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High-efficiency expression/cloning of epidermal growth factor–receptor-binding proteins with Src homology 2 domains

(cloning of receptor targets/growth factor receptor-bound/λEXlox/ras GTPase-activating protein)

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ABSTRACT **Src homology 2 domains bind to tyrosine-phosphorylated growth factor receptors and are found in proteins that serve as substrates for tyrosine kinases, such as phospholipase C-γ1 and ras GTPase-activating protein. We have previously described the cloning of phosphatidylinositol 3′-kinase-associated p85 from expression libraries with the tyrosine-phosphorylated epidermal growth factor receptor as a probe. We have now modified this technique by using T7 polymerase-based expression libraries, which significantly improves sensitivity of the method. In one screening of such a library, we identified five different murine Src homology 2 domain-containing proteins, which we call GRBs (growth factor receptor-bound proteins). Two of these proteins represented the tyrosine kinase fyn and the mouse homologue of phospholipase C-γ1, whereas two genes encoded proteins similar to v-crk and NCK. We also isolated the gene for GRB-7, which encodes a protein of 535 amino acids. In addition to a Src homology 2 domain, GRB-7 also has a region of similarity to the noncatalytic domain of ras GTPase-activating protein and is highly expressed in liver and kidney. Use of this expression/cloning system should increase our ability to identify downstream modulators of growth factor action.**

Activation of receptor-linked and cytoplasmic tyrosine kinases leads to cellular growth and transformation (for reviews, see refs. 1 and 2). It is hypothesized that tyrosine kinases phosphorylate specific intracellular signaling molecules in the initial steps leading to mitogenesis, but molecular characterization of these tyrosine kinase substrates has proven difficult. Recently, it has been appreciated that several substrates of growth factor receptor-linked tyrosine kinases, such as phospholipase C-γ1 (PLC-γ1) and ras GTPase-activating protein (ras-GAP) contain Src homology 2 (SH2) and SH3 domains (for reviews, see refs. 3–5). The SH2 domain mediates binding of these proteins to tyrosine-phosphorylated growth factor receptors, and for PLC-γ1, there is evidence that this binding promotes the tyrosine phosphorylation and activation of this enzyme (3–6). Another group of proteins such as v-crk (7) and NCK (8) are virtually composed only of SH2 and SH3 domains. Expression of one of these proteins, v-crk, which is encoded by an oncogene, markedly increases cellular tyrosine phosphorylation, although this protein is not a tyrosine kinase (7). Thus, SH2 domain-containing proteins are a diverse group of molecules important in tyrosine kinase signaling.

With this in mind, our laboratory set out to develop a method to clone additional proteins that contain SH2 domains, relying on the fact that these proteins can bind to autophosphorylated growth factor receptors (9–11). Using a method we called CORT (cloning of receptor targets), we screened a human brainstem λgt11 expression library with the autophosphorylated region of the epidermal growth factor receptor (EGFR) and cloned two proteins that we termed GRBs (for growth factor receptor-bound) (12). GRB-1 was also cloned by conventional methods and shown to be phosphatidylinositol 3′-kinase-associated p85 (13, 14). The second molecule, GRB-2, represents a 25-kDa protein that contains only SH2 and SH3 domains and can be grouped with v-crk and NCK (46). Interestingly, we have recently found that the gene encoding GRB-2 represents a human homologue of *sem-5*, a gene crucial for *Caenorhabditis elegans* vulval development (15).

In this paper, we report a modification in the CORT methodology, which markedly increased sensitivity of the technique. This increase in sensitivity was obtained by using a T7 polymerase-based library that yielded higher expression of library proteins. By screening such a library with the tyrosine-phosphorylated carboxyl terminus of the EGFR, we identified five different murine SH2 domain proteins. One of these genes, which we call GRB-7,§ is a distinctive SH2 domain protein expressed primarily in liver and kidney. This modified CORT methodology should allow routine and rapid cloning of SH2 domain-containing proteins crucial for signal transduction by tyrosine kinases.

MATERIALS AND METHODS

PLC-γ1 Expression in Different Phage Vectors. In the initial stages of this project, different fragments of PLC-γ1 cDNA were subcloned in λgt11 or λEXlox (Novagen, Madison, WI), a phage system that allows expression of proteins in bacteria with T7 polymerase (16). The DNA fragments containing the human PLC-γ1 (17) were amplified by PCR with primers that incorporated *Eco*RI sites and permitted the correct reading frame for protein expression. The amplified DNA was cut with *Eco*RI and ligated into *Eco*RI-digested λgt11 (Promega) or λEXlox. After packaging (Gigapack, Stratagene), the phages were plated and screened with PLC-γ1 antibody (18) by using standard techniques (19). This phage was then tested for binding to a cyanogen bromide-generated ^{32}P-labeled fragment from EGFR as described (12).

λEXlox Library Screening. A λEXlox library from a 16-day mouse embryo was plated at 40,000 phages per plate in *Escherichia coli* strain BL21(DE3)pLysE, according to manufacturer's instructions. After growth for 8 hr, plates were covered with nitrocellulose impregnated with 1 mM isopropyl β-D-thiogalactoside. Plates were grown overnight, and the

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Abbreviations: SH2, Src homology 2; EGFR, epidermal growth factor receptor; PLC-γ1, phospholipase C-γ1; GRB, growth factor receptor-bound protein; CORT, cloning of receptor targets; ras-GAP, ras GTPase-activating protein.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M94450).

filters were probed as described (12). Positive clones were selected and reprobed until plaques were purified. Phages were then converted to plasmids by using bacterial strain BM25.5, according to manufacturer's instructions. These plasmids were used to transform bacterial strain DH5α, and the resultant plasmids were subjected to double-stranded sequencing by using standard techniques (Sequenase version 2, United States Biochemical).

To obtain the full-length clone of GRB-7, an *Eco*RI fragment was cut from clone 66 and labeled with [^{32}P]dCTP (New England Nuclear/DuPont; 3000 Ci/mmol; 1 Ci = 37 GBq) by using a random-primed labeling kit (United States Biochemical). This fragment was then used to reprobe the 16-day mouse library at high stringency with conventional methods (20). For this screening, the λEXlox phage was plated with *E. coli* ER1647, which does not express T7 polymerase. Positive clones were then purified, and those with additional 5′ sequence as identified by PCR were sequenced.

Sequence Alignment. All DNA and protein data bases were searched with the Genetics Computer Group sequence analysis software package (21). The SwissProt and GenBank/European Molecular Biology Laboratory data bases were searched with FASTA and TFASTA, respectively (22). Proteins were aligned with the Genetics Computer Group programs LINEUP, PILEUP, PRETTY, and BESTFIT. Conservative substitutions were defined as a score of $\geq$0.8 by using the scoring table of Schwartz and Dayhoff (23), as modified by Gribskov and Burgess (24).

RNA Blot Analysis. RNA blots were analyzed as described (25). Briefly, mRNA was extracted from the tissue of 6-week-old mice with SDS and proteinase K and purified on oligo(dT)-cellulose. Approximately 3 μg of mRNA was run on a 1.2% agarose-formaldehyde gel and transferred to nylon (Schleicher & Schuell). The blot was probed with a [^{32}P]dCTP-labeled DNA fragment that encodes amino acids 297–515 of GRB-7. Blots were probed in 0.5 M sodium phosphate, pH 7.2/7% SDS/1 mM EDTA/salmon sperm DNA at 100 μg/ml at 65°C overnight. Blots were washed in 40 mM sodium phosphate, pH 7.2/1% SDS/1 mM EDTA at 65°C.

RESULTS

Optimization of CORT Cloning Methodology with PLC-γ1 as a Model System. After cloning GRB-1 and GRB-2 from a human brainstem library (12), we attempted to clone additional SH2 domain proteins from other λgt11 libraries but were unsuccessful. Surprisingly we were unable to identify phages encoding proteins, such as PLC-γ1, that were known to bind to the EGFR and were widely expressed. To further understand the exact methodologic problem, we cloned different fragments of PLC-γ1 into λgt11 and screened these phages with the ^{32}P-labeled carboxyl-terminal fragment of the EGFR (12). The first clone encoding PLC-γ1 from amino acids 399–1290 (which contains two SH2 domains) did not bind to the EGFR fragment in the plaque-binding assay in spite of producing PLC-γ1 protein as monitored with antibody screening. When this phage was used to infect bacteria and protein expression was monitored, we noted that this clone produced much less β-galactosidase fusion protein than the original GRB-1 and GRB-2 λgt11 phages (results not shown). In contrast, when we expressed just the SH2 domains of PLC-γ1 (amino acids 532–764), we achieved higher expression and binding to the tyrosine phosphorylated carboxyl terminus of the EGFR (Fig. 1*A*). Thus, we hypothesized that low expression of proteins in the λgt11 library might account for our inability to detect additional SH2 domain proteins.

In an effort to solve this problem we turned to a different phage library system where the proteins are expressed under the powerful T7 polymerase (ref. 16; λEXlox vector). This system is based on the pET (plasmid for expression by T7 RNA polymerase) system developed by Studier and coworkers (26), in which cDNA clones are fused to a fragment of the T7 capsid protein T10 under control of the T7 promoter (16, 26). These phages are then used to infect *E. coli* harboring the T7 polymerase under lacUV5 control. Induction with isopropyl β-D-thiogalactoside generates the T7 polymerase, which then initiates transcription of the fusion protein encoded by the phage library. To test this technique, we cloned the SH2 domain fragment of PLC-γ1 (amino acids 532–764) into this phage and analyzed binding of the phosphorylated carboxyl terminus of the EGFR. As can be seen, more uniform and strong binding of the ^{32}P-labeled probe was seen with the T7 polymerase system, as compared with the λgt11 (compare Fig. 1 *A* and *B*). We also cloned a longer fragment of PLC-γ1, including amino acids 532–1290 into the T7 system, and this fragment also bound the labeled EGFR (Fig. 1*C*). This last result suggested that we could detect clones that encompass the carboxyl terminus and SH2 domains of PLC-γ1 in oligo(dT)-primed λEXlox libraries. We observed that the λEXlox plaques, although mostly smaller than the λgt11 plaques, gave stronger signals. This result makes this system particularly suitable for library screening when thousands of small plaques occur per plate. The major advantage of this system is the high level of protein expression from the greater activity of the T7 polymerase versus *E. coli* RNA polymerase (26). Also the fusion proteins using the smaller T10 gene

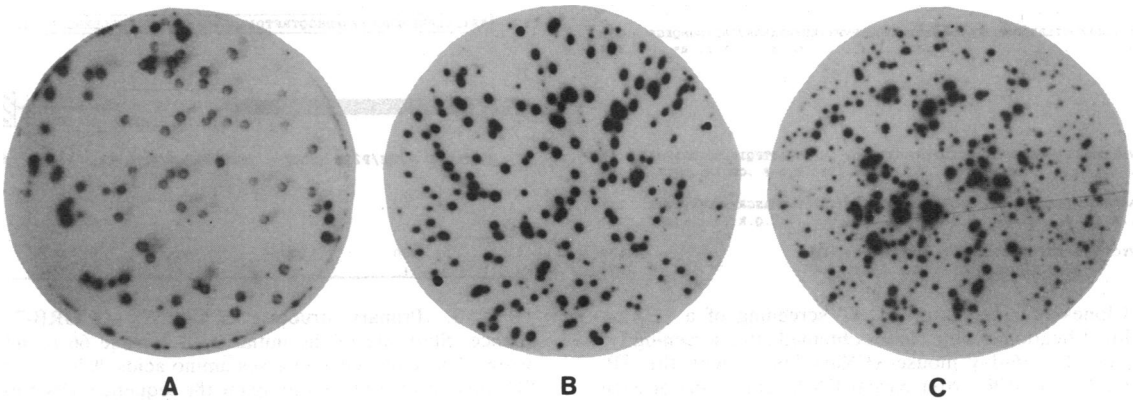

FIG. 1. Comparison of binding of the phosphorylated EGFR carboxyl terminus to PLC-γ1 fragments expressed in λgt11 or T7 polymerase expression libraries. The SH2 domains of PLC-γ1 (amino acids 532–764) were cloned in λgt11 (*A*) or λEXlox (*B*) and probed with the ^{32}P-labeled carboxyl terminus of the EGFR as described (12). (*C*) A longer fragment of PLC-γ1 (amino acids 532–1290) was cloned into λEXlox vector and screened similarly. After screening, filters were washed with Tris-buffered saline/0.1% Triton X-100 and exposed 12 hr for autoradiography.

fragment (26 kDa versus the 110-kDa β-galactosidase of λgt11) may yield more stable expression, and its hydrophobic character may promote binding to nitrocellulose. In addition to directional cloning, the λEXlox phages also allow for automatic conversion to a pET plasmid (16), which can be useful for expression of a fusion protein for antibody production. All of these reasons suggest that screening a λEXlox library might give superior results to screening a λgt11 library for this type of cloning strategy.

Screening a T7 Polymerase-Based Library. When we screened 1.6 million clones of a directional oligo(dT)-primed mouse λEXlox library, we obtained nine positive clones. All nine clones encoded proteins with SH2 domains; six of these clones encoded proteins similar or identical to other known proteins (Fig. 2*A*). The comparison of two of these proteins, GRB-3 and GRB-4, to their known counterparts is displayed in Fig. 2 *B* and *C*. Partial sequence of GRB-3 revealed that it was closely related to the protein encoded by the avian oncogene v-*crk* (7). GRB-3 has a high degree of identity with v-crk beginning with the methionine at residue 32, which is the start site of avian c-crk (47). Beginning at this methionine, the available sequence of GRB-3 is 77% identical at the protein level (Fig. 2*B*) and 80% similar at the DNA level to v-crk. Another clone, GRB-4, is similar to NCK (ref. 8; Fig. 2*C*), identified as a human protein composed of three SH3 domains and one SH2 domain. Our clone contained one SH3 domain and one SH2 domain and was 74% identical at the protein level and 66% similar at the DNA level in the open reading frame. We also cloned two SH2 domain-containing proteins with intrinsic enzymatic activity. Partial sequencing of one clone, GRB-5, revealed identity to mouse fyn (27), whereas GRB-6 was identified as the mouse homolog of PLC-γ1 (refs. 17, 28, and 29; sequences not shown).

A

SH2 DOMAIN PROTEIN	CLONES ISOLATED	DESCRIPTION
GRB-3	#19, #76, #80	*crk*-like
GRB-4	#64	*nck*-like
GRB-5	#63B	*fyn*
GRB-6	#88	PLC-γ1
GRB-7	#63A, #66, #86	novel protein

B

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GRB-3    1  PDTGAGPLGAGARAGGARVPAAAQRESAEAAMAGNFDSEERSSWYWGRLSRQEAVALLQG   60
v-crk  205  QPRAGRGA.HRGLRRP.GRGQRVRPAGGA.L...Q....D.G.........GD..S....  264

GRB-3   61  QRDGVFLVRDSSTSPGDYVLSVSENSRVSHYIINSSGPRPPVPPSPAQP-PPGVSPSRLR  119
v-crk  265  ..E.T......GSI...F.......S.......V..L..AGGRRAGGEG.GA..LN.T.FL  324

GRB-3  120  IGDQEFDSLPALLEFYKIHYLDTTTLIEPVARSRQGSGVILRQEEAEYVRALFDFNGNDE  179
v-crk  325  ....V.....S.....................S....N.........V.........K...D  384

GRB-3  180  EDLPFKKGDILRIRDKPEEQWWNAEDSEGKRGMIPVPYVEKYRPASASVSALIGGMQEGS  239
v-crk  385  G..........K..............MD.............C..S.....T.T..R*      444
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C

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GRB-4    1  VIEKPENDPEWWKCKNARGQVGLVPKNYVVVLSDGP---ALEPAETPQISYTGPSASGRF   57
nck    219  ..............RKIN.M.........T.MQNN.LTSG.E.S-P..CD.IR..LT.K.   277

GRB-4   58  AGREWYYGNVTRHQAECALNERGVEGDFLIRDSESSPSDFSVSLKASGRNKEFKVQLVDS  117
nck    278  ..NP....K.......M......E.............N.........Q.K.........KET  337

GRB-4  118  VYCIGQRRFESMDELVEHYKKAPIFTSEHGEKLYLVRALQ*                     157
nck    338  .......K.ST.E..............Q.......KE.S*                       377
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FIG. 2. Clones obtained after CORT screening of a λEXlox library. (*A*) Identification of nine clones obtained after screening 1.6 million plaques of a 16-day mouse λEXlox library with the ^{32}P-labeled fragment of EGFR. After partial DNA sequencing of each clone, the SwissProt and GenBank/European Molecular Biology Laboratory data bases were searched for similar proteins. (*B*) Comparison of a partial sequence from GRB-3 to avian v-crk (7). (*C*) Comparison of a partial sequence from GRB-4 to human NCK (8). Dots indicate amino acid identity.

GRB-7 Represents a Gene with Homology to ras-GAP. The three remaining clones were identical and encoded a protein with a distinctive SH2 domain that we call GRB-7. To obtain a full-length cDNA clone, the λEXlox library was reprobed with a 700-base-pair (bp) *Eco*RI fragment from clone 66. Several overlapping clones were identified that were used for DNA sequencing to deduce the full-length GRB-7 protein sequence of Fig. 3*A*. The primary sequence of GRB-7 is schematically displayed in Fig. 3*B* and depicts the regions of similarity to the known proteins discussed below. GRB-7 is composed of 535 amino acids and has one SH2 domain at its extreme carboxyl terminus. In Fig. 4*A*, the SH2 domains of GRB-3, GRB-4, and GRB-7 are compared with other SH2 domains, including avian c-src (32), mouse fyn (27), human PLC-γ1 (17), and human p85α (12). The SwissProt and GenBank/European Molecular Biology Laboratory data bases were scanned to search for other protein motifs in GRB-7. Interestingly, amino acids 242–339 of GRB-7 were similar to a sequence from the central noncatalytic region of ras-GAP (30, 33). Over this region of 92 amino acids from ras-GAP, GRB-7 has 26% identity and 43% similarity allowing for conservative substitutions (Fig. 4*B*). This region of ras-GAP lies between the SH2/SH3 domains and the GTPase-activating carboxyl-terminal region and has not been assigned a specific function (34). The amino-terminal sequence of GRB-7 was proline-rich and, thus, is similar to many other proline-rich proteins. GRB-7 does have an extended region of limited similarity to the catalytic domain of protein phosphatase 2B (34), including the proline-rich region (Fig. 4*C*), but no significant similarity was found to other serine/threonine phosphatases, such as protein phosphatase 1 or 2A (35).

RNA Analysis of GRB-7. RNA blot analysis of GRB-7 in murine tissues is presented in Fig. 5. We probed oligo(dT)-selected mRNA with the same DNA fragment used to isolate full-length GRB-7. The major transcript was seen at 2.4 kb, which closely corresponds to the longest cDNA clone. The strongest signal for GRB-7 message was found in liver and

A

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  1  MELDLSPTHLSSSPEDVCPTPATPPETPPPPDNPPPGDVKRSQPLPIPSSRKLREEEFQA   60
 61  TSLPSIPWPFPELCSPPSQKPILGGSSGARGLLPRDSSRLCVVKVYSEDGACRSVEVAAG  120
121  ATARHVCEMLVQRAHALSDESWGLVESHPYLALERGLEDHEFVVEVQEAWPVGGDSRFIF  180
181  RKWFAKYELFKSPPETLFPEKMVSSCLDAQTGISHEDLIQNFLMAGSFPEIQGFLQLRGS  240
241  GRGSGRKLWKRFFCFLRRSGLYYSTKGTSKDPRHLQYVADVHESNVYVVTQGRKLYGMPT  300
301  DFGFCVKPHKLRNGEKGLHIFCSEDEQSRTCWLAAFRLFKYGVQLYKNYQQAQSRHLRLS  360
361  YLGSPPLRSVSDWTLVAMDFSGHAGRVIDNPREALSAAMEEAQAWRKKTNHRLSLPTTCS  420
421  GSSLSAAIERTQPWFHGRISREESQRLIGQQGLVDGVFLVRESQRNPQGFVLSLCHLQKV  480
481  KHYLILPSEDEGCLYFSMDEGQTRFTDLLQLVEFHQLNRGILPCLLRHCCARVAL       535
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B

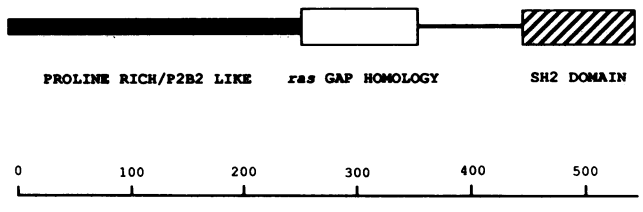

FIG. 3. Primary structure of GRB-7. (*A*) GRB-7 protein sequence. Start site of the initial GRB-7 clone 66 is indicated with arrow. This clone encompasses amino acids 297–535, including the SH2 domain (boxed). To complete the sequence, DNA hybridization was used to select several additional clones, the longest being a full-length clone of 2.3 kilobases (kb). This clone was sequenced in both directions with the open reading frame beginning at base 369. (*B*) Schematic representation of GRB-7, including various sequence motifs.

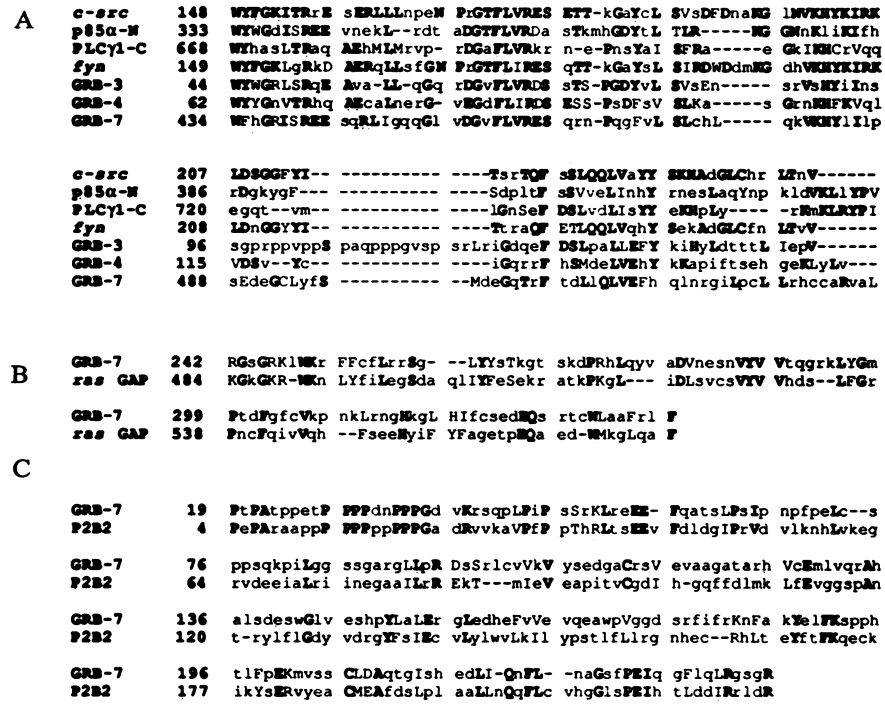

FIG. 4. Similarity of GRB-7 to other proteins. (*A*) Alignments of SH2 domains from avian c-src, human GRB-1/p85α, human PLC-γ1, mouse fyn, and GRB-3, GRB-4, and GRB-7. For GRB-1/p85α, N indicates amino-terminal SH2 domain, and for PLC-γ1, C indicates carboxyl-terminal SH2 domain. Alignment was done with multiple sequence alignment programs LINEUP, PILEUP, and PRETTY with c-Src as the reference sequence. (*B*) Similarity in sequence between the noncatalytic central region of human ras-GAP (30) and GRB-7. (*C*) Alignment of human protein phosphatase 2B (P2B2; ref. 31) and GRB-7 protein sequences. Alignments in *B* and *C* were done with BESTFIT. Capital letters indicate conservative substitutions, whereas boldface letters indicate identity; conservative substitution is defined in text. Dashes represent gaps inserted to optimize alignments.

kidney, and a lower level of GRB-7 mRNA was detected in ovary and testes. On longer exposure, a weak signal was detected in lung but was not detected in heart, muscle, spleen, or brain.

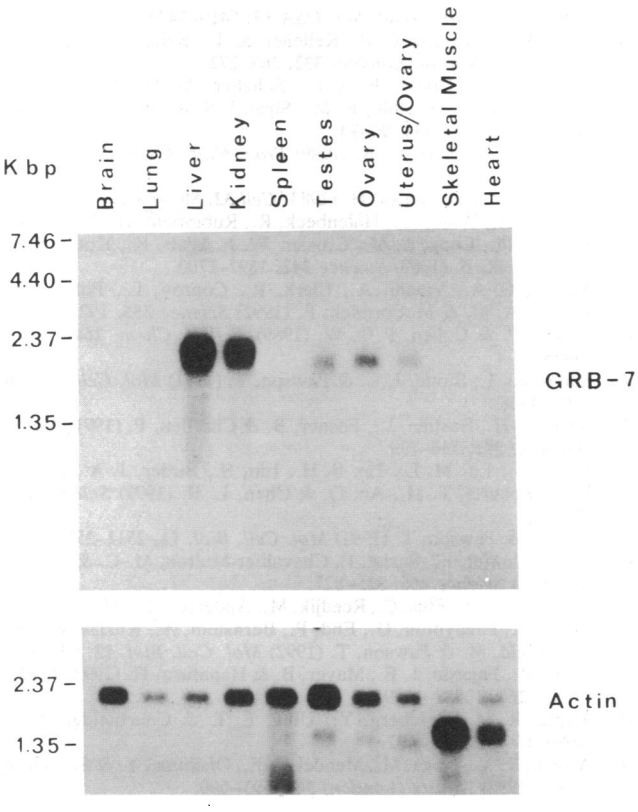

FIG. 5. RNA blot analysis of GRB-7 mRNA. The mRNA was extracted from 6-week-old murine tissues, separated on a 1.2% agarose-formaldehyde gel, and transferred to nylon membrane. The blot was then probed with a DNA fragment of GRB-7, as described. After exposure of the GRB-7 blot for 96 hr, blots were stripped, reprobed with actin, and exposed for an additional 36 hr. Kbp, kilobase pairs.

DISCUSSION

GRB-7 represents the third gene cloned using the CORT technology. It belongs to a relatively rare group of proteins with SH2 domains but with no SH3 domain including the fps tyrosine kinase (36), protein tyrosine phosphatase 1C (37), and possibly tensin (38). At this point, no functional properties can be attributed to GRB-7, but its limited tissue distribution suggests a specialized function for it. GRB-7 has two regions of similarity to ras-GAP, one being the SH2 domain and the other being a region that encompasses the central portion of ras-GAP and GRB-7. The ras-GAP homology region lies just carboxyl terminal to a tyrosine phosphorylation site (39) and has been proposed to have similarities with the adenylate cyclase of *Saccharomyces cerevisiae* (30). However, in our search of DNA and protein data bases, no other proteins significantly similar to this region of GRB-7 or ras-GAP could be identified. It has been hypothesized that ras-GAP may be a downstream effector of *ras*, in addition to its effect on the GTPase activity of *ras* (34, 40). It is possible that the region of similarity between ras-GAP and GRB-7 might serve such an effector function; however, others have localized an effector domain to the SH2 and SH3 regions (34). Thus, the function of this region remains unknown, and further studies on GRB-7 function will be necessary.

It is unclear how many SH2 domain proteins might exist, but in our screening six of nine clones were identical or highly similar to previously identified proteins. Two of these proteins, GRB-5 and GRB-6, were encoded by known genes, *fyn* and the mouse homologue encoding PLC-γ1, respectively. For GRB-3 and GRB-4, it is difficult to ascertain whether we have cloned the mouse homologue of crk and nck or whether they are distinct members of the same family. Certainly there is significant divergence in the sequence of the human NCK from murine GRB-4, and further analysis is required to resolve this issue.

Although the CORT methodology does not give clues to protein function, it points to proteins that might interact with the EGFR and lie downstream of the EGFR-signaling pathway. In general, *in vitro* associations between SH2 domain and tyrosine-phosphorylated proteins correlate with interactions in living cells (41). Indeed, several of the proteins cloned

in this work are known to associate with the EGFR. Certainly, the interaction of PLC-γ1 and the EGFR is well-established (4), and it has been demonstrated that crk (42) and NCK (W. Li, P. Hu, E.S., and J.S., unpublished results) can also interact with the EGFR. Fyn has not been demonstrated to interact with the EGFR, but it does bind to the platelet-derived growth factor receptor (43). Convincing evidence that the CORT methodology can yield important downstream signaling components of growth factor receptors is the finding that Sem-5 is a *C. elegans* homologue of human GRB-2. The *sem-5* gene was shown crucial for *C. elegans* vulval development (15), a process that also requires the activity of *let-23*, an EGFR-like tyrosine kinase (44). A reasonable hypothesis is that *sem-5* lies downstream of the activated *let-23* and that GRB-2 serves a similar crucial function in EGFR signaling (46). Accordingly, examining the interaction of GRB-7 with EGFR and other growth factor receptors will be interesting. Preliminary results indicate that the GRB-7 SH2 domain expressed as a GST fusion protein can bind both platelet-derived growth factor receptor and EGFR. One fact that should be considered, however, is the interaction between EGFR and p85/GRB-1. We could clone p85/GRB-1 by CORT on the basis of its binding to EGFR, and when overexpressed in cells, p85/GRB-1 binds well to the EGFR (12, 45). However, in nontransfected cells, p85/GRB-1 binds poorly to the EGFR, probably due to interactions between p85/GRB-1 and the 110-kDa subunit of phosphatidylinositol 3′-kinase (13).

In summary, we have expanded the use of CORT to identify additional SH2 proteins that interact with the EGFR. To date, we have identified seven different SH2 domain proteins with this method, and these molecules, such as p85/GRB-1 and GRB-2, appear to have important signaling functions. With the use of the T7 polymerase-based library, this methodology may be more easily applied to other growth factor receptor systems. Moreover, T7 polymerase-based libraries should prove beneficial to others applying expression/cloning techniques to study protein–protein or DNA–protein interactions.

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