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Inhibitors of RAS Signal Transduction as Antitumor Agents

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ABSTRACT. Anarchic cell proliferation, observed in some leukemia and in breast and ovarian cancers, has been related to dysfunctioning of cytoplasmic or receptor tyrosine kinase activities coupled to p21 Ras. The growth factor receptor-bound protein 2 (Grb2) adaptor when complexed with Sos (Son of sevenless), the exchange factor of Ras, conveys the signal induced by tyrosine kinase-activated receptor to Ras by recruiting Sos to the membrane, allowing activation of Ras. This review shows how it is possible to stop the Ras-deregulated signaling pathway to obtain potential antitumor agents. Grb2 protein is comprised of one SH2 surrounded by two SH3 domains and interacts by means of its Src homology (SH2) domain with phosphotyrosine residues of target proteins such as the epidermal growth factor (EGF) receptor or the Shc adaptor. By means of its SH3 domains, Grb2 recognizes proline-rich sequences of Sos, leading to Ras activation. Inhibitors of SH2 and SH3 domains were designed with the aim of interrupting Grb2 recognition. On the one hand, using structural data and molecular modeling, peptide dimers or "peptidimers", made up of two proline-rich sequences from Sos linked by an optimized spacer, were developed. On the other, using the structure of the Grb2 SH2 domain complexed with a phosphotyrosine (pTyr)-containing peptide and molecular modeling studies, a series of N-protected tripeptides containing two phosphotyrosine or mimetic residues, with one pTyr sterically constrained, were devised. These compounds show very high affinities for Grb2 *in vitro*. They have been targeted into cells showing selective antiproliferative activity on tumor cells. These results suggest that inhibiting SH2 or SH3 domains of signaling proteins might provide antitumor agents. BIOCHEM PHARMACOL **60**;8:1165–1169, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Grb2; SH2; SH3 domain inhibitors; antitumor agents; Ras signaling

GRB2 IN THE RAS SIGNALING PATHWAY AS A POTENTIAL ANTITUMOR TARGET

Grb2† is a small adaptor protein that is essential in the Ras signaling pathway [1]. It is made up of one SH2 domain of approximately 100 aminoacids surrounded by two SH3 domains, each containing about 60 aminoacids. The SH2 domain interacts with target proteins at the level of phosphotyrosine residues and the SH3 domains with proteins at the level of proline-rich sequences [2]. The key role of Grb2 is shown in Fig. 1, which illustrates the events triggered by the binding of a growth factor, e.g. EGF, to its receptor at the surface of the cell. The receptor is dimerized and phosphorylated on its C-terminal tyrosine residues. This allows recruitment under the membrane near the Ras protein of the Grb2/Sos complex. Sos, the exchange factor of Ras, is thus able to activate Ras under its GTP form. Then, Ras can recruit Raf and activate the MAPK cascade including the extracellular signal-regulated kinase (ERK) 1 and ERK 2 kinases, which can translocate into the nucleus after phosphorylation on tyrosine and threonine residues. In the nucleus, MAPKs activate early transcription factors, which promote cell division and/or differentiation, depending on MAPK level stimulation [3].

Grb2 constitutes a target for the design of therapeutic agents such as antitumor agents, because several proteins are found to be overexpressed or expressed by oncogenes in the Ras signaling pathway, which is activated by proteins with tyrosine kinase activity [4]. This is the case of the EGF receptor or its oncogenic analog HER2/neu, found in breast or ovarian cancers with a poor prognosis [5]. This is also the case of the oncogenic protein BCR-Abl, a product resulting from a translocation between the *BCR* and *Abl* genes, giving the Philadelphia chromosome [6]. This protein is found in 95% of patients suffering from chronic myeloid leukemia, and its transforming potential is related to stimulation of the Ras signaling pathway. Moreover, transfection, in HER2-transformed cells, of Grb2 mutants deleted from their N-terminal SH3 domain is able to reverse

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† *Abbreviations:* Grb2, growth factor receptor-bound protein 2; Shc, SH2 domain-containing adaptor protein; EGF, epidermal growth factor; Sos, Son of sevenless; MAPK, mitogen-activated protein kinase; ER22, EGF receptor-overexpressing clone 22; pY(pTyr), phosphotyrosine; and mAz, meta-amino benzyloxycarbonyl.

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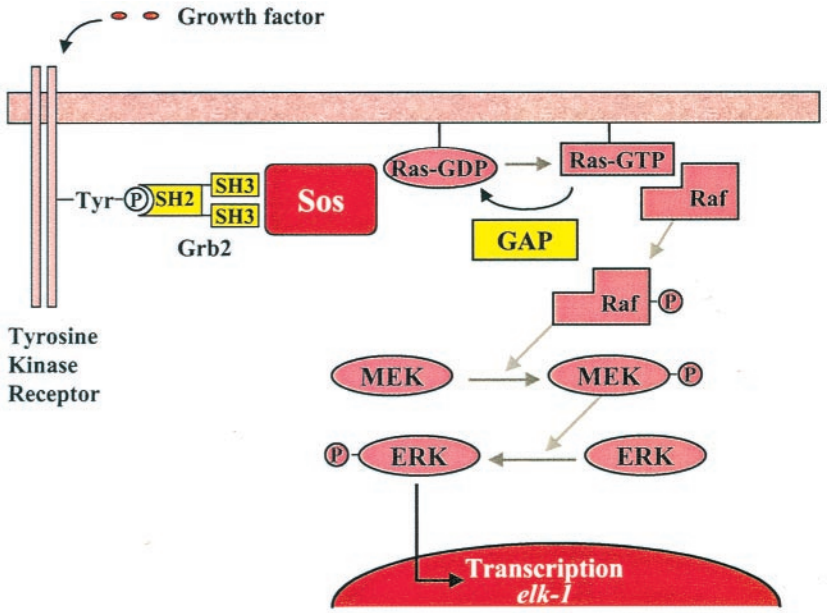

FIG. 1. Ras signaling pathway. This figure shows the key role of Grb2 in the Ras signaling pathway in response to growth factor receptor stimulation. After receptor dimerization and tyrosine transphosphorylation, the Grb2/Sos complex is recruited near the membrane, allowing the activation of Ras in its GTP form. When activated, Ras stimulates the MAP kinase cascade including Raf, MEK (MAPK kinase), and extracellular signal-regulated kinase (ERK) proteins. Phosphorylated forms of ERK translocate into the nucleus, activating early transcription factors such as *elk*-1, leading to cell proliferation and differentiation. The GTPase-activating protein (GAP) is a negative regulator of Ras, which allows Ras-GTP to Ras-GDP cycling.

the cellular phenotype [7]. All these observations support the fact that Grb2 is an oncogenic mediator and a good target for the design of antitumor drugs. There are two possible ways of inhibiting the Ras signaling pathway at the level of Grb2, by inhibiting either its SH3 domains or its SH2 domain. We first describe our strategy to inhibit Grb2/Sos interaction at the level of its SH3 domains and then report the results obtained in blocking Grb2 SH2 domain interaction with EGF-R or Shc.

INHIBITION OF GRB2 SH3 DOMAINS

The Sos sequence is comprised of four proline-rich regions. Each one shows a low affinity for the Grb2 SH3 domain (10^{-4} to 10^{-6} M), making it difficult to inhibit Grb2/Sos interaction by mimicking proline-rich peptides. The interactions between Grb2 SH3 domains and proline-rich peptides have been characterized in our laboratory and by other groups via NMR [8, 9]. The SH3 domains adopt a beta-barrel form stabilized by two perpendicular beta-sheets, each one made up of three beta-strands. In the complex with the Grb2 N-SH3 domain, the proline-rich peptide, which has the sequence VPPPVPPRRR, adopts a helical poly-L-proline II conformation. In this conformation, each aminoacid as i and i + 3 has its side chain in a parallel orientation. Therefore, Pro2, Val5, and Arg8 point towards the SH3 domain and interact with a specific recognition platform [9]. Moreover, Grb2 has been crystallized alone, its structure showing that the two SH3 domains are in close proximity [10]. Taking advantage of this proximity and of the geometry of the proline-rich peptide complexed with a Grb2 SH3 domain, we designed proline-rich peptide dimers which interact with both SH3 domains. When they can bind twice as monomers do, dimers are expected to have a high affinity for their target, which is the product of each monomer [11].

Using molecular modeling, two proline-rich peptides were docked, interacting with each SH3 domain according to the geometry determined by NMR, with the carboxy-terminal part of these peptides appearing adjacent to each other (Fig. 2). Thus, we linked them by a connector containing two amino groups, which was optimized as a lysine [12]. While the monomer (VPPPVPPRRR) is characterized by low affinity (K_d around 18 μM), the dimer, comprised of two sequences linked by their C-terminal carboxyl group by the amino groups of a lysine ($(VPPPVPPRRR)_2K$), shows an affinity for Grb2 in the range of 40 nM, which constitutes a more than 400-fold increase. The specificity of this interaction is shown by the fact that if the peptide dimer has the same content of aminoacids but one of the monomers does not have the

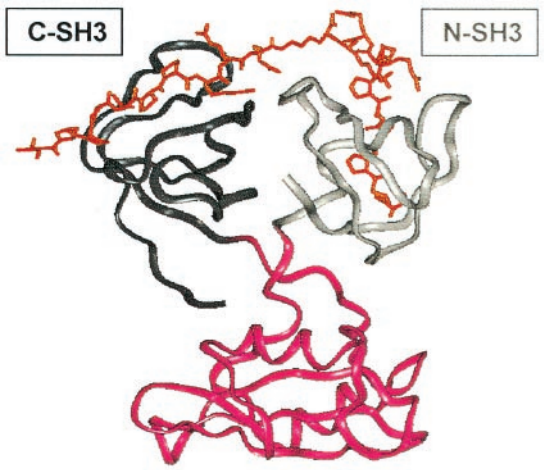

FIG. 2. Molecular modeling of Grb2 complexed with the proline-rich peptidimer [$(VPPPVPPRRR)_2K$] [12]. Grb2 is represented as ribbon (N-SH3 domain in gray, C-SH3 domain in black, and SH2 domain in pink), and the peptidimer backbone is in red.

consensus sequence PXXP, the affinity for Grb2 remains similar to that of a monomer [12]. These dimers, called "peptidimers," have the highest affinity reported so far for Grb2, which they share with compounds of a peptoid series. These compounds, reported by Nguyen *et al.*, were obtained from a combinatorial approach based on the YEVPPPVP-PRRR sequence in which the authors tested the importance of each aminoacid so as to improve SH3 recognition [13]. It was shown that replacing X by an N-(*S*)-phenylethyl glycine in the YEVPPPVXPRRR sequence gave a peptide with an affinity for the N-SH3 domain of Grb2 in the range of 40 nM. However, until now, no additional results concerning a potential effect of these compounds either *in vivo* or *in vitro* have been reported.

The subsequent evaluation of peptidimer effects on a cellular model was carried out. The monomer and peptidimer, containing one (VPPPVPPRRR) or two proline-rich motifs, respectively, were tested for their capacity to inhibit Grb2/Sos association by treatment of a homogenate of ER22 fibroblasts at increasing concentrations. The Grb2/Sos complex was pulled down from the cell lysate by using an Shc-derived phosphotyrosine peptide previously coupled to Sepharose beads and specifically binding the Grb2 SH2 domain, and revealed with an antibody directed towards Sos [12]. The Grb2/Sos complex was completely inhibited with 5 μM of the monomer while 50 nM of the dimer was sufficient, attesting to the superior efficiency of the peptidimer [12].

CELLULAR TARGETING

Although they were quite efficient on a cellular homogenate, peptidimers did not show cellular effects by themselves. Thus, it was decided to couple the VPPPVPPRRR dimer to a vector molecule. An increasing number of sequences have been reported which allow cell diffusion of their "cargo" molecule, e.g. penetratin [14], signal sequences [15], or Tat-derived sequences [16]. Penetratin, a peptide consisting of 16 aminoacids issued from the homeodomain of Antennapedia, a *Drosophila* transcription factor, was linked to a peptidimer [12]. The conjugate thus obtained was tested for its ability to inhibit the Grb2/Sos complex on ER22 fibroblasts. The cells were treated for 15 min with the conjugate and successively stimulated with EGF for 5 min. The cellular homogenate was subsequently tested for the presence of Grb2 complexed with Sos. This association was found to be completely disrupted by 10 μM pretreatment with the conjugate. Moreover, MAPK phosphorylation was largely decreased as expected (60%). Nevertheless, the division of ER22 cells, which are not tumoral cells, was not modified by the conjugate treatment, suggesting that other pathways might compensate for the interruption of Ras signaling at the level of Grb2.

In order to test the effect of the peptidimer conjugate on a tumor model of non-attached cells, its capacity to inhibit cellular growth on soft agar gel of NIH3T3 cells transfected with the oncogenic tyrosine kinase receptor HER2 was studied. The conjugate inhibited formation of colonies by the tumor cells with an efficient ED_{50} value around 1 μM. In this case, the interruption of Ras signaling, due to oncogenic tyrosine kinase receptor overexpression, is efficient and inhibits cell growth.

To conclude this first section, our results show that peptidimers conjugated with penetratin enter cells, inhibit Grb2/Sos interaction and MAPK phosphorylation, and display an antiproliferative effect on tumoral cells involving the Ras signaling pathway in response to tyrosine kinase protein activation. Very interestingly, the peptidimers do not seem to develop toxicity in systems which do not overexpress the Ras signaling pathway. Moreover, one of the advantages of this approach is the topologically controlled selectivity of Grb2 recognition.

INHIBITION OF THE GRB2 SH2 DOMAIN

In the second part, we investigated the possibility of inhibiting Grb2 at the level of its SH2 domain, using a model of molecular interaction with its target. This approach was expected to be easier because small phosphopeptides were reported to have affinities for SH2 domains in the 10^{-8}–10^{-7} M range. However, these peptides are negatively charged and do not enter cells easily. Moreover, cellular phosphatases are likely to degrade the phosphotyrosine group that is necessary for phosphopeptide activity. In our approach, we again used the three-dimensional structure of the Grb2 SH2 domain, complexed with a phosphopeptide [17], to design peptidomimetic inhibitors. The SH2 domains are highly folded structures containing one central beta-sheet crossing at the surface of the domain with two helices on each side. A recognition groove for phosphopeptides appears on the surface of the domain, where the pTyr (pY) side chain can interact with basic groups of the groove and the aminoacid C-terminal to pY can interact with other groups, providing the specificity of the recognition. In the case of the Grb2 SH2 domain, the presence of a tryptophan residue in the groove induces a large steric hindrance, allowing only phosphopeptides having a β-turn structure involving pY carbonyl and pY + 3 NH to be recognized. In order to design small molecules able to inhibit Grb2 interaction, we evaluated the importance of recognition parameters starting from the structure of Grb2 complexed with KPFpYVNV [17] in an attempt to improve them. One aminoacid N-terminal and two aminoacid C-terminals to pY, especially the Asn residue, appeared essential for Grb2 SH2 domain recognition. This was also inferred by Furet *et al.*, who designed different series of N-protected compounds, including the pY-X-N sequence with X as hydrophobic aminoacid and a *meta*-amino-benzyloxycarbonyl group (mAZ) as N-protection [18].

In our case, we exploited the fact that the Shc 239/240 sequence contains two Tyr residues followed by an Asn, which could be considered as consensus motif (pYXN) if X is replaced by a tyrosine or phosphotyrosine residue. Ac-

TABLE 1. Phosphopeptide affinities for Grb2

Peptide	K_d (nM)
mAZ-pTyr-pTyr-Asn-NH_2	60 ± 10
mAZ-pTyr-(α-Me)pTyr-Asn-NH_2	3 ± 1
mAZ-pTyr-(α-Me)Phe(4-CO_2H)-Asn-NH_2	45 ± 10
mAZ-pTyr-(α-Me)Phe(4-CH_2CO_2H)-Asn-NH_2	60 ± 10
mAZ-pTyr-(α-Me)Phe(4-PO_3H_2)-Asn-NH_2	4.5 ± 1.2
mAZ-pTyr-Ac_6c-Asn-NH_2*	30 ± 5

*mAZ-pTyr-Ac_6c-Asn-NH_2 was designed by the group of Novartis [20]. We have synthesized this compound for comparison with other derivatives in the same test. Ac_6c, 1-aminocyclohexanecarboxylic acid.

The affinity of the phosphopeptides for Grb2 has been measured using a fluorescent test [19] and is expressed as K_d (dissociation constant) value in nM.

cordingly, compounds with the sequence pY-pY-N, ended by mAz as N-terminal-protecting moiety and by an amino group in the C-terminal part to allow formation of a β-turn, were designed. The series was further optimized to increase the affinity for the Grb2 SH2 domain [19]. Both to this end and to promote the β-turn structure, an α-methyl substitution was included on the second pY. The affinity of the phosphopeptides was measured through a fluorescent test and compared to that obtained by Garcia-Echeverria *et al.* [20] for mAz-pY-Ac_6c-N-NH_2 (Ac_6c: 1-aminocyclohexanecarboxylic acid).

The phosphopeptide mAZ-pY-(αMe)pY-N-NH_2 exhibits one of the highest affinities for the SH2 domain of Grb2 reported so far. In order to explain its important increased affinity (Table 1), the peptide was docked on the surface of the SH2 domain. Reduction in peptide flexibility appears to provide a favorable orientation for additional stabilizing interactions between the phosphate group of the (αMe)pTyr and the Asn 143 and Arg 142 residues [19]. These results were recently confirmed by x-ray data of the complex.* The examination of this complex will make it possible to optimize the design of peptido-mimetics and cyclic analogs as was done in the case of Src SH2 domain inhibitors [21].

Other modifications were performed in our series, such as the replacement of the phosphate group of the (αMe)pY by a carboxylate or a methylcarboxylate substituent. This led to only an approximate 10-fold lower affinity for the SH2 domain. This decrease is likely related to the presence of only one negative charge on the carboxylate group, which could reduce the interactions with the Asn 143 and Arg 142 side chains [19]. Recently, we obtained a new and exciting result [22] by introducing a phosphonate group in the *para* position of the (αMe)pY, which provides a direct binding of the phosphorus atom to the aromatic ring. This compound has a very high affinity for the Grb2 SH2 domain (Kd = 4.5 nM).

Finally, the compound mAZ-pY-(αMe)pY-N-NH_2, which showed the highest affinity for Grb2, was chosen in order to prepare a prodrug, in which the phosphate groups were protected with *S*-acetyl thioethyl ester (SATE). This modification was used by the group of Imbach [23] to deliver the HIV antiviral drug into cells, and we successfully used it to deliver phosphopeptides into tumor cells [24]. The prodrugs thus obtained are very hydrophobic and can easily enter cells, where they are degraded by esterases, which cut the thioester group, and the chemically unstable intermediates liberate the active drugs. The twice-modified prodrug, mAZ-pY(SATE)$_2$-(αMe)pY(SATE)$_2$-N-NH_2, has no affinity for the SH2 domain of Grb2 in this prodrug form. However, it is able at a very low dose ($ED_{50} < 0.1$ μM) to inhibit the growth of NIH3T3 cells transfected by the oncogene *HER2* in colonies on soft agar [22].

In conclusion, these results show that inhibiting Grb2 SH2 or SH3 domains provides antiproliferative compounds and support the assumption that these molecules may constitute new potential antitumor agents.

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