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# SIGNIFICANT COMPENSATORY ROLE OF POSITION Y-2 CONFERRING HIGH AFFINITY TO NON-PHOSPHORYLATED INHIBITORS OF GRB2-SH2 DOMAIN

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**Abstract**: Systematic modification of amino acid at position Y-2 of a library-derived non-phosporylated thioether-cyclized peptide, cyclo($CH_2CO$-$Glu^{-2}$-Leu-$Tyr^{0}$-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide, aided by molecular modeling, demonstrates that the $Glu^{-2}$ sidechain compensates for the absence of $Tyr^{0}$ phosphorylation in retaining effective binding to Grb2-SH2 domain. Replacement of $Glu^{-2}$ with γ-carboxy-glutamic acid produced a high affinity inhibitor, the first example with submicromolar affinity ($IC_{50}$ = 640 nM).


**Introduction**: Growth factor receptor-bound protein 2 (Grb2) mediates intracellular signaling by its Src homology 2 (SH2) domain binding to phosphotyrosyl (pTyr) motifs on growth factor (GF) receptors such as EGFR and members of *erb*B family, and this leads to downstream activation of the mitogenic Ras pathways.[1] One approach toward inhibiting Grb2 function, and thereby down regulating its associated mitogenic effects, is to block the binding of its SH2 domain to pTyr target residues, which provides a promising target for development of antitumor agents.[2] Indeed, an impressive number of such inhibitors have been reported recently, but still containing pTyr or pTyr mimics as a key requirement in peptides or peptidomimetically modified structures.[3-7] Finding potent non-phosphorylated inhibitors of Grb2-SH2 domain has continued to be a challenge,[8] one example being a family of actinomycin derivatives.[9] These antibiotics, however, also possess unrelated and strong biological properties. Our approach is based on our earlier reported discovery of phage library-based non-phosphorylated cyclic peptide prototype that bound to the Grb2-SH2 protein with 10~25 μM affinity, and was comprised of a 9 a.a. long sequence motif, **$E^{-2}$-L-$Y^{0}$-E-$N^{+2}$-$V^{+3}$-G-M-Y,** flanked by 2 terminal disulfide linked cysteines.[10,11] This sequence has only $N^{+2}$ and $V^{+3}$ in common with **-P-S(E)-pY-X-N-V-** found in Grb2-binding phosphorylated regions of proteins,[10] but **Glu** in position Y-2 is unique in this novel non-

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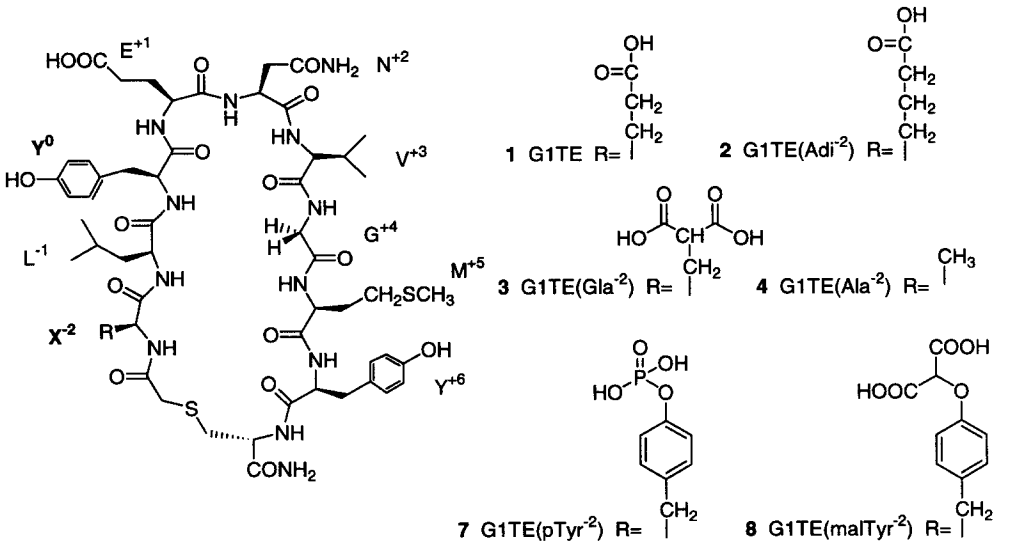
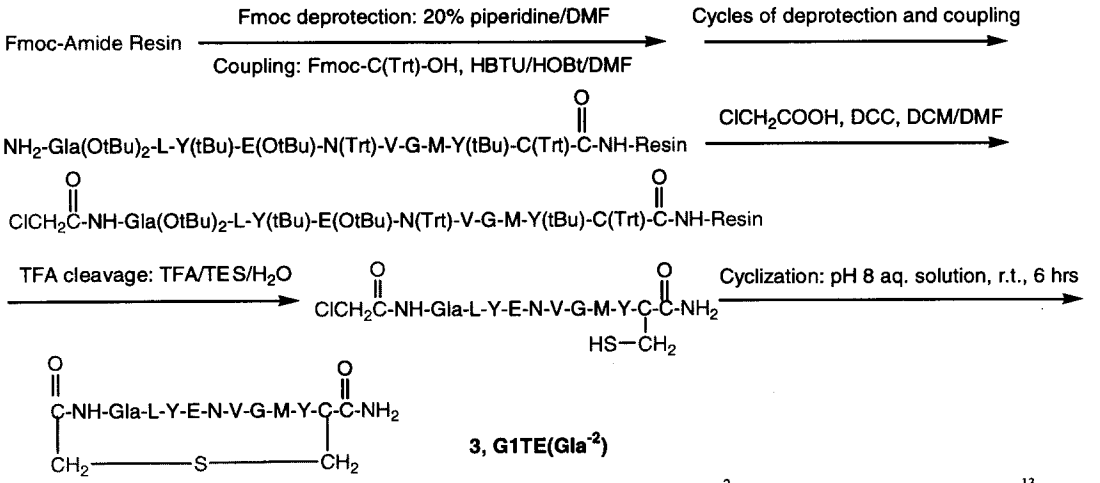
phosphorylated ligand. Starting from its redox-stable thioether cyclized analog termed **G1TE** (Figure 1), which exhibited equipotent binding but in addition inhibited the association of the Grb2 protein with the growth factor receptor $p185^{erbB\text{-}2}$ in cell lysates derived from the breast cancer cell line MDA-MB-453,[10,12] we initiated more comprehensive structure~activity studies in order to further improve the activity and explore the salient molecular features required by this new type of SH2 domain binding motif in a pTyr independent manner. Contrary to the conventional efforts on the amino acids C-terminal to phosphotyrosine in ligands of SH2 domains, our present work discloses the functional importance of specific amino acids N-terminal to the concensus sequence of **$-Y^0$-X-$N^{+2}$-** in the non-phosphorylated cyclic peptide. We report here the significant compensatory function of $X^{-2}$ for phosphotyrosine, thus providing high affinity inhibitors of Grb2-SH2 domain without phosphate or phosphate mimicking functionality.

**Figure 1.** The structures of G1TE and its analogs modified in position -2




**Materials and Methods:**

**Peptide ligands.** The synthesis of cyclic thioether peptides **1~8** was carried out in a manner similar to that reported previously (Scheme 1).[12] The PAL amide resin and Fmoc derivatives of standard amino acids were obtained from Perkin~Elmer/Applied Biosystems Division. Fmoc-γ-carboxy-L-Glu(O*t*Bu)$_2$-OH, Fmoc-L-α-aminoadipic acid-δ-*t*-butyl ester and Fmoc-L-tyrosine(O-malonyl-di-O*t*Bu)-OH were purchased from BACHEM (Torrance, CA, USA). Fmoc-L-tyrosine(PO(OH,Obzl))-OH from NOVAbiochem (La Jolla, CA, USA) was used for the synthesis of phosphotyrosine-containing analogs.




**Scheme 1.** The general synthetic scheme for cyclic peptide **3** ($X^{-2}$ = L-γ-carboxyglutamic acid)[13]

**Binding affinity measurements using Surface Plasmon Resonance (SPR).** The competitive binding affinity of ligands for the Grb2-SH2 protein was assessed using Biacore Surface Plasmon Resonance (SPR) methods. $IC_{50}$ values were determined by mixing the inhibitor with recombinant GST-Grb2 SH2 protein and measuring the amount of binding at equilibrium to an immobilized SHC(pTyr-317) phosphopeptide in a manner similar to that reported previously.[10]

**Molecular Modeling.** Simulations were performed with the Insight II 97/Discover 3.0 modeling package from Molecular Simulations Inc., San Diego, CA, with the cff91-force field. The X-ray structure of the KPFpYVNV peptide ligand bound to the Grb2-SH2 was used as a starting geometry.[14] The positions of the backbone atoms, and the sidechain atoms of those residues in the turn region of the reference peptide sequence, (F-$pY^0$-V-$N^{+2}$-$V^{+3}$) that are identical in G1TE, were used as the initial atom positions of the sub-sequence L-$Y^0$-$E^{+1}$-$N^{+2}$-$V^{+3}$ in G1TE, since a turn structure has been predicted for this sub-sequence also.[15] The turn comprising consensus atoms include $Asn^{+2}$, which possesses extraordinary specificity for Grb2-SH2 binding of phosphopeptides.[3] Then, the remaining residues of G1TE were added to this model. The turn comprising atoms, – CO(Y), E, N, NH(V) –, and the protein atoms were kept fixed during the following minimization and 50 simulated annealing (SA) simulations. In each SA run a different random seed was used. The same minimized starting geometry was subjected to an MD simulation at 2000 K for 10 ps and consecutively cooled in 5K increments to 5 K during 195 ps. The final structure with the lowest energy was taken. The same procedure was repeated for the mutant $Gla^{-2}$ peptide **3**.

**Results and Discussion:** Lead peptide G1TE (**1**) and its various analogs substituted in position Y-2 were synthesized (Table 1). These include, extending the sidechain of Glu with a $CH_2$ moiety ($X^{-2}$ = α-aminoadipic acid, **2**); attaching one more carboxyl group to the Glu sidechain ($X^{-2}$ = γ-carboxyglutamic acid, **3**); or replacing

Glu with Ala in the presence or absence of phosphate on $Tyr^0$ to compare the interactive effects of $X^{-2}$ and $Y^0$ (**1,4,5,6**). Additional modifications included the strongly acidic relatively rigid pTyr residue, and the pTyr mimicking O-malonyl-tyrosine at position Y-2 (**7** and **8**). Our earlier Ala scan studies with the non-phosphorylated disulfide linked cyclic peptide indicated that replacement of $Glu^{-2}$ with Ala significantly decreased the binding affinity to Grb2-SH2.[6] The Ala substituted peptide **4** in the current study confirms those findings ($IC_{50}$ = 100 μM). Predictably, phosphorylation of $Tyr^0$ in G1TE greatly improved the affinity by a factor of 150-fold (**5**, $IC_{50}$ = 0.13 μM). Surprisingly, when $Glu^{-2}$ was replaced by Ala in the pTyr peptide **5**, an even higher affinity was observed (**6**, $IC_{50}$ = 0.023 μM), suggesting that the polar sidechain of $Glu^{-2}$ competes for the conformational space occupied by the $pTyr^0$ phosphate functionality. In further developments, extending the sidechains of $Glu^{-2}$ by incorporation of α-aminoadipic acid or of Gla into that position remarkably improves the binding efficacy by 6-fold and 31-fold, respectively, in the non-phosphorylated peptides **2** ($IC_{50}$ = 3.45 μM) and **3** ($IC_{50}$ = 0.64 μM). These results confirm the similar and overlapping roles of Glu (Adi, Gla) in position Y-2 and of the $pTyr^0$ sidechains. However, the $X^{-2}$ sidechains can not fully occupy the $pTyr^0$ binding pocket in the protein. For example, incorporation of pTyr (**7**, $IC_{50}$ = 27.5 μM) or pTyr mimic such as malonylTyr (**8**, $IC_{50}$ = 64.5 μM) in position Y–2 results in reduction in binding potency. The rigid aromatic moiety, shared by pTyr and malonylTyr, may be responsible for the loss of compensatory interaction with $pTyr^0$ because of a disfavored positioning of the malonyl or phosphate terminal groups. More likely, amino acids in position Y–2 that possess flexible alkyl chains can orient the polar carboxyl groups to an optimal position for bonding interaction with the protein.

**Table 1.** Grb2-SH2 Domain Inhibitory Activity of the Peptides **1~8**[a].

O=C-NH-$X^{-2}$-L-$Y^0$-E-N-V-G-M-Y-C-Amide (S—S)

| Compound | $X^{-2}$ | $Y^0$ | $IC_{50}$ (μM) |
|---|---|---|---|
| **1** | Glu | Tyr | 20 ± 5 |
| **2** | N-α-aminoadipic acid | Tyr | 3.45 ± 0.15 |
| **3** | γ-carboxyglutamic acid | Tyr | 0.64 ± 0.10 |
| **4** | Ala | Tyr | 100 ± 20 |
| **5** | Glu | pTyr | 0.13 ± 0.01 |
| **6** | Ala | pTyr | 0.023 ± 0.01 |
| **7** | pTyr | Tyr | 27.5 ± 6.5 |
| **8** | malonylTyr | Tyr | 64.5 ± 2.5 |

[a]The experiments were performed on a BIAcore 2000 instrument by the method described previously.[10] The results represent mean value of at least two independent experiments and are expressed as the concentration at which half-maximal competition was observed ($IC_{50}$).

Thermodynamic,[16] NMR[17], and X-ray[14]analyses have shown that when pTyr containing peptides bind to Grb2 SH2 domains, a β-turn conformation is taken up in the region of -$X^{-1}$-pTyr-$X^{+1}$-Asn- in these ligands. Also, it has been found consistently that the negatively charged pTyr oxygens form key binding interactions with the Arg67 (αA-helix) and Arg86 (βC-strand) residues of the protein and the three Ser residues 88, 90 and 96 of the protein, and consensus amino acid $Asn^{+2}$ of the ligands forms well conserved interactions as well. We have docked G1TE (**1**) and G1TE($Gla^{-2}$)(**3**) into the Grb2-SH2 binding pocket, assuming that $Tyr^{0}$ and $Asn^{+2}$ occupy near proximal binding sites before dynamic simulation, as found previously for phosphopeptides.[14] The energy minimized adduct structures display the most probable binding mode for G1TE (Figure 2), in which the $Glu^{-2}$ carboxyl group together with $Tyr^{0}$ interacts with both Arginines and with Serines 88 and 90 of the binding pocket. The docking results for G1TE($Gla^{-2}$) confirm that γ-carboxyglutamic acid in position Y–2 is favored over Glu, because the second carboxyl group can undergo additional interactions within the pTyr binding pocket.

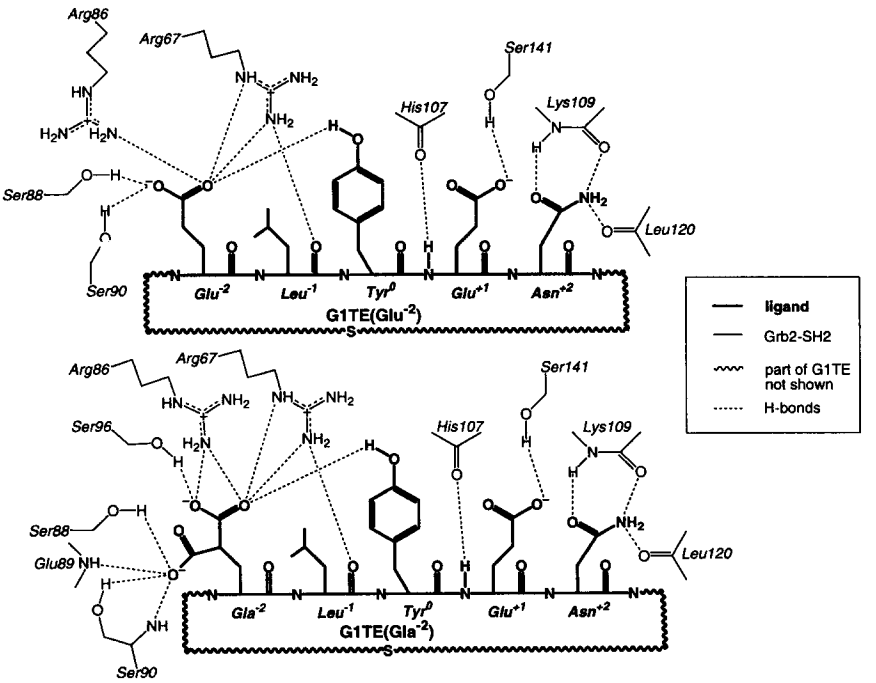



**Figure 2.** Schematic depiction of interactions between Grb2-SH2 and peptides **1** and **3**, based on molecular modeling.

**Conclusions:** In this study, we have successfully explored the functional importance of N-terminal Glu in the Y-2 position of the non-phosphorylated ligand, cyclo($CH_2CO$-$Glu^{-2}$-Leu-$Tyr^{0}$-Glu-Asn-Val-Gly-Met-Tyr-Cys)-amide with respect to its binding interactions with the Grb2-SH2 protein, and determined that the carboxyl sidechain in that position provides compensation for the absence of phosphate on $Tyr^{0}$. When extending the

sidechain of $Glu^{-2}$ by one $CH_2$ moiety ($X^{-2}$ = Adi, **2**), or adding one more carboxyl group to the sidechain ($X^{-2}$ = Gla, **3**), the resulting modified carboxyl sidechain(s) provide for more favorable ionic interactions with the guanidino functionalities of Arg67 and Arg86 in the protein. This results in moderate binding enhancement for peptide **2,** and significant increase in binding affinity for peptide **3.** These new agents are among the most potent non-phosphorous- and non-pTyr-mimic containing Grb2-SH2 domain inhibitors yet reported.

It is possible that regions of native proteins with clusters of acidic amino acids in the N-terminal vicinity of specific tyrosines can bind to SH2 domains in a pTyr independent manner. A convincing case was demonstrated recently by Rojas et al,[8] whereby a cell permeabilized non-phosphorylated peptide segment ($^{312}$F-D-D-P-S-Y-V-N-V-Q-N-$L^{323}$) of the Shc protein inhibited EGF induced Shc/Grb2 association in cells. Related mutation studies have also implicated the importance of $Asp^{313}$, $Asp^{314}$, and $Tyr^{317}$ for effective binding.

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11. Ligand residues are numbered relative to the position of phosphotyrosine, or putative phosphotyrosine, which is denoted 0. Positive numbers are used for amino acids C-terminal to phosphotyrosine, and negative numbers for amino acids N-terminal to phosphotyrosine. In our case, the consensus sequence for binding to Grb2-SH2 is YXN, in which Y is denoted 0.
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