KINASE ACTIVATING DEPENDENT CYCLIN PROTEIN KINASES, AND THEIR USES

The present invention relates to a new cycline-dependent protein kinase-activating kinase from *Candida albicans* and to its uses.

Cycline-dependent protein kinases (Cdk) are cell division cycle regulators in eukaryotes which are essential both at the level of the G1/S transition and the G2/M transition of the cell cycle. The CDC28 of *Saccharomyces cerevisae* and the CDC2 of *Schizosaccharomyces pombe* are the first Cdks which have been identified.

The activation of the Cdks requires both the attachment of a molecule of cycline, and the phosphorylation of the Cdk on a conserved threonine residue situated in a region called: "T loop".

It has been shown that this phosphorylation is carried out by a kinase called: "Cdk-activating kinase" (CAK), which, in vertebrates, exists in the form of a heterotrimer comprising a catalytic subunit called Cdk7, a subunit of the cycline type, called cycline H, and a factor MAT-1 [for a review, cf. SOLOMON, Trends Biochem. Sci. 19, 496-500 (1994)]. The Cdk7-cycline H complex is in addition a component of the TFIIH complex, which is necessary for the basal transcription of genes by RNA polymerase II, and is involved in the phosphorylation of the repeat sequences of the carboxy-terminal domain (CTD) of the large subunit of this polymerase.

In the fission yeast *Schizosaccharomyces pombe*, a complex similar to Cdk7-cycline H, comprising a catalytic subunit called Crk1, and a regulatory cycline called Mcs2, has been identified. It has been shown that the *Crk1* gene was essential for cell viability, and it has been observed *in vitro* that the complex Crk1-Mcs2 was associated with the CAK activity and with the CTD-kinase activity [BUCK et al., EMBO J., 14(24),

6173-83 (1995); DAMAGNEZ et al., EMBO J., 14(24), 6164-72, (1995)].

In the budding yeast *Saccharomyces cerevisiae*, a complex comprising a kinase (Kin28) and a cycline (Ccl1) respectively related, at the level of their sequence, to the kinases Cdk7 and Crk1, and to the regulatory proteins cycline H and Mcs2, has also been identified. The complex Kin28-Ccl1 forms part of the complex TFIIH and has a CTD-kinase activity, but is not involved in the CAK activity.

Recently, the inventors have identified a kinase responsible for the CAK activity in *Saccharomyces cerevisiae*. This kinase was called CIV1 (CAK *in vivo*), and the corresponding gene was called *CIV1* (THURET et al., Cell, 86(4), 1996). These results have been confirmed by other teams [KALDIS et al., Cell, 86(4), 553-564 (1996); ESPINOZA et al., Science, 273(5282), 1714-1717 (1996)]. The *Saccharomyces cerevisiae* CAK is overall related to the serine-threonine-kinase family, and in particular to the protein kinases CDC2 and CDC28, and differs from the CAKs previously identified in other organisms by the absence of the glycine-rich conserved motif GxGx(Y/F)GxV, which is present in most protein kinases, the presence of inserts of 5 to 29 amino acids, which are situated between the components of secondary structure which are conserved in the Cdk family, and by the fact that its CAK activity does not require its incorporation into an enzymatic complex.

The CAK activity of CIV1 being essential for cellular division and survival, the inventors have undertaken to investigate if there are, in pathogenic yeasts, genes homologous to *CIV1*, encoding protein kinases possessing a CAK activity. Indeed, in this case, the obtaining of means of regulating this activity, and in particular of inhibitors, would be of great interest from an industrial or therapeutic point of view, mainly for the production of fungicides.

With this aim in view, the inventors first undertook the screening of DNA libraries from the pathogenic yeast *Candida albicans* using probes derived from various regions of the *Saccharomyces cerevisiae* *CIV1* gene. However, none of the probes used have made it possible to detect the presence of homologous sequences in the *Candida albicans* genome.

The inventors have, however, investigated whether *Candida albicans* possibly possesses a functional analogue of the *Saccharomyces cerevisiae* CAK by examining if there are in *Candida albicans* one or more genes capable of restoring in *Saccharomyces cerevisiae* the CAK function in a heat-sensitive mutant of the *CIV1* gene. They thus succeeded in identifying a *Candida albicans* gene capable of complementing on its own the deficient CAK function in the mutant.

The sequence of this gene, called *CaCIV1*, has been determined; it is represented in the sequence listing in the annex under the number SEQ ID NO:1; the sequence of its translational product, called CaCIV1, is represented under the number SEQ ID NO:2.

Figure 1 represents the comparison of the amino acid sequence (1-letter code) of CaCIV1 with that of the *Saccharomyces cerevisiae* CAK (called ScCIV1), and with that of the *Saccharomyces cerevisiae* kinase CDC28 (called ScCDC28). The residues conserved in ScCIV1 and CaCIV1 are in bold characters.

Legend to the annotations of Figure 1:

k = residue conserved in most protein kinases;

• = residue often present in the Cdk family;

o = residue always present in the Cdk family;

+ = residue present in the Cdk family and in ScCIV1;

secondary structures: a = α helix; b = β sheet.

CaCIV1 only exhibits at the level of the overall amino acid sequence a 28% identity with the CAK of *Saccharomyces cerevisiae*, ScCIV1.

However, the similarities observed between ScCIV1 and CaCIV1 make it possible to define a kinase

family, called hereinafter CIV1, grouping together proteins having the following characteristics:

- they lack the motif GxGx(Y/F)GxV, in which G represents glycine, x represents any amino acid, Y/F represents either tyrosine or phenylalanine, V represents valine;

- they possess a non-cycline-dependent CAK activity.

The present invention encompasses the protein kinases belonging to the CIV1 family as defined above, with the exception of the *Saccharomyces cerevisiae* CAK ScCIV1.

According to a preferred embodiment of the present invention, the said protein kinase is capable of being obtained from an ascomycete, advantageously a hemiascomycete, and preferably *Candida albicans*.

A protein kinase in accordance with the invention is for example represented in the sequence listing in the annex under the number SEQ ID NO:2.

The subject of the present invention is also a nucleic acid sequence encoding a protein kinase in accordance with the invention.

A nucleic acid sequence in accordance with the invention consists for example of the sequence SEQ ID NO:1 of the sequence listing in the annex.

The subject of the present invention is also nucleic acid fragments of at least 18 bp, homologous or complementary to a nucleic acid sequence encoding a peptide sequence specific to the CAK in accordance with the invention.

These fragments may in particular be used as hybridization probes, and/or amplification primers, to isolate and/or to clone using *Candida albicans*, a nucleic acid sequence encoding a CAK in accordance with the invention.

The present invention also encompasses nucleic acid fragments of at least 15 bp, preferably of at least 18 bp, homologous or complementary to a nucleic acid sequence encoding a peptide sequence conserved in

the protein kinase family defined by the CAK CaCIV1 in accordance with the invention, and the *Saccharomyces cerevisiae* CAK ScCIV1.

These fragments may in particular be used as hybridization probes, and/or amplification primers, to detect the existence, in organisms other than *Saccharomyces cerevisiae* and *Candida albicans*, of sequences encoding kinases related to CAK CaCIV1 and ScCIV1 and to isolate and/or to clone the genes thus identified. The invention also encompasses the nucleic acid sequences obtained in this manner, and the protein kinases of the CAK CaCIV1 and ScCIV1 family which are encoded by these sequences.

The subject of the present invention is also any recombinant vector, and in particular any expression vector, resulting from the insertion of at least one nucleic acid sequence in accordance with the invention into an appropriate vector. The choice of an appropriate vector can be easily made by persons skilled in the art, among numerous available vectors, depending on the host cell chosen to multiply and/or to express a nucleic acid in accordance with the invention.

The invention also encompasses prokaryotic or eukaryotic cells transformed with a nucleic acid sequence in accordance with the invention. These transformed cells can be used in particular to express a kinase in accordance with the invention, for example so as to purify it from cell cultures, for example using techniques similar to those previously described by the inventors for *Saccharomyces cerevisiae* CIV1 (THURET et al., 1996, publication cited above), or alternatively so as to detect its activity by an appropriate test of cell viability.

The demonstration, by the inventors, of the functional homology of the kinases ScCIV1 and CaCIV1 makes it possible to envisage numerous applications for this kinase family.

In particular, because it appears that the non-cycline-dependent CAK activity of this kinase family is essential for cell division and survival, substances inhibiting this activity may be used as fungicides, either as medicaments or on an industrial level.

For example, to screen fungicidal substances such as substances which are active on *Candida albicans*, the kinase activity of CaCIV1, or of one of its functional homologues consisting of a non-cycline-dependent CAK of the CIV1 family, is measured in the presence of each of the products for which it is desired to determine the fungicidal properties, and the products having an inhibitory effect on this activity are selected.

Such a screening can be carried out by measuring the kinase activity of a CAK of the CIV1 family, in the presence of potential activators or inhibitors to be tested. The kinase activity may, for example, be measured *in vitro*, either directly by detecting the phosphorylation of a peptide or of a substrate protein, for example CDC28 or Cdk2, or the protein MBP (myelin basic protein), in an appropriate reaction mixture, or indirectly, by detecting and/or measuring the activity of the substrate protein when the latter depends on phosphorylation.

The kinase activity can also be measured *in vivo*, by a test of cell viability; for example, the kinase activity of CaCIV1 can be advantageously measured in cells of a mutant of *Saccharomyces cerevisiae* not expressing CAK ScCIV1, which are transformed with the *CaCIV1* gene.

The invention also encompasses the use of a product selected as indicated above for its inhibitory properties of a non-cycline-dependent CAK of the CIV1 family for the production of a fungicide.

The present invention will be understood more clearly with the aid of the additional description which follows, which refers to an example illustrating

the detection of the CAK activity of CaCIV1, and the cloning of the corresponding gene.

Example:

The *Saccharomyces cerevisiae* strain, CMY975 (genotype *CIV1-2 ura3 leu2 trp1 lys2 ade2 ade3*) carries a heat-sensitive mutation of the *CIV1* gene and, because of this, grows at 24°C, but not at 37°C.

A culture of this strain was transformed by the lithium acetate method [SCHIESTL and GIETZ, Current Genetics, *16*, 339-346, (1989)] with a library of *Candida albicans* Sau3A genomic fragments cloned into the BamHI site of the vector YEp24 (multicopy-URA3) [Botstein et al., Gene *8*, 17-24, (1979)].

The CMY975 cells are plated on dishes containing a uracil-free synthetic medium, and cultured at 37°C.

Ten colonies of CMY975 growing under these conditions were obtained. These plasmids YEp24 containing *Candida albicans* inserts were recovered from each of these colonies, and amplified in *Escherichia coli*.

The restriction map of each of these plasmids was established, and made it possible to observe that all the inserts came from the same region of the *Candida albicans* genome. Sequencing of this region made it possible to identify the same open reading frame encoding a protein kinase of 339 amino acids, which shows that the 10 inserts separately obtained correspond to one and the same *Candida albicans* gene. This gene was called *CaCIV1*.

The sequence of *CaCIV1* is represented in the sequence listing in the annex under the number SEQ ID NO:1, and that of the protein CaCIV1 is represented under the number SEQ ID NO:2. Comparison of the sequence of the protein CaCIV1 with the sequences available in data bases reveals that this protein has only 28% of amino acids identical with the CAK of *Saccharomyces cerevisiae* ScCIV1, and 24% of amino acids

identical with the Cdk ScCDC28 of *Saccharomyces cerevisiae* and the CaCDC28 of *Candida albicans*.

A BamHI-ClaI genomic fragment of 3 kb containing the *CaCIV1* gene was subcloned into the centromeric plasmid TRP1 pRS414 [SIKORSKI and HIETER, Genetics, 122, 19-27 (1989)]. This plasmid was used to transform a *Saccharomyces cerevisiae* mutant in which the *ScCIV1* sequence is deleted from the genome and containing the *ScCIV1* gene on a replicative plasmid also carrying the selectable gene *URA3* (strain CMY116 of the genome *ura3 leu2 trp1 lys2 civ1 LEU2/pJG43 (URA3-ScCIV1)*). The cells transformed with the plasmid pRS414-*CaCIV1* are viable after counterselection and the loss of the plasmid pJG43 (*URA3-ScCIV1*), which confirms that the *CaCIV1* gene can function in place of the essential *ScCIV1* gene. Therefore, *CaCIV1* encodes a functional homologue of *ScCIV1*, and is sufficient to restore the CAK activity.



CLAIMS

1) Protein kinase belonging to the family called CIV1 defined by the following characteristics:

- it lacks the motif GxGx(Y/F)GxV, in which G represents glycine, x represents any amino acid, Y/F represents either tyrosine or phenylalanine, V represents valine;

- it possesses a non-cycline-dependent CAK activity;

with the exception of the *Saccharomyces cerevisiae* CAK ScCIV1.

2) Protein kinase according to Claim 1, characterized in that it is capable of being obtained from *Candida albicans*.

3) Protein kinase according to either of Claims 1 and 2, characterized in that it corresponds to the sequence represented in the sequence listing in the annex under the number SEQ ID NO:2.

4) Nucleic acid encoding a protein kinase according to any one of Claims 1 to 3.

5) Recombinant vector resulting from the insertion of a nucleic acid according to Claim 4 into an appropriate vector.

6) Method of screening fungicidal products, characterized in that it comprises a step in which the kinase activity of a non-cycline-dependent CAK of the CIV1 family defined in Claim 1 is measured in the presence of each of the products for which it is desired to determine the fungicidal properties, and the products having an inhibitory effect on this activity are selected.

7) Use of a product selected by the process according to Claim 6 for the production of a fungicide.