Short Communication

Effect of host Src-kinase inhibition on replication of Coxsackievirus B3 *in vitro*

Maghsoudi Nader, Zeinoddini Mehdi, Hosseini Amini S. M. Saeid

Biotechnology Research Center, P.O. Box: 19395-1949,Tehran, Iran

Received 30 January 2003; accepted 19 November 2003

Abstract
There are evidence showing a relationship between host Src kinase activation and viral (CVB3) replication, which are based on the observation that inhibition in the enzyme activity could result in inhibition of viral replication. The present study assessed the effect of Src kinase inactivation on viral replication at different stages of infection. It was observed that the Src kinase activity is necessary for the initiation of viral replication. In this study HeLa cell lines were treated with 5 and 10 µM herbimycin A (Src kinase inhibitor) with a time schedule of -90´, -60´, -30´, 0´, +15´, +30´, +45´, ... +210 minutes. All cultures were infected with CVB3 at zero-minute (+ve sign indicates that herbimycin A was added after infection with CVB3). The reaction was terminated after 24 h, cells were then detached from petri plates with trypsin/EDTA. Viral replication was monitored using a set of specific primers and the plaque formation unit (PFU) count. In cells pretreated with herbimycin A before infection viral replication was inhibited. However addition of herbimycin A after infection did not affect viral replication.
Keywords: *Coxsackie B virus, Herbimycin, Src Kinase, RT-PCR, PFU*

Coxsackieviruses of group B (CVB) are enteroviruses of the family Picornaviridae and the causative agents of a variety of human diseases, from minor common cold to fatal myocarditis and meningitis (Kandolf and Hofchneider, 1989; McManus and Kandolf, 1991). Coxsackievirus B3 (CVB3), a cytolytic virus, has been shown to be capable of inducing persistent heart muscle infection by restricted virus replication (Kandolf *et al*., 1987). CVB3 is a small, non-enveloped and icosahedral enterovirus containing a positive-sense, single-stranded RNA genome containing about 7400-nucleotides (Klump *et al*., 1990). Infection of a permissive host cell starts with the attachment of the virus to a specific cell surface receptor (Raab *et al*., 1995). Following entry into the cell, genomic RNA is replicated by the virus-encoded RNA-dependent RNA polymerase generating full-length, intermediate, negative-strand RNAs. New infectious positive-strand RNAs are subsequently produced by the replication of negative-strand RNAs. Translation occurs by a cap-independent mechanism, yielding the viral polyprotein, which is processed by virus-encoded proteinases. Interactions of viral and cellular components are necessary for the production of an infectious cycle with respect to the correct formation of protein-protein and protein-RNA complexes in the course of infection.

The main question raises is whether or not the above-mentioned processes are regulated by the constituent of the cellular signal transduction pathways as there are several reports indicating src kinase play role in some viral replications. Kinases of the Src family are proteins with 500-530 amino acid residues and three domains, namely SH_2, SH_3 and the catalytic domains (sometimes called SH_1). In their inactive form, these kinases are phosphorylated at tyr-527 and for activation tyr-527 phosphor is removed by a phosphatase activity, and another site (the tyr-416) becomes phosphorylated. Both the tyr-416 and tyr-527 are conserved amino acids. The Src kinases has been reported to be related to the viral replication process (Cohen *et al*., 1992; Fukami *et al*., 1986; Majumder *et al*., 1990). For example, in Epstein-Barr virus-infected human B-lym-

Correspondence to: **Nader Maghsoudi, Ph.D**
Tel: +98 212959490*, Fax: +98 21* 2959493
E-mail: maghsudi@yahoo.com

phocytes, rapid Ca^{2+}-dependent tyrosine phosphorylation of the cytosolic tyrosine kinase $P56^{LCK}$ has been reported (Lacy *et al*., 1991; Flint *et al.*, 2000). To verify such relationship, in present study, the course of viral replication in presence of the Src kinase inhibitors, before and after virus infection was investigated. HeLa cell line obtained from the National Cell Bank of Iran (NCBI C115) were placed in six-well plates in RPMI-1640 containing 10% fetal bovine serum and incubated for 24 h at 37ºC to reach 80% confluency. HeLa cells were used for src kinase inhibitory studies and Vero cells (NCBI C101) for PFU study. CVB3 (Nancy strain, ATCC # VR-30; American Type Culture Collection) was maintained by passage through HeLa cells. Inhibitory effect of herbimycin A, specific tyrosine kinase inhibitor, was performed by adding it (5-10 µM), before and after CVB3 infection. Herbimycin A was added at -90´, -60´, -30´, 0´, +15´, +30´, +45´,…,+240 min. All cultures were infected with 1×10^5 PFU of CVB3 at time zero (negative and positive signs indicate the addition of inhibitor, before and after infection). After 24 h, virus was recovered from the cell suspension following three cycles of freezing and thawing. This was followed by total RNA extraction from infected HeLa cells using RNAX kit (Cinagen company, Iran). Complementary-strand DNA (cDNA) was synthesized using random hexanucleotide forward and reverse primers, cDNA was amplified by PCR using primers specific to a conserved region of the 2A proteinase. In order to detect CVB3 proliferation in cells, the plaque formation unit (PFU) of each sample was measured. For plaque assay, Vero cells (2×10^5/well) were cultured overnight at 37ºC under 5% CO_2 in a 24-well plate prior to infection. Cells were 90-100% confluent at the time of titration. The medium was aspirated, and 200 µl of serially diluted virus was added to each well. Infected plates were placed back in the incubator for 1h. Then the cells were overlaid with 1ml of 1X DMEM in 0.5% agar (1:1 mixture of 2X DMEM at 37ºC and 1% agar at 55ºC). 72 h after infection, cells were fixed by adding 1 ml of 10% formalin in phosphate buffered saline (PBS) to each well and left the plates at room temperature for 1 h. The fixative was poured off, and agarose plugs were removed. Cells were stained with 500 µl 0.5% crystal violet in 20% ethanol per well. Plates were rinsed with tap water and plaques were counted (Fig. 1).

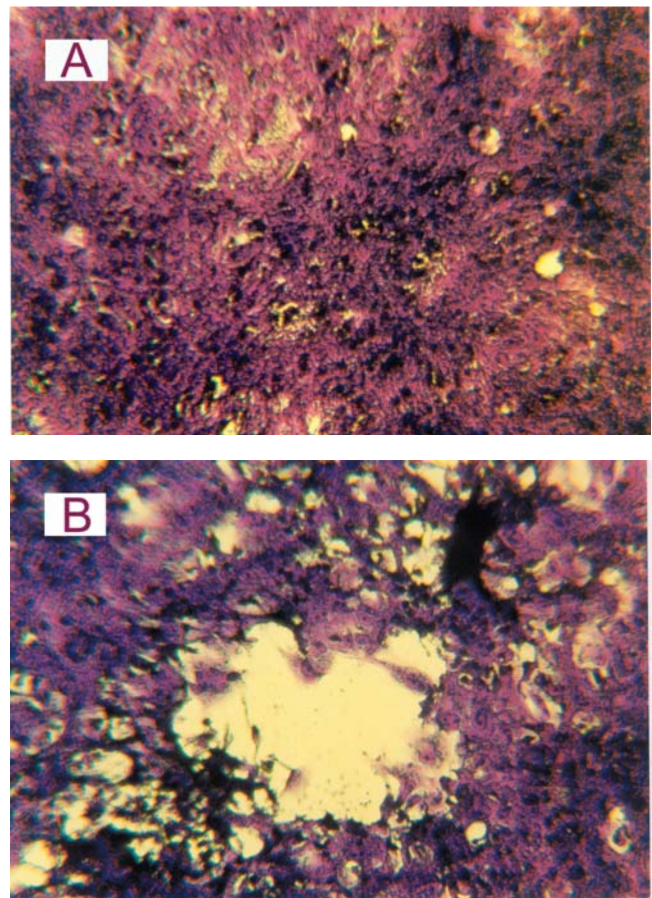

Figure 1: CVB3 plaque on vero cells (A: Normal cells, B: Infected cells)

Results related to the detection of viral RNA in infected HeLa cells was qualitative which is based on the fact that, as CVB3 is a RNA(+) virus, appearance of RNA(-), in the cell lysate can be used as index of viral progeny production, and hence an indication of viral replication. This was confirmed by using only forward primer for RT-PCR. Analysis of the PCR product on agarose gel (1.5%) shows a 460 bp fragment suggesting the presence of 2A proteinase cDNA of CVB3 (Fig. 2). Several studies show the relation between the activity of the Src kinases and viral replication (Liu *et al*., 2000; Huber *et al*., 1997). Incubation of the culture media in presence of herbimycin A before viral infection revealed that there is no viral replication (absence of viral negative strand as judged by PCR shown in figure 2). In contrast, addition of the inhibitor after the virus infection had no effect on viral replication and proliferation (Fig. 2 and Table 1). These results further indicate the direct effect of the Src kinase on viral replication, and support previous reports (Lacy, 1991; Isakov and Bisinger, 2000). It has been observed that the viruses of the Papovaviridae (Bolen, 1984) and Herpesviridae (Kulwichi, 1998)

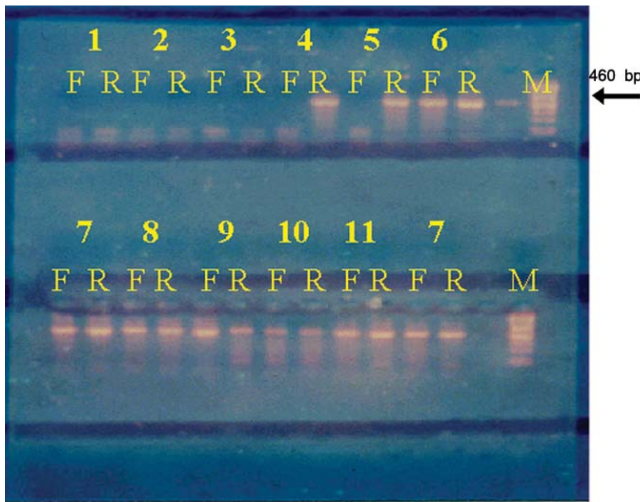

Figure 2: Analysis of RT-PCR production (reverse and forward primers were used). Time of herbimycin A treatment is: (1)-90, (2)-60, (3)-30, (4) 0, (5)+30, (6)+60, (7)+90, (8)+120, (9)+150, (10)+210 minute, (11) normal HeLa cell (control), (M) Molecular size marker.

families code proteins which permanently bind members of the Src kinase family and cause continuous activation of the Src signal pathways, which in turn facilitates viral replication. A regulatory role for the Src kinase family in Coxsackievirus B3 replication has been reported by Huber and co-workers (1997). Liu *et al*. (2000) pointed out the role of P56^{LCK} (a Src kinase family member) in CVB3 replication in HeLa cells, T-cells and mice heart muscle tissue. They observed no viral replication and no pathogenecity in P56$^{LCK-/-}$ (P56^{LCK} gene is knocked out) mice.

In the present study the role played by the Src kinase family in CVB3 replication seems to be related mostly to the early phase of replication, therefore addition of inhibitors following infection failed to affect the viral replication. Once replication starts, the influence of the Src kinase seems negligible, as the addition of inhibitors could not affect viral proliferation, at least qualitatively. In order to confirm the results, the process can be checked quantitatively which can show the exact role played by Src kinase in CVB3 replication. In this study, the shortest intervals for studing Src kinase inhibitory action was 30 minutes before and after the infection. Hence it appears that critical time for effects of Src kinase inhibitor has been missed. It is suggested that for better assessment of molecular mechanism(s) of this interaction, the inhibitor to be added in shorter time intervals for example ±5 minutes. By understanding this molecular mechanism it is possible to design molecules which can interfere with the process and leading to block viral proliferation.

Table 1: PFU result of infected HeLa cells in presence of herbimycin A.

Sample	Herbimycin A (Time)	10^5 PFU/ml
1	-90	0.15
2	-60	0.26
3	-30	0.33
4	0	0.22
5	+30	0.27
6	+60	0.12
7	+90	1.5
8	+120	8.5
9	+150	1.45
10	+210	1.8

References

Bolen JR, Thiele CJ, Israel MA, Yonemoto W, Lipsich LA, Brugg JS. (1984) Enhancement of cellular src gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. *Cell,* 38:767-77.

Cohen D, Tani I, Tian H, Boone E, Samelson LE, Lane HC. (1992) Participation of Tyrosine phosphorylation in the cytopatic effect of human Immunodeficiency virus. I. *Science*, 256: 542-545.

Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka Am. (2000) *Principals of Virology*, ASM Press, American Society for microbiology, chapter 16, PP: 578.

Fukami Y, Nakamura T, Nakamura A, Kanehisa T. (1986) Phosphorylation of Tyrosine residues of calmodulin in Rous satcoma virus – Transformed cells. *Proc. Natl. Acad, Sci, USA* 83: 4190 – 4193.

Huber M, Selinka HC, Kandolf R. (1997) Tyrosine phosphorylation events during coxsachievirus B3 replication. *J. Virol*, 71(1): 595-600.

Isakov N, Biesinger B. (2000) LCK protein kinase is a key regulator of T cell activation and a target for signal intervention by Herpesvirus saimiri and other viral gen products. *Eur. J. Biochem*. 267(12):3413-3421.

Kandolf R, Ameis OD, Kirschner P, Canu A, Hofschneider PH. (1987) In situ detection of entroviral genomes in myocardial cells by nucleic acid hybridisation: an approach to the diagnosis of viral heart disease. *Proc. Natl. Acid. Sci. USA* 84: 6272-6276.

Kandolf R, Hofchneider PH. (1989) Viral heart disease. Springer Semin. *Immunopathol*. 11: 1-13.

Klump WM, Bergmann I, Muller BC, Amies D, Kandolf R. (1990) Complete nucleotide sequence if infectious coxsackievirus B3 cDNA: Two initial 5´ uridine residues are regained during plus – Strand RNA synthesis. *J.*

Virol. 64(4): 1573-1583.

Kulwichi W, Edwards RH, Davenport EM, Baskar JF, Godfrey Y, Rabb-Traub N. (1998) Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc, Natl. Acad. Sci. USA*, 95(20):11963-11968.

Lacy J, Bartiss A, Coleman DL. (1991) Conversion of a human B cell lymphoma line by Epstin–Bar virus is associated with increased Trosine phosphorylation of a 50 kilodalton cytosolic protein. *Oncogene Res.* 5: 329-335.

Liu P, Aitken K, Kong YY, Oparsky MA, Martino T, Dawood F, Wen WH, Kozieradzki I, Bachmaier K, Straus D, Mak TW. Penninger JM. (2000) The tyrosine kinase P56LCk is essential in coxsackievirus B3-mediated heart disease. *Nat. Med.* 6(4):429-434.

Majumder S, Ray P, Besmer P. (1990) Tyrosine protein activity of the HZ4-Feline sarcoma Virus P80 gag–kit transforming protein. *Oncogene Res.* 5: 329-335.

McManus BM, Kandolf R. (1991) Evolving concepts of cause, consequence and control in myocarditis. *Curr. Opin. Cardiol*. 6: 418-427.

Raab de Verdugo, U, Selinka HC, Huber M, Kramer B, Kellerman J, Hofschneider PH, Kandolf R. (1995) Characterization of a 100 kilodalton binding protein for six serotypes of coxsackie B viruses. *J. Virol.* 69: 6751-6757.