Neuroglobin as a regulator of mitochondrial-dependent apoptosis: A bioinformatics analysis

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Received September 19, 2013; Accepted November 1, 2013

DOI: 10.3892/ijmm.2013.1564

Abstract. Apoptosis represents the key mechanism for the removal of surplus, damaged, or aged cells, and deregulated apoptosis has been implicated in the etiology of diverse pathologies. There are two main pathways which are known to initiate apoptosis: the death receptor-dependent (extrinsic) pathway and the mitochondrial-dependent (intrinsic) pathway. In the intrinsic pathway, as a response to diverse signals from the cellular environment, a permeabilization of the mitochondrial outer membrane occurs, followed by the release of cytochrome *c* and the activation of the effector caspases, which leads to cell death. Recently, increased attention has been paid to the possible role of the protein neuroglobin, in the regulation of the apoptotic process, and data have been provided, demonstrating the ability of the protein to inhibit the intrinsic pathway of apoptosis by interacting with mitochondrial proteins. The molecular details of these interactions, however, remain largely undefined. In the present study, well recognized bioinformatics methods were applied to predict the possible interaction interfaces which the protein can exploit to interact with relevant proteins of the mitochondrial-dependent pathway of apoptosis. In the search for therapeutic approaches based on the modulation of apoptosis, such a computational prediction could represent a first, guiding step, for the design of strategies aimed at modulating these interactions, and tune the apoptotic process.

Introduction

Apoptosis represents the key mechanism for the removal of surplus, damaged and aged cells. It can be activated under disease conditions, constituting a protective mechanism in a multi-cellular organism by eliminating potentially dangerous cells or cells that have lost their functional capabilities. It is an evolutionarily conserved form of cell death carried out by a highly complex molecular signaling pathway. In particular, there are two main pathways which are known to initiate apoptosis: the death receptor-dependent (extrinsic) pathway and the mitochondrial-dependent (intrinsic) pathway, both of which converge on activating the execution caspases (caspase-3 and caspase-7) (1,2). In the extrinsic pathway, death receptor ligation leads to the recruitment of adaptor molecules that activate an initiator caspase (caspase-8), which directly cleaves and activates the execution caspases. In the intrinsic pathway, the mitochondria respond to diverse signals (such as DNA damage, low nutrient levels, increased calcium levels, receptor signaling, oxidative stress and intracellular aggregation of misfolded proteins) emanating from other cell compartments. In response to these signals, a mitochondrial outer membrane permeabilization (MOMP) occurs, followed by the release of cytochrome *c* (Cyt-C) that binds apoptotic protease-activating factor 1 (APAF1), leading to the formation of a caspase activation platform (apoptosome). The apoptosome recruits and activates an initiator caspase (caspase-9), which, in turn, cleaves and activates the execution caspases. Crosstalk between the extrinsic and intrinsic pathways occurs through the caspase-8-mediated cleavage of proteins of the Bcl-2 family, leading to MOMP (2). Thus, the opening of channels in the outer mitochondrial membrane, which induces the release of Cyt-C, represents the most frequent final step leading to apoptotic death.

In recent years, some proteins have emerged as important regulators of the apoptotic process. The most well characterized is the cellular tumor antigen, p53 (3,4). It has been referred to as the 'guardian of the genome' due to its role in protecting cells from DNA damage, but it is also a key factor in transmitting programmed cell death signals to the mitochondrion, which, in turn, regulates the activity of p53 through the generation of reactive oxygen species (4). In addition, increased attention has been paid to the possible role of neuroglobin (NGB) in the regulation of the apoptotic process. NGB (5) is a member of the vertebrate globin superfamily that is mainly expressed in the central and peripheral nervous system, cerebrospinal fluid, retina and endocrine tissues, where it exerts a clear-cut neuroprotective role (6). Structural analyses (7) have indicated

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Key words: mitochondria, protein-protein interaction, cytochrome *c*, voltage-dependent anion channel, bioinformatics

that human NGB displays the typical globin fold, comprised of 151 amino acids (molecular mass, 17 kDa), with only 20-25% of sequence identity with myoglobin and hemoglobin. It is a particularly highly conserved protein, with mouse and human NGB differing in only 6% of the amino acid positions and it has a substitution rate almost four-fold lower than that of other vertebrate globins. This suggests that its functions are of basic importance to some types of tissues and possibly an enriched scenario for NGB functions has been obtained during evolution. In fact, although NGB reversibly binds oxygen with an affinity higher than that of hemoglobin, storing and supplying oxygen to neurons may be one, but not the only one, of its functions (5).

Of particular interest is the evidence that NGB is both physically and functionally related to mitochondrial functions (8,9). NGB may play a role in oxygen sensing and ATP production (10), and of particular interest is the ability of the protein to interfere with the release of Cyt-C from the mitochondria during cell death, leading to an inhibition of the intrinsic pathway of apoptosis (1). In addition, NGB scavenges damaging reactive oxygen or nitrogen species (11), and appears able to regulate G protein-coupled receptor (GPCR)-triggered signal transduction pathways, by inhibiting the dissociation of GDP from G protein α (12). Thus, as Brittain *et al* (13) suggest, NGB emerges as a critical player regulating key mitochondrial events in the intrinsic pathway of apoptosis, opening new avenues for therapeutic interventions in a number of disorders. Recent findings identifying proteins involved in energy metabolism and mitochondrial function as NGB-interacting proteins (14), provide further experimental support for this view.

The molecular details of this network of NGB interactions, however, remain largely undefined. Bioinformatics methods may provide some clarification to this specific issue and this type of approach is the focus of the present study. In particular, the NGB structure is analyzed by well recognized bioinformatics methods in order to identify the possible interaction interfaces it can exploit to interact with relevant proteins of the mitochondrial-dependent pathway of apoptosis.

Materials and methods

NGB datasets. The three-dimensional structure of human NGB, as determined by X-ray diffraction (7), was obtained from Protein Data Bank (PDB) and the biological assembly 1 stored in the deposited pdb file (code: 1oj6) was considered as representative of monomeric NGB, that is the biologically active form of the protein (15,16). The NGB structure is illustrated in Fig. 1A.

Protein-protein interaction propensities. To predict possible protein-protein interaction sites on the NGB structure, the meta-PPISP (17) and meta-PPI (18) methods were used. Both follow a consensus strategy (i.e., they combine the results from multiple predictors) to increase prediction robustness and accuracy. Meta-PPISP is built on three individual predictors, cons-PPISP (19), promate (20) and PINUP (21), and a linear regression method, using the raw scores of the three component methods as input, was trained on a set of 35 non-homologous proteins to derive the final predictor. Meta-PPI is based on the individual predictions provided by the SPIDER (22) and ConSurf (23) methods, that are combined into a final confidence score assigned to each residue. In the present study, a weighted mean of the output scores obtained from the two abovementioned consensus methods was considered as an index of the propensity of each NGB residue to take part in protein-protein interactions. The applied weights were simply given by the number of individual predictors on which each consensus method relies (i.e., three for meta-PPISP and two for meta-PPI).

The network of known and predicted protein-protein interactions involving human NGB was obtained from the STRING database (24) and the results it provided were complemented with information from recent literature (14). From the set of proteins identified by this procedure, only those involved in mitochondrial-dependent apoptosis and characterized by a known three-dimensional structure were selected for further analysis.

Prediction of interaction interfaces between NGB and the selected protein set. To predict the most probable interface exploited by NGB to interact with each of the selected proteins, a docking analysis was performed. For this purpose, the following docking methods were applied:

i) PatchDock (25) is a geometry-based molecular docking algorithm. It is aimed at finding docking transformations that yield good molecular shape complementarity. Such transformations, when applied, induce both wide interface areas and small amounts of steric clashes.

ii) With GRAMM-X (26), the best surface match between molecules is determined by a correlation technique (27) using fast Fourier transform (FFT). It uses a smoothed Lennard-Jones potential on a fine grid during the global search FFT stage, followed by a refinement optimization in continuous coordinates. An important feature of GRAMM-X is the ability to smooth the protein surface representation to account for possible conformational change upon binding within the rigid body docking approach.

iii) ZDOCK (28) is also based on a grid-based representation of two proteins and a 3-dimensional FFT to efficiently explore the rigid-body search space of docking positions. Its scoring function includes shape complementarity, electrostatics, and a pairwise atomic statistical potential developed using contact propensities of transient protein complexes.

For each of the analyzed interactions the five best solutions provided by each method were considered, leading to a set of 15 predictions for each of the selected proteins. They were then submitted to PDBePISA (29) to obtain an estimate of their stability, and for each protein the solution showing the lowest energy docked structure was taken for the final prediction of the interface with NGB. The main characteristics of the used predictors (all available as Web servers) are summarized in Table I.

Results

Protein-protein interaction propensity of NGB. The interaction network provided by the STRING database for NGB is illustrated in Fig. 2, together with additional interactions recently identified experimentally (14). The spectrum of proteins that can interact with NGB appears quite broad and diverse. It

Table I. Bioinformatics predictors.

Predictor	Description	URL	Refs.
Meta-PPISP	Built on three individual web servers: cons-PPISP, PINUP and promate. A linear regression method, using the raw scores of the three servers as input, was trained on a set of 35 non-homologous proteins	http://pipe.scs.fsu.edu/meta-ppisp.html	(17)
Meta-PPI	Uses the prediction results from the SPPIDER and ConSurf servers to build up the final consensus prediction	http://projects.biotec.tu-dresden.de/metappi/	(18)
PatchDock	Geometry-based molecular docking algorithm	http://bioinfo3d.cs.tau.ac.il/PatchDock/	(25)
GRAMM-X	The best surface match between molecules is determined by a correlation technique using fast Fourier transform (FFT), followed by a refinement optimization	http://vakser.bioinformatics.ku.edu/resources/gramm/grammx/	(26)
ZDOCK	A FFT based protein docking program	http://zdock.umassmed.edu/	(28)
PDBePISA	An interactive tool for the exploration of macromolecular interfaces	http://www.ebi.ac.uk/msd-srv/prot_int/	(29)

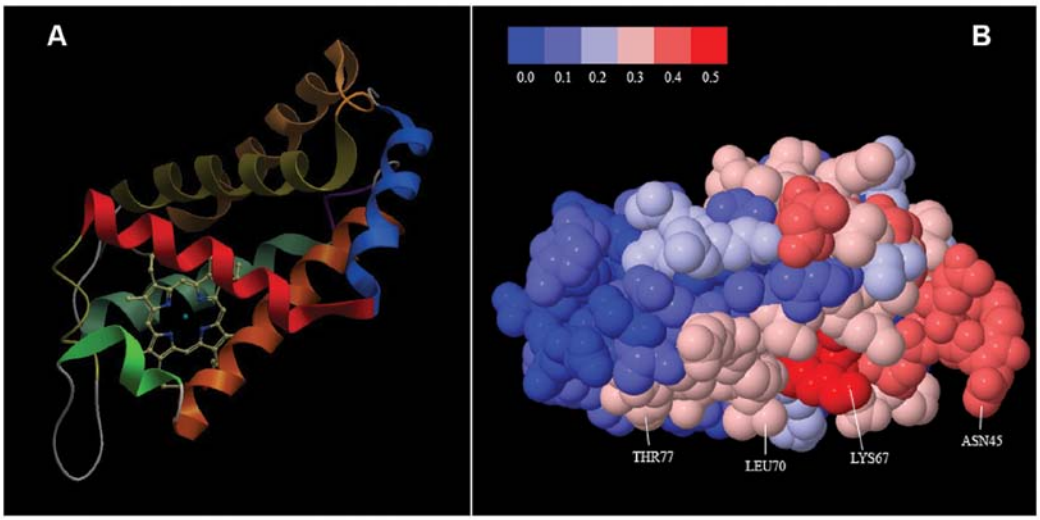

Figure 1. (A) Tertiary structure of neuroglobin (NGB) as derived from crystallographic data (7). The heme group is highlighted. (B) The surface amino acids of NGB are shown coded in colors according to the scale at the top of the image. It expresses the propensity score to take part in protein-protein interactions as derived by the combined use of meta-PPSIP and meta-PPI methods.

involves plasma membrane proteins (such as flotillin-1) fundamental for the formation of caveolae or caveolae-like vesicles, globulins involved in the oxygen transport mechanisms and enzymes. Moreover, of particular importance to the present study is the possibility for NGB to interact with a number of proteins involved in the regulation of the apoptotic process, such as the release of Cyt-C, voltage-dependent anion channel (VDAC) and elements of signal transduction mechanisms (such as G proteins). Furthermore, most of the indicated interactions are predicted by STRING as direct interactions, involving the binding of the protein to NGB.

The result of the combined use of meta-PPISP and meta-PPI predictors is consistent and provides further support to this view. It is summarized in Fig. 1B, where the NGB molecule is shown with the individual amino acids coded in color according to a score scale ranging from 0 to 0.5 expressing increasing propensity to participate in protein-protein interaction. As illustrated, >80% of the amino acids on the molecule surface have non-null propensity, suggesting that NGB can exploit a number of different sites for interaction with other proteins.

Prediction of the interaction interfaces with a selected set of proteins. From the interaction network identified by STRING, and according to the criteria specified in 'Materials and methods', the set of proteins presented in Table II was

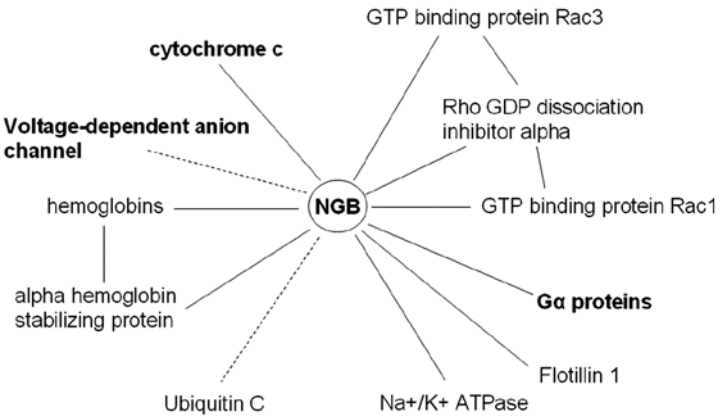

Figure 2. Simplified schematic representation of the network of protein interactions available to neuroglobin (NGB). Only the interactions indicated by STRING as direct (i.e., involving binding between the molecules) are shown, complemented by interactions recently identified (14) by experimental procedures (dashed lines). In bold are highlighted the proteins selected for the docking analysis.

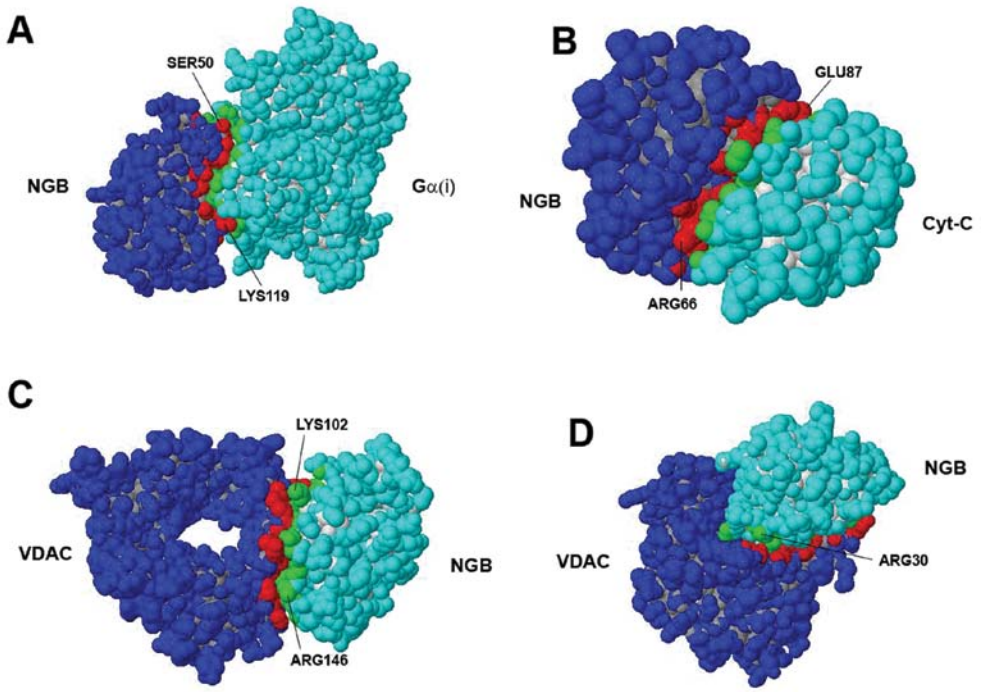

Figure 3. Lowest energy docked structures between neuroglobin (NGB) and (A) Gα(i), (B) Cyt-C and (C) voltage-dependent anion channel (VDAC). The interface between NGB and Ga(i) involves regions on the Ga(i) molecule (V233-D251; T284-E289; Y302-K312) that appear available when the Gα subunit is present in the GPCR complex (38). As far as the interface between NGB and VDAC is concerned, a second possibility (energetically less favoured and less stable) is shown in (D). It involves an interaction of NGB at the level of residues located at the boundary of the pore formed by the VDAC molecule. Cyt-C, cytochrome *c*.

selected for docking analysis. The set included human guanine nucleotide-binding protein G_i subunit α-1 [Ga(i)], human VDAC and human Cyt-C. The identified sites of interaction of NGB with each of the abovementioned proteins identified by the docking analysis are reported in Table III, together with some biophysical characteristics (as provided by PDBePISA) of the predicted interface. The identified complexes with NGB are illustrated in Fig. 3. As far as the NGB-VDAC complex is concerned, it should be noted that the analysis identified as the most probable configuration (Fig. 3C), an interaction of NGB with a region of the VDAC molecule that, however, is usually buried within the mitochondrial outer membrane. Although less energetically favored (and likely unstable), an alternative suggested configuration involved the binding of NGB to residues located at the boundary of the pore formed by the VDAC molecule. This assembly, of potential interest from a functional point of view, is also presented in Table III and illustrated in Fig. 3D.

Discussion

Significant evidence has accumulated, demonstrating that NGB protects cells from stroke damage, amyloid toxicity and anoxic injury (32-34). Although the exact mechanisms by which NGB

Table II. Set of analyzed proteins.

Protein	PDB code	Refs.
Human neuroglobin	1oj6	(7)
Human guanine nucleotide-binding protein G_i subunit α-1	3ums	(41)
Human voltage-dependent anion channel	2jk4	(42)
Human cytochrome *c*	3nwv	(43)

PDB, Protein Data Bank.

achieves this protective abiltiy remain controversial, a major role it plays is certainly in the interception of the mitochondrial pathway of apoptosis (1). A recent study by Yu *et al* (9) suggests that NGB is a migrating protein capable of moving from the cytoplasm to the mitochondria under physiological resting conditions, and this phenomenon is modulated by the oxygen-glucose deprivation condition of the cell. In both environments, NGB can establish a physical interaction with mitochondrial proteins, some of which have been recently identified using yeast two-hybrid screening and confirmed by co-immunoprecipitation (14). The bioinformatics analysis performed in the present study provides further support and adds molecular details to this view. Since, in general terms, bioinformatics predictions benefit from the combined use of multiple methods (30,31), all the bioinformatics investigations were performed not only by using available, well recognized procedures, but also by organizing them according to a consensus strategy. They allowed the identification on the globulin surface of possible interaction interfaces with a set of proteins relevant for mitochondrial-dependent apoptosis.

The obtained prediction concerning the interaction of NGB with Cyt-C is consistent with previously reported experimental and computational data (1), showing that NGB reacts very rapidly with Cyt-C, and that the interface residues, Glu60 and Glu87, bind the residues, Lys72 and Lys25, on Cyt-C, that are mandatory for the binding of Cyt-C to APAF-1 (1,35). A particularly intriguing suggestion from the analysis presented in our study, however, concerns a predicted configuration of the complex NGB-VDAC that involves an interaction at the level of the channel aperture, opening the possibility for NGB to directly modulate the permeability of the outer mitochondrial membrane (43,44), which represents the key event leading to apoptotic cell death (2). Another possible mechanism through which NGB regulates apoptosis has been suggested by experimental data showing that NGB modulates GPCR signaling (6) by interacting with the Gα subunits of the trimeric G protein. In fact, evidence exists that GPCR-mediated events can trigger (36) or inhibit (37) the apoptotic process. In the present study, the possible docking between NGB and Ga(i) was analyzed. Interestingly, the Ga(i) molecular regions involved in the predicted interface appear available when the molecule is complexed in the GPCR (38), a result in line with available experimental data showing that NGB binds to the GDP-bound state of the G protein α subunit, and inhibits the exchange of GDP for GTP (39).

Table III. Predicted interaction interfaces of NGB.

Protein	NGB
Cyt-C	ARG10, TRP13, ARG18, PRO20, PRO59, GLU60, LEU62, ARG66, LYS67, LEU70, VAL71, ASP73, ALA74, VAL76, THR77, ASN78, SER84, LEU85, GLU87, TYR88, ALA90, SER91, LEU92, ARG94, LYS95, HIS96
AA (n)	26
Surface (%)	11.3
Δ^iG (p-value)	0.312
VDAC	ARG3-GLU5, LEU8, LEU34-ASP37, LYS102, LEU103, SER104, SER107, THR108, GLY110, GLU111, LEU114, GLU118, PRO127, ALA128, ARG130-ALA132, SER134, GLN135, TYR137-ALA139, VAL141, GLN142, SER145, ARG146, TRP148, ASP149
AA (n)	33
Surface (%)	14.5
Δ^iG (p-value)	0.138
	ARG3-PRO6, ARG10, LEU34-ASP37, ARG30, VAL79, GLU80, LEU114, TYR115, GLU118, PRO123, PRO127, ARG130, ALA131, SER134, GLN135, GLN142
AA (n)	18
Surface (%)	7.0
Δ^iG (p-value)	0.706
Gα(i)	THR25, VAL26, PHE28-ARG30, PHE32-ASP37, LEU39, PRO40, GLN48-GLU53, LEU56, PHE61, GLU111, TYR115, GLU118, LYS119
AA (n)	25
Surface (%)	11.4
Δ^iG (p-value)	0.125

Δ^iG (p-value) is a measure (in energy terms) of interface specificity: P<0.5 indicates that the interface surface can be interaction-specific. P>0.5 indicates that the interface is likely to be unstable or unspecific. NGB, neuroglobin; VDAC, voltage-dependent anion channel; Cyt-C, cytochrome *c*.

Deregulated apoptosis has been implicated in the etiology of diverse pathologies. In a simplified manner, the diseases in which apoptosis has been involved can be divided into two categories (40): those in which there is an increase in cell survival (or diseases associated with the inhibition of apoptosis, such as cancer and autoimmune diseases), and those in which there is an excess in cell death (and hence hyperactive apoptosis, as in neurodegenerative diseases and ischemic damage). Thus, the search for therapeutic approaches based on the modulation of apoptosis is of particular importance in

medicine, and NGB may be a target deserving specific attention for the future development of therapeutic strategies. In this respect, the results of the present study, based on information from bioinformatics analyses, can only suggest possibilities and a theoretical insight. However, the interaction interfaces between NGB and mitochondrial proteins identified in the present study may represent a first, guiding step, for the design of strategies aimed at modulating these interactions, and tune the mitochondrial-dependent pathway of apoptosis.

Acknowledgements

The study was financially supported by a grant from the University of Padova (ex 60%) to D.G. and C.T.

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