STAUROSPORINE INHIBITS TYROSINE-SPECIFIC PROTEIN KINASE ACTIVITY OF ROUS SARCOMA VIRUS TRANSFORMING PROTEIN p60

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Protein kinases that phosphorylate tyrosine residue have been identified as products of retroviral oncogenes including *src* gene of Rous sarcoma virus (RSV), *fps* of Fujinami sarcoma virus, *yes* of Y73 avian sarcoma virus, *ros* of UR2 avian sarcoma virus, *erb*-B of avian erythroblastis virus, *fgr* of Gordner-Rasheed feline sarcoma virus, *fms* of Susan McDonough feline sarcoma virus and *abl* of Abelson murine sarcoma virus. In addition to the viral oncogenes, the growth factor receptors for epidermal growth factor (EGF), for platelet-derived growth factor, for insulin and for insulin like growth factor I have the same kind of protein kinase activity.[1] The nucleotide sequence of a complementary DNA of *onc*-D (*trk*), a transforming gene from a human colon carcinoma, have the striking homology with the catalytic domain of tyrosine-specific protein kinases.[2] Tyrosine-specific protein kinase activity is present even in normal cells and phosphotyrosine is detectable as a minor component of phosphoamino acids in the total cellular protein. From these findings, tyrosine-specific protein kinase has been suggested to play vital roles in cellular proliferation.

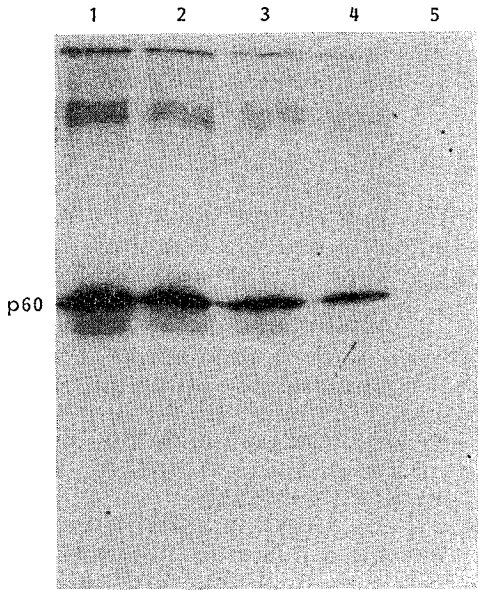
We have reported that staurosporine, a microbial alkaloid,[3] inhibits markedly phospholipid/Ca^{++}-dependent protein kinase (protein kinase C) and shows the strong cytotoxic activity on the growth of some tumor cell lines.[4] Because staurosporine inhibits strongly protein kinase C from rat brain with IC_{50} value of 2.7 nM and also inhibit cyclic AMP-dependent protein kinase from bovine heart with IC_{50} value of 8.2 nM, we have been interested in determining if staurosporine exert similar inhibitory effects on tyrosine specific protein kinase activity. In this paper we report that staurosporine inhibits the protein-tyrosine kinase activity of $p60^{v\text{-}src}$ *in vitro* with an IC_{50} value of 6.4 nM.

For the measurement of protein kinase activity of $p60^{v\text{-}src}$, we used an autophosphorylating reaction of $p60^{v\text{-}src}$ in the immunocomplex with antibacterial p60 serum. Secondary cultures of chicken embryo fibroblasts (CEF) were infected with wild type Rous sarcoma virus (SR-A) and grown in MEM medium supplemented with 5% calf serum and 10% Tryptose phosphate broth. Fully transformed cells were lysed in the RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) as described previously.[5] Cell extracts were immunoprecipitated with an excess amount of anti-p60 serum raised against $p60^{v\text{-}src}$ which is produced in *Escherichia coli*;[6] 4 μl of anti-p60 serum was added to 80 μl of lysate (prepared from 1/20 of one 90 mm-dish culture) and kept on ice for 1 hour. After protein A-Sepharose 4B-CL were added, the mixture were rotated at 4°C for 90 minutes. The immunoprecipitates were washed five times with RIPA buffer and further washed three times with 40 mM Tris-HCl (pH 7.2). Washed immunoprecipitates were resuspended in 30 μl of kinase assay buffer containing 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 10 μCi [γ-^{32}P]ATP (3,000 Ci/mmol) and 0.5% DMSO, with staurosporine at the concentration indicated. After 30 minutes incubation at 20°C, the reaction was terminated by adding 1 ml of RIPA (4°C). The immunoprecipitates were washed three times with 1 ml of RIPA buffer (0°C) to remove [γ-^{32}P]ATP. In the uninhibited condition, about 1% of initial ^{32}P was incorporated to the immunoprecipitate from ATP. The final pellets were resuspended in 30 μl of SDS gel buffer boiled for 3 minutes, and analyzed by 10% SDS-polyacrylamide gel as described previously[7] and phosphoprotein bands were detected by autoradiography. Quantitative determinations of protein phosphorylation were obtained by excising the $p60^{v\text{-}src}$ bands from the gel and counting as described.[8] By this reaction, $p60^{v\text{-}src}$ is phosphorylated on tyrosine-416[9] and this residue is the site of phosphorylation of

Fig. 1. Autoradiogram showing inhibition of *in vitro* auto-phosphorylation of $p60^{v\text{-}src}$ by staurosporine.

$p60^{v\text{-}src}$ were immunoprecipitated from the lysates of CEF infected with RSV by an anti-p60 serum. The immunoprecipitates were incubated with kinase assay buffer (20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$ and 10 μCi [γ-^{32}P]ATP) for 30 minutes at 20°C. Staurosporine was dissolved, diluted in DMSO and added to the assay buffer at the concentrations indicated. The reaction products were analyzed on a SDS-10% acrylamide gel. The dried gel was exposed to X-ray film for 90 minutes.

Key to lanes: 1; Control (DMSO), 2~5; staurosporine 1 ng/ml (lane 2), 3 ng/ml (lane 3), 10 ng/ml (lane 4), 100 ng/ml (lane 5).

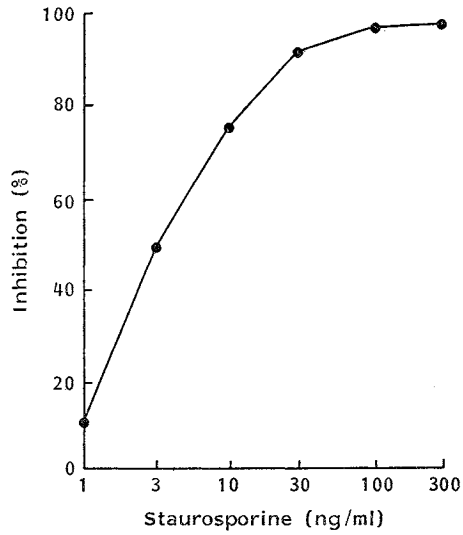


$p60^{v\text{-}src}$ *in vivo*. By the screening of several $p60^{v\text{-}src}$ mutants, it was shown that extent of the auto-phosphorylation in this system has good correlation to the level of phosphotyrosine of total celluler protein, 34K protein and p60 itself (tyrosine-416) in cells expressing each p60.[5,6]

Fig. 1 shows that auto-phosphorylation of $p60^{v\text{-}src}$ was detectable in the autoradiogram and staurosporine strongly inhibited the tyrosine kinase activity of $p60^{v\text{-}src}$ immunocomplex. The serum used here can also immunoprecipitate endogenous $p60^{c\text{-}src}$ in the cells. The protein band which migrated close to the p60 was found to be degraded p60.[7] The minor bands which migrated as high molecular weights were not reproducible. We think that auto-phosphorylation reaction detected here is attributable exclusively to $p60^{v\text{-}src}$, because no band of phosphorylated protein was detectable when lysates from uninfected CEF were analyzed by the same procedure. Phosphopeptide map analysis and phosphoamino acid determination confirmed that the band is derived from $p60^{v\text{-}src}$ and tyrosine-416 is the site of phosphorylation (data not shown). ^{32}P-Labeled $p60^{v\text{-}src}$ were excised from the appropriate region of the SDS-polyacrylamide gels and counted for ^{32}P. Fig. 2 shows that the IC_{50} value of staurosporine was 3 ng/ml (6.4 nM).

Fig. 2. Inhibition of $p60^{v\text{-}src}$ phosphorylation by staurosporine.

The $p60^{v\text{-}src}$ bands were excised from the SDS-polyacrylamide gels like shown Fig. 1 and ^{32}P count was quantitated. The percent inhibition of the protein kinase activity was calculated from the average of two independent experiments.



From these result, we conclude that staurosporine is a potent inhibitor of tyrosine specific protein kinases as well as serine and threonine-specific protein kinases which include phospholipid/Ca^{++}-dependent protein kinase and cyclic AMP-dependent protein kinase. Staurosporine may bind to the catalytic domain of these protein kinases which shares an homologous region.[8] It is noteworthy that extremely low levels of staurosporine (1~8 nM) caused the 50% inhibition of phosphate transfer from ATP under the assay conditions containing much higher concentrations of ATP (100 μM). It suggests that staurosporine can inhibit protein phosphoryla-

tion *in vivo* even if ATP concentrations are higher than 1 mM under physiological conditions.[9] Other known inhibitors of $p60^{src}$ protein-tyrosine kinase have reported with IC_{50} values of ~100 μM for tosyl-lysyl chloromethyl ketone,[10] 15 μM for Ap_4A.[11] Erbstatin[12] and genistein[13] have isolated as inhibitor of tyrosine kinase activity of EGF receptor with IC_{50} values of 3 μM for both compound. Thus staurosporine is a more potent inhibitor of protein-tyrosine kinase than other known inhibitors.

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