blocked in the G_2 phase by NCS[8,15] and several other antibiotics such as bleomycin[16], mitomycin C[17], adriamycin[18] and cytochalasin B[19]. We examined the effect of these G_2 blockers on histone H2a phosphorylation by the kinase *in vitro*. Table 2 shows that low levels

Table 1. Requirements for the activity of microtubule-associated kinase *in vitro*.

Requirements	Protein kinase activity (pmol)
Complete	96.0
Complete−Mg^{2+}	0.1
Complete−Mg^{2+}+Ca^{2+}	0.3
Complete−Histone H2a	0.3
Complete−cAMP	18.7
Complete−cAMP+cGMP	23.0
Complete−Dithiothreitol+NEM	83.6
Complete+NCS	55.4
Complete+NPC	8.8
Complete+PC	95.7
Complete+NPC (2 μg)+PC (20 μg)	60.7

The complete reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 8 mM magnesium acetate, 1 μM cAMP, 10 μg of bovine serum albumin, 20 μg of histone H2a, 0.2 μg of purified microtubule-associated kinase and 100 μM [γ-^{32}P]ATP (2,000 cpm/pmol).

The reaction mixtures were incubated for 10 minutes at 37°C in the presence or absence of various substances. The concentrations of Mg^{2+}, Ca^{2+}, dithiothreitol, N-ethylmaleimide (NEM), cAMP, cGMP, NCS, NPC and PC were 8 mM, 1 mM, 4 mM, 2 mM, 1 μM, 1 μM, 5 μg, 2 μg and 100 μg respectively. The [^{32}P] radioactivity of the trichloroacetic acid precipitate was measured.[13]

Table 2. Effect of G_2 blocker on histone H2a phosphorylation by microtubule-associated kinase *in vitro*.

	G_2 blockers	Concentration (μg)	Inhibition (%)
None (control)		—	0.0
G_2 block antibiotics	NCS	1	21.3
		10	60.8
	NPC	1	78.2
		10	98.2
	Bleomycin	1	5.4
		10	12.5
	Mitomycin C	1	2.3
		10	18.8
	Adriamycin	1	8.3
		10	19.6
	Cytochalasin B	1	6.5
		10	25.0
	Actinomycin D	1	9.6
		10	15.0
	Puromycin	1	4.8
		10	15.6
Mitotic inhibitors	Vinblastin-sulfate	1	0.1
		10	1.8
	Colcemid	1	3.1
		10	17.5

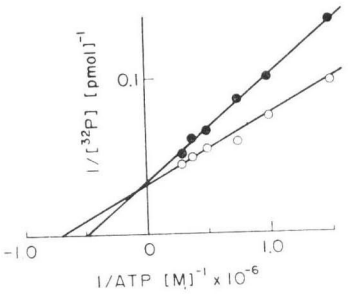
The kinase (0.2 μg) was assayed in the presence of various inhibitors at the indicated concentrations for 10 minutes at 37°C under the conditions as described in Table 1. 100 % Activity (0 % inhibition) of the kinase corresponds to the incorporation of 96.0 pmol of [γ-^{32}P]ATP into histone H2a.

of NCS and NPC highly inhibit the phosphorylation, whereas no significant inhibition by other tested G_2 blockers including mitotic inhibitors on the phosphorylation was observed at even 10-times higher concentration than that of NPC. A similar effect of NPC on the phosphorylation was observed when other phosphate acceptors (histone H1, H3, H4 and 13,000 NHCP) were used instead of histone H2a (data not shown). These results suggest the possibility that the mechanisms of NCS-induced G_2 block may be different from that of other G_2 blockers such as bleomycin, which is considered to inhibit DNA synthesis in growing cells in a manner similar to that of NCS.[6,20]

The kinetic experiments showed that an apparent *Km* value for ATP of the kinase was 1.89×10^{-7} M, whereas that of the kinase in the presence of NPC (1 μg) was 2.75×10^{-6} M (Fig. 1). Although no exact *Km* value for histone H2a of the kinase was determined, since the kinase itself was also phosphorylated under the conditions, the obtained *Km* value was greatly reduced when the kinase was incubated with histone H2a in the presence of NPC (1 μg). Moreover, 5~25% glycerol density gradient centrifugation showed that the activity of the sedimented kinase, which was preincubated with different concentrations, was reduced depending on the NPC concentrations (Fig. 2). A similar result was observed when the kinase incubated with NPC was dialyzed against a suitable buffer before ultracentrifugation. These experimental results suggest that the NPC-induced inhibition

Fig. 1. *Km* value of microtubule-associated kinase for ATP.

The kinase activity was assayed in the presence (●) or absence (○) of NPC (1 μg) using histone H2a as a phosphate acceptor.



of histone H2a phosphorylation by the kinase *in vitro* seems to be due to the binding of NPC to the kinase.

Finaly, the present data and our previous reports[8,9] suggest the possibility that NCS may bind to the microtubular proteins on cell membrane which consequently affects the microtubule-associated kinase *in vivo*, resulting in the inhibition of microtubular paracrystal formation, cell surface capping and cell spreading. The future study of the detailed mechanism of the NCS and NPC effect on microtubule-associated kinase activity *in vivo* may lead to an understanding of the biological role of the kinase and also should provide a new clue to study the biochemical mechanism of the cell cycle in mammalian cells.

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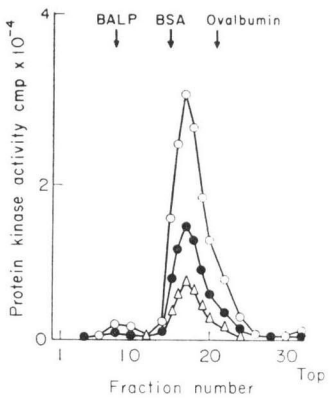
Fig. 2. Glycerol density gradient sedimentation of microtubule-associated kinase preincubated with NPC.

The kinase (5 μg) was incubated with different concentrations of NPC in the absence of [γ-32P]-ATP and the phosphate acceptor for 10 minutes at 37°C, then analyzed by 5~25% (v/v) glycerol density gradient centrifugation. After centrifugation (165,000×*g* for 17 hours at 4°C), the gradients (5.0 ml) were fractionated separately into 32 fractions. Aliquots (5 μl) of the indicated fractions were assayed for the kinase activity using histone H2a as a phosphate acceptor (10 minutes at 37°C). Arrows indicate bacterial alkaline phosphatase (BALP, Mr=92,500, 6.3S), bovine serum albumin (BSA, M 67,000, 5.0S) and ovalbumin (Mr = 45,000, 3.6S).

Control (○), 5 μg of NPC (●) and 25 μg of NPC (△).



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