Ras recruits mitotic exit regulator Lte1 to the bud cortex in budding yeast

Satoshi Yoshida, Ryuji Ichihashi, and Akio Toh-e

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan

A Cdc25 family protein Lte1 (low temperature essential) is essential for mitotic exit at a lowered temperature and has been presumed to be a guanine nucleotide exchange factor (GEF) for a small GTPase Tem1, which is a key regulator of mitotic exit. We found that Lte1 physically associates with Ras2-GTP both in vivo and in vitro and that the Cdc25 homology domain (CHD) of Lte1 is essential for the interaction with Ras2. Furthermore, we found that the proper localization of Lte1 to the bud cortex is dependent on active Ras and that the overexpression of a derivative of Lte1 without the CHD suppresses defects in mitotic exit of a Δlte1 mutant and a Δras1 Δras2 mutant. These results suggest that Lte1 is a downstream effector protein of Ras in mitotic exit and that the Ras GEF domain of Lte1 is not essential for mitotic exit but required for its localization.

Introduction

Budding yeast Ras1 and Ras2 are homologues of mammalian Ha-ras oncogene and have redundant function (Kataoka et al., 1985). The only well-understood yeast Ras function is to produce cAMP by activation of adenylate cyclase Cyr1 (Shima et al., 2000). cAMP binds to the protein kinase A (PKA) regulatory subunit Bcy1 and releases catalytic subunits (Tpk1, Tpk2, and Tpk3), thereby activating PKA (Broach, 1991). Activated PKA carries out many functions, including the regulation of G1–S transition. The Ras–cAMP pathway is also a negative regulator of the stress response pathway (Marchler et al., 1993), and cells with the dominant active Ras2 mutation ras2 cyr1, the Ras GAP mutation ira1-1, or the deletion of the Bcy1 gene show a heat shock–sensitive phenotype (Broach, 1991). The Ras–cAMP pathway is the only essential pathway of Ras because the overexpression of the TPK1 gene or the deletion of the Bcy1 gene rescues the lethality of the Δras1 Δras2 double null mutant, but these cells are defective in mitotic exit at a higher or lower temperature, suggesting that Ras proteins have another target functioning in mitotic exit (Morishita et al., 1995). To date, how Ras is involved in mitotic exit remains entirely unknown.

Lte1 (low temperature essential) is a protein with homology to Ras guanine nucleotide exchange factor (GEF) protein Cdc25 and is required for mitotic exit at a lowered temperature (Shirayama et al., 1994a). Lte1 was identified as a multicopy suppressor of the heat shock sensitivity of the ira1-1 and RAS2Val19 mutants (Shirayama et al., 1994a). As Lte1 contains the Cdc25 homology domain (CHD), Lte1 was supposed to interfere with the Ras–cAMP pathway by direct interaction with Ras, not downstream of PKA. On the other hand, LTE1 was also identified as a multicopy suppressor of a late mitotic defect of the Δras1 Δras2 cyr1 mutant (Morishita et al., 1995). How the overexpression of LTE1 suppressed both dominant and deletion mutants of yeast RAS is an important question to understand the role of Lte1.

A possible role of Lte1 was suggested by the identification of Tem1 GTPase, which is essential for mitotic exit, as a GEF for Tem1 protein, Lte1 would be a GEF for Tem1, and assuming this, an attractive model, in that the interaction of Lte1 at the bud cortex with Tem1 GTPase at the spindle pole determines the timing of mitotic exit, has been proposed (Bardin et al., 2001). However, it was reported that Lte1 is not involved in the timing of mitotic exit at 30°C or above (Adames et al., 2001), and there is no positive biochemical evidence indicating that the Ras GEF domain of Lte1 possesses GEF activity toward Tem1 (Geymonat et al., 2002). Recent studies have revealed that the Lte1 localization at the bud cortex is dependent on the Cdc42 GTPase and its effector Cla4 kinase, the phosphorylation state of Lte1, cell...
polypeptide and septin, but is independent of the microtubule or actin cytoskeleton (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002). Hofken and Schiebel (2002) reported that Cdc42 and Cla4 are essential for priming Lte1 at the bud cortex; however, it remains unknown what anchors Lte1 there. We report here that active Ras2-GTP anchors Lte1 at the bud cortex via the direct interaction through the CHD of Lte1. We also found that the Ras GEF domain of Lte1 is essential for localization but not essential for mitotic exit, indicating that the CHD of Lte1 does not act as a GEF of Tem1.

**Results**

Lte1 associates with Ras protein in a GTP-dependent manner

As Lte1 contains a Ras GEF at its COOH terminus, we presumed that there is a GTPase interacting with it. To seek for a binding partner of the Lte1 CHD, we tested whether a two-hybrid interaction could be observed between the Lte1 CHD and yeast small GTPases Tem1, Ras2, or Bud1, because these GTPases are known to have a genetic interaction with Lte1 (Shirayama et al., 1994b; Morishita et al., 1995). We took advantage of the bacterial two-hybrid system to neglect possible indirect interactions that are often observed in the yeast two-hybrid system and found that the Lte1 CHD interacted with Ras2, but not with either Tem1 or Bud1, in this system (Fig. 1 a). The physical interaction of Lte1–5HA with Ras2 was confirmed by a coimmunoprecipitation experiment in yeast cells when both proteins are expressed at a native level (Fig. 1 b). The membrane localization of Ras2 is essential for the interaction with Lte1 because the ras2Ser318 mutation, in which the cysteine residue essential for the farnesylation of Ras2 is mutated to serine, abolished the Lte1 interaction (Fig. 1 b, lane 2), suggesting that Ras2 and Lte1 encounter each other at the membrane.

To clarify the role of guanine nucleotides in the interaction between Ras2 and Lte1, we examined the association of purified Lte1 (Fig. 1 d) with Ras2 in vitro. Ras2 protein purified from the insect cells specifically interacted with purified Lte1 in the presence of GTPγS, a nonhydrolyzable analogue of GTP, but the interaction was hardly detectable in the presence of GDP or in the absence of nucleotide (Fig. 1 e). We also found that the Ras2Val19 protein, which is defective in GTP hydrolysis, thereby forming a stable complex with GTP, interacted more strongly with the CHD of Lte1 than wild-type Ras2 in the two-hybrid system (Fig. 1 a), indicating that GTP-bound Ras2 has a higher affinity for Lte1. However, the amount of Ras2Val19 protein immunoprecipitated with Lte1–5HA was almost the same as that of Ras2 protein pulled down with Lte1–5HA in the yeast cells when these proteins were expressed at an endogenous level (Fig. 1 b, lanes 3’ and 4’). This consequence can be explained by the fact that the amount of Lte1 is extremely low compared with that of Ras2 and would be rate limiting because the amount of Ras2Val19 protein precipitated with Lte1–2HA was greater than that of wild-type Ras2 when Lte1–2HA was overexpressed (Fig. 1 c). Also, Lte1–2HA protein was more effectively precipitated with GFP–Ras2Val19 than with GFP–Ras2 when Lte1 was overexpressed (Fig. 1 c). These observations suggest that the interaction of Ras2Val19 with Lte1–2HA is stronger than that of Ras2 with Lte1–2HA in the yeast cells. We also found by coimmunoprecipitation that Ras2Asn22, which is frozen in the GDP form but is localized to the membrane (see Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200301128/DC1), did not associate with Lte1–2HA even when overexpressed (Fig. 1 c, lane 3’). This observation further supports the importance of GTP binding to Ras2 in the Lte1–Ras2 interaction. Above all, we conclude that Lte1 binds to Ras2 both in vivo and in vitro in a GTP-dependent manner.

**RAST functions upstream of LTE1 in mitotic exit**

The physical interaction between Ras2 and Lte1 raised the possibility that Ras regulates mitotic exit in concert with Lte1. One of the reasons why Δras1 Δras2 Δbcy1 cells, in which BCY1 is deleted to keep a double mutant alive by bypassing its defect in the Ras–CAMP pathway, are defective in mitotic exit is the failure of Cdc14 liberation, a critical step of mitotic exit, because Cdc14–5GFP release from the nucleolus is partially defective in Δras1 Δras2 Δbcy1 cells compared with that in Δbcy1 cells cultured at 10°C (Fig. 2 a). It has been reported that Cdc14 liberation is governed by at least two steps; one is by the Cdc14 early anaphase release (FEAR) and the other is by the mitotic exit network (MEN) (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). Lte1 is known to act at the top of the MEN and shows synthetic lethality in combination with a null mutation in a FEAR component spo12 (Stegmeier et al., 2002). We found by tetrad dissection of SAY510 (ras1/ras1, ras2/ras2, bcy1/bcy1, SPO12/spo12) cells that Δras1 Δras2 Δbcy1 Δspo12 segregants were inviable whereas Δras1 Δras2 Δbcy1 Δlte1 segregants were viable (Fig. 2 c). We also found that the low temperature sensitivity of Δras1 Δras2 Δbcy1 as well as that of Δlte1 was partially suppressed by the deletion of the BUB2 gene (Fig. S1 b). These genetic interactions indicate that Ras and Lte1 function in the same pathway.

Although Lte1 contains the CHD, Lte1 does not seem to be a GEF for Ras2 because Lte1 binds to GTP-bound, but not GDP-bound, Ras2 (Fig. 1), in contrast to the Cdc25 protein (Lai et al., 1993). Several lines of genetic evidence place LTE1 downstream of RAS in mitotic exit because the low temperature sensitivity of Δlte1 was not suppressed by overexpression of RAS2 or dominantly active RAS2Val19 (unpublished data), whereas overexpression of LTE1 suppressed the temperature sensitivity of the Δras1 Δras2 mutant (Morishita et al., 1995; Fig. 2 b). Therefore, yeast Ras proteins function upstream of LTE1, and Lte1 would fail to exert its function in the absence of Ras proteins.

**Ras is essential for Lte1 localization at the bud cortex**

To investigate the role of Ras in the regulation of Lte1, we examined the bud cortex localization of Lte1 in the Δras1 Δras2
mutants because Ras proteins exist at the plasma membrane and Lte1 and Ras proteins interact with each other. In the \( \Delta \text{ras1}, \Delta \text{ras2}, \text{or} \Delta \text{bcy1} \) cells, Lte1–2HA protein localized properly to the bud cortex as in wild-type cells (Fig. 3 a), but in \( \Delta \text{ras1} \Delta \text{ras2} \Delta \text{bcy1} \) cells, although \( \text{LTE1}–2\text{HA} \) was expressed at a same level, Lte1–2HA localization at the bud cortex was abolished and Lte1–2HA was diffused to the cytoplasm (Fig. 3 a) (unpublished data). This observation indicates that Ras proteins regulate Lte1 localization to the bud cortex, and Ras1 and Ras2 share a redundant role in Lte1 localization.

To determine the role of Ras in more detail, we examined the effect of various \( \text{RAS} \) mutations on Lte1 localization. We took advantage of using SAY627 cells (\( \text{ras1} \text{ras2} \text{bcy1} \text{pLTE1-902ET} \)) with the indicated \( \text{RAS} \) plasmid, where plasmid-born Ras is the only Ras protein in the cell (Fig. 3 b). In the SAY627 cells, Lte1–2HA was diffused to the cytoplasm when transformed with an empty vector, but the Lte1 localization defect was fully cured when \( \text{RAS1}, \text{RAS2}, \text{or RAS2Val19 plasmid was introduced} \) (Fig. 3 b). We also found that Lte1 localization was not recovered by the introduction of a \( \text{ras2Ser318} \) or \( \text{ras2Asn22} \) plasmid (Fig. 3 b). These observations indicate that both the GTP-binding ability and the membrane localization of Ras are essential for Lte1 localization at the bud cortex.
Ras and Cdc42–Cla4 pathways have a separate role in Lte1 localization

The recent finding that Cdc42 and its effector Cla4 kinase are essential for the hyperphosphorylation and for the bud cortex localization of Lte1 (Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002) raises a possibility that Ras regulates Lte1 localization indirectly via activation of the Cdc42–Cla4 pathway because Ras2 has recently been reported to regulate actin polarity and Cdc42 localization at a higher temperature (Ho and Bretscher, 2001).

To examine the phosphorylation level of Ras-bound Lte1, Lte1–2HA was pulled down with GFP–Ras (Fig. 4 a). By analyzing cell extracts, we found in ras1 ras2 bcy1 cells that the hyperphosphorylated form of Lte1–2HA was not so obvious as in the cla4 cells (Fig. 4 b), suggesting that the Cla4-dependent hyperphosphorylation of Lte1 is partially impaired in the absence of Ras. There are two possibilities that can explain the reason why Lte1 hyperphosphorylation is impaired in the absence of Ras: (1) the Cdc42–Cla4 pathway (or Cla4 kinase activity) is not active in the ras mutant, and (2) Cla4 cannot interact with Lte1 simply because Lte1 is diffused in the cytoplasm in the absence of Ras. To examine whether Lte1 mislocalization in the absence of Ras is due to a failure in Cdc42–Cla4 activation, we introduced an extra copy of CDC42 or dominantly active CDC42Val12 under an inducible promoter in the SAY627 (ras1 ras2 bcy1 pLTE1–2HA) cells and found that the induction of neither CDC42 nor CDC42Val12 recovered the Lte1 localization at the bud cortex at all (Fig. 4 c). This observation indicates that the Lte1 mislocalization in the ras mutant was not due to the lack of Cdc42 activation. We also found that Cla4–GFP was localized at the bud cortex in the /H9004 ras1 /H9004 ras2 /H9004 bcy1 cells as well as in /H9004 bcy1 cells (Fig. 4 d). Judging from the normal morphology of /H9004 ras cells and normal localization of Cdc12 septin shown in Fig. 4 d, Cla4 seems functional in the absence of Ras. Thus, we think that impaired hyperphosphorylation of Lte1 is not due to the misregulation of the Cdc42–Cla4 pathway but due to the absence of Lte1 around Cla4 residing at the bud cortex. We also found that the hyperphosphorylation of Lte1 is not required for the Lte1–Ras2 interaction because the Lte1 protein immunoprecipitated with GFP–Ras2 was not hyperphosphorylated (Fig. 4, a and b). Assuming that Lte1 and Ras2 interact at the plasma membrane, the hyperphosphorylation of Lte1 is not required for its localization.

Besides Cdc42–Cla4, it is reported that a bud cortex protein Kel1 and a septin ring component Cdc12 are involved in Lte1 localization. We found that Kel1 was localized at the bud cortex and bud neck in the ras1 ras2 bcy1 cells as in wild-type cells (Fig. 4 d). Also, the septin ring appeared normal in the ras1 ras2 bcy1 cells, as judged by the localization of Cdc12–GFP (Fig. 4 d). Above all, we conclude that the hyperphosphorylation of Lte1 is not required for its localization.
mislocalization of Lte1 in the ras1 ras2 bcy1 mutant is not due to the failure of Cdc42–Cla4 activation or mislocalization of Cla4, Kel1, or septin.

**Ras GEF homology domain of Lte1 is essential for the interaction of Lte1 with Ras2**

Lte1 contains the Ras GEFN (GEF at the NH2 terminus) domain at its NH2 terminus and the Ras GEF CHD at its COOH terminus, but the role of each domain is not yet clear. We constructed various truncated mutants of LTE1 to determine the domains of Lte1 that are essential for the interaction of Lte1 with Ras2 in vivo (Fig. 5 a). We found that deletion of 103 amino acids from the COOH terminus or of 500 amino acids from the NH2 terminus of Lte1 abolished the Lte1–Ras2 interaction, as judged by immunoprecipitation, even when these truncated versions of Lte1 were overproduced (Fig. 5 b). In contrast to the bacterial two-hybrid system (Fig. 1 a), the CHD alone is not sufficient for the interaction with Ras2 in vivo (Fig. 5 b). These results indicate that both NH2- and COOH-terminal GEF homology domains of Lte1 are essential for in vivo association of Lte1 with Ras2.

We also found that the overexpression of any one of the truncated mutant forms of Lte1 that does not interact with Ras2 failed to localize at the bud cortex (Fig. 5 c). All of the GFP–truncated Lte1 proteins tested showed similar cytoplasmic localization to each other. Furthermore, none of the truncated forms of Lte1 suppressed the heat shock sensitivity of RAS2Val19 either (Fig. 5 d), suggesting that the physical interaction between Lte1 and Ras is important for Lte1 localization and suppression of the heat shock sensitivity of the RAS2Val19 mutant.

**Ras GEF domain of Lte1 is not essential for mitotic exit**

If Lte1 is the GEF of Tem1 GTPase, the CHD of Lte1 is functioning both for the activation of Tem1 and for anchoring itself to the bud cortex via Ras binding. We attempted to determine an essential domain of Lte1 for mitotic exit. If the CHD of Lte1 acts as a Tem1 GEF, a mutant protein lacking this domain would not fulfill the function of Lte1 in mitotic exit. We found that overexpression of the CHD domain of Lte1 could not suppress the low temperature sensitivity of lte1, but to our surprise, overexpression of the internal region of Lte1 (LTE1-mini) that lacks both NH2- and COOH-terminal Ras GEF homology domains cured the cold sensitivity shown by lte1 cells (Fig. 5 e). This was further substantiated by following the length of the spindle during synchronized cultures of Δlte1 GFP–TUB1 cells; the cell cycle of the cells anti–mouse IgG goat antibody. At least 200 budded cells were counted for the Lte1 localization in each strain. A representative population is presented below the graph. (b) SAY607 (ras1 ras2 bcy1) cells containing a multicopy number plasmid Lte1–2HA driven by the LTE1 promoter (pLTE1-902ET) were transformed with a low copy number plasmid harboring various RAS mutations, and Lte1–2HA localization was examined as in a. At least 300 cells were counted. Representative cells are shown below the graph. We noted that a small population of the cells showed a faint bud tip localization of Lte1–2HA even in the absence of Ras activity, most possibly due to the overproduction of Lte1–2HA on a multicopy number vector.

Figure 3. **Lte1 localization at the bud cortex depends on Ras.**
(a) Strains containing a low copy number plasmid expressing Lte1–2HA under the LTE1 promoter (pLTE1-902CU) were fixed and processed for the indirect immunofluorescence method using mouse monoclonal anti-HA antibody 16B12 and FITC-conjugated

mislocalization of Lte1 in the ras1 ras2 bcy1 mutant is not due to the failure of Cdc42–Cla4 activation or mislocalization of Cla4, Kel1, or septin.

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containing Lte1-mini progresses more or less like those containing genuine Lte1 (Fig. 5 f). We also found that overexpression of \( \text{LTE1-mini} \) as well as full-length \( \text{LTE1} \) rescued the temperature sensitivity of the \( \text{ras1 ras2} \) mutant (Fig. 5 a). Lte1d10 lost activities expressed by the Lte1mini (Fig. 5, a and e), indicating the importance of the amino acid sequence 868–926 for Lte1mini activity. These results indicate that the \( \text{LTE1-mini} \) domain is sufficient for transmitting the signal of mitotic exit to its downstream target, and the Ras GEF homology domain of Lte1 is essential only for its proper localization by association with Ras and is dispensable when the mini domain of Lte1 is overproduced.

**Discussion**

**A role of Ras in mitotic exit**

In this study, we identified Lte1 as a direct target of Ras involved in mitotic exit. It is obvious that Ras2 is essential for Lte1 localization, and the reason why the \( \text{ras1 ras2} \) mutant delays in mitotic exit is partly due to the mislocalization of Lte1 because overexpression of \( \text{LTE1} \) suppressed the mitotic delay of the \( \text{ras1 ras2} \) mutant (Fig. 2 b). We think that the bud cortex localization of Lte1 is directly regulated by Ras proteins because the loss of Ras function disrupts Lte1 localization (Fig. 3 b) but does not affect the localization of Lte1 regulators (Fig. 4 d). The asymmetric localization of Lte1 is due to the localization of active Ras proteins because Ras2Val19 protein was localized to the entire cell cortex (Fig. S1 a). Therefore the asymmetric localization of Lte1 to the bud is regulated not by Ras, but by cell polarity proteins such as Cdc42–Cla4 or septin, and Ras may function as an anchor of Lte1 at the bud cortex.

We think there is another target of Ras involved in mitotic exit because the \( \text{ras1 ras2 bcy1} \) cells are defective in mitotic exit not only at a higher but also at a lower temperature, whereas \( \text{bcy1} \) cells are defective in mitotic exit only at a lower temperature. Although it is also possible that active Ras inhibits, and at the same time promotes, mitotic exit because the active Ras–cAMP pathway does some harm on mitotic exit by inhibiting the anaphase-promoting complex (APC) (Anghileri et al., 1999; Irriger et al., 2000). It requires further study to elucidate the role of Ras in mitotic exit.
Figure 5. **Domain analysis of Lte1.** (a) Summary of the domain analysis. Amino acid number is written in the figure. Ras2-I.P., coimmunoprecipitation of Ras2 with 2HA-tagged Lte1 as judged by the results shown in b and by results not depicted; Loc., localization of 2HA-tagged protein at the bud cortex. Representative figures are shown in c. RAS2Val19, suppression of the heat shock sensitivity of the RAS2Val19 mutant judged by the data shown in d; lte1, suppression of the low temperature sensitivity of the Δlte1 strain by overexpression from a multicopy vector at 12°C as judged by the results shown in e and by results not depicted. O, positive; X, negative; --, not tested. (b) Immunoprecipitation of truncated Lte1–2HA. Full-length Lte1 (lane 2), full-length Lte1–2HA (lane 3), Lte1dC–2HA (lane 4), Lte1d2–2HA (lane 5), Lte1dN–2HA (lane 6), and Lte1GEF–2HA (lane 7) were expressed from a multicopy number plasmid in the SAY611 ( Δlte1) cells. Lane 1 of the top gel was loaded with molecular marker proteins. HA-tagged proteins were precipitated with anti-HA antibody 16B12 and protein A–Sepharose beads.
The role of Lte1

Our findings strongly support the idea that Lte1 functions as an effector of Ras because (a) Lte1 binds to Ras2-GTP, and this interaction is essential for Lte1 localization, and (b) overexpression of Lte1 rescues the mitotic exit defect of a \( \Delta \text{lte1} \) \( \Delta \text{ras2} \) mutant whereas active \( \text{RAS2Val19} \) mutation does not rescue the cold sensitivity shown by the \( \Delta \text{lte1} \) mutant. Lte1 has long been regarded as a GEF for Tem1 because Lte1 contains a putative Ras GEF domain and Lte1 functions upstream of Tem1. But our finding that overexpression of the \( \text{LTE1-mini} \) (659–926) domain, which lacks both NH\(_2\)– and COOH-terminal Ras GEF homology domains, fulfills the \( \text{LTE1} \) function (Fig. 5, e and f) suggests that Lte1 is not necessarily a GEF for Tem1.

Our finding that Lte1 physically associated with active Ras2 strongly supports the idea that overexpressed Lte1 blocks the Ras–CAMP pathway by titrating out the excess amount of active Ras. The facts that overexpression of \( \text{LTE1} \) suppressed the heat shock sensitivity of the active Ras mutation \( \text{RAS2Val19} \) and \( \text{ira1}^{-1} \), but not that of the \( \text{bcy1} \) mutation (Shirayama et al., 1994a), and that truncated Lte1 mutants that failed to interact with Ras2 also failed to suppress the heat shock sensitivity of \( \text{RAS2Val19} \) (Fig. 5 d) further support the idea that Lte1 acts directly with Ras. However, it is unlikely that Lte1 functions as an inhibitor of the Ras–CAMP pathway in a physiological state because \( \Delta \text{lte1} \) cells did not show the heat shock–sensitive phenotype (unpublished data).

Genetic data indicate that Lte1 possibly functions upstream of Tem1 (Shirayama et al., 1994b), but it remains unclear how Lte1 regulates the Tem1 pathway. Recent studies have revealed that Lte1 physically associates with Kel1/Kel2, and the deletion of either protein rescues the mitotic defect of \( \Delta \text{lte1} \) cells (Hofken and Schiebel, 2002). These interactions suggest that the function of Lte1 is to inactivate Kel1/Kel2.

Finally, we would like to emphasize that the CHD of Lte1 has a unique property that prefers to bind to GTP-bound Ras2. It is of interest whether there are other proteins whose exchange factor domain does not act as an exchange factor but acts as if it is an effector.

Materials and methods

Yeast techniques

Yeast strains and plasmids used in this study are listed in Tables S1 and SII, respectively (available at http://www.jcb.org/cgi/content/full/jcb.200301128/DC1). Standard yeast techniques for the cell culture, cell synchronization, and indirect immunofluorescence method were performed as described in Adams et al. (1997) and in the figure legends. Yeast cells were fixed with 4% formaldehyde for 20 min and washed with PBS (pH 7.5, 140 mM NaCl, 2.7 mM KCl, 3.8 mM NaHPO\(_4\)) before microscopic observation or application of the indirect immunofluorescence method. α-Factor and nocodazole were used at 10μg/ml. Microscopic photos were taken by an Olympus IX70, UPlanApo100x/1.35 objective with a SENSYS III (Nippon Roper) cooled CCD camera using IP Lab software. Images were analyzed with IP Lab software, and the figures were created with Adobe Photoshop 5.0\(^\circ\) software. Immunoprecipitation of Lte1 or Ras2 was performed as described in the report by Asakawa et al. (2001) using modified lysin buffer for Ras2 extraction (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl\(_2\), 0.5% Triton X-100).

Purification of flag-Lte1

SAY623 cells overexpressing flag-Lte1 under the GAL1 promoter in YPGalactose medium were lysed in lysin buffer LBI (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100) and incubated with anti-flag M2 beads. The beads were subsequently washed with LBI (50 mM Tris-HCl, 500 mM NaCl, 1.5% Triton X-100) and LBII (50 mM Tris-HCl, 1.5 M NaCl, 0.1% Triton X-100), and then flag-Lte1 was eluted from the beads with 0.1 M glycine (pH 3.4). Eluted flag-Lte1 was neutralized and separated by anion exchange mono-Q column (Amersham Biosciences) and used for the Ras2 binding assay.

In vitro binding of Ras2–Lte1

A two-hybrid test using the BacterioMatch system (Stratagene) was performed per the manufacturer's instructions. XL1-Blue MRF' Kan (Stratagene) was used as the host for the bacterial two-hybrid assay. The amp\(^+\) gene was used as a reporter gene whose expression was detected by resistance to carbenicillin. Purified flag–Lte1 was precipitated with M2 beads, and 10 μl of the aliquots (containing 30 pmol flag–Lte1) was incubated with 6 pmol each of various forms of purified Ras2 protein, which had been loaded with GTP\(^\gamma\)S or GDP, in 30 μl of buffer A (20 mM Tris-HCl, pH 7.4, 40 mM NaCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol PX) (Shima et al., 2000) for 30 min with continuous mixing at 30°C and subsequently washed three times with buffer A. The bound Ras2 was separated by SDS-PAGE and detected by Western immunoblotting with the anti-Ras2 antibody (Santa Cruz Biotechnology, Inc.).

Online supplemental material

The supplemental figure and tables are available at http://www.jcb.org/cgi/content/full/jcb.200301128/DC1. The localization of Ras2 and its derivatives, the suppression of the ras2 \( \text{ras2} \) bcy1 low temperature–sensitive phenotype by \( \text{bub2} \), and a detailed domain analysis of Lte1 are included in the figure. The yeast strains and plasmids used in this study are listed in the tables.

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Bead-associates proteins were analyzed by Western blotting using 16B12 (top) and goat anti-Ras2 antibody (bottom). Total yeast lysate was loaded in lane 1 of the bottom panel to confirm the position of Ras2 protein. (c) Localization of truncated Lte1 proteins. A truncated Lte1 protein with a 2HA tag at its COOH terminus was expressed from a multicopy number plasmid in SAY611 cells (\( \text{lte1} \)). The cells were fixed with formaldehyde and processed for the immunofluorescence method using 16B12 and FITC-conjugated anti–mouse IgG goat antibody. pLTE1-902ET (\( \text{lte1} \), i), pDC-902ET (\( \text{lte1} \), ii), pDN-902ET (\( \text{lte1}\)D1, iii), and pTS902ET (empty, iv). (d) Suppression of the heat shock sensitivity of \( \text{RAS2Val19} \) cells. SAY617 cells (\( \text{RAS2Val19} \) with a multicopy vector expressing various versions of the Lte1 mutant were plated on YPD medium and incubated at 30°C for 2 d (left) or incubated at 30°C for 3 d after heat shock for 50 min at 55°C (right). 1, pLTE1-902ET; 2, pDC-902ET; 3, pDC-902ET; 4, pdc-902ET; 5, pdc-902ET; 6, pDN-902ET; 7, pdc-902ET; 8, pmini-902ET; 9, pGFR-902ET; 10, pNM-902ET; 11, pNG-902ET; 12, pNC-902ET. Constructs not shown in panel a are shown in Fig. S1b (available at http://www.jcb.org/cgi/content/full/jcb.200301128/DC1). (e) Suppression of the cold temperature sensitivity of \( \text{lte1} \). SAY611 (\( \text{lte1} \)) cells containing the indicated plasmids were streaked on selective SC-Trp medium and incubated for 2 d at 30°C (left) or 13 d at 12°C (right). (f) Overproduction of Lte1-mini promotes mitotic exit at a low temperature. SAY661 cells (\( \Delta \text{lte1}\) GFP–\( \text{UCA1} \)) were arrested in G1 phase with mating pheromone for 3 h and released in fresh medium at 10°C, and the budding index and spindle length were examined at each time point. At least 300 cells were counted. Budding, cells with a bud; metaphase, cells with a short spindle \(~1–4\) μm long; anaphase, cells with a spindle \(~2–4\) μm.
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