Involvement of tyrosine phosphorylation in HMG-CoA reductase inhibitor-induced cell death in L6 myoblasts

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Abstract Our previous studies have shown that the HMG-CoA reductase (HCR) inhibitor (HCRI), simvastatin, causes myopathy in rabbits and kills L6 myoblasts. The present study was designed to elucidate the molecular mechanism of HCRI-induced cell death. We have demonstrated that simvastatin induces the tyrosine phosphorylation of several cellular proteins within 10 min. These phosphorylations were followed by apoptosis, as evidenced by the occurrence of internucleosomal DNA fragmentation and by morphological changes detected with Nomarski optics. Simvastatin-induced cell death was prevented by tyrosine kinase inhibitors. The MTT assay revealed that the addition of mevalonic acid into the culture medium partially inhibited simvastatin-induced cell death. Thus, these results suggested that protein tyrosine phosphorylation might play an important role in the intracellular signal transduction pathway mediating the HCRI-induced death of myoblasts.

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Key words: Hydroxymethylglutaryl coenzyme A reductase inhibitor; Tyrosine phosphorylation; Apoptosis; L6 myoblast

1. Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HCR) is known to be rate limiting in the biosynthesis of cholesterol. Therefore, its inhibitor (HCRI) is a powerful and widely used drug for the treatment of hypercholesterolemia [1,2]. HCR catalyzes the conversion of HMG-CoA to mevalonate, which is an important precursor of all isoprenoids. Isoprenoids serve as important lipids for the normal posttranslational modification of membrane-associated proteins such as Ras and Rac. Lovastatin, a lipophilic HCRI, is reported to block cell cycling in G1 and G2/M phase progression and therefore suppress the growth of tumors in vitro [3,4]. Our previous studies have revealed that simvastatin, another lipophilic HCRI, but not pravastatin, a hydrophilic HCRI, causes electrical myotonia and muscle cell degeneration in intact rabbits and kills L6 myoblasts in culture [5–7]. The L6 myoblast cell line was originally developed from rat thigh muscle [8] and has retained the characteristics of skeletal muscle [9]. Interestingly, intracellular Ca^{2+} mobilization from the Ca^{2+} pool in the cell preceded cell death, suggesting that Ca^{2+} mobilization is involved in the cell killing mechanism in L6 myoblasts [7].

Increased tyrosine phosphorylation caused by increased activity of either tyrosine kinases or by decreased activity of protein tyrosine phosphatase has been shown to play a pivotal role in the intracellular signal transduction pathway of many extracellular signals for cellular proliferation and differentiation [10]. It has been suggested that protein tyrosine phosphorylation is also involved in the signal transduction pathway mediating apoptosis in blood cancer cells [11].

In this study, we examined the intracellular signalling events mediating HCRI-induced cell death in L6 myoblasts as a model system. Simvastatin rapidly induces the tyrosine phosphorylation of cellular proteins in L6 myoblasts, and then causes apoptotic cell death. Herbimycin A and genistein, inhibitors of protein tyrosine kinases, prevented the occurrence of simvastatin-induced myoblasts cell death. These results strongly suggest that tyrosine phosphorylation might play an important role in the development of myopathy by HCRI in vivo.

2. Materials and methods

2.1. Cell culture and treatment

L6 myoblasts were cultured as monolayers in culture dishes with Dulbecco's modified Eagle's medium (DMEM) containing 7.5% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin [7]. Cells were treated with various concentrations of simvastatin (a generous gift from Sankyo Pharmaceutical Co. Ltd.) for appropriate periods of time at 37°C. Control sister cultures were prepared in a similar way except for the absence of simvastatin. In some experiments, the cells were pretreated with protein tyrosine kinase inhibitors such as herbimycin A (10 μM) (BioMol, USA) and genistein (75 μM) (Extrasynthese, France) for 1 h. The cells were then exposed to simvastatin. For morphological studies, L6 myoblasts were cultured either in 12-well culture plates or on coverslips at 37°C [12,13]. For Nomarski optic observations, cell monolayers grown on special coverslips (Flexyperm, Toyobo Engineering Co. Ltd., Tokyo, Japan) were examined under differential interference contrast optics of Nomarski type (confocal laser microscope, Olympus, Tokyo, Japan) as reported previously [14].

2.2. MTT assay

To estimate the survival rate of L6 myoblasts treated with simvastatin, the colorimetric 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed on cells treated with simvastatin or with simvastatin plus mevalonate (0.1, 1 mM) according to the method of Jacobson and Evans [15].

2.3. DNA extraction and electrophoresis

After exposure to simvastatin for the indicated times at 37°C with or without preincubation with tyrosine kinase inhibitors, total cellular DNA was extracted from the cells and quantitated by spectrophotometric absorption at 260 nm as described [13]. Total DNA (10 μg) was loaded onto a 1.8% agarose gel and run at 50 V, then stained with ethidium bromide and examined under UV light.

2.4. Detection of protein tyrosine phosphorylation and immunoblot analysis

L6 myoblasts were exposed to 30 μg/ml of simvastatin for the indicated times. Then cells were rinsed briefly with chilled phosphate-buffered saline (PBS) containing 1 mM *p*-nitrophenylphosphate and lysed with SDS sample buffer [16]. The samples were heated at 100°C for 2 min and equal amounts of protein (100 μg per lane) were subjected to 10% SDS-PAGE followed by transfer to polyvinylidene di-

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fluoride (PVDF) membranes as previously described [16]. Protein tyrosine phosphorylation was detected with an antibody against phosphotyrosine (α-pY, 4G10, UBI, USA) using the ECL detection system (Amersham, UK) [12,13,16]. Protein concentration was determined using the Bradford reagent (Bio-Rad) with γ-globulin as a standard [17].

2.5. Assay of protein tyrosine phosphatase activity

Protein tyrosine phosphatase activity was assayed according to the method of Yousefi et al. [18]. Briefly, 2×10^6 cells were incubated with various concentrations of simvastatin for the indicated times at 37°C. The cell pellets were lysed on ice for 15 min using 20 μl of TKM buffer (50 mM Tris, pH 7.5/25 mM KCl/5 mM $MgCl_2$/1 mM EGTA) containing 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. After centrifugation at 14 000 rpm for 15 min at 4°C, the supernatant was diluted 10-fold with TKM buffer without Triton X-100. Aliquots of 100 μl were distributed in a 96-well plate and 10 mM *p*-nitrophenylphosphate was added and the plate incubated for 30 min at room temperature. The phosphatase activity was estimated by measuring the absorbance of *p*-nitrophenol at 405 nm.

3. Results

3.1. Morphology

We examined the morphology of simvastatin-treated L6 myoblasts by Nomarski optics and phase contrast microscopy. In the absence of simvastatin, the cells were spindle-shaped in appearance with normal nuclei (Fig. 1-1). On the other hand, in the presence of simvastatin, the cells showed some intracytoplasmic vacuolations and a rounded phenotype within 1 h (Figs. 1-2 and 2-2). When estimated by the trypan blue dye exclusion assay, more than 90% of the cells treated with 30 μg/ml simvastatin appeared to be dead by 2 h after its addition. At a lower concentration of simvastatin, it took a longer time to detect these morphological changes. In contrast, a hydrophilic HCRI, pravastatin, which cannot enter

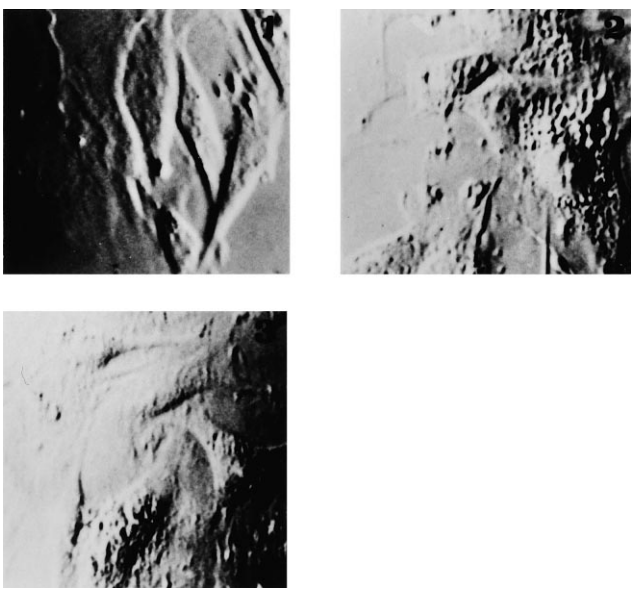

Fig. 1. Morphology of simvastatin-treated L6 myoblasts examined by Nomarski optics. L6 myoblasts were cultured on special coverslips with regular culture medium and were treated with either 30 μg/ml simvastatin (2), 60 μg/ml pravastatin (3) or left untreated for 1 h at 37°C (1). These cells were examined under differential contrast optics of the Nomarski type. Note that simvastatin-treated, but not pravastatin-treated cells, showed nuclear refractory and cytoplasmic vacuolation.

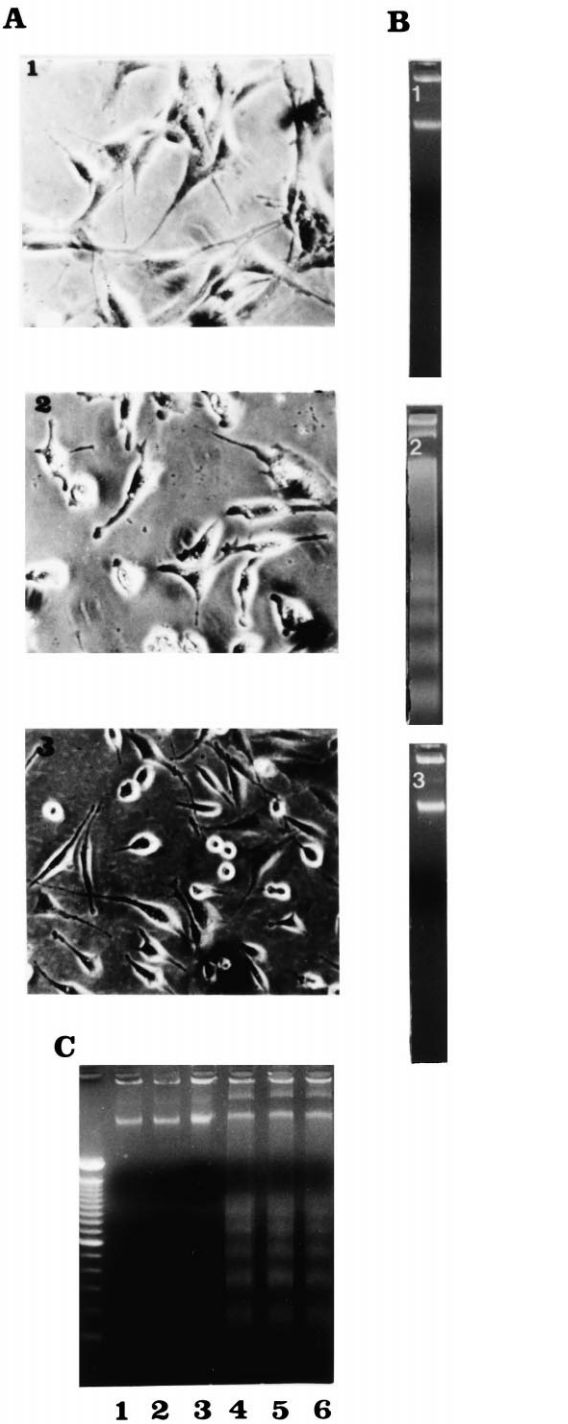

Fig. 2. Herbimycin A effect on morphological degeneration and DNA fragmentation induced by simvastatin. L6 myoblasts were pretreated with 10 μM herbimycin A, a potent protein tyrosine kinase inhibitor, for at least 3 h at 37°C (1), then exposed to either simvastatin (30 μg/ml) (1 and 2) or pravastatin (60 μg/ml) (3) for 30 min. These cells were examined by phase contrast microscopy (A) and by agarose gel electrophoresis of DNA (B). C: Agarose gel electrophoresis of DNA obtained from cells treated with 60 μg/ml pravastatin for either 30 (2) or 60 min (3) and treated with either 3 μg/ml (4) or 30 μg/ml simvastatin (5, 6) for either 30 (5) or 60 min (4, 6).

the cells, did not cause any morphological changes (Figs. 1-3 and 2-3). Pretreatment of the cells with protein kinase inhibitors such as herbimycin A (10 μM) (Fig. 2-1) or genistein (75 μM) (data not shown) for at least 3 h at 37°C prevented these morphological alterations induced by simvastatin. These concentrations have been shown to inhibit cellular protein tyrosine kinases in other systems [19,20].

3.2. DNA fragmentation

We extracted total DNA from L6 myoblasts cultured in the presence of simvastatin (3 and 30 μg/ml) for various times. We detected an internucleosomal fragmentation pattern typical for apoptosis as early as 30 min after addition of 30 μg/ml simvastatin (Fig. 2-B2 and C5). At a lower concentration of simvastatin (3 μg/ml), we detected internucleosomal DNA fragmentation 60 min after addition, as we did with the higher concentration (30 μg/ml) (Fig. 2-C4 and C6). Pravastatin (60 μg/ml), a hydrophilic HCRI, failed to induce DNA laddering even 60 min after its addition to culture medium (Fig. 2-B3 and C3). At concentrations higher than 60 μg/ml of simvastatin, we observed a smearing of DNA on agarose gels, which suggests necrotic cell death. On the other hand, we did not observe such DNA laddering in herbimycin A-pretreated cells (Fig. 2-B1 and C2).

3.3. MTT assay

We also examined cell survival by the MTT assay of cells treated with simvastatin. Simvastatin treatment for 45 min reduced the numbers of living cells to about 30% of control, whereas supplementation with 1 mM mevalonate, a product of the HMG-CoA reductase, rescues cells up 80% of the cells (Fig. 3).

3.4. Protein tyrosine phosphorylation and immunoblot analysis

To determine whether or not protein tyrosine phosphorylation is involved in the intracellular signal transduction pathway leading to cell death, we examined the change in total cellular protein tyrosine phosphorylation with an anti-phosphotyrosine (a-pY) antibody. Tyrosine phosphorylation of several proteins with molecular weights of 140–150 kDa, 120–130 kDa, 110 kDa, 70–80 kDa, 50–60 kDa, and 40 kDa was observed at a concentration of 30 μg/ml simvastatin within 10 min after its addition to culture medium (Fig. 4-A4). Even as little as 0.3 μg/ml simvastatin clearly induced tyrosine phosphorylation of several proteins (Fig. 4-A2). The time course of the activation of tyrosine phosphorylation by simvastatin showed a maximum at 10 min after addition (Fig. 4-B4). On the other hand, pravastatin did not induce protein tyrosine phosphorylation (Fig. 4-C). Moreover, addition of different concentrations of mevalonate to the culture medium caused a decrease of simvastatin-induced tyrosine phosphorylation in a dose-dependent manner (Fig. 4-C).

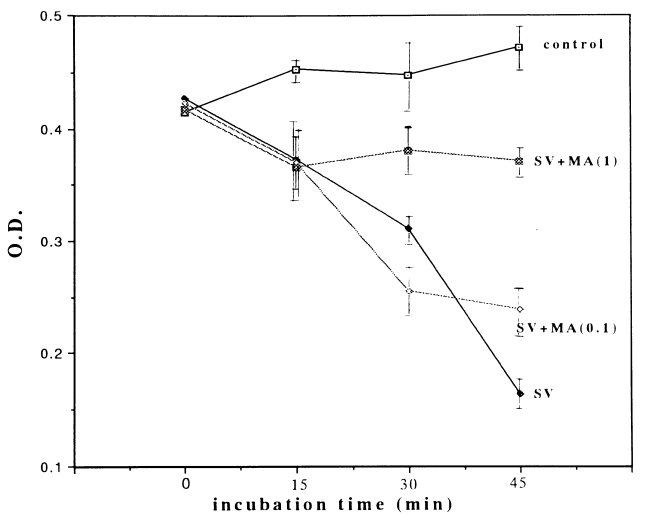

Fig. 3. Survival of simvastatin-treated L6 myoblasts. Cell survival was measured by the MTT assay. Each point represents the percentage mean aborbance ± S.D. from three wells against the mean absorbance at 0 time. The cells were preincubated with 0.1 mM and 1 mM mevalonate (SV+MA(0.1), SV+MA(1)) for 30 min before the addition of 30 μg/ml simvastatin (SV, SV+MA(0.1), SV+MA(1)) or left untreated (control).

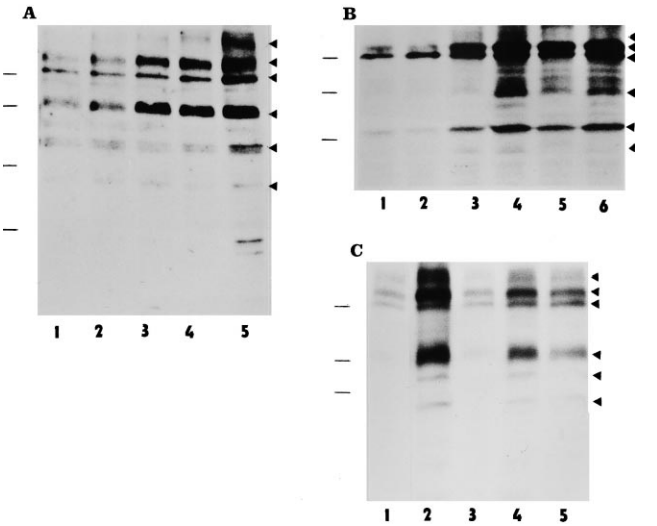

Fig. 4. Simvastatin effect on tyrosine phosphorylation of cellular proteins. A: L6 myoblasts were treated with 0.3 μg/ml (2), 3 μg/ml (3), 15 μg/ml (4), and 30 μg/ml (5) simvastatin or left untreated (1) for 10 min at 37°C. Total cell-free lysates were obtained as described in Section 2 and subjected to 10% SDS-PAGE, transferred to PVDF membranes and probed with an anti-pY antibody. B: L6 myoblasts were pretreated with 10 μg/ml herbimycin A (1) or left untreated (2–6), then treated with (1, 3–6) or without (2) 30 μg/ml simvastatin for 5 min (3), 10 min (1, 4), 30 min (5), and 60 min (6). Then, these cells were washed with chilled PBS containing *p*-nitrophenylphosphate and lysed with SDS sample buffer. Samples were normalized for protein and 100 μg were subjected to 12.5% SDS-PAGE followed by Western blotting probed with an anti-pY antibody. C: L6 myoblasts were precultured in the presence or absence of with 0.5 mM (4) and 1 mM (5) mevalonate for 30 min at 37°C and then with pravastatin (60 μg/ml) (1) or simvastatin (30 μg/ml) (2, 4, 5) for 10 min or left untreated (3) at 37°C. Lines on the left side of each panel represent positions of molecular weight markers (107 kDa, 68 kDa, 46 kDa, 28 kDa for A, and 107 kDa, 68 kDa, 46 kDa for B and C). The data represent a typical response from three different samples with similar results.

3.5. PTPase activity

To elucidate the molecular mechanism for simvastatin-induced tyrosine phosphorylation, we assayed PTPase activity in simvastatin-treated cells. No inhibition of PTPase activity was observed (Fig. 5).

4. Discussion

The most important finding of this study is that simvastatin

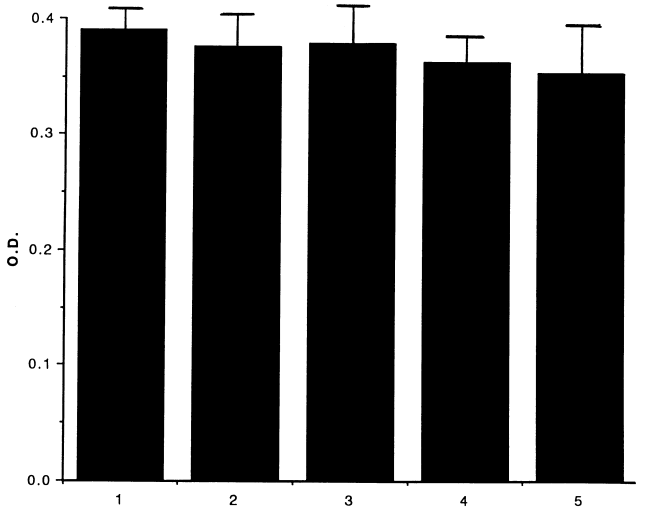

Fig. 5. Effect of simvastatin on protein tyrosine phosphatase (PTPase) activity. L6 myoblasts were cultured in 10 cm culture dishes and incubated in the presence of 3 μg/ml simvastatin (2, 4) and 30 μg/ml simvastatin (3, 5) for 5 min (2, 3) and 15 min (4, 5). Equal amounts of protein from cell-free lysates prepared as described in Section 2 were assayed for PTPase activity. The data represent the values of three different samples each done in duplicate (mean ± S.D.). Data of each experiment are not statistically significantly different.

rapidly induces tyrosine phosphorylation of several cellular proteins even before any morphological change becomes apparent, and well before any induction of apoptotic cell death. Herbimycin A or genistein, specific inhibitors of protein tyrosine kinases, showed an inhibitory effect on protein tyrosine phosphorylation induced by simvastatin, and a subsequent inhibition of DNA fragmentation and cell death. These results suggest a close relationship between protein tyrosine phosphorylation and the induction of apoptosis by simvastatin. In other words, these data strongly suggest that the simvastatin-induced protein tyrosine phosphorylation response is an obligatory signal transduction mechanism for cell death but not a signal for protecting themselves. Tyrosine phosphorylation is known to be regulated by either protein tyrosine kinase or protein tyrosine phosphatase activity. The simvastatin-induced tyrosine phosphorylation observed here is probably due to the activation of protein tyrosine kinase(s), because simvastatin apparently did not affect protein tyrosine phosphatase activity. Such an obligatory role of protein tyrosine phosphorylation in cell death has been observed only in blood cells and ovarian tumor cell systems [11,18,21,22].

What is the primary target of simvastatin on L6 myoblasts? First of all, we should remember that only the lipophilic form of HCRI, which can cross the plasma membrane, can induce the apoptotic cell death of L6 myoblasts. Pravastatin, a hydrophilic HCRI, did not induce any biological effect on these cells. Pravastatin is known to be unable to get into the cells unless its specific receptor is expressed on the plasma membrane. These results might indicate that simvastatin does its job inside the cells. Previous studies employing a lipophilic HCRI also revealed apoptotic cell death in cells other than muscle cells (i.e. brain tumor cells and leukemic cells) [4,23]. These reports highlighted the significance of the impaired maturation of Ras protein in the event of an apoptotic cell death, because HCRI induced inhibition of the isoprenylation of Ras protein and, therefore, the Ras protein could not be properly targeted to the plasma membrane. Such inhibition in the targeting of the Ras protein to the plasma membrane in turn disrupts Ras-mediated signal transduction in the cells, although these previous reports did not look for changes in cellular protein tyrosine phosphorylation. In this study, we examined the effect of the addition of mevalonate to the culture medium and found that it rescues cells from simvastatin-induced cell death incompletely but significantly, and that it inhibits simvastatin-induced tyrosine phosphorylation of cellular proteins, as well. These results indicate that the impairment in the biosynthesis of mevalonate and the possible decrement of isoprenoids caused by simvastatin makes a major contribution the development of cell death, although we cannot exclude the possibility of a direct effect on protein tyrosine kinase(s). We recently observed a decreased amount of Ras protein in the plasma membrane fraction in simvastatin-treated L6 myoblasts within 10 min after its treatment [24]. These observations strongly suggest that simvastatin caused an impairment of the Ras-mediated intracellular signaling pathway. Previous studies have indicated that the intracellular signaling cascade involving growth factor-induced MAPK activation is dependent on the sequential activation of receptor-type tyrosine kinase-Ras-Raf 1-MEK (MAPK kinase) [25]. It is now well known that this pathway plays a critical role in signaling for cell proliferation and survival as well as for cellular differentiation. Therefore, it is reasonable to assume that a change in protein tyrosine phosphorylation and a loss of the Ras-mediated signaling cascade work cooperatively to kill the cells. The relationship between tyrosine phosphorylation of cellular proteins and an impairment of the Ras-mediated intracellular signaling pathway is not clear at present. We are now engaged in trying to determine the target(s) of this tyrosine phosphorylation response, which might reveal the molecular mechanism by which tyrosine phosphorylation can interact with Ras-mediated signaling in the cells.

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