Critical intracellular signals in normal and malignant cells are transmitted by the adapter protein Grb2 by means of its Src homology 2 (SH2) domain, which binds to phosphoryltyrosyl (pTyr) residues generated by the activation of tyrosine kinases. To understand this important control point and to design inhibitors, previous investigations have focused on the molecular mechanisms by which the Grb2 SH2 domain selectively binds pTyr containing peptides. In the current study, we demonstrate that the Grb2 SH2 domain can also bind in a pTyr independent manner. Using phage display, an 11-amino acid cyclic peptide, G1, has been identified that binds to the Grb2 SH2 domain but not the src SH2 domain. Synthetic G1 peptide blocks Grb2 SH2 domain association (IC50 10–25 μM) with a 9-amino acid pTyr-containing peptide derived from the SHC protein (pTyr317). These data and amino acid substitution analysis indicate that G1 interacts in the phosphopeptide binding site. G1 peptide requires a YXX sequence similar to that found in natural pTyr-containing ligands, and phosphorylation of the tyrosine increases G1 inhibitory activity. G1 also requires an internal disulfide bond to maintain the active binding conformation. Since the G1 peptide does not contain pTyr, it defines a new type of SH2 domain binding motif that may advance the design of Grb2 antagonists.

The binding characteristics of Src homology 2 (SH2) 1 domains determine their important role as regulators of intracellular signaling (1, 2). Signal flow requires a phosphoryltyrosyl (pTyr) residue in the target protein for binding by the SH2 domain (1, 3, 4). Interaction of SH2 domains with specific pTyr-containing proteins activates distinct signaling pathways. SH2 domains modulate the activities of c-src (5), alter the substrate specificity of c-abl proto-oncoproteins (6, 7), and transduce signals initiated at growth factor receptors (8) and cellular attachment systems (9). SH2 domains have been suggested as promising sites for therapeutic intervention (10). Consequently, there has been significant effort to understand the structural basis of SH2 domain binding to pTyr-containing targets (11–21).

The Grb2 SH2 domain binds pTyr-containing motifs within several proteins including the adapter proteins SHC (22, 23), growth factor receptors such as members of the erbB family (23–27), morphology-determining proteins such as FAK (9), and cellular oncoproteins such as BCR-abil (25, 28). SH2 domain binding leads to activation of important downstream pathways by bringing the nucleotide exchange factor SOS1 to the membrane environment of p21ras (29). Other pathways may be initiated through action of the Grb2 SH3 domain as well. These pathways are suggested by experiments showing that the SH3 domains of Grb2 can bind to other proteins including dynamin (30), Vav (31, 32), Cbl (33), and several as yet unidentified targets (34). A particularly important role for Grb2 in human cancer has been proposed for cells transformed by high levels of erbB2 (HER-2 or neu) expression (35, 36). In these cells, the SH2 domain of the Grb2 protein is primarily associated with pTyr residues on p52SHC and on the p185erbB2 (34–36). Recent studies have indicated that Grb2 function is required for cell transformation by the neu and bcr-abil oncoproteins (37, 38). Moreover, communication by the epidermal growth factor receptor to the mitogen-activated protein kinase can be inhibited by interference at the Grb2 SH2 domain (39). In this study we identify a new small nonphosphorylated peptide motif that can selectively bind the Grb2 SH2 domain and block its function.

**EXPERIMENTAL PROCEDURES**

Phage Display—The library was constructed to contain a variable 9-amino acid peptide flanked by cysteine residues inserted into the GeneIII protein of the phage fUSE5 (40). The Grb2 protein used to isolate the G1 phage was generated as a GST fusion protein using recombinant expression vectors in Escherichia coli (34). To isolate Grb2 binding phage, we used standard methodology (40). Briefly, over 1010 phage particles of the library containing over 109 individual members were allowed to bind to the recombinant GST-Grb2 protein. The GST-Grb2 proteins and bound phage were collected on glutathione-Sepharose. Unbound phage were removed by washing. Bound phage were eluted and allowed to infect E. coli, and a mixture of phage were collected from the resulting tetracycline-resistant colonies. Phage capable of interacting with GST or glutathione-Sepharose were then allowed to bind to GST-loaded glutathione-Sepharose and discarded. Binding of phage to GST-Grb2 was repeated twice. Following the third GST-Grb2 binding step, 18 phage clones were isolated and subjected to nucleotide sequencing. The variable region of each phage showed an identical nucleotide sequence and predicted protein sequence. The G1 phage was shown to bind to immobilized GST-Grb2 in an ELISA assay. No binding was seen with immobilized GST or recombinant GST fusion proteins containing the...
G1Cys-Ser peptide, an open chain analogue of G1, contains serines in place of cysteine residues, and G1 Cys-Ser peptide was cyclized by intramolecular nucleophilic displacement of the chloro group by cysteine thiol. The peptide was attached to SA5 chips at 2 nM. Binding of GST-Grb2 was conducted at 200 nM in HBS buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% P-20 surfactant, Pharmacia Biotech) at flow rate of 5 μl/min for 10 min. Total Ru change for GST-Grb2 or GST-Grb2SH2 binding to SHC phosphopeptide in the absence of inhibitors was 200–500 Ru. Peptides at the indicated concentrations were pre-mixed with the GST-Grb2 prior to introduction onto the SPR chip. The SHC phosphopeptide, DDPSpYVNVQ, was obtained from Quality Control Biochemicals. The G1 peptide was shown to be in the disulfide-linked form by mass spectrometry (molecular weight = 1453) by mass spectrometry. This peptide was attached to SA5 chips at 2 nM. Binding of GST-Grb2 was conducted at 200 nM in HBS buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% P-20 surfactant, Pharmacia Biotech) at flow rate of 5 μl/min for 10 min. Total Ru change for GST-Grb2 or GST-Grb2SH2 binding to SHC phosphopeptide in the absence of inhibitors was 200–500 Ru.

Synthesis of G1TE—Generation of G1TE used methods previously described for the generation of thiocysteine cyclized peptides (42). Briefly, the peptide ELYENVGMYS was synthesized by standard solid phase methods. Cleavage from the resin was preceded by chloroacetylation of the peptide ELYENVGMYC was synthesized by standard solid phase methods. Cleavage from the resin was preceded by chloroacetylation of appropriate molecular weight (1453) by mass spectrometry. The peptide was attached to SA5 chips at 2 nM. Binding of GST-Grb2 was conducted at 200 nM in HBS buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% P-20 surfactant, Pharmacia Biotech) at flow rate of 5 μl/min for 10 min. Total Ru change for GST-Grb2 or GST-Grb2SH2 binding to SHC phosphopeptide in the absence of inhibitors was 200–500 Ru. Peptides at the indicated concentrations were pre-mixed with the GST-Grb2 prior to introduction onto the SPR chip. The SHC phosphopeptide, DDPSpYVNVQ, was obtained from Quality Control Biochemicals. The G1 peptide was shown to be in the disulfide-linked form by mass spectrometry (molecular weight = 1321) and C18 chromatography. The G1 Cys-Ser peptide, an open chain analogue of G1, contains serines in place of cysteine residues, i.e. SELYENVGMYS. The SHC control peptide, DDPSpYNVQ, was nonphosphorylated. Equilibrium binding Ru was determined at 20 s following the last GST-Grb2 flowing across the chip. Two experiments were conducted using separate sensor chips and peptide dilutions.

**Synthesis of G1TE**—Generation of G1TE used methods previously described for the generation of thiocysteine cyclized peptides (42). Briefly, the peptide ELYENVGMYS was synthesized by standard solid phase methods. Cleavage from the resin was preceded by chloroacetylation of the terminally deprotected Glu residue. After cleaving and deblocking, the peptide was cyclized by intramolecular nucleophilic displacement of the chloro group by cysteine thiol.

**G1TE Inhibition of Grb2 SH2 Domain Function**—Cell lysates were prepared from serum-treated erbB2 overexpressing breast (MDA-MB-453) cancer cells using 1% Triton X-100 in PBS containing 0.2 mM NaVO<sub>4</sub>. Lysates were incubated with 3.1–400 μM of G1TE or 200 μM of SHC phosphopeptide or G1 Cys-Ser control peptide for 20 min. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate (5 mg) with anti-Grb2 antibodies and collected using protein A-Sepharose using methods previously described (34). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on 8–16% gradient gels (Novagen). pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies. The major pTyr-containing proteins in Grb2 immunoprecipitates are indicated by arrows. Previous experiments have shown that the major tyrosine-phosphorylated protein of this size in these cells is the p185<sup>erbB2</sup>, which is overexpressed as a consequence of gene amplification (43).

**RESULTS**

**Identification of Grb2 SH2 Domain Binding Peptides by Phage Display**—We screened a random peptide phage display library using a recombinant GST fusion protein of Grb2. Since the goal was to identify small molecular weight binding structures, the library was generated with a variable 9-amino acid region flanked by cysteine residues. These cysteines can undergo intramolecular disulfide bond formation, thereby resulting in cyclized peptides with limited conformational flexibility compared with linear peptides. Such phage display libraries have been used to isolate high affinity ligands for other proteins including integrins (44, 45) and cell surface receptors (46,
47). The library used in this study contained over 10^7 different sequences. Standard techniques were used to screen the phage library in three rounds of affinity selection (40). We determined the nucleotide sequence of 18 independent phage isolates that bind to GST-Grb2 and all were identical (Fig. 1). No binding to the N-terminal or C-terminal SH3 domains or GST alone was found using an ELISA type assay (data not shown) suggesting that the phage interacted with the SH2 domain. No tyrosine phosphorylation was present on the G1 phage using antiphosphotyrosine antibodies (data not shown). A disulfide bond in G1 is required for efficient binding of Grb2 to the G1 phage as pretreatment of phage with DTT reduced binding (Fig. 2). DTT did not reduce binding of Grb2 to the SHC phosphopeptide. We examined the binding of GST-Src fusion proteins to the G1 phage using an ELISA assay. As shown in Fig. 3, the GST-Grb2 protein binds well to the G1 phage immobilized on the plate, whereas the src-GST protein exhibits no apparent binding. This suggests that the affinity of the Src SH2 domain is at least 100-fold less than that of Grb2 SH2 domain for the G1 phage.

**Isolated G1 Peptides Require Conformational Constraint and a YXN Motif**—We chemically synthesized a peptide corresponding to the peptide displayed on the G1 phage and isolated a form containing the cysteine residues oxidized to a disulfide bond. This G1 peptide was used as an inhibitor in surface plasmon resonance (Biacore) studies to monitor the Grb2/SHC phosphopeptide interaction. In these experiments, the SHC phosphopeptide was attached to the solid surface and GST-Grb2 was allowed to bind. These methods have been previously used for analysis of Grb2 binding to SHC (48) as well as other SH2 domain interactions (41, 49, 50). The IC_{50} values obtained are measures of the solution phase interaction of Grb2 SH2 domains with the inhibiting peptides. The SPR serves as a detector of free active SH2 domain. The method does not suffer from overestimation of affinity encountered in some methods where association and dissociation rates at the surface are used to calculate K_D. As shown in Fig. 4, A and B, when the G1 peptide and SHC phosphopeptide are premixed in solution with the GST-Grb2 SH2 domain, they inhibit interaction with the SHC phosphopeptide at the surface. Fig. 4C suggests an IC_{50} of approximately 25 μM for the G1 peptide. Premixing of soluble SHC phosphopeptide with GST Grb2(SH2) provides a standard (IC_{50}...
approximately 2 µM). Fig. 5 shows a similar analysis in which peptides inhibit binding of recombinant protein containing the intact Grb2 fused to GST. An IC50 of approximately 10 µM is obtained for the G1 peptide. Similar results are observed for recombinant intact Grb2 obtained by cleaving with thrombin (data not shown). These experiments indicate that the G1 peptide sequence binds in nonphosphorylated form to the Grb2 SH2 domain in a manner that blocks binding to a relatively short SHC phosphopeptide ligand. This suggests that G1 binds in, or very close to, the phosphopeptide binding pocket. No affinity was measurable for a nonphosphorylated SHC peptide sequence or for a peptide in which the cysteine residues of G1 were replaced by serine (Fig. 5). This confirms results shown in Fig. 2 indicating that the disulfide bond of G1 is required for Grb2 interaction of the free peptide as well as the phage bound G1 peptide. The results demonstrate that the conformational constraint imparted by the disulfide bond in the G1 peptide is required for the affinity of the G1 peptide for the Grb2 SH2 domain.

The alignment of the G1 sequence, shown in Fig. 1, with Grb2 phosphopeptide ligands suggests the importance of tyrosyl and asparagine residues at positions 4 and 6, respectively. These residues have also previously been shown to be required for high affinity binding of pTyr-containing peptides to Grb2 SH2 domain (51). We tested the requirement of these amino acids in the G1 peptide sequence by synthesizing peptides with alanine substitutions at these positions. As shown in Fig. 6, replacement of the tyrosine or asparagine residues of G1 greatly diminish its ability to bind Grb2 and block association with SHC phosphopeptide. No inhibiting activity was seen at up to 500 µM. These results strongly suggest that G1 binds the ligand binding region of the Grb2 SH2 domain using some of the same amino acid contacts as pTyr-containing peptides. Alanine substitutions were also made at other positions in the G1 peptide. The ability of these peptides to interfere with Grb2 binding to the SHC phosphopeptide is shown in Fig. 7. A comparison of the effects of alanine substitution is shown in Fig. 8. Except for replacement of glycine 8, all substitutions reduced but did not eliminate the inhibitory activity of the peptide. The elimination of binding activity by alanine substitution of position 4 tyrosine and position 6 asparagine strongly suggest they are directly involved in binding the SH2 domain. The other substitutions may alter the conformation of the G1 peptide, or they may eliminate important side chain interactions that improve Grb2 SH2 domain binding.

To confirm that the G1 peptide binds directly in the phosphopeptide binding pocket, we synthesized a G1 peptide with a phosphotyrosyl residue at position 4. As shown in Fig. 9, the resulting peptide inhibits Grb2 interaction more potently than G1 or the SHC phosphopeptide. These results indicate that G1 binds directly in the phosphopeptide binding pocket of the Grb2 SH2 domain. They also suggest that the constrained conformation or additional side chain contacts of G1 provide for additional affinity in comparison to the SHC phosphopeptide.
A Stabilized G1, G1TE, Blocks SH2 Domain Function in Cell Extracts

To verify that the interaction between G1 and Grb2 is sufficient to inhibit the Grb2 SH2 domain association with intact phosphoproteins, we conducted experiments in cell extracts. We used the cell line MDA-MB-453 in which an overexpressed p185\textsuperscript{erbB-2} (43) generates abundant autophosphorylation (34). Since we were concerned that the G1 peptide requires a disulfide bond and reduction might occur in the cell lysate conditions, we synthesized a similar molecule, G1TE, in which the disulfide structure has been replaced by a thioether bond. Binding of G1TE to GST-Grb2 and inhibition of SH2 function \textit{in vitro} demonstrate similar IC\textsubscript{50} values compared with G1 (Fig. 10). When added to cell lysates, the G1TE molecule is able to diminish the amount of p185 pTyr-containing protein that co-immunoprecipitate with Grb2. SHC phosphopeptide also showed similar ability, SH2 domains associate with phosphopeptides with high affinity but display very fast dissociation and association kinetics. Thus a "preformed" complex of Grb2 bound to p185\textsuperscript{erbB-2} can be sensitive to peptides that prevent the reassociation component of the equilibrium. The relative effectiveness of G1TE in these assays is comparable to that seen in the SPR analysis with partial interference at 50 \textmu M (Fig. 10). No effect of peptides was seen on binding of Grb2 to SOS1, an SH3 domain mediated interaction (34, 52–54). These results indicate that the association of Grb2 with p185\textsuperscript{erbB-2} can be prevented by small nonphosphorylated peptides like G1TE.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Comparison of the activity of alanine substituted G1 peptides. The results shown in Figs. 7 and 8 are displayed for inhibition at 100 \textmu M peptide. The inhibition by unsubstituted G1 peptide represents 100\%.
}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Demonstration that phosphorylation of G1 at position 4Y increases binding affinity. The binding of peptide to the Grb2 SH2 domain was measured by surface plasmon resonance (Pharmacia Biacore). The percent inhibition was calculated by comparison of the Ru\textsubscript{eq} in the presence of peptide with the Ru\textsubscript{eq} with no inhibiting peptide present.
}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig10.png}
\caption{Inhibition of complexes formed by the Grb2 SH2 domain in cellular extracts by G1TE peptide. A, the structure of the G1TE molecule containing a thioether linkage. B, the inhibition of Grb2 binding to the SHC phosphopeptide using surface plasmon resonance methods. C, addition of G1TE and SHC phosphopeptide to lysates of cells inhibits the immunoprecipitation of complexes between Grb2 and p185\textsuperscript{erbB-2}. The binding of Grb2 to SOS1 is unaffected by peptide addition. Cell lysates were derived from the breast cancer cell line MDA-MB-453, which contains an amplified \textit{erbB-2} gene and overexpressed p185\textsuperscript{erbB-2} (43).
}
\end{figure}
Our results are unexpected in that they show that small nonphosphorylated peptides can bind to SH2 domains with micromolar affinity. This raises several interesting possibilities for the study of SH2 domains. First, our findings suggest new approaches toward the design of SH2 domain antagonists. Based on previous indications of the requirement for pTyr residues within SH2 domain ligands, synthetic efforts have focused on creating nonhydrolyzable pTyr analogues. Compounds containing a difluoromethyl group in place of the phosphate ester oxygen of pTyr (F2PMP) are stable and bind well to the targeted SH2 domain (55). However, the activity of these and other similar compounds when applied to intact cells is limited (56). Alternatively, other nonphosphorus-containing pTyr mimetics, such as O-malonyl tyrosine (57), are highly charged at physiological pH, and would not be expected to exhibit potent effects on intact cells. Therefore, our identification of G1 addresses one of the primary limitations of current strategies for the development of antagonists of SH2 domains, namely, poor cell penetration due to the highly charged moiety. We do not consider the G1 or G1TE molecules to be cell permeable drugs in their current form, since peptides can have limitations in vivo. Alternatively, we see the G1 interaction with the Grb2 SH2 domain as a means to assess and potentially exploit the contribution of interactions not requiring a ligand phosphate group. To this end, design and synthesis of peptide mimetics of G1 are in progress.

A second novel aspect of small nonphosphorylated peptide binding to SH2 domains is that it suggests that SH2 domains may have naturally occurring peptide ligands that are non-phosphorylated. Very little is known about whether such proteins exist. Some clues are present in previous studies that suggest that the specificity for phosphotyrosyl-containing proteins is not absolute. The SH2 domain of lyn and blk can bind phosphorylated serine or threonine residues (58, 59). A recent report of a nonphosphorylated protein binding to p56

\[ \alpha _{56} \] indicates that such interactions can exist as do phosphotyrosyl-independent interactions of the amyloid precursor protein with the phosphotyrosine binding domain of FE65 (60). Since the original submission of this manuscript, a version of the SHC

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