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Inhibition of Branched-Chain α-Keto Acid Dehydrogenase Kinase and Sln1 Yeast Histidine Kinase by the Antifungal Antibiotic Radicicol

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ABSTRACT

The 90-kDa heat shock family (HSP90) of protein and two-component histidine kinases, although quite distinct at the primary amino acid sequence level, share a common structural ATP-binding domain known as the Bergerat fold. The Bergerat fold is important for the ATPase activity and associated chaperone function of HSP90. Two-component histidine kinases occur in bacteria, yeast, and plants but have yet to be identified in mammalian cells. The antifungal antibiotic radicicol (Monorden) has been shown to bind to the Bergerat fold of HSP90 and to inhibit its ATPase activity. The structural similarity between the Bergerat fold of HSP90 and bacterial two-component histidine kinases prompted our inquiry into whether radicicol could be a potential inhibitor of histidine kinase-like proteins. Structural homology searches suggest that the ATP-binding domains of the yeast histidine kinase Sln1 and the mammalian, branched-chain α-keto acid dehydrogenase kinase are very similar to that of other Bergerat fold family members. On the basis of structural homology, we tested radicicol as a potential inhibitor of Sln1 and branched-chain α-keto acid dehydrogenase kinase (BCKDHK) and propose a mechanism of inhibition of these kinases. Although BCKDHK has been shown to have serine autophosphorylation activity, we speculate, based on the results from this study and other supporting evidence, that BCKDHK may also have intrinsic histidine kinase activity.

Protein kinases that phosphorylate target proteins on histidine residues are not as well studied as serine, threonine, or tyrosine kinases. There are reports that histidine phosphorylation may account for up to 6% of the total protein phosphorylation events in mammalian cells (Matthews, 1995), thus making a potentially significant contribution to cell signaling compared with <1% representation for tyrosine phosphorylation (Hunter and Sefton, 1980). The defining absence of reports of histidine kinases and histidine phosphorylation primarily stems from the acid-labile nature of the histidine-phosphate bond.

Histidine phosphorylation occurs via a phosphoramidate bond to either of the two nitrogen atoms of the imidazole moiety. This phosphoramidate bond is very susceptible to hydrolysis at moderate to low pH, often making biochemical identification, protein purification, and kinase assays difficult. However, known histidine kinases prevail in the two-component signal transduction pathways of bacteria, cyanobacteria, fungi, yeast, and plants but have yet to be identified in mammalian cells (Swanson et al., 1994).

A two-component signal transduction pathway typically involves a receptor TCHK that autophosphorylates on a histidine residue. This high-energy phosphate group is then transferred onto an aspartate residue of an associated response regulator. In prokaryotes, this simple system of signaling provides rapid adaptation to environmental changes by altering cellular behavior (Stock et al., 1990). Given that histidine phosphorylation has traditionally been difficult to determine biochemically and that TCHKs are not represented in any mammalian signaling systems, the fact remains that phosphohistidine is present in mammalian cells (Besant and Attwood, 2000; Besant et al., 2000). Although certain mammalian metabolic enzyme reaction intermediates possess phosphohistidine (Rose et al.,1975; Ostrowski, 1978; Pilkis et al., 1983; Williams et al., 1985; Fothergill-Gilmore and Watson 1989; Krivanek and Novakova, 1991;

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ABBREVIATIONS: TCHK, two-component histidine kinase; BCKDHK, branched-chain α-keto acid dehydrogenase kinase; PDHK, pyruvate dehydrogenase kinase; HSP90, 90-kDa heat-shock protein; NCBI, National Center for Biotechnology Information; RPS-BLAST, reverse position-specific blast; DMSO, dimethyl sulfoxide; GST, glutathione-*S*-transferase; PVDF, polyvinylidene difluoride; 6xHis, hexahistidine fusion tag; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

Webb et al., 1995), the presence of TCHKs in mammalian cells still remains in question. This question has become increasingly important as highlighted by research into putative anti-infective agents that may block two-component signaling (Deschenes et al., 1999). If it can be definitively shown that TCHKs do not occur in mammalian cells, then pharmaceutical companies may target these kinases in an effort to combat the ever-increasing antibiotic resistance of pathogenic bacteria.

Current homology studies have yet to reveal any functional bacterial-like histidine kinases in mammalian cells. However, two mammalian enzymes, BCKDHK and pyruvate dehydrogenase kinase (PDHK), do contain prototypical TCHK motifs (Popov et al., 1992; Davie et al., 1995). BCKDHK and PDHK are components of a mitochondrial enzyme complex involved in the regulation of the oxidative decarboxylation of the branched-chain α-keto acids derived from leucine, isoleucine, valine, and pyruvate, respectively. In vitro, both of these enzymes are known to autophosphorylate on a serine residue, but there has been conjecture as to whether BCKDHK also has intrinsic histidine kinase activity (Davie et al., 1995; Thelen et al., 2000). Why an enzyme involved in a metabolic pathway should possess a bacterial-like histidine kinase motif is an interesting question. From an evolutionary standpoint, the fact that BCKDHK is a mitochondrial enzyme and has bacteria-like histidine kinase motifs makes sense with respect to the hypothesis put forward by evolutionary biologists of a primordial symbiosis of a bacterial-like cell becoming what we now recognize as a mitochondrion.

In a recent article by Koretke et al. (2000), the evolution of TCHKs was examined using a bioinformatics approach. The possibility of a distant evolutionary relationship between bacterial TCHKs and eukaryotic serine/threonine protein kinases is inferred through a phylogenetic tree, which has at its origin an ancestral "protokinase". This phylogenetic tree is bifurcated early in the evolutionary process, with one early branch leading to a diverse family of ATPase proteins described in a recent review by Dutta and Inouye (2000).

Dutta and Inouye investigate the functional role of an ATP-binding domain unique to a diverse family of proteins from a structural perspective. This family of proteins comprises TCHKs, HSP90, Mut L DNA mismatch repair enzymes, and type II DNA topoisomerases. The unique structure of this ATP-binding motif, known as the Bergerat fold, is essentially superimposable in all four families of proteins, yet each family shares neither any functional similarity nor any significant primary sequence homology ($<$15%).

The Bergerat fold captures ATP for the kinase activity associated with TCHKs and the ATPase activity associated with the remaining members of this family. This novel fold possesses an α/β-sandwich structure, which consists of a four-stranded mixed β-sheet and three α-helices, as deduced from the solved structures of representative members of the Bergerat fold protein family (Dutta and Inouye, 2000). This differs from conventional serine, threonine, and tyrosine kinase ATP-binding domains, which have an $\alpha/\beta/\alpha$-layer with four open parallel β-sheets surrounded by two α-helices on either side (Traut, 1994). Closer examination of the Bergerat fold also reveals an external loop or "ATP-lid", which has a slightly different conformation in each of the four family members. Among the four Bergerat fold family of proteins, HSP90 and TCHKs share the closest conformation, with both having the ATP-lid in an open position (Dutta and Inouye, 2000). Because the Bergerat fold of these two proteins seems structurally related, it was thought that exploitation of this relationship may be useful in identifying mammalian TCHK-like proteins.

To determine whether BCKDHK was part of this Bergerat family of proteins, structural motif searches based on the recently solved crystal structure of rat BCKDHK (Machius et al., 2001) were performed using the ATP-binding domain of BCKDHK as the input sequence in the NCBI RPS-BLAST (conserved domain search with three-dimensional structure) database. Although there is little direct biochemical evidence to suggest that BCKDHK belongs to the TCHK family, there are reports that speculate its inclusion. Two of these reports (Wynn et al., 2000; Machius et al., 2001) not only suggest that the ATP-binding domain of BCKDHK is similar to the Bergerat fold family of proteins to which TCHKs belong but also that the structure of BCKDHK may be the prototype for all mitochondrial protein kinases. RPS-BLAST database searching revealed that the BCKDHK ATP-binding sequence, matched to numerous TCHKs (e.g., Sln1, EnvZ), HSP90, and DNA gyrase B, all of which are members of the Bergerat fold family. Given the homologous structural relationship between the Bergerat folds of HSP90 and histidine kinases, we sought to exploit this relationship by using the known HSP90 inhibitor radicicol as a potential inhibitor of BCKDHK and a known TCHK, Sln1. The TCHK Sln1 is found upstream of the yeast equivalent of the mitogen-activated protein kinase pathway that controls osmoregulation.

The inhibitor radicicol (Monorden) (Fig. 1) is a macrocyclic antifungal antibiotic first isolated from *Monosporium bonorden* (Delmotte and Delmotte-Plaquee, 1953). Radicicol was initially thought to be a direct inhibitor of many signaling proteins (Shulte et al., 1999) but has since been shown to act via the inhibition of the HSP90 protein family. It has been reported that radicicol does not inhibit the serine/threonine kinases of the mitogen-activated protein kinase pathway but may affect some tyrosine kinases in vivo (Roe et al., 1999). Whether the in vivo effect on tyrosine kinases results from radicicol binding directly to the kinase, or from the ATPase inhibition of kinase-associated HSP90 chaperones is unknown. This was the case for the tyrosine kinase c-src. Radicicol was originally thought to be directly involved in the inhibition of c-src tyrosine kinase activity. However, it was later shown that the effect of radicicol inhibition was directed to the associated HSP90, which was responsible for main-

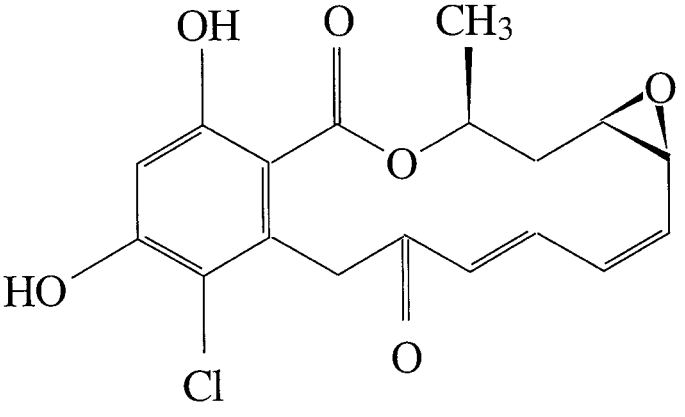

Fig. 1. Structure of radicicol.

taining c-src integrity (Bijlmakers and Marsh, 2000). Radicicol's mode of inhibition for HSP90 is through direct competition with ATP for the N-terminal Bergerat fold, thereby preventing the ATPase activity necessary for its role as a chaperone. Radicicol binds to HSP90 with a K_d of 19 nM (Roe et al., 1999) making it a potent inhibitor of HSP90 compared with the K_d for ATP of only 400 μM (Prodromou et al., 1997).

Although searching the genome for common histidine kinase sequences provides important information with regard to identifying common motifs, it does not necessarily translate into protein function. Given that the structure ultimately defines the function of any protein, the presence of mammalian TCHKs might only be found by examining structure-function relationships. Based on the predicted structural homology of the Bergerat fold of HSP90, we present evidence that demonstrates the efficacy of radicicol as an inhibitor of autophosphorylation for mammalian BCKDHK and a known yeast TCHK, Sln1. Given the apparent homologous structural relationship among HSP90, BCKDHK, Sln1, and the Bergerat family, we also speculate whether BCKDHK is a putative TCHK and whether the inhibitory effect of radicicol is modulated through direct binding to this unique ATP-binding domain.

Materials and Methods

Radicicol was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in either 50% (v/v) DMSO or 50% (v/v) absolute ethanol at a concentration of 500 μg/ml and stored at −20°C. GST-Sepharose was from Amersham Biosciences (Piscataway, NJ). The PVDF membrane was from Millipore Corporation (Bedford, MA). ATP-Sepharose was from Upstate Biotechnology (Lake Placid, NY). GST-Sln1 catalytic subunit (from Dr. Haruo Saito, Harvard Medical School) and GST-TGFβR kinase (from Dr. Rik Derynck, University of California, San Francisco) were both purified according to a previously published protocol (Posas et al., 1996). Insulin receptor kinase was purchased from Calbiochem (San Diego, CA).

Protein Expression. The 6xHis-BCKDHK (amino acids 1–382 of the rat BCKDHK) fusion protein was expressed using the pQE-32 (QIAGEN, Valencia, CA) plasmid. *Escherichia coli* DH5α cells were initially grown in 2× yeast-tryptone media at 37°C overnight. For protein expression, the cells were expanded in 500 ml of 2× yeast-tryptone media, and expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside at room temperature. After 3 h, the cells were centrifuged at 2,000 rpm for 20 min, and the cell pellet was immediately transferred into liquid nitrogen. Cells were lysed in a solution of PBS, 0.5% (v/v) Tween 20, 1 M NaCl, 1 mM PMSF, and 10 mM dithiothreitol and sonicated for 1 min at 4°C. The lysate was centrifuged at 14,000 rpm, and the supernatant was applied to 0.5 ml of nickel-NTA-Sepharose. After loading, the column was washed with 20 ml of PBS, pH 8, 1% (v/v) Nonidet P-40, 80 mM imidazole, and 10 mM Tris-Cl, pH 8.

RPS-BLAST Analysis. Sequence homology with three-dimensional structure was performed using the RPS-BLAST analysis program on the NCBI Blast database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The algorithm for this program can be found in Altschul et al. (1997).

The input sequence A was the ATP-binding domain of the BCKDHK: 271-YILPELLKNA MRATMESHLD TPYNVPDVVI TIANNDVDLI IRISDRGGGI AHKDLDRVMD YHFTTAEAST QDPRISPLFG HLDMHSGAQS GPMHGFGFGL PTSRAYAEYL GGSLQLQSLQ GIGTDVYLRL-400.

The input sequence B was the ATP-binding domain of the yeast histidine kinase Sln1: 680-G DSNRIIQIVM NLVSNALKFT PVDGTVDVRM KLLGEYDKEL SEKKQYKEVY IKKGTEVTEN LETTDKYDLP TLSNHRKSVD LESSATSLGS NRDTSTIQEE ITKRNTVANE SIYKKVNDRE KASNDDVSSI VSTTTSSYDN AIFNSQFNKA PGSDDEEGGN LGRPIENPKT WVISIEVEDT GPGIDPSLQE SVFHPFVQGD QTLSRQYGGT GLGLSICRQL ANMMHGTMKL ESKVGVGSKF TFTLPLNQ-928.

Kinase Reaction for BCKDHK. Ni-NTA beads containing approximately 5 μg of 6xHis-BCKDHK (50% slurry) were incubated in kinase reaction buffer A (50 mM Tris-Cl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol) together with radicicol at various concentrations, dependent upon the experiment. A control of 50% (v/v) of an equal volume of DMSO was also used. The samples were incubated at 25°C for 1 h, after which ATP to a final concentration of 0.2 mM and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) were added. The kinase reaction proceeded for 1 h at 25°C and was then stopped by the addition of 2× SDS sample buffer. All samples were then separated on 4 to 20% SDS-PAGE. For quantitation, the proteins were then electroblotted onto PVDF membrane, which was subsequently exposed to X-ray film for 1 h. The membrane was briefly stained with Amido Black, and the protein bands corresponding to BCKDHK were excised. The protein bands were then Cherenkov-counted for 10 min.

Kinase Reaction for Sln1. Glutathione beads containing approximately 5 μg of GST-Sln1 (50% slurry) were incubated in kinase reaction buffer A together with increasing concentrations of radicicol. A control of an equal volume of 50% (v/v) ethanol was also used. The samples were incubated at 25°C for 2 h, after which ATP to a final concentration of 0.2 mM and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) were added. The kinase reaction continued for 1 h at 25°C and was then stopped by the addition of 2× SDS sample buffer. All samples were subsequently separated on 4 to 20% SDS-PAGE. The proteins were then electroblotted onto PVDF membrane and treated as described above for BCKDHK.

Radicicol Effect on Other Autophosphorylating Protein Kinases. Five micrograms of GST-Sln1, 10 μg of GST-TGFβRK, 5 μl of insulin receptor kinase (1 mg/ml), and 5 μg of BCKDHK were incubated with kinase reaction buffer A together with either 275 μM radicicol or the equivalent volume of vehicle control, in a total volume of 50 μl for 2 h at 25°C. After incubation with radicicol, ATP to a final concentration of 0.2 mM and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) were added. The kinase reactions proceeded for 1 h at 25°C and were then stopped by the addition of 2× SDS sample buffer. All samples were subsequently separated on 4 to 20% SDS-PAGE, which was then exposed to X-ray film for 1 h.

ATP Competition. Direct competition of radicicol binding by ATP involved the phosphorylation of BCKDHK that was pretreated with radicicol. Approximately 5 μg of 6xHis-BCKDHK (50% slurry) was incubated in kinase reaction buffer A, together with either 55 μM radicicol or vehicle control. The samples were incubated for 1 h at 25°C, after which increasing concentrations of ATP containing 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) tracer were added. The reactions were again incubated for 1 h at 25°C and then stopped by the addition of 2× SDS sample buffer. All samples were separated on 4 to 20% SDS-PAGE, and the gel was exposed to X-ray film for 1 h.

ATP Binding Affinity. For ATP-binding studies, 6xHis-BCKDHK was eluted from the Ni-NTA resin using 400 mM imidazole in PBS, pH 8. Fifty micrograms of 6xHis-BCKDHK was diluted in 10 ml of buffer A to obtain a final imidazole concentration of 40 mM. Thirty microliters of ATP-Sepharose (in a 50% slurry) was added to the diluted 6xHis-BCKDHK and allowed to bind overnight at 4°C. The next morning, the resin was briefly centrifuged at 10,000 rpm and washed with 2× 500 μl of fresh buffer A. To elute the 6xHis-BCKDHK, 100 μl of elution buffer containing 500 μM radicicol in buffer A was added to the resin and incubated at 25°C for 2 h. The eluate was dried using a speed vacuum to a volume of 30 μl and separated by 4 to 20% SDS-PAGE.

Results

The ATP-binding domain of BCKDHK is known to be homologous to many of the bacterial TCHKs. A homology search of motifs with conserved domains can be performed using the NCBI RPS-BLAST analysis program (Altschul et al., 1997). This conserved domain search compares a protein sequence against the conserved domain database with the RPS-BLAST program and allows known functional and structural domains to be identified on protein query sequences. Using the ATP-binding domain of BCKDHK as the input sequence, there is a distinct relationship (score 48.9, E value = 2×10^{-7}) between the ATP-binding domain of BCKDHK and the members of the Bergerat fold family. A similar result is obtained with the input sequence for Sln1. Here the relationship is even stronger (score 92.8, E value = 2×10^{-20}) because Sln1 belongs to the histidine kinase subgroup of the Bergerat fold family.

At the primary sequence level for the ATP-binding domains shown in Fig. 2, there is very little overall homology between BCKDHK and the various members of the Bergerat fold family. The difference in the outcome of homology searches between a primary sequence-based or structure-function-based search is striking. On the basis of total primary sequence using a simple BLAST analysis, there seems to be very little in common among the proteins that make up the Bergerat family (<15% homology). From a structural perspective of the conserved domains, the known members of the Bergerat family share an important ATP-binding fold that is essentially superimposable among the four family members (Dutta and Inouye, 2000). This highlights the importance of identifying functional relationships that are not based solely on the primary sequence.

However, it is important to note that each Bergerat family member does possess small motifs within the ATP-binding fold that do align at the primary sequence level. From the solved crystal structures of representative Bergerat family members, these four motifs (Fig. 2) define the ATP-binding domain (Dutta and Inouye, 2000). Together they help coordinate the Mg-ATP complex, either through direct interaction with ATP or through maintaining the structural integrity of the Bergerat fold (Dutta and Inouye, 2000). The predicted structural relationship, as determined by RPS-BLAST analysis of the ATP-binding domains of BCKDHK, Sln1, and HSP90, was the basis for testing radicicol as an inhibitor of BCKDHK activity. Radicicol had previously been shown to inhibit the ATPase activity of HSP90 through direct binding to its Bergerat fold (Sharma et al., 1998; Roe et al., 1999; Shulte et al., 1999) and by inference, should similarly inhibit BCKDHK activity.

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          ...|....*....|....*....|....*....|....*....|....*....|.....
HSP90)NKEIFLRELISNASDALDKIRYKSLSDPKQLETEPDLFIRITPKP---------------- 80
GyrB) TNIGGLHHMIYEVVDNSIDEAMAG--------HCDTIDVEITT----------------- 64
Topo) TDERALHHLFAEVLDNSMDEAVAG--------FAKTIEVKLDA----------------- 86
CheA) DLLDPIMHCVRNSLDHGIEAPEVRARS--GKPAQGTLLLRASNEG--------------- 562
BCK)  -------YILPELLKNAMRATME----------SILDTPYNVPDVVITIANNDVDLIIR 306
Sln1) GDSNRIIQIVMNLVSNALKFTPV---------DGTVDVRMKLLGEYDKELSEKKQYKEV 729
EnvZ) TGQEMPMEMADLNAVLGEVIAAESGYEREI------------------------------ 59
                N-box

          ....*....|....*....|....*....|....*....|....*....|....*....|.
81    -EQKVLEIRDSGIGMTKAELINN----------LGTIAKSGTKAFMEALSAGA-DVSMI 127
65    --EGSCIVSDNGRGIPVDMHPTE----------NMPTLTVVLTVLHAGGKFDK-DTYKV 110
87    --DGFLSVKDDGRGMPVDPHPKY----------PGKSALEVIMTVLHAGGKFTG-KAYET 133
563   -NMIVIEVADDGRGIDVEAVKTKAVERGVLHPGKNLTEVEAFQLIFAPGFSTSR-SVSNV 620
307   -------ISDRGGGIAH----------KDLDRVMDYHFTTAE-------------ASTQ 334
850   TWVISIEVEDTGPGIDP----------------------SLQESVFHPFVQGDQTLSRQY 887
75    ------QVRDDGPGIAPEQRKHLFQP---------------------------------- 107
            G1-Box

          ...*....|....*....|....*....|....*....|....*....|....*....|.
 128  GQ--FGVGFYSLFLVADRVQVISKSNDDEQYIWESNAGGSFTVTLDEVNERIGRGTILRL 185
 111  SGGLHGVGVSVVNALSKKLVATVERNGEIYRQEFSEGKV--ISEFGVIGKSKKTGTTIEF 168
 134  SGGLHGVGASVVNALSERVEVTVWRDGFEHLQVFSRGKP--LGPIQQVAPSKKKGTMVRF 191
 621  SG--RGVGLDVVKTHIERLNGTVSVFSE----------------------VQKGTRFVI 655
 354  PMHGFGFGLPTSRAYAEYLGGSLQLQSI----------------------QGIGTDVYL 364
 888  GG--TGLGLSICRQLANMMHGTMKLESK----------------------VGVGSKFTF 922
 108  TISGTGLGLAIVQRIVDNHNGMLELGTSE----------------------RGGLSIR 144
        G2-Box                                              G3-Box
```

Fig. 2. Primary sequence alignment of representative Bergerat family members HSP90 (row 1), DNA gyrase (2), topoisomerase (3), CheA bacterial histidine kinase (4), mammalian BCKDHK (5), Sln1 (6), and *E. coli* Env Z (7). Letters highlighted in bold represent conserved amino acid residues that make up the Bergerat fold motifs.

As a preliminary experiment to gauge the inhibitory effect of radicicol on BCKDHK activity, equal amounts of 6xHis-BCKDHK were treated with either 27.5 μM radicicol or an equal volume of reaction buffer containing the equivalent volume of DMSO. After radicicol treatment for 1 h, [γ-^{32}P]ATP was added to the reaction mix and incubated for a further 30 min, and the samples were separated by SDS-PAGE. At this concentration, there seems to be no autophosphorylation activity of BCKDHK (Fig. 3). Subsequent to our preliminary observation, we examined the extent of radicicol inhibition by titrating with increasing concentrations of radicicol.

From the autoradiograph shown in Fig. 4A, the inhibition of kinase activity seems to begin at a radicicol concentration of 2.75 μM. To verify that the apparent inhibitory effect was not caused by uneven protein loading, the membrane was briefly stained with Amido Black (Fig. 4B). Although notable inhibition of histidine kinase activity in Fig. 4A appears at 2.75 μM, this is only a qualitative estimate. To acquire a quantitative measure of radicicol inhibition, the protein bands corresponding to BCKDHK were excised from the membrane and Cherenkov counted. The counts for each radicicol-treated sample were then compared against the control (DMSO) sample. The results were calculated from two inde-

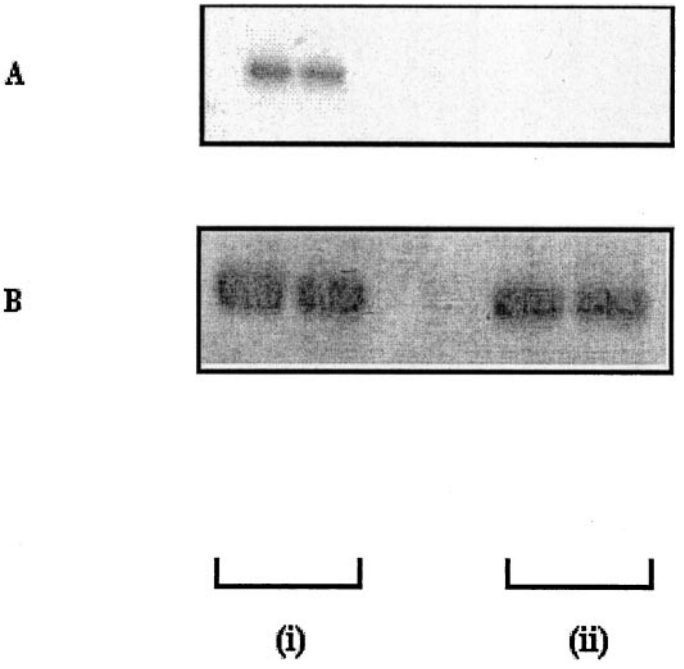

Fig. 3. A, autoradiograph of BCKDHK activity after treatment with DMSO control (i) or 27.5 μM radicicol (ii), for 10 min. B, Coomassie-stained gel of A showing protein loading.

pendent experiments and displayed graphically in Fig. 4C. Calculation of the data indicates 50% inhibition (IC_{50}) of kinase activity to be at a radicicol concentration of 635 nM with complete inhibition at 50 μM.

A similar experiment was performed using the yeast TCHK, Sln1. From the autoradiograph shown in Fig. 5A, inhibition of histidine kinase activity seems to begin at a radicicol concentration of 275 μM. To verify that the apparent inhibitory effect was not due to uneven protein loading, the membrane was again stained with Amido Black (Fig. 5B). A quantitative measure of radicicol inhibition based on the results from two independent experiments is displayed graphically in Fig. 5C. The data were calculated to show IC_{50} of kinase activity at a radicicol concentration of 127 μM and close to complete inhibition at 1 mM. Although radicicol is not as potent as an inhibitor of Sln1 autophosphorylation as it is for BCKDHK autophosphorylation, it should be pointed out that only the catalytic domain of Sln1 is expressed as a GST-fusion protein compared with the full-length 6xHis-BCKDHK. Consequently, the overall structure of Sln1 may

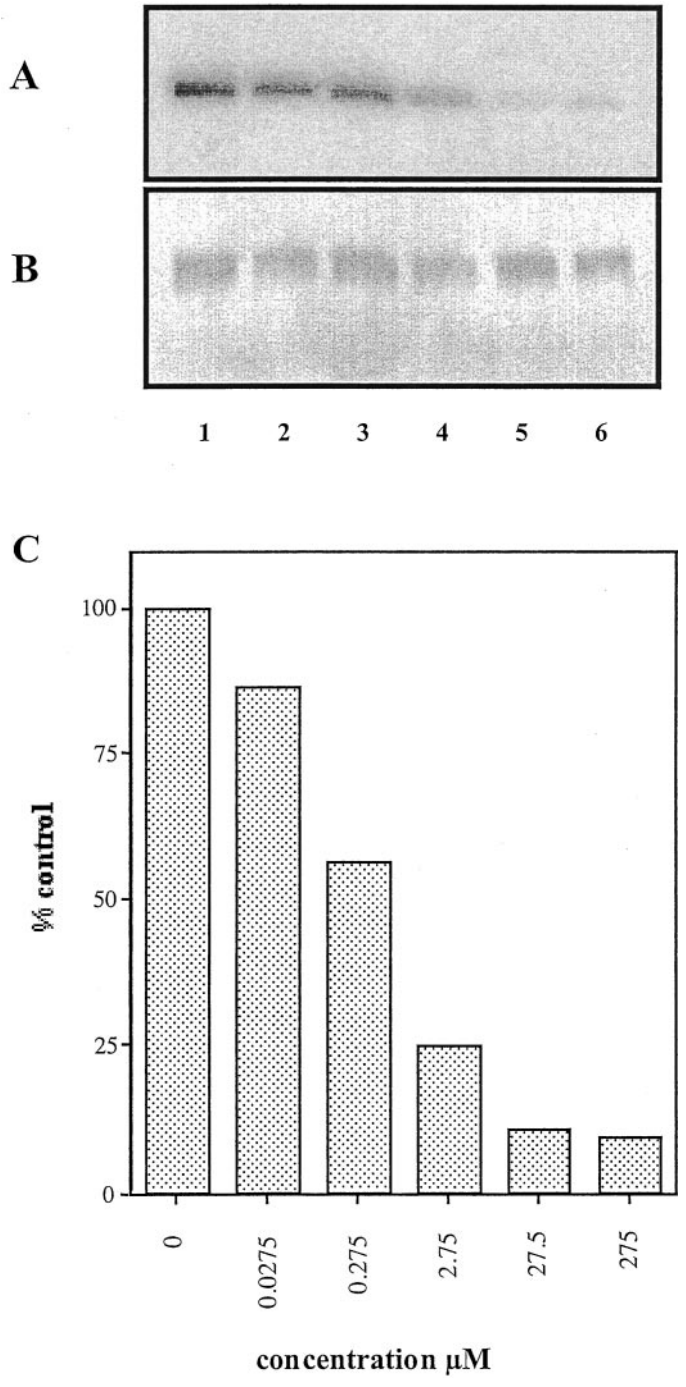

Fig. 4. A, autoradiograph illustrating the titration of radicicol inhibition of BCKDHK activity. 1, DMSO control; 2, 27.5 nM; 3, 275 nM; 4, 2.75 μM; 5, 27.5 μM; and 6, 275 μM. B, Amido Black-stained membrane of A showing protein loading. C, quantitative measure of radicicol inhibition of BCKDHK activity. Inhibition for each concentration is calculated as a percentage of the control.

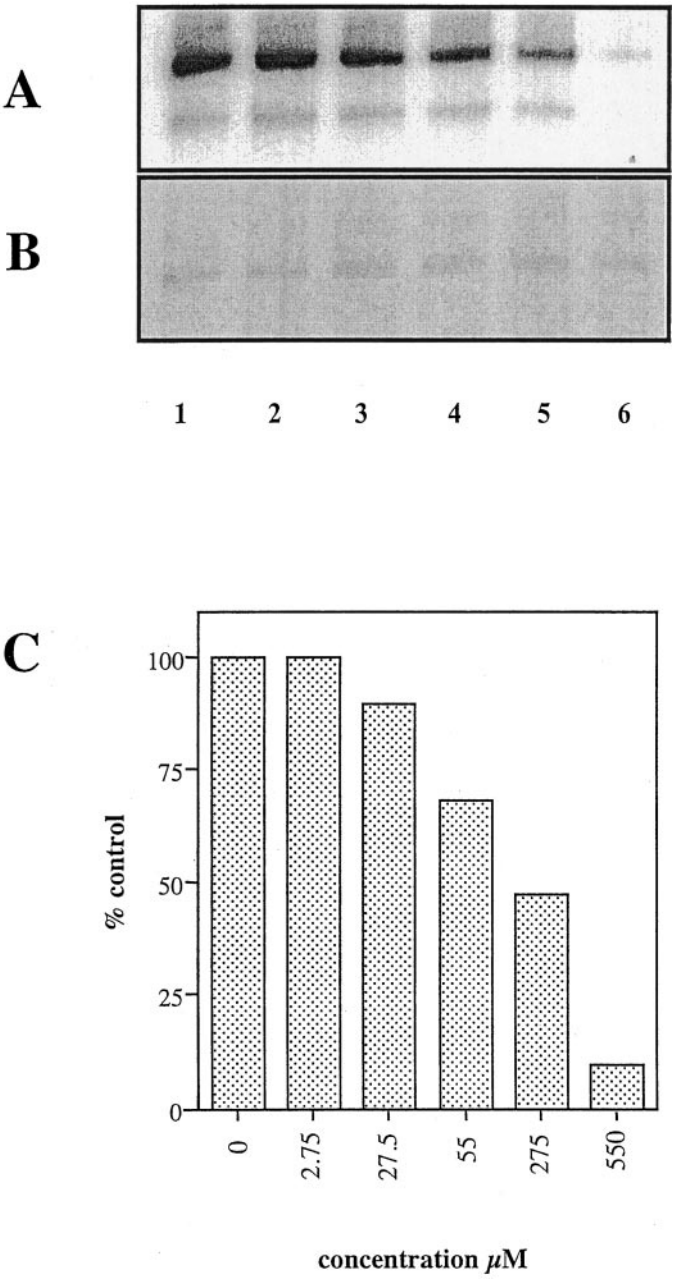

Fig. 5. A, autoradiograph illustrating the titration of radicicol inhibition of Sln1 kinase activity. 1, control; 2, 2.75 μM; 3, 27.5 μM; 4, 55 μM; 5, 275 μM; and 6, 550 μM. B, Amido Black-stained membrane of A showing protein loading. C, quantitative measure of radicicol inhibition of Sln1 kinase activity. Inhibition for each concentration is calculated as a percentage of the control.

be compromised with respect to radicicol binding, thus potentially affecting its potency as an inhibitor.

Having established a radicicol concentration-dependent profile for the inhibition of BCKDHK and Sln1 kinase activity, we sought to test the inhibitory effect of radicicol on other protein kinases also known to autophosphorylate. TGFβR kinase and insulin receptor kinase represent two of many autophosphorylating serine/threonine and tyrosine kinases, respectively. The ATP-binding domain of these other kinases is structurally distinct from the Bergerat fold. To determine whether radicicol inhibits these kinases, they were incubated with either 500 μM radicicol or an equal reaction volume containing vehicle control. The autoradiograph shown in Fig. 6A illustrates that at radicicol concentrations sufficient to almost completely inhibit BCKDHK and Sln1 kinase activity, the serine/threonine and tyrosine kinases seem unaffected. To ensure even protein loading, the proteins were also visualized by Coomassie staining (Fig. 6B). Although by no means properly validated for every serine/threonine and tyrosine protein kinases, these data suggest that radicicol may be specific for only those proteins that contain a Bergerat fold.

Inhibition of other bacterial histidine kinases has previously been demonstrated with different inhibitors at concentrations similar to those displayed by radicicol (Stephenson et al., 2000). However, the mechanism of inhibition of these other compounds remains unclear (Deschenes et al., 1999; Stephenson et al., 2000). It has been suggested that these other histidine kinase inhibitors are not ATP mimetics and elicit their effects via structural perturbation of the histidine kinase. Radicicol however, is known to mimic Mg^{2+}-ATP/ADP in the inhibition of HSP90 ATPase activity, through direct competition for the Bergerat fold (Roe et al., 1999). Because of the structural relationship between HSP90, BCKDHK, and Sln1, radicicol most probably inhibits these kinases via the same mechanism.

Competition of radicicol for the ATP-binding domain of BCKDHK was tested in two ways: 1) by direct competition for radicicol binding with ATP, and 2) indirectly by radicicol competition of BCKDHK bound to ATP-Sepharose. The direct competition experiment in Fig. 7A involved phosphorylating BCKDHK that had been previously treated with 55 μM radicicol with increasing concentrations of ATP. The amount of radioactive ATP tracer was maintained at the same level for each sample (10 μCi). This results in a reduced signal, as expected with increasing ATP concentrations in the DMSO control group. Phosphorylation of the control samples was still clearly visible up to a concentration of 10 mM ATP. Having established competition of the control samples with ATP, a similar competition experiment was performed on identical samples pretreated with radicicol. By comparison, the same sample treated with 10 mM ATP in the control clearly shows no phosphorylation when pretreated with 55 μM radicicol. This was the case for all the samples treated with 55 μM radicicol and illustrates that ATP at concentrations several orders of magnitude greater than the 55 μM radicicol were still insufficient to compete off radicicol that was bound to BCKDHK. Although these data suggest that radicicol binds directly to the ATP-binding domain of BCKDHK, it is by no means unequivocal. A more comprehensive

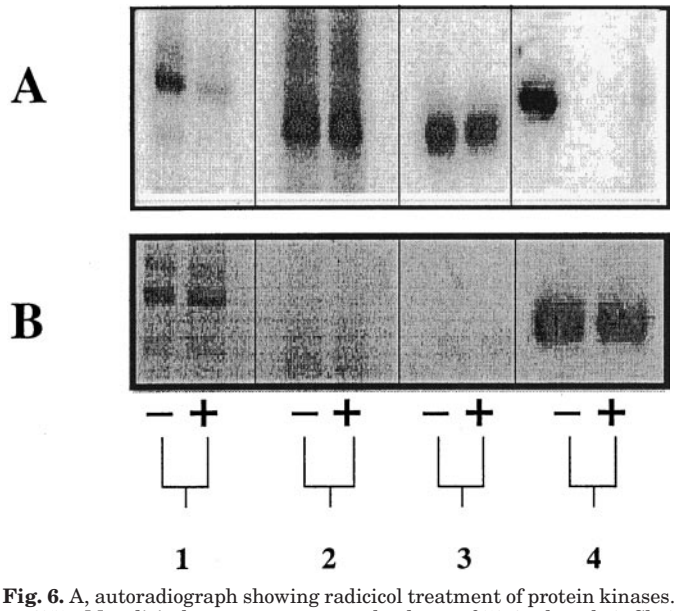

Fig. 6. A, autoradiograph showing radicicol treatment of protein kinases. +, 275 μM radicicol treatment; −, equal volume of 50% ethanol. 1, Sln1 histidine kinase; 2, TGFβR serine/threonine kinase; 3, insulin receptor tyrosine kinase; and 4, BCKDHK. B, Coomassie-stained gel of A to indicate even protein loading for each protein kinase pair.

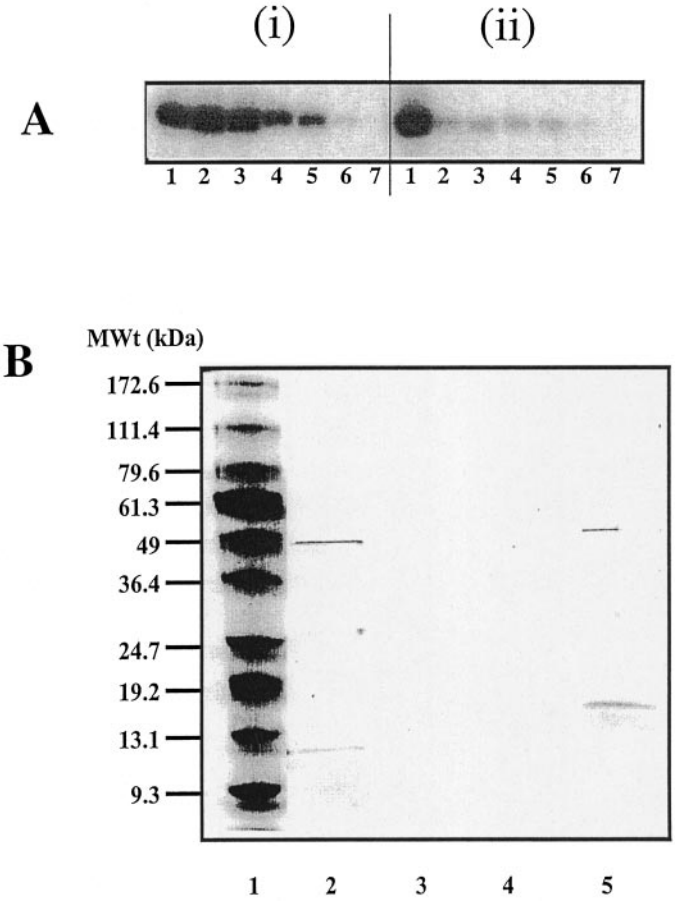

Fig. 7. A, autoradiograph of ATP competition for DMSO control (i) and BCKDHK (ii) pretreated with 55 μM radicicol. Samples were treated with the following concentrations of ATP: 1, control; 2, 0 mM; 3, 0.1 mM; 4, 1 mM; 5, 10 mM; 6, 100 mM; and 7, 1000 mM. B, Coomassie-stained SDS gel showing the elution of BCKDHK from ATP-Sepharose. Lane 1, molecular mass standards; lane 2, purified 6xHis-BCKDHK bound to ATP-Sepharose; lanes 3 and 4, 500-μl kinase buffer washes; and lane 5, 6xHisBCKDHK eluted from the ATP-Sepharose by 500 μM radicicol.

study on the kinetics of BCKDHK inhibition by radicicol is currently on-going.

The indirect method of establishing the competition of radicicol for the ATP-binding site of BCKDHK is shown in Fig. 7B. ATP-Sepharose-bound BCKDHK was washed with buffer and subsequently eluted from the affinity resin using 500 μM radicicol. This ability to elute BCKDHK from ATP-Sepharose, together with similar evidence from structure-function experiments of HSP90 (Sharma et al., 1998; Roe et al., 1999; Shulte et al., 1999), again suggests that the mode of radicicol inhibition for BCKDHK is via direct competition for the ATP-binding domain.

Discussion

The Bergerat fold is limited to a unique subset of proteins that happen to possess vastly different functions. Each protein uses ATP as the energetic currency to drive its respective enzymatic reactions, but this family of proteins is functionally very diverse. The inhibition of HSP90 ATPase activity by the antifungal radicicol, is achieved through direct competition with the Bergerat fold of this protein. Based on their similarity to the Bergerat fold of HSP90, we demonstrate that radicicol can also inhibit BCKDHK and Sln1 autophosphorylation activity. Like HSP90, preliminary indications for BCKDHK suggest that inhibition by radicicol is through competition for the ATP-binding domain. Machius et al. (2001) and others (Bowker-Kinley and Popov, 1999; Wynn et al., 2000) have also noted that the ATP-binding domain of BCKDHK is similar to the Bergerat fold family of proteins to which TCHKs belong. On the basis of this structure-function homology relationship, further supporting evidence for the idea that BCKDHK is not only a Bergerat fold protein but also a putative TCHK, was demonstrated by Thelen et al. (2000). This group reported that the closely related PDHK enzyme from *Arabidopsis thaliana* shows bacterial TCHK-like activity based on mutagenesis and modification of canonical histidine residues thought to be involved in phosphotransfer (Mooney et al., 2000). Steussy et al. (2001) have also reported a TCHK/ATPase nucleotide-binding domain in PDHK, but unlike Thelen's group, they claim PDHK has only serine kinase activity.

Another HSP90 ATPase inhibitor, geldanamycin, from the benzoquinone ansamycin class of antitumor fungal antibiotics, also has its inhibitory function through direct competition with ATP for the Bergerat fold (Roe et al., 1999). Geldanamycin presumably would also inhibit BCKDHK via the same mechanism as radicicol. In this study, we chose radicicol as an inhibitor whose mode of inhibition had previously been defined for a protein with a distinctly unrelated function, yet happens to belong to a family that possesses a common structural motif. Because BCKDHK is also known to autophosphorylate on a serine residue as part of its regulatory function in this protein complex (Davie et al., 1995), we chose other kinases to test with radicicol that are similar to BCKDHK and Sln1 in respect to the fact that they also autophosphorylate. Although by no means universally true for all serine, threonine, and tyrosine kinases, radicicol does not seem to inhibit those kinases that we tested. This evidence lends further support to the hypothesis that BCKDHK, like PDHK, might be a histidine kinase.

Radicicol inhibits BCKDHK and Sln1 kinase activity in a concentration-dependent manner, having a calculated IC_{50} of 635 nM and 127 μM, respectively. Testing the effects of radicicol in vivo would prove difficult because the HSP90 family and potentially other members of the Bergerat fold family will also be inhibited. However, the apparent specificity of radicicol for Bergerat fold proteins may prove useful for the purposes of protein purification. An affinity matrix comprising immobilized radicicol may be a useful step in purifying previously unrecognized histidine kinases on the basis of structure homology to the Bergerat protein family. A similar protein purification approach using a derivative of radicicol has been used successfully to isolate HSP90 and ATP citrate lyase (Ki et al., 2000), which interestingly has a phosphohistidine intermediate as part of its enzymatic function (Williams et al., 1985).

It will be interesting to see whether TCHKs appear in mammalian cells in the form of Bergerat proteins. Homology searches for mammalian TCHKs have traditionally been done by comparing the primary sequences of common bacterial TCHKs to mammalian sequence databases. Given the structural homology of TCHKs to the Bergerat family of proteins, the use of immobilized radicicol or radicicol derivatives maybe one option in the quest for identifying mammalian TCHKs that is worth pursuing. The work by Dutta, Inouye, and others has been important in identifying protein structure-function relationships. The use of radicicol as inhibitor of BCKDHK and Sln1 arose from comparisons between the structurally homologous ATP-binding domains of BCKDHK, Sln1 and the functionally distinct protein, HSP90. Other proteins that are identified or suspected of containing a Bergerat fold may also have their enzymatic function inhibited by radicicol.

We now have enough evidence to suggest that radicicol is a useful inhibitor of BCKDHK. Our future goals will be to establish a stronger kinetic evaluation of radicicol as an inhibitor of BCKDHK and also test its efficacy on other known TCHKs. We are currently re-examining the biochemistry of BCKDHK as a putative histidine kinase through mutation of suspected histidine residues and phosphopeptide mapping. Given our preliminary observations, we are also in the process of testing mammalian cell extracts with a radicicol affinity matrix. It is hoped that by applying a proteomics approach together with modeling programs, we can identify not only those proteins in mammalian cells that possess Bergerat folds but also those that may possess histidine kinase activity. If radicicol proves useful as an inhibitor of TCHKs in pathogenic bacteria, then for the purposes of an antimicrobial agent, radicicol will be a good starting point for a structure-activity relationship program for pharmaceutical applications. However, it is important that effects of radicicol and similar compounds do not compromise the function of potential mammalian histidine kinases or other members of their structurally related family.

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