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STAUROSPORINE IS A POTENT INHIBITOR OF p34^{cdc2} AND p34^{cdc2}-LIKE KINASES

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Summary: We previously demonstrated that nontransformed cells arrest in the G1 phase of the cell cycle when treated with low concentrations (21 nM) of staurosporine (1). Both normal and transformed cells are blocked in the G2 phase of the cell cycle when treated with higher concentrations (160 nM) of staurosporine (1,2). In the present study, we show that staurosporine inhibits the activity of fractionated p34^{cdc2} and p34^{cdc2}-like kinases with IC_{50} values of 4-5 nM. We propose that the G2 phase arrest in the cell cycle caused by staurosporine is due, at least in part, to the inhibition of the p34^{cdc2} kinases.

We have shown that low concentrations of the kinase inhibitor staurosporine cause nontransformed cells to block in the G1 phase of the cell cycle whereas, under the same experimental conditions, transformed cells remain cycling (1). However, both normal and transformed cells will arrest in the G2 phase when treated with higher concentrations of staurosporine (1,2). Although staurosporine is often referred to as a specific protein kinase C inhibitor (3), it is more accurately a nonspecific kinase inhibitor which inhibits several other kinases including the insulin receptor tyrosine kinase (4), cAMP-dependent protein kinase (5), cGMP-dependent protein kinase (6), Ca^{2+}-calmodulin-dependent kinase (5), and *src* tyrosine kinase (7) in the nanomolar range *in vitro*. Recently, a significant amount of data has accumulated which sheds doubt on the

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presumption that staurosporine affects solely protein kinase C *in vivo* (6, 8-14).

The cdc2 gene encodes a 34kD protein kinase which functions to control the onset of mitosis (for review, see 15). Temperature-sensitive mutations in cdc2 from both yeast and mouse cause the cells to arrest in G2 phase of the cell cycle when incubated at the nonpermissive temperature and the failure of *cdc2* mutants to enter mitosis correlates with the loss of $p34^{cdc2}$ kinase activity (16, 17). With this in mind we determined if the staurosporine-induced G2 block could be related to cdc2 function. We find that staurosporine is a potent inhibitor of partially purified $p34^{cdc2}$ and $p34^{cdc2}$-like kinases *in vitro*. These kinases retain only 10% of their activities when treated with 64 nM staurosporine, a concentration that is 2-3 fold lower than that required to bring about G2 phase arrest. Our results indicate that additional sites of action for staurosporine are the $p34^{cdc2}$ kinases, and that the cell cycle arrest in the G2 phase induced by staurosporine may be due in large part to inhibition of the $p34^{cdc2}$ kinases.

Materials and Methods

Materials. Stock solutions of staurosporine (Kamiya Biomedical Company, Thousand Oaks, CA) were prepared in dimethylsulfoxide (DMSO) and stored at -20°C.

Cell Culture and H1 Kinase Fractionation. The FM3A mouse mammary carcinoma line was provided by Masa-atsu Yamada (Faculty of Pharmaceutical Sciences, University of Tokyo) and Hideyo Yasuda (Faculty of Pharmaceutical Sciences, Kanazawa University). Cells were grown in spinner flasks at 32°C in Hepes-buffered RPMI 1640 medium containing 10% bovine calf serum. The $p34^{cdc2}$ and $p34^{cdc2}$-like kinases were fractionated from crude extracts by Mono S cation exchange chromatography as described[a].

Kinase Assays The H1 kinase activity of the $p34^{cdc2}$ and $p34^{cdc2}$-like kinases were assayed using a synthetic peptide substrate, S1

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[a]Hamaguchi, J.R., Tobey, R.A., Pines, J., Crissman, H.A., Hunter, T., and Bradbury, E.M. (1991) Submitted for publication.

(AAKAKKTPKKAKK)[a]. The S1 peptide contains a growth-associated site of phosphorylation of histone H1 typical of the phosphorylation sites in the N- and C-terminal tails of H1 (18). Each reaction (25 μl) contained 2.5 μl of enzyme, 50 mM Tris-HCl, pH 7.4, pH 7.4, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM [γ-^{32}P]ATP (1,100 dpm/pmol), 20 μg/ml S1 peptide, 2.5% DMSO and different concentrations of staurosporine. Reactions were incubated at 32°C for 10 min, then stopped by the addition of phosphoric acid to a concentration of 75 mM. Aliquots were spotted on P81 phosphocellulose filter paper, which were then washed four times in 75 mM phosphoric acid, once in acetone, and counted in a scintillation counter. These values were corrected for background autophosphorylation which was determined in reactions performed without peptide substrate or staurosporine. Activity in the presence of staurosporine was normalized to a reaction containing 2.5% DMSO without staurosporine.

Results and Discussion

We tested the ability of staurosporine to inhibit the H1 kinase activity of three different $p34^{cdc2}$ or $p34^{cdc2}$-like kinases isolated from FM3A mouse mammary carcinoma cells. Fraction F2A contains a complex of $p34^{cdc2}$ and cyclin A, fraction F2B contains $p34^{cdc2}$-like protein associated with cyclin A, and fraction F2C contains a complex of $p34^{cdc2}$ and cyclin B1[a]. These kinases were chosen for study because it is known that their activities peak in the G2 phase of the cell cycle[a] and would therefore be candidate targets for staurosporine inhibition in the G2 phase. At least 80% of the activity in F2A, F2B, and F2C can be immunodepleted using antibodies specific to $p34^{cdc2}$ in the case of F2A and F2C or antibodies to cyclin A in the case of F2B[a]. Therefore although these kinases have not been purified to homogeneity, most if not all activity is due to the $p34^{cdc2}$ and $p34^{cdc2}$ -like kinases. Another degree of specificity for H1 kinase activity is achieved in these assays by using the S1 peptide as a substrate for enzyme activity. The S1 peptide contains a growth associated site of phosphorylation present in histone H1 (18) and

[b]Kamijo, M., Yasuda, H., Yau, P.M., Inagaki, M., Yamashita, M., Nagahama, Y., and Ohba, Y. Personal communication.

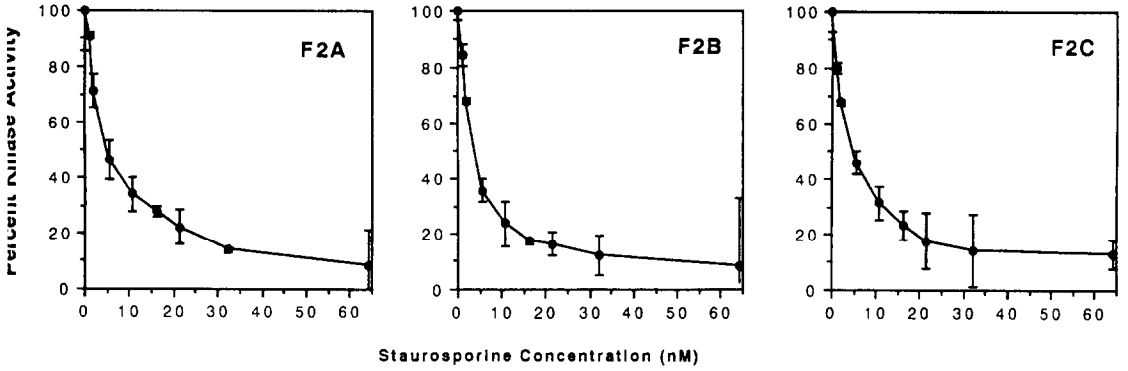

Fig. 1. **Staurosporine inhibition of fractionated $p34^{cdc2}$ or $p34^{cdc2}$-like kinases.** The experiment was carried out as described in the experimental procedures section. Each point represents the average of two assays with each assay divided among two phosphocellulose papers. Error bars represent the standard deviation of the data. F2A, inhibition of fraction F2A ($p34^{cdc2}$ and cyclin A). 100% activity is equal to 42 units of activity where 1 unit of activity is defined as the amount of enzyme which catalyzes the transfer of one pmol of phosphate per minute. F2B, inhibition of F2B ($p34^{cdc2}$-like protein and cyclin A). 100% activity is equal to 17 units of activity. F2C, inhibition of F2C ($p34^{cdc2}$ and cyclin B1). 100% activity is equal to 18 units of activity.

is not phosphorylated by cAMP-dependent protein kinase or Ca^{2+}-phospholipid-dependent protein kinase C[b].

The results shown in Figure 1 demonstrate that the H1 kinase activities in F2A, F2B, and F2C are very sensitive to staurosporine with half of the activity being lost at about 4-5 nM staurosporine. At concentrations of 64 to 430 nM, staurosporine reduces each of the H1 kinase activities about 10-fold. The amount of staurosporine required to cause a G2 arrest in FM3A cells is 160 nM so it quite likely that the $p34^{cdc2}$ and $p34^{cdc2}$-like kinases are inhibited *in vivo* at this concentration.

The IC_{50} values for staurosporine inhibition of other protein kinases are shown in Table 1 along with the IC_{50} values we obtained for the $p34^{cdc2}$ and $p34^{cdc2}$-like kinases. Comparable concentrations of staurosporine cause 50% inhibition of the $p34^{cdc2}$ and $p34^{cdc2}$-like kinases, protein kinase C, and *src* tyrosine kinase, whereas 2 to 12-fold higher concentrations of staurosporine are required to inhibit 50% of the activities of the other known staurosporine-sensitive kinases.

Table 1. *Staurosporine Inhibition of Purified Kinases*

Kinase	IC50 (nM)	Reference
F2A ($p34^{cdc2}$/cyclin A)	4	Figure 1
F2B ($p34^{cdc2}$-like/cyclin A)	4	Figure 1
F2C ($p34^{cdc2}$/cyclin B1)	5	Figure 1
Protein Kinase C	2.7	3
src tyrosine kinase	6.4	7
cGMP protein kinase	8.5	6
cAMP dependent protein kinase	22	5
calmodulin-dependent kinase	25	5
insulin receptor tyrosine kinase	61	4

Values for $p34^{cdc2}$ and $p34^{cdc2}$-like kinases were obtained in this paper as described in the experimental procedures section whereas values for other kinases were obtained from the literature.

Since staurosporine inhibits a number of protein kinases *in vitro*, the *in vivo* targets may be numerous. There is some evidence to suggest that protein kinase C is required for cell cycle progression through G2 phase (19). In addition, the *spk*1$^+$ gene, which encodes a protein kinase having sequence similarity to the MAP2 kinase, was found to suppress the drug sensitivity of staurosporine-sensitive yeast mutants (20). Given the acute sensitivity of $p34^{cdc2}$ kinases to staurosporine and the established requirement for $p34^{cdc2}$ function in regulating the onset of mitosis (16,17), we suggest that additional sites of action for staurosporine are the mitotic $p34^{cdc2}$ kinases and that the G2 phase arrest of mammalian cells induced by staurosporine may be due to the inhibition of the $p34^{cdc2}$ kinases *in vivo*.

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