Lfc and Lsc Oncoproteins Represent Two New Guanine Nucleotide Exchange Factors for the Rho GTP-binding Protein*

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Lfc and Lsc are two recently identified oncoproteins that contain a Dbl homology domain in tandem with a pleckstrin homology domain and thus share sequence similarity with a number of other growth regulatory proteins including Dbl, Tiam-1, and Lbc. We show here that Lfc and Lsc, like their closest relative Lbc, are highly specific guanine nucleotide exchange factors (GEFs) for Rho, causing a >10-fold stimulation of [3H]GDP dissociation from Rho and a marked stimulation of GDP-[35S]GTPγS-bound states. None of the other Dbl family proteins including Dbl, Tiam-1, and Lbc are able to act catalytically in stimulating the guanine nucleotide exchange activity, such that a single molecule of each of these oncoproteins can activate a number of molecules of Rho. Neither Lfc nor Lsc shows any ability to stimulate GDP dissociation from other related GTP-binding proteins such as Rac, Cdc42, or Ras. Thus Lbc, Lfc, and Lsc appear to represent a subgroup of Dbl-related proteins that function as highly specific GEFs toward Rho and can be distinguished from Dbl, Ost, and DbS which are less specific and show GEF activity toward both Rho and Cdc42. Consistent with these results, Lbc, Lfc, and Lsc each form tight complexes with the guanine nucleotide-depleted form of Rho and bind weakly to the GDP- and GTPγS-bound states. None of these oncoproteins are able to form complexes with Cdc42 or Ras. However, Lfc (but not Lbc nor Lsc) can bind to Rac, and this binding occurs equally well when Rac is nucleotide-depleted or is in the GDP- or GTPγS-bound state. These findings raise the possibility that in addition to acting directly as a GEF for Rho, Lfc may play other roles that influence the signaling activities of Rac and/or coordinate the activities of the Rac and Rho proteins.

The Dbl family constitutes a group of oncoproteins and growth regulatory factors that have been implicated in a diversity of biological responses. Among the members of this family is the prototype Dbl oncoprotein, as well as Cdc24, a Saccharomyces cerevisiae cell-division-cycle protein involved in bud-site assembly (1, 2), Bcr, the breakpoint cluster region protein that has been implicated in the development of certain human leukemias (3), Tiam-1, which was first identified as a gene product involved in cell invasiveness and metastasis (4), and the vav (5), ost (6), ect2 (7), tim (8), fgd1 (9), abr (10), dbs (11), lbc (12), lfc (13), and lsc (14) oncogene products. The Dbl oncoprotein was first discovered when transfecting the DNA from diffuse B cell lymphomas into NIH 3T3 fibroblasts (1). Analysis of the primary amino acid sequence of the Dbl protein indicated that it contained a region of ~250 amino acids that shared homology with Cdc24 and Bcr. Given that genetic evidence placed Cdc24 upstream of Cdc42 in the bud-site assembly pathway in S. cerevisiae, it seemed plausible that Dbl regulated the actions of the human Cdc42 protein (Cdc42Hs). This led to the biochemical demonstration that Dbl is a guanine nucleotide exchange factor (GEF) for Cdc42Hs and Rho (15, 16) and that Cdc24 is a highly specific GEF for the S. cerevisiae Cdc42 protein (Cdc42Sc) (2). It also was shown that the region of sequence similarity that Dbl shared with Cdc24 was critical for both GEF activity and for cellular transformation (16). More recent sequence analysis has subdivided this region of sequence similarity into two domains that are shared among all of the members of the Dbl family. The first domain, designated the Dbl homology (DH) domain, is essential for the GEF activity of Dbl, and the second domain, which shares homology with the platelet protein pleckstrin (designated the PH domain), is critical for the proper cellular targeting of Dbl and related proteins (13, 17).

Based on the initial biochemical studies performed on Dbl and Cdc24, it has been generally assumed that all proteins that contain a DH domain-PH domain in tandem will be GEFs for Rho-subtype proteins. In some cases this has been borne out. For example, Tiam-1 shows in vitro GEF activity toward Rac, Rho, and Cdc42 (18), and Lbc is a specific GEF for Rho (19). However, in a number of other cases, no GEF activity has yet been associated with the Dbl-related protein. This raises some important questions. 1) Are all DH domains involved in GEF activity or do they serve other biological roles, for example in the recruitment of Rho-related GTP-binding proteins to particular cellular locations and/or signaling complexes? 2) What accounts for the presumed specificity in the functional coupling of Rho-like proteins to Dbl-related molecules? In some cases in vivo specificity is probably mediated by cellular targeting, as accomplished by the individual PH domains of the different Dbl proteins. However, there also are clear indications that certain DH domains couple with high specificity to GTP-binding proteins, as exemplified by the interaction between Lbc and Rho. Overall, a better understanding of the regulation of Rho-re-

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† The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; GST, glutathione S-transferase; JNK, c-Jun kinase; GTPγS, guanosine 5'-O-(3-thiotriphosphate); Mant, N-methylanthraniloyl; PH, pleckstrin homology.

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labeled GTP-binding proteins by members of the DbI family is of broad significance, given the highly coordinated actions of the Rho proteins in mediating a series of cytoskeletal alterations including filopodia and lamellipodia formation and the generation of stress fibers (20–23), as well as in stimulating DNA synthesis (24–29) and promoting cell morphology changes and cell motility (30).

In the present study, we have begun to address the questions raised above concerning the functions of DbI-related proteins. To do this we have taken advantage of what appears to be a subgroup within the DbI family, for which the prototype is Lbc and which includes the recently identified Lfc and Lsc oncoproteins. Lfc and Lsc (i.e. the “first” and “second” cousins of Lbc) were initially identified along with Dbs (“DbI’s big sister”), as the products of cDNAs that induce transformation, by using a retroviral vector-based expression system to transfer a library of cDNAs from the murine 32D or BOSUTA1 hematopoietic cell lines into NIH 3T3 fibroblasts (31). Both Lfc and Lsc share the highest sequence similarity with the Lbc oncoprotein, within the regions of the DH and PH domains. In this work, we first set out to determine if like Lbc, the Lfc and Lsc proteins were capable of stimulating guanine nucleotide exchange on Rho-related proteins. We also were interested in determining if Lfc and Lsc demonstrated a high degree of specificity in their binding and GEF activities. The demonstration that Lfc and Lsc, as well as Lbc, were highly specific in their interactions with Rho proteins could lead to new insights regarding the elements within DH domains that impart the ability to recognize individual members of the Rho family.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—The GST-Lbc and GST-Lfc proteins were prepared by first inserting the cDNAs encoding a fragment of Lfc (fragments 208–573) and Lsc (fragments 333–778), which encompasses the DH and PH domains of these proteins, into the pGEX2T vector, and then the glutathione S-transferase (GST) constructs were cloned into the BamHI site of pAX142 (13, 14). The GST constructs were digested from pAX142 at the XbaI and SalI sites; the fragment was then blunt-ended and inserted into the BamHI/SmaI sites of the baculovirus transfer vector pVL1393. Spodoptera frugiperda cells (SF-21) were infected with recombinant baculovirus; the cells were collected 8 h after infection and lysed in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The GST fusion proteins were purified by glutathione-agarose affinity chromatography. Preparation of GST-Cdc24 and GST-Lbc and their expression in SF21 cells has been previously described (2, 19) as the product of cDNA that induce transformation, by using immobilized GST-Lsc and GST-Lfc, and as positive controls, GST-Lbc, GST-Cdc24, and GST-GFP were bound to glutathione-agarose beads to co-purify precipitated His-tagged (clipped) GTP-binding proteins. Initially all interactions were assayed using the nucleotide-free state of the GTP-binding proteins. Co-precipitations were performed in 500-µl volumes of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (incubating for 2 h at 4°C). The agarose beads were washed in the precipitation buffer three times by centrifugation and resuspended in Laemmli buffer, subjected to SDS-polyacrylamide gel electrophoresis, and Western blotted. Co-precipitations were performed for each GTP-binding protein individually, and interactions were detected using anti-RhoA monoclonal antibody (Santa Cruz Biotechnology), or anti-Rac polyclonal antibody (Santa Cruz Biotechnology), anti-Ras monoclonal antibody (Santa Cruz Biotechnology), or anti-Cdc42 antibody (raised against the carboxyl-terminal 23 amino acids), by the ECL method (Amer sham Corp.).

In order to fully characterize the nucleotide dependence of the interactions occurring between GEFs and GTP-binding proteins in the nucleotide-free state, we repeated co-precipitations using RhoA and Rac bound to GDP and GTP-S. These experiments were performed as described above except that the GDP-binding proteins were preloaded with the appropriate nucleotide, and the EDTA was replaced with 10 mM MgCl2 in order to stabilize nucleotide binding to the GTP-binding proteins.

RESULTS

The principal aim of these studies was to determine whether the Lfc and Lsc proteins were capable of functionally coupling to the members of the Rho subgroup of GTP-binding proteins. Previous studies have shown that the DH domain of DbI is essential for both its GEF activity and transforming capability (16). All members of the DbI family also contain a PH domain, which is immediately carboxyl-terminal to the DH domain, and recent findings suggest that the PH domain is important for cellular targeting rather than for GEF activity (17). However, because of the possibility that the PH domains of DbI proteins may have other regulatory functions, and because we have found that the presence of surrounding sequences includ-
ing the PH domain may be necessary to achieve the proper folding of DH domains, we expressed and purified forms of the Lfc and Lsc proteins that include both the DH and PH domains, as well as some additional flanking sequences. Fig. 1A shows the schematic representations of both the full-length Lfc and Lsc proteins and the glutathione S-transferase (GST) fusion proteins that were expressed and assayed for GEF activity. D4 Lsc and D10 Lfc refer to constructs that have been described previously (13, 14). B, expression and purification of Lfc, Lsc, and Lbc as GST fusion proteins. SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) of the purified GST fusion proteins which were prepared from SF21 insect cell lysates infected with recombinant viruses that encode the Lfc, Lsc, and Lbc proteins. C, comparison of the Dbl homology domains from Lbc, Lfc, Lsc, and Dbl. Alignment of residues 78–304 of Lbc to similar sequences of Lfc, Lsc, and Dbl is shown. Boxed residues represent sequence identities.

**FIG. 1.** Expression of Lfc and Lsc as GST fusion proteins. A, schematic representation of full-length Lfc and Lsc and the GST-Lfc and GST-Lsc fusion proteins that were expressed and assayed for GEF activity. D4 Lsc and D10 Lfc refer to constructs that have been described previously (13, 14). B, expression and purification of Lfc, Lsc, and Lbc as GST fusion proteins. SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) of the purified GST fusion proteins which were prepared from SF21 insect cell lysates infected with recombinant viruses that encode the Lfc, Lsc, and Lbc proteins. C, comparison of the Dbl homology domains from Lbc, Lfc, Lsc, and Dbl. Alignment of residues 78–304 of Lbc to similar sequences of Lfc, Lsc, and Dbl is shown. Boxed residues represent sequence identities.

**Determination of Guanine Nucleotide Exchange Activity for Lfc and Lsc**—Comparisons of the Dbl domains of Lfc and Lsc with other members of the Dbl family show that these domains are most similar to those for Lbc (Fig. 1C). Given that Lbc is a highly specific GEF for Rho, we examined whether the Lfc and Lsc proteins were capable of similar biochemical activities. Fig. 2, A and B, shows that this is the case. Both Lfc (Fig. 2A) and Lsc (Fig. 2B) were highly effective in stimulating the dissociation of [$^{3}H$]GDP from *E. coli*-expressed RhoA. In the absence of any regulatory protein, the half-time for the dissociation of
GDP from RhoA is relatively slow, i.e. >30 min at room temperature. However, both Lfc and Lsc proteins were able to markedly accelerate the rate of GDP dissociation by at least 10-fold such that the half-time for GDP dissociation was 2–3 min in the presence of these regulatory proteins.

Fig. 3, A and B, shows that the Lfc and Lsc proteins also strongly stimulated the exchange of GDP for $[^3H]GTP_S$. Here again in the absence of any regulatory factor, RhoA is capable of little if any guanine nucleotide exchange over a period of 20 min. However, both Lfc and Lsc catalyzed the complete exchange of GDP for GTP$_S$ within ~10 min, thus indicating that these proteins qualify as effective GEFs for the RhoA protein.

We have closely compared the initial rates of guanine nucleotide exchange on RhoA that are catalyzed by Lfc, Lsc, and Lbc, under conditions where RhoA was present in at least a 6-fold excess over the GEFs, using a very sensitive fluorescence spectroscopic assay (Fig. 4A). This assay is based on the finding that the fluorescence emission of Mant-GTP is enhanced upon binding to GTP-binding proteins. Thus, under conditions where guanine nucleotide exchange is catalyzed, the presence of Mant-$\gamma$GTP in a cuvette solution containing RhoA will result in an exchange of the GDP that was originally bound to RhoA for the Mant-nucleotide and thereby provide a real-time assay for...
the exchange event, as monitored by the enhancement of Mant fluorescence. Fig. 4A shows that the purified recombinant Lfc, Lsc, and Lbc proteins each stimulated the enhancement of Mant fluorescence as an outcome of catalyzed GDP-Mant-dGTP exchange on RhoA. The time courses for the fluorescence changes stimulated by Lbc and Lfc were virtually identical for each of the three different GEF concentrations assayed. While we consistently found Lsc to stimulate guanine nucleotide exchange at a rate that was 
\[2\]\text{-fold slower compared with the rate of exchange stimulated by Lbc and Lfc, it is difficult to know how much significance to attach to these differences because of the difficulties in estimating the protein concentrations for the amount of functional GEF present in the assay.}

In order to further compare the catalytic potential of the Lfc and Lsc proteins, we assayed \[^{3}H\]GDP dissociation from RhoA as catalyzed by different concentrations of these GEFs. We found that guanine nucleotide exchange occurred rapidly when the GEF and GTP-binding proteins were present in equimolar concentrations. As expected, decreasing the concentration of Lfc or Lsc such that RhoA was in 2- or 4-fold excess (over [GEF]) resulted in slower half-times of dissociation. However, it appeared that complete dissociation of \[^{3}H\]GDP from RhoA will occur at each of the concentrations of Lfc and Lsc assayed, indicating that both of these proteins act catalytically in stimulating the guanine nucleotide exchange reaction.

**Specificity of Lfc and Lsc as GEFs**—The Lbc protein was shown to be a highly specific GEF for Rho and did not stimulate the guanine nucleotide exchange activity of Cdc42Hs, Rac, or Ras (19). Thus, we examined whether Lfc and Lsc showed similar GEF specificity. The data presented in Fig. 5 indicate that like Lbc, both Lfc and Lsc are highly specific for RhoA when assaying \[^{3}H\]GDP dissociation after 15 min at room temperature. Neither Lfc nor Lsc showed any GEF activity toward Ras under these conditions, whereas recombinant GST-Ras-GRF strongly stimulated GDP dissociation from Ha-Ras. Similarly neither Lfc nor Lsc showed any activity toward Cdc42Hs, again under conditions where Cdc24 effectively stimulated GDP dissociation. Both Lfc and Lsc were ineffective in stimulating GDP dissociation from Rac. The inability of Lfc and Lsc to serve as GEFs for Ras, Cdc42Hs, and Rac also was observed when complete time courses for \[^{3}H\]GDP dissociation were obtained (i.e. between 2.5 and 30 min (data not shown)). It is interesting that although the DH domains of oncogenic Dbl

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**Fig. 4. Comparison of Lfc, Lsc and Lbc: initial rates of exchange on RhoA and catalytic potential.** A, fluorescence spectroscopic analysis of initial rates for guanine nucleotide exchange on RhoA (650 nm) catalyzed by Lbc (1), Lfc (2), and Lsc (3) at 25, 50, and 100 nm. The rate of change of fluorescence was estimated using linear fits to the first 50 s after addition of GEFs. B, kinetics of guanine nucleotide exchange catalyzed by Lfc (top panel) and Lsc (bottom panel), using equimolar concentrations of RhoA and GEF (●); ▲ represents a 2-fold excess of RhoA to GEF; □ represents the GST control.
Do Lfc and Lsc Show Similar Binding Specificity for Rho Family Members?—Some members of the Dbl family are able to bind to Rho-related GTP-binding proteins but do not stimulate their guanine nucleotide exchange activity (6, 7). This suggests that in some cases the DH domains serve as binding motifs, perhaps functioning only to recruit GTP-binding proteins. Following this line we wanted to examine whether Lbc, Lfc, and Lsc acted strictly as GEFs, such that they showed similar binding specificity as that exhibited in the GEF assays, or if one or more of these Dbl proteins within the Lbc subfamily were capable of binding to other GTP-binding proteins, in addition to Rho. These experiments were carried out using Lbc, Lfc, and Lsc expressed as GST fusion proteins and immobilized on glutathione-agarose beads. We first assayed the binding specificities of these proteins for different GTP-binding proteins in their guanine nucleotide-depleted state, since this should be the preferred state for binding to GEFs. Fig. 6 shows that as expected, GST-Lbc and GST-Lsc selectively associated with RhoA but not with Rac, Cdc42, or Ras. Also as expected, GST-Ras-GRF formed a complex with Ras and GST-Cdc24 co-immobilized on glutathione-agarose. However, it was surprising that although GST-Lbc bound RhoA as effectively as Lbc and Lsc, the GST-Lfc protein also associated with Rac (as did GST-Cdc24).

We next examined the nucleotide specificity for the binding of RhoA and Rac to the Lbc subfamily members. The results presented in Fig. 7 show that Lbc, Lf, and Lsc bound specifically to the guanine nucleotide-depleted state of RhoA, again, as is typically the case for GEFs (16). However, the interaction between Lfc and Rac did not demonstrate this specificity, such that Lfc effectively associated with both the GDP- and GTPγS-bound states of Rac as well as with the nucleotide-depleted form of the protein. Neither Lbc nor Lsc showed any binding capability for Rac, regardless of nucleotide state. Based on the finding that Lfc was able to associate with both the GDP- and GTP-bound forms of Rac, we assayed the ability of Lfc to inhibit GDP dissociation (i.e. act as a GDP dissociation inhibitor) or influence GTP hydrolysis (data not shown). All such assays were negative, that is we found no detectable GDP dissociation inhibitor activity for Lfc nor did we find that Lfc could alter the intrinsic or GTPase-activating protein-stimulated GTP hydrolysis of the Rac protein.

**DISCUSSION**

The Lfc and Lsc oncoproteins are two recently discovered members of the Dbl family of growth regulatory proteins. Each of the members of this family share two motifs, a Dbl homology (DH) domain of ~150 amino acids and a pleckstrin homology (PH) domain that contains ~100 amino acids. Various members of this family including the prototype, Dbl, as well as Cdc24, Tiam-1, and Lbc have been shown to act as guanine...
nucleotide exchange factors (GEFs) by stimulating the exchange of GTP for GDP on Rho subfamily GTP-binding proteins (2, 15, 18, 19). In two cases, Cdc24 and Lbc, the GEF activity is highly specific, with Cdc24 serving as the substrate for Cdc24 and Rho serving as the substrate for Lbc. Given the fact that the DH domains of Lfc and Lsc are most similar to that of Lbc, we were interested in the possibility that these two oncoproteins might also act as specific GEFs for Rho and thus together with Lbc comprise a specific sub grupo of the larger family of Dbl-related proteins. The data presented here suggest that this in fact is the case. Both Lfc and Lsc appear to be family of Dbl-related proteins. The data presented here suggest that of Lbc, we were interested in the possibility that these two oncoproteins represent two new GEFs for Cdc24 and Rho serving as the substrate for Lbc. Given the activity is highly specific, with Cdc42 serving as the substrate.

Specifically, it appears that phosphatidylinositol 4,5-bisphosphate may act cooperatively with Dbl (since Dbl, alone, is fully capable of stimulating GDP-GTP exchange on Cdc42 or Rho), it will be interesting to see if phosphatidylinositol 4,5-bisphosphate or other lipids can act synergistically with Lfc to influence the activation state of Rac. The location of a potential lipid binding domain on Lfc raises other possibilities regarding lipid factors that might influence the functional coupling of this oncprotein to Rac-like GTP-binding proteins. Thus, it may be that the family of Dbl-related proteins provide a broader group of functions than originally anticipated, not only acting directly as specific GEFs but also specifying the cellular sites where other factors can promote the activation of GTP-binding proteins and/or where targets can bind to GTP-binding proteins and mediate downstream signaling events.

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