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(54) Title: INHIBITION OF P70 S6 KINASE

(57) Abstract

A method of screening for an antiproliferative or immunosuppressive agent, which method includes the steps of (1) contacting a eukaryotic cell with a candidate antiproliferative or immunosuppressive composition; and (2) determining the level of activity of a serine/threonine kinase or a serine/threonine phosphatase in the p70 S6 kinase cascade of said cell in the presence of the candidate composition, wherein a level of said activity that results in a lower p70 S6 kinase activity in the presence of the composition than in the absence of the composition is an indication that the composition is antiproliferative or immunosuppressive agent; and methods of treatment using such compositions.

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INHIBITION OF P70 S6 KINASE

The invention was made in the course of work funded in part by a grant from the U.S. government, which has certain rights in the invention. The field of the invention is antiproliferative or immunosuppressive agents.

Background of the Invention

A conserved response of many eukaryotic cell types to mitogenic signals is the phosphorylation of multiple serine residues on the 40S ribosomal subunit protein S6 (Erikson, J. Biol. Chem. 266:6007-6010, 1991). This phosphorylation increases the efficiency of protein synthesis, which appears to be required in several steps of cell cycle progression (Erikson, *supra*). Recently, two families of S6 kinases have been characterized at the enzymatic and molecular levels: the 85-90 kDa (rsk) S6 kinase family (Jones et al., Proc. Natl. Acad. Sci. USA 85:3377-3381,, 1988), referred to herein as p90 S6 kinase, and the 70 kDa S6 kinase family (Banerjee et al., Proc. Natl. Acad. Sci. USA 87:8550-8554, 1990; Kozma et al., Proc. Natl. Acad. Sci. USA 87:7365-7369, 1990), referred to herein as p70 S6 kinase. The activity of both types of kinases is regulated by serine/threonine phosphorylation (Erikson, *supra*), and both are serine/threonine kinases themselves.

Eukaryotic cells contain many different kinases as part of various cascades that transmute signals from cell-surface receptors to effector molecules within the cell. In some cases the cell-surface receptor is itself a kinase that is activated upon binding its ligand; in other cases, the receptor is associated with a separate protein that acquires kinase activity when the receptor

binds its ligand. The newly-activated kinase then phosphorylates the next member of the relevant cascade, thereby activating (or in some cases, deactivating) it. Phosphatases also form an integral part of the cascade, acting to remove the phosphate groups added by the kinases, and thereby deactivating (or in some cases, activating) the substrate polypeptide. In general, each member of such a cascade transmutes signals by sequentially phosphorylating (if it is a kinase) or dephosphorylating (if it is a phosphatase) certain critical residues in the next member of the cascade, thereby activating or deactivating such next member, as the case may be.

In T cells expressing the interleukin-2 (IL-2) receptor, binding of IL-2 to this receptor triggers a response culminating in proliferation of the T cell (Smith, Ann. Rev. Immunol. 2:319-333, 1984). Evans et al. observed that one aspect of the IL-2 generated response is an increase in S6 phosphorylation and a concomitant increase in the rate of protein synthesis (J. Biol. Chem. 262:4624-4630, 1987). Similarly, the multi-faceted response of insulin-dependent H4 hepatoma cells to stimulation by insulin includes an increase in the level of S6 phosphorylation, apparently attributable at least in part to increased activity of the S6 kinases.

Rapamycin and FK506 are macrolide antibiotics which are potent immunosuppressive agents. Although the two compounds share certain structural features (see Fig. 9) and are capable of binding to the same family of cellular proteins (termed FK506-binding proteins, or FKBPs) to form a biologically active complex, their mechanisms of immunosuppression differ significantly. The FK506/FKBP complex inhibits a very early step in antigen-induced T cell activation, preventing proliferation of activated T cells by blocking the

induction of cytokine gene transcription. Liu et al. (Cell 66:807-815, 1991) have recently shown that this occurs through the binding of the FK506/FKBP complex to calcineurin, a calcium-dependent phosphatase thought to play a role in T cell signal transduction, thereby blocking the phosphatase activity of this enzyme and short-circuiting signal transduction along this pathway. In contrast, the rapamycin/FKBP complex apparently does not bind to or affect the activity of calcineurin, and acts by blocking a later step in antigen-induced T cell activation, one that occurs subsequent to binding of IL-2 to its receptor (i.e., at a point after the initial induction of cytokine gene transcription). The entity directly targeted by the rapamycin/FKBP complex has not been identified, although it has been shown not to be the IL-2 receptor itself.

Summary of the Invention

As disclosed herein, it has now been shown that the addition of rapamycin to an IL-2-stimulated T cell line results in the inhibition of p70 S6 kinase, which kinase may constitute the direct target of the rapamycin effector complex, or may be downstream of the actual target. Furthermore, experiments are herein disclosed which demonstrate that rapamycin will inhibit both basal and insulin-stimulated proliferation and p70 S6 kinase activity in an insulin-dependent hepatoma cell line, an observation that has broad implications with respect to novel medical applications for rapamycin. The information linking p70 S6 kinase inhibition to the immunosuppressive and newly discovered general antiproliferative effects of rapamycin may be utilized to design an assay for screening for immunosuppressive and/or antiproliferative agents which act by the same or a related mechanism to that of rapamycin: i.e., which

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