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Changes in structural dynamics of the Grb2 adaptor protein upon binding of phosphotyrosine ligand to its SH2 domain

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Abstract

Growth factor receptor-bound protein 2 (Grb2) is an extensively studied adaptor protein involved in cell signaling. Grb2 is a highly flexible protein composed of a single SH2 domain flanked by two SH3 domains. Here we report on the structural dynamic effects upon interaction of a phosphopeptide ligand derived from the recognition sequence of the Shc adaptor protein with (i) the isolated SH2 domain of Grb2 (Grb2 SH2) and (ii) the full-length Grb2 protein. From kinetic studies using surface plasmon resonance, it was deduced that a conformation change occurred in the SH2 protein as well as the full-length Grb2 after binding. Measurements of hydrogen/deuterium exchange (HDX) in the isolated SH2 domain and full-length Grb2 protein as monitored by electrospray mass spectrometry, showed that binding reduces the overall flexibility of the proteins, possibly via slightly different mechanisms for the single SH2 domain and the full-length Grb2 protein.

Keywords: Grb2; SH2; Binding kinetics; Surface plasmon resonance; H/D mass spectrometry; Protein structural dynamics

1. Introduction

The idea of a rigid "lock and key" model for receptor–substrate interactions, originally introduced by Emil Fischer over 100 years ago, has evolved into an increased understanding in how flexibility and allosteric changes regulate biological processes. In signal transduction pathways, proteins frequently act as multiple functional modular domains. There is an increasing number of cases in which it is understood how these domains function as modular allosteric switches to repress or activate the protein [1]. In Src family kinases, focal adhesion kinase, and SH2-containing phosphatases, allosteric interactions involving SH2- and SH3-domains regulate the repressed and activated state of the catalytic domain [1].

The adaptor protein Grb2 (growth factor receptor-bound protein 2) has no catalytic domain and consists of a central SH2 domain flanked by two SH3 domains. The SH2 domain of Grb2 recognizes specific phosphotyrosine (pY) sequences containing pYXNX (X being any amino acid) [2], the SH3 domains generally bind to proline-rich sequences. Grb2 is involved in the activation of numerous receptors: receptor tyrosine kinases, e.g., the epidermal growth factor receptor (EGFR) [3], receptors from hematopoietic cells like the T-cell receptor [4], the FcεRI receptor [5] and the B-cell receptor [6]. Furthermore, Grb2 is involved in

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; Grb2, growth factor receptor-bound protein 2 (Q13588); *m/z*, mass-to-charge ratio; SPR, surface plasmon resonance; HDX, hydrogen/deuterium exchange; pY, phosphorylated tyrosine; MTL, mass transport limitation; SH2, Src homology domain 2; SH3, Src homology domain 3; RSSq, residual standard deviation as returned by the CLAMP program indicating the quality of the fit; R, change in SPR signal upon binding; R_{max}, maximum binding capacity

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changes in the cytoskeleton following adhesion [7]. Adaptor proteins such as the Grb2 protein have the function to recruit various proteins to the membrane region during the process of receptor activation [8,9]. Grb2 functions in the activation of guanine nucleotide exchange by Ras and proteins of the Rho/Rac families, by binding to the guanine nucleotide exchange factors Sos and Vav, respectively [3,10]. An important target protein for Grb2 is the adaptor protein Shc, which functions as an intermediate between membrane receptors and Sos activation by binding to Grb2 [4]. Shc contains several tyrosines that become phosphorylated in response to a wide variety of extracellular signals. One of these tyrosines (^{317}Y) in the sequence YVNV forms a consensus recognition sequence for binding to the Grb2 SH2 domain, and from this sequence the Shc peptide used in this study was derived.

Backbone dynamics of Grb2 from NMR relaxation data showed Grb2 as a flexible protein in which the SH3–SH2 linker regions are flexible [10]. The Grb2 crystal structure shows a compact dimer structure with intramolecular contact between two SH3 domains [11]. Comparing the free Grb2 SH2 domain structure with that complexed with a pYVNV containing peptide, Nioche et al. [12] concluded that structural differences exist between the free and complexed Grb2 SH2.

In view of these observations, we proceeded to investigate whether structural changes occur on binding to phosphotyrosine ligands, in either the isolated SH2 domain or the full-length Grb2. With this purpose, we used analysis of the real-time surface plasmon resonance (SPR) signal based on various kinetic models for binding. Additionally, by using solution-phase hydrogen/deuterium exchange (HDX) in combination with electrospray mass spectrometry, we evaluated the global structural changes of the SH2-domain and full-length Grb2 upon phosphotyrosine peptide binding.

2. Results

With the SPR technique, a binding process can be followed in real time. Kinetic analysis of the SPR signal, assuming predefined binding models, can be a powerful tool to study details of molecular interactions [13]. For this, Morton and Myszka [14] have developed the computer program, CLAMP, that performs global fits of SPR curves, i.e., the kinetic parameters derived from the binding model applies to all curves included in the analysis. As the values of kinetic parameters derived from CLAMP analysis are frequently correlated with each other, more reliable parameters can be obtained from the fits if experimental values from independent experiments can be introduced. In particular, the use of experimental values for the dissociation rate constant (k_{off}) seemed to be important, and therefore, separate experiments to determine k_{off} were performed (see below).

2.1. Kinetics of the dissociation phase

The dissociation rate has been assayed in the presence of a high amount of competing peptide to prevent rebinding of released analyte [15]. From initial experiments, it appeared that at peptide concentrations $>10^{-4}$ M, the dissociation rate did not increase further and that dissociation proceeds with first-order kinetics. Therefore, dissociation of the SH2 domain, as well as that of full-length Grb2 protein from immobilized Ahx-PSpYVNVQN-NH_2, was monitored immediately after addition of 10 μl of a 1 mM Ac-PSpYVNVQN-NH_2 (Shc peptide) solution to 35 μl of a 1 μM protein solution in the sample cell (Fig. 1). In these experiments, a high sampling rate of 5 data points per second was used. From control experiments with a non-Grb2 SH2 binding peptide, it appeared that addition of 1 mM of nonbinding peptide induces a bulk effect, which takes approximately 1 s to come to equilibrium. Therefore, the data points of the first second were discarded. The dissociation rate in both cases was extremely rapid. For the SH2 domain, the dissociation phase could hardly be discerned from the bulk effect caused by the nonbinding peptide, and its k_{off} value is estimated to be at least 10 s^{-1}. Fit of the data according to a monophasic exponential function yields a k_{off} value of approximately 1.3 s^{-1} for full-length Grb2 protein. These values are very high; being even somewhat higher than those obtained for p85 SH2 domains [16], and previously by us for Lck SH2 [15],

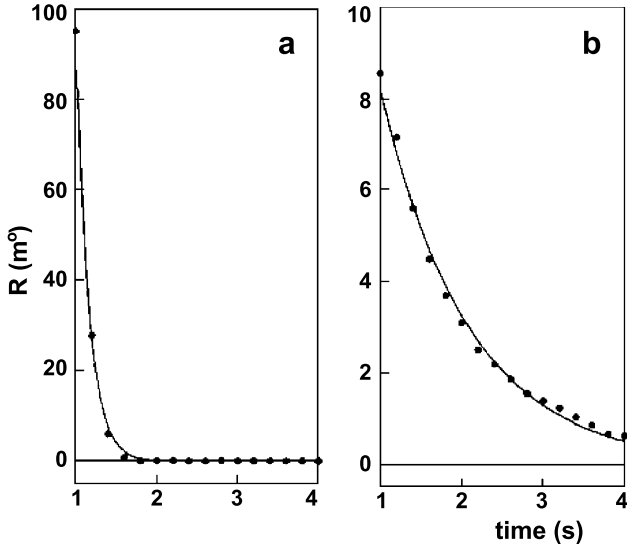


Fig. 1. Dissociation of Grb2 SH2 domain (a) and full-length Grb2 (b) from Shc peptide immobilized to the sensor surface, immediately after addition of 10 μl of 1 mM of Shc peptide to 35 μl of protein solution (1 μM) in the sample cell. In panel a, the data points are shown immediately after addition of the peptide; in panel b, the data points are shown 1 s after addition of peptide. The lines depict monophasic exponential fits.

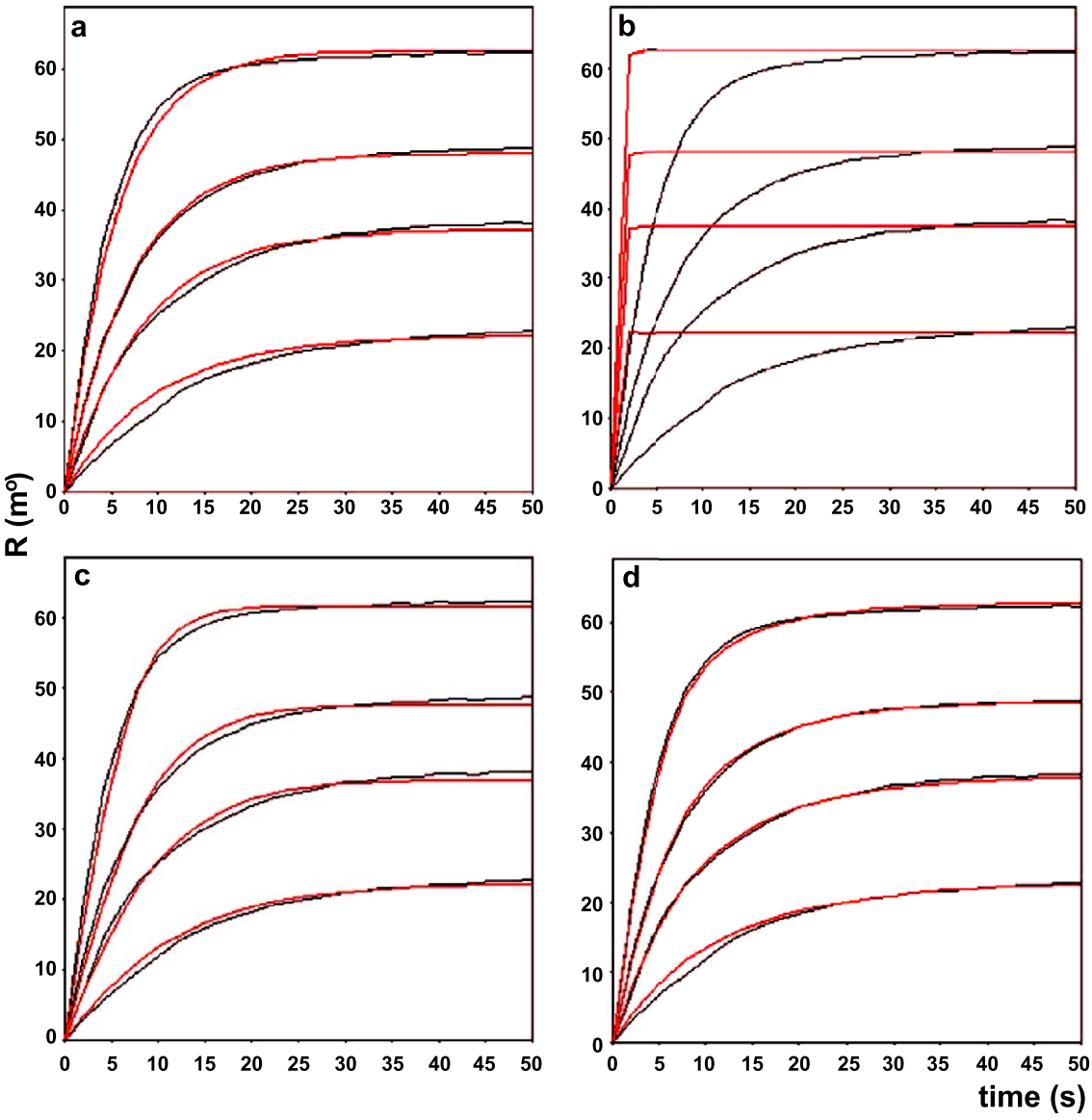


Fig. 2. SPR sensorgrams of the association phase for binding of Grb2 SH2 to immobilized Shc peptide. SH2 concentrations are 500, 300, 200, and 100 nM. The light gray (red) curves are the results from global fit of the data with the program CLAMP of various binding models. (a) Bimolecular interaction (Eq. (1)). (b) Simulation of the bimolecular model with k_{on} calculated from $k_{on}=k_{off}/K_D$, and with a k_{off} of 10 s^{-1}. (c) Mass transport model (Eq. (2)). (d) Mass transport model with conformational change after binding (Eq. (3)). The parameters from the fits are included in Table 1. For colour, see online version of figure.

which were in the tenths of s^{-1}. The derived k_{off}-values have been used in the CLAMP kinetic analyses of the association phase.

2.2. Kinetic analysis of the association phase

In a binding experiment, the change in SPR angle (R) represents a change in refractive index, which is directly related to a change in mass at the sensor surface, and can be measured in real time. Therefore, the signal contains kinetic information. Based on a specified binding model, the differential rate equations (dR/dt) are defined. By numerical integration of these equations, the program CLAMP fits the experimental data and generates kinetic parameters (k_{on} and k_{off}) as well as the maximum binding capacity (R_{max}) for the model.

The binding of the Grb2 proteins to the Shc peptide was analyzed adopting different models. Initially we investigated (i) a simple bimolecular model (Eq. (1)); (b) a simulation of the bimolecular model with experimental rate constants; (c) a bimolecular model, including a step for diffusion of the analyte from the bulk to the sensor surface (Eq. (2)), and (d)

Table 1
Parameters from kinetic global analysis of Grb2 SH2 with CLAMP using various models

	Bimolecular interaction fit a	Bimolecular interaction fit b	Transport limited model fit c	Transport-limited conformation model fit d
k_{tr} (m s^{-1})	–	–	$1.06(\pm 0.03)\times 10^{-5}$	$1.27(\pm 0.03)\times 10^{-5}$
k_{on} (M^{-1} s^{-1})	$1.98(\pm 0.04)\times 10^{5}$	2.41×10^{7} (f)[a]	$2.53(\pm 0.13)\times 10^{7}$	$1.82(\pm 0.11)\times 10^{7}$
k_{off} (s^{-1})	0.082 ± 0.002	10 (f)	10 (f)	10 (f)
k_{conf} (s^{-1})	–	–	–	0.046 ± 0.014
k_{-conf} (s^{-1})	–	–	–	0.100 ± 0.014
K_D (nM)[b]	414 ± 12	414	395 ± 18	376 ± 52[c]
R_{max} (m°)	114 ± 2	114 (f)	111 ± 3	110 ± 1
RSSq[d]	0.97	not determined	0.95	0.54

The indications of the fits correspond with the panels in Fig. 2. The errors are as indicated by the program.
[a] (f) indicates fixed experimental value in the fit.
[b] Dissociation binding constant (k_{off}/k_{on}).
[c] Calculated with Eq. (4) (see Materials and methods).
[d] Residual standard deviation as calculated by CLAMP indicating the quality of the fit (see Materials and methods).

a model including a transportation step and conformational change after binding (Eq. (3)).

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB \tag{1}$$

$$A_{bulk} \underset{-k_{tr}}{\overset{k_{tr}}{\rightleftharpoons}} A_{chip}$$
$$A_{chip} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB \tag{2}$$

$$A_{bulk} \underset{-k_{tr}}{\overset{k_{tr}}{\rightleftharpoons}} A_{chip}$$
$$A_{chip} + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB \tag{3}$$
$$AB \underset{k_{-conf}}{\overset{k_{conf}}{\rightleftharpoons}} (AB)^*$$

We only analyzed the association phase of the sensorgrams with CLAMP, as the dissociation appears to proceed too rapidly to be included properly in the analysis (see above). Results of the CLAMP fits for Grb2 SH2 are shown in Fig. 2 and the derived parameters are presented in Table 1.

At first sight, the simple bimolecular model (Eq. (1)) gives satisfying results (Fig. 2a). Nevertheless, the k_{off} value extracted from this model deviates several orders in magnitude from the experimental value of approximately 10 s^{-1} (see Table 1). With the experimental parameters R_{max}, k_{off}, and k_{on} (calculated by $K_D = k_{off}/k_{on}$), a simulation of the bimolecular model was performed (Fig. 2b). It is obvious that, to accommodate the high dissociation rate, the association should be much faster than what is actually observed. This shows that mass transport of the analyte from the bulk to the sensor surface is rate limiting. Mass transport limitation (MTL) occurs when a surface has a high demand of analyte. This can be caused by the use of high-capacity surfaces and/or systems with fast association rate constants [17]. For the present case, it is calculated that R_{max} should be smaller than 1 m° to make the transport flux comparable to the reaction flux [15]. A MTL model including transport (diffusion) from the bulk to the sensor matrix is given in Eq. (2). The fit of the data according to this model is only slightly better than that to the bimolecular model as appears from the RSSq value, which is the residual standard deviation of the data from the fit (see Materials and methods) (Fig. 2c, Table 1); however, this model is able to cope with high dissociation rates.

Yet, there seems to be a systematic deviation between the MTL model and the experimental data; that is, after a rapid initial increase, the SPR signal increases further at a much slower speed. In view of possible structural changes upon binding, a model including a conformational change upon binding was also explored (Eq. (3)). As shown in Fig. 2d and Table 1, this model describes the experimental data very well, with a significant reduction of the RSSq value.

Similar kinetic analyses were also performed for the full-length Grb2 protein. Sensorgrams for binding of Grb2 protein to immobilized Shc peptide are shown in Fig. 3. Also, in this case, the association appears to proceed very rapidly. To obtain a manageable rate of the association phase and to be able to perform kinetic analyses, an experiment was performed with relatively low protein concentrations ($<K_D$). In the fits with CLAMP of the data for the Grb2 protein, k_{off} was kept at the experimental value of 1.3 s^{-1}. More clearly than for the isolated SH2 domain, the association of the Grb2 protein appears to proceed biphasic. As a consequence of this, the deviation of the fitted MTL bimolecular model (Eq. (2)) is larger (Fig. 3a). Also, for full-length Grb2, the model including a transport step and conformational change is able to fit the biphasic association phase quite well (Fig. 3b; Table 2). The K_D values for binding of the SH2 domain and the full-length Grb2 protein to immobilized Shc peptide are found to be similar, despite the difference in k_{off} for both proteins (Tables 1 and 2), and compare well to that obtained from SPR experiments by

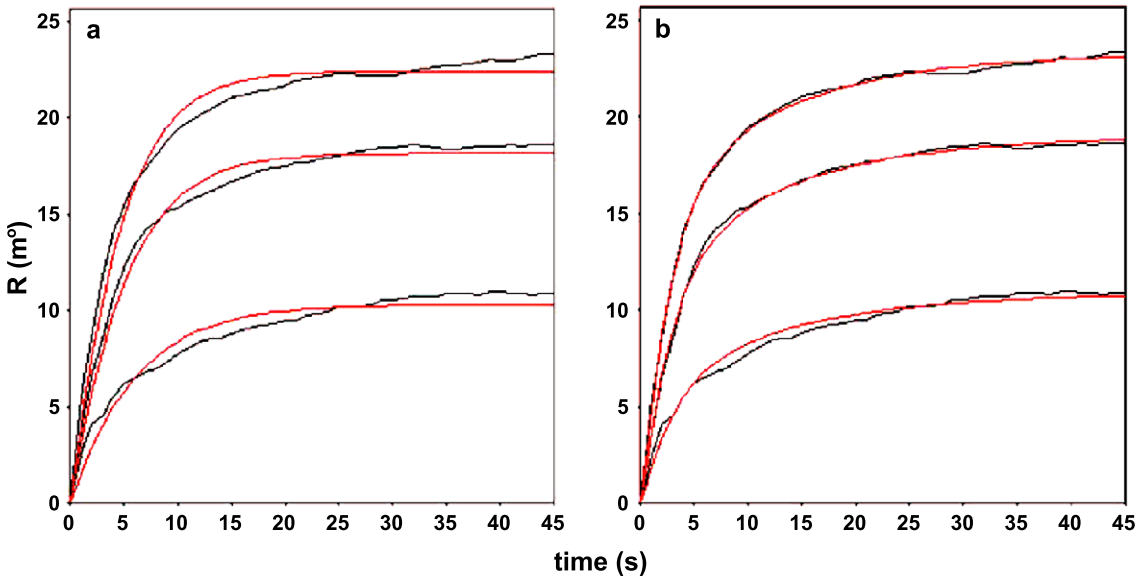


Fig. 3. SPR sensorgrams of the association phase of Grb2 protein binding to immobilized Shc peptide. Grb2 concentrations are 222, 167, and 83 nM. The light gray (red) curves are the results from global fit of the data with the CLAMP program. (a) Mass transport limited bimolecular interaction model (Eq. (2)). (b) Model including a conformation change upon binding (Eq. (3)). The parameters from the fits are included in Table 2. For colour, see online version of figure.

Rahuel et al. [33] for a somewhat longer Shc-derived pYVNV-peptide.

The absolute values of the rate constants k_{conf} and k_{-conf} given in Tables 1 and 2 are not completely reliable as they are correlated in the fits (correlation coefficient 0.940, for SH2 domain as well as for full-length protein). $K_{conf} = k_{-conf}/k_{conf}$, corresponding to [AB]/[AB]* (see Eq. (3)) is a more reliable parameter. Interestingly, identical values for K_{conf} of 2.2 for SH2 domain and full-length Grb2 protein were obtained.

Although the data are well described by the transport-conformation model (Eq. (3)), it is possible that also other models could describe the data. In view of the apparent larger protein dynamics before binding detected by the HDX experiments (see below), we explored a "conformation heterogeneity before binding" model with CLAMP (Eq. (4)).

$$\begin{aligned} &A_{bulk} \underset{-k_{tr}}{\overset{k_{tr}}{\rightleftharpoons}} A_{chip} \\ &A_{chip} \underset{k_{-conf}}{\overset{k_{conf}}{\rightleftharpoons}} A^{*}_{chip} \\ &A_{chip} + B \rightleftharpoons AB \\ &A^{*}_{chip} + B \rightleftharpoons AB \end{aligned} \quad (4)$$

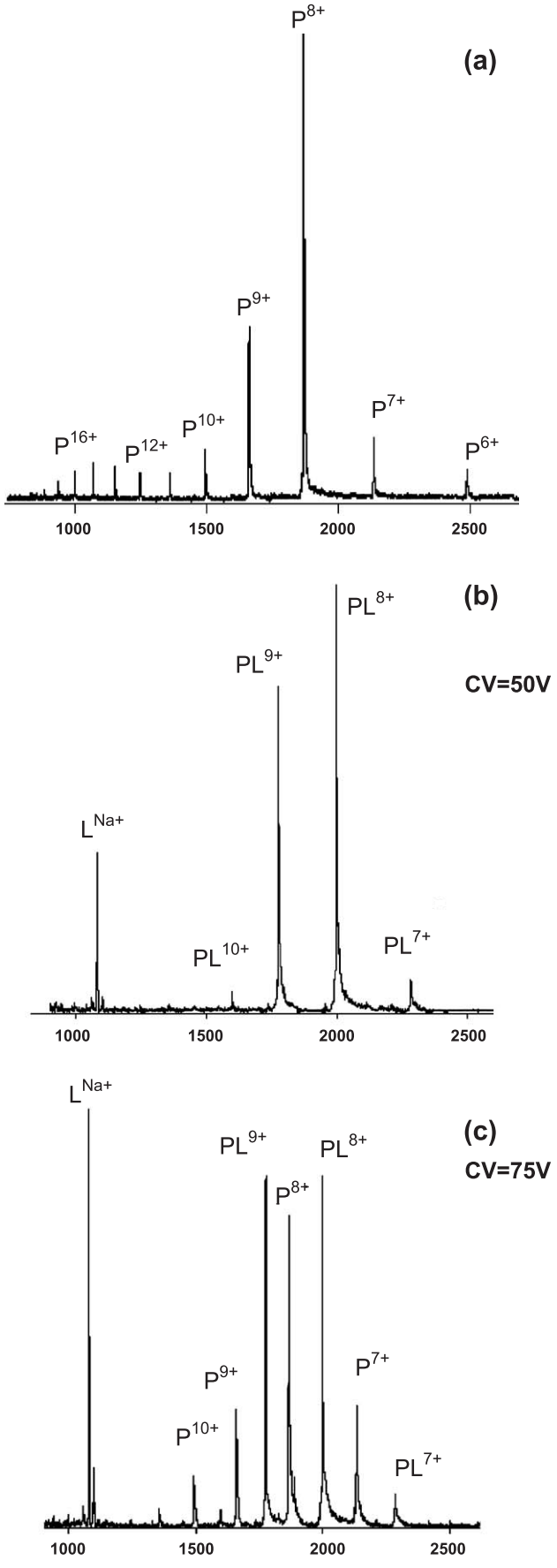
For the full-length protein as well as the SH2 domain, this model only converged if k_{conf} was $>10^4$ times k_{-conf}. The solution conformation heterogeneity model fitted the experimental data with residual standard deviations (RSSq) of 0.95 and 0.58, for Grb2 SH2 and full-length Grb2, respectively. The quality of the fits is comparable to the transport-limited model (Eq. (2); Tables 1 and 2) but of lower quality than the fits to the two state conformational change model (Eq. (3); Tables 1 and 2).

In view of the report on dimer formation in the crystal structure of the Grb2 SH2-pYVNV complex [18], we also explored the possibility of dimerization with a 'dimerization after binding' model. This model is similar to the two-state conformational model shown in Eq. (3), but now the last step is an equilibrium between two species of the complex AB and the dimer $(AB)_2$. The dimerization upon binding model fitted the experimental data with RSSq's of 0.70 and 0.29, for Grb2 SH2 and full-length Grb2, respectively. The quality of the fits approaches that of the fits to the transport limited-conformation model (Eq. (3); Tables 1 and 2). On the other hand, our mass spectrometric data revealed that binding to the Shc peptide of either protein had no influence in the oligomerization state of the protein; that is, they

Table 2
Parameters from CLAMP analysis of full length Grb2, binding to immobilized Ahx-PSpYVNVQN-NH_2

Model	Transport limited model (Eq. (2)) fit a	Transport limited conformation model (Eq. (3)) fit b
k_{tr} (m s^{-1})	7.2 ($\pm$ 0.2) $\times$ 10^{-6}	1.0 ($\pm$ 0.03) $\times$ 10^{-5}
k_{on} (M^{-1} s^{-1})	2.4 ($\pm$ 0.2) $\times$ 10^{6}	1.92 ($\pm$ 0.07) $\times$ 10^{6}
k_{off} (s^{-1})	1.3 (f)[a]	1.3 (f)[a]
k_{conf} (s^{-1})	–	0.037 $\pm$ 0.006
k_{-conf} (s^{-1})	–	0.082 $\pm$ 0.008
K_D (nM)	540 $\pm$ 50[b]	470 $\pm$ 40[c]
R_{max} (m°)	76.5 $\pm$ 4.0	72.5 $\pm$ 1.5
RSSq[d]	0.58	0.25

Values $\pm$ standard error as indicated by the fit program.
[a] (f) indicates fixed experimental value in the fit.
[b] Dissociation binding constant (is k_{off}/k_{on}).
[c] Calculated with Eq. (4) (see Materials and methods).
[d] Residual standard deviation as calculated by CLAMP indicating the quality of the fit (see Materials and methods).



remained in their monomeric form (see below). Yet, the protein concentration at the surface of the SPR chip may be several orders of magnitude higher than the micromolar concentrations used in electrospray mass spectrometry (see below). Based on a layer thickness of 100 nm and a change in SPR angle of 120 m° for 1 ng/mm^2, the concentration of Grb2 protein in the hydrogel is calculated to be 2×10^{-4} M. At the latter concentration, we loaded the protein complex on a size exclusion chromatography (SEC) column (Superdex-75, Amersham Pharmacia-Biotech) and on a native PAGE-gel (PhastGel system, Amersham Pharmacia-Biotech). In neither experiment, evidence for dimerization was found as only (>97%) the monomeric complex was observed (results not shown). Altogether, the latter techniques ruled out the dimerization upon binding model, and left the two-state conformational change model as the best description of the binding process by the SPR data analysis.

Additionally, we studied the effect on protein dynamics upon binding by H/D exchange mass spectrometry.

2.3. H/D exchange mass spectrometry

Typical electrospray mass spectra (ESI-MS) of the Grb2 SH2 domain protein either ligand-free or bound to the Shc peptide are shown in Fig. 4a and b, respectively. Both the free and the bound SH2 domain presented as the most intense peaks the charge states corresponding to the 8- and 9-fold charged species (Fig. 4a,b). Similarly, the ESI spectra of the full-length Grb2, with and without ligand, displayed as the most intense peaks the 10- and 11-fold charged ions (spectra not shown). Yet, the spectrum of the unbound protein(s) presented a bimodal charge state distribution (Fig. 4a), while the charge state distribution of the protein complex was monomodal (Fig. 4b) [19]. Molecular masses of the SH2 domain and full-length Grb2 were measured to be 14721 ($\pm$ 1) and 25318 ($\pm$ 2) a.m.u., respectively (see Materials and methods), in good agreement with the expected masses.

To further probe any change in structural dynamics of the SH2 domain or full-length Grb2 protein upon binding to the phosphopeptide ligands, we performed H/D exchange (HDX) mass spectrometry. Deuterium intake was monitored by ESI-MS as the time-dependent increase in molecular mass of the protein [20]. The total numbers of exchangeable hydrogens in the SH2 domain and the full-length Grb2 protein were calculated from their amino acid sequences: being 235 and 410, respectively. To verify that the proteins

Fig. 4. ESI-MS spectra of Grb2 SH2 domain protein in a 95% D_2O solution. Five micromolars of the Grb2 SH2 domain protein in a 95% D_2O solution containing ammonium acetate at a concentration of 65 mM (neutral pD) after 15-min exposure: (a) as ligand-free protein; (b) in complex with the Shc peptide at a cone voltage of 50 V; and (c) identical conditions as in (b), but the protein is partially in complex with the Shc peptide and partially peptide-free by applying a higher cone voltage (CV, 75 V). "P" stands for protein, "L" for the Shc peptide ligand, and "PL" for the complex protein–ligand; the number of charges in the respective ions is given in superscript.

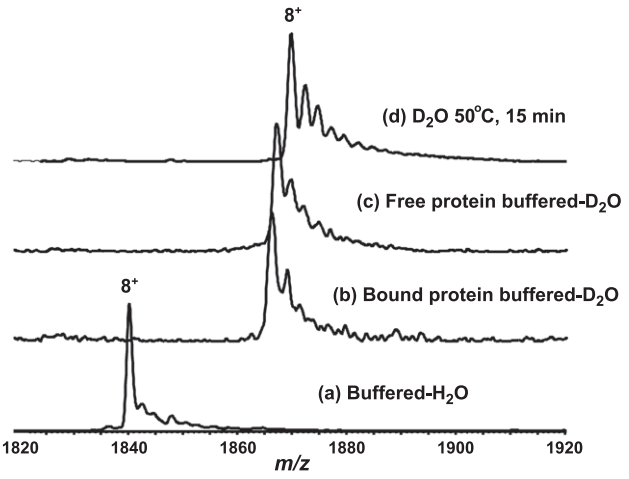


Fig. 5. Zoomed-in ESI-MS spectra of about 5 μM of the isolated SH2 protein showing the 8-fold charged ions at different conditions: (a) protein in 65 mM ammonium acetate H_2O solution; (b) protein from the complex with the Shc peptide in 65 mM ammonium acetate D_2O solution after 15-min exposure (cone voltage ≥ 75 V); (c) free protein in 65 mM ammonium acetate D_2O solution after 15-min exposure; and (d) protein in D_2O after incubation at 50 °C for 15 min. The satellite peaks on the right of the main peaks originate from adduct ions such as Na^+ and K^+.

are indeed capable of exchanging all these hydrogens, we incubated each of the proteins for 15 min in 95% D_2O in a somewhat denaturing environment, namely at 50 °C. Subsequently, the protein masses were analyzed by ESI-MS and the mass of the full (95%)-deuterated protein compared to the mass of the deuterium-free proteins. As an example, we show in Fig. 5 the shift in mass-to-charge ratio (*m/z*) of the 8^+ ions of the Grb2 SH2 domain obtained from the ESI data taken under the different applied experimental conditions. A dramatic *m/z* shift is observed when comparing the deuterium-free protein (Fig. 5a) to the fully deuterated one (Fig. 5d) obtained when heating the protein solution for 15 min at 50 °C. We found that 229 (± 2) hydrogens had been exchanged for the SH2 domain and similarly, 391 (± 2) hydrogens for the full-length Grb2, in good agreement with the expected 95% D-incorporation values, showing that these proteins can exchange their hydrogens nearly up to completion. Subsequently, we studied the H/D exchange of the ligand-free SH2 domain and full-length Grb2 at different time-points by incubating each of them in a 65 mM ammonium acetate D_2O solution (neutral pD). Similarly, we set out to monitor the effect of ligand binding on the HDX kinetics. Therefore, we prepared and electrosprayed solutions containing protein to ligand ratios of 1 to 3. Typically, in these spectra, only noncovalent complexes of protein–peptide were observed and no appreciable ligand-free protein signals (Fig. 4b). To measure the deuterium content in the protein, the noncovalent protein–ligand complex was dissociated in the gas phase by applying high cone voltage (Fig. 4c) and the mass of the so-released protein monitored in time [21]. Comparing the mass of the protein–peptide complex with the mass of the released protein, we obtained the mass of the full (95%)-deuterated peptide, i.e. 1057 ± 1 a.m.u. (1058 a.m.u., from sequence). In Fig. 5b and c, we show the shift in *m/z* of the 8^+ ions of the Grb2 SH2 domain obtained from the ESI data taken after 15 min of incubation in aqueous (D_2O) ammonium acetate in the presence (Fig. 5b) and absence (Fig. 5c) of peptide. As is clear from Fig. 5b and c, a significant amount of hydrogens in the proteins are shielded from exchange when more physiological buffer solutions are used.

The mass of the deuterated protein was calculated from and averaged over different charge states and, in this way, the deuterium content was monitored as a function of time. The so-calculated protein deuterium uptake along time is displayed in Fig. 6; the deuterium uptake of the protein from the ligand-free and the ligand-saturated solutions is depicted in solid and open triangles, respectively. Looking at the deuterium incorporation (Fig. 6), it appears that the HDX is very rapid, within 1 min over 80% of the available protons have been exchanged. It can be directly extracted from the curves that peptide binding has a diminishing effect on the HDX for both the isolated Grb2 SH2 domain and the full-length Grb2. Interestingly, the HDX data indicate that, for both the SH2 domain as well as full-length Grb2, the exchange difference observed between the ligand-bound and ligand-free protein seems to be larger at shorter incubation times: for Grb2 SH2 domain, the difference is 10 (± 1) at 1 min, whereas for full-length Grb2 it is 13 (± 1). After 15 min, the figures are 5 (± 1) and 9 (± 1), respectively. Our data reveal that the Grb2 SH2 domain and the full-length protein display a comparable degree of hydrogen protection upon binding to the ligand, although a slightly higher number of hydrogens (3 to 4) seemed to be protected in bound full-length Grb2 protein.

3. Discussion

The association phase of binding of Grb2 protein to Shc peptide immobilized on the SPR sensor surface is characterized by a biphasic course: First, there is an initial rapid increase of binding, followed by a slower increase (Fig. 3). For Grb2 SH2, a similar behavior was observed, although somewhat less pronounced (Fig. 2). This biphasic association phase could be well described by a two-step model in which a second event after initial binding was included, next to a diffusion step of the analyte from the bulk solution to the sensor surface, to deal with the high dissociation rates (Eq. (3)). This event might be a conformational change or a change in protein dynamics leading to higher affinity. Such a binding model is only a first approach of the real situation, as proteins are dynamic entities, which may be present in more than one specific conformation.

The kinetic analysis suggests that binding affects the structure of the Grb2 proteins. HDX experiments using mass spectrometry with gentle ionization is a suitable method to study protein dynamics [20,22,23]. These studies show a

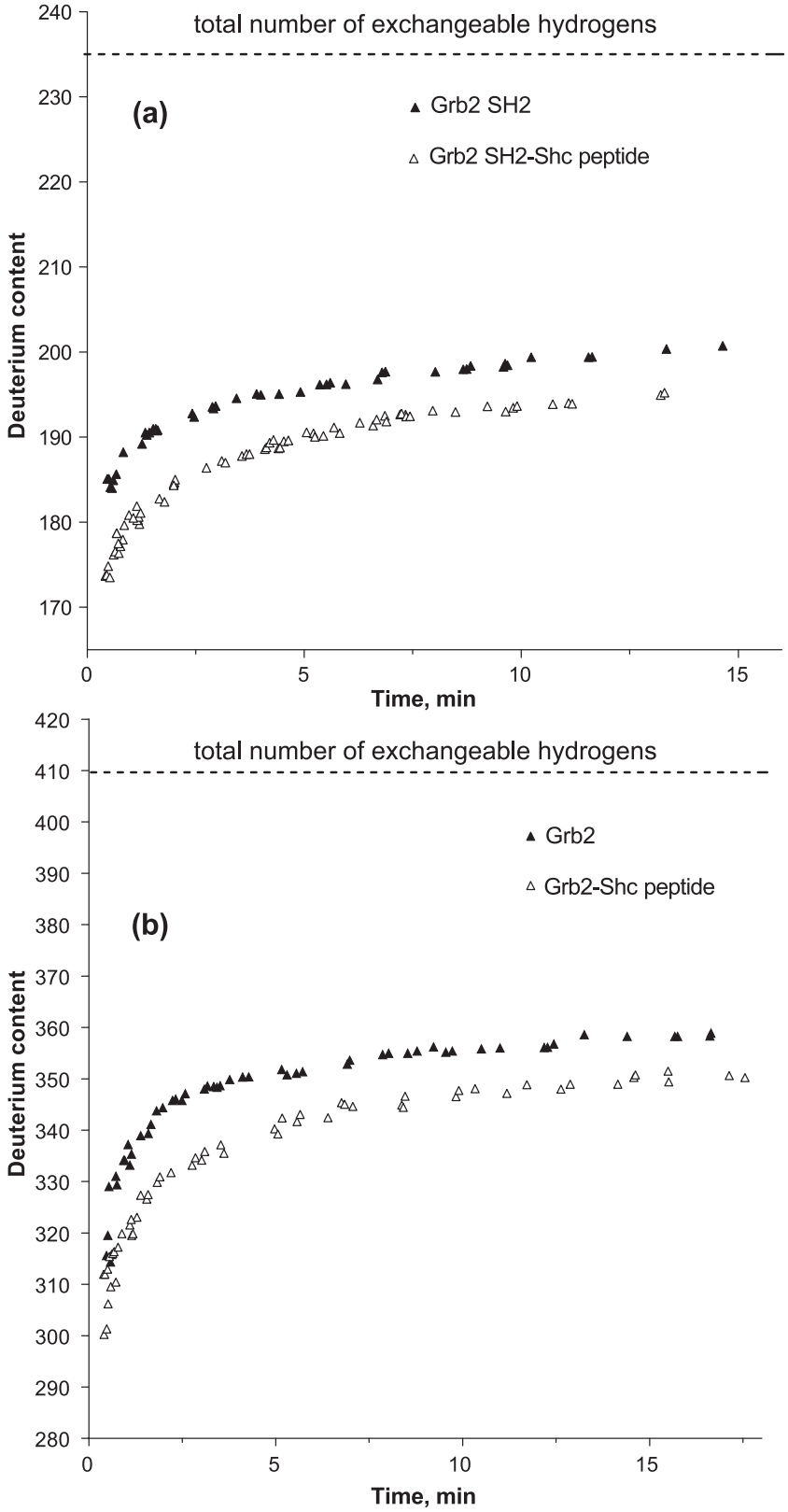


Fig. 6. Time-dependent change in deuterium content of (a) Grb2 SH2 domain and (b) full-length Grb2. The proteins were exposed to buffered D_2O, alone (full triangles) and in complex with the Shc peptide (open triangles).

marked change in dynamics upon binding. For both full-length Grb2 as well as the SH2 domain, decrease in deuterium incorporation upon binding was observed (Fig. 6).

Several explanations for such a decrease on HDX upon binding can be given: (i) shielding of exchangeable hydrogens from the solvent by the ligand, and (ii) changes in conformation and overall dynamics of the protein. Shielding will only have a modest effect as only a small part of the surface of the protein is covered by the ligand in β-turn conformation. Moreover, the ESI mass spectrum of the free protein(s) presents a bimodal charge state distribution (Fig. 4a), which turns monomodal when the protein is in complex with the peptide (Fig. 4b) [19]. The bimodal charge state distribution might be interpreted as reflecting, to some extent, the ensemble heterogeneity of conformational states present in solution [21,24–26] when the protein is unbound. The clear change in charge state distribution upon ligand binding would re-enforce the findings with the HDX experiments; that is, a clear reduction of the overall protein dynamics upon binding.

Comparable effects on HDX as observed here have also been reported for other SH2 domains. We believe that in line with the HDX observations for Src and Hck SH2, binding to the SH2 domain reduces the flexibility and increases the conformational stability of the Grb2 proteins [20,27]. Also, from NMR experiments, it has been concluded that ligand binding reduces the dynamics of several SH2 domains [28–30].

The biphasic kinetic behavior of the Grb2 proteins is deviant from a phosphotyrosine peptide binding to the Src SH2 protein: We have found that the binding to v-Src SH2 protein can be well fitted to a bimolecular model only including a diffusion step as described by Eq. (2) [31]. For the Src SH2 domain, no major structural change upon binding has been observed [32], making a conformational change for Src SH2 upon binding unlikely. The binding of phosphotyrosine ligands to Grb2 SH2 is deviant from the Src family of SH2 domains, as the ligand binds in a β-turn conformation, because the bulky Trp in position EF1 (W121) prevents binding of the ligand in an extended conformation [33]. Furthermore, structural differences exist between the free and bound Grb2 SH2 protein. Nioche et al. [12] concluded that upon binding of a pYVNV-containing peptide, indeed structural changes occur within Grb2 SH2: The entire BG loop moves and comes closer to the CD loop, coinciding with a change in the Trp EF1 rotamer. Such structural changes are compatible with the observed biphasic association kinetics.

The large majority of the exchanging protons exchange very rapidly (Fig. 6). The fast exchanging protons are believed to be on side chains and backbone amides at the surface of the protein. The kinetic analysis with CLAMP indicates very high on and off rates (Tables 1 and 2); the conformational change also proceeds rapidly. Although not very accurate due to correlation of k_{conf} and k_{-conf}, using the Monte Carlo procedure of the program CLAMP, these rates can be estimated in the range of 2 to 10 min^{-1} for full-length Grb2 and Grb2 SH2. This implies that if the binding influences the rate of HDX, this will especially affect very fast exchanging protons. Interestingly, Fig. 6 shows that at the shortest time intervals, the decrease in HDX due to binding is largest, confirming that the fast exchanging protons are more sensitive for the binding process.

The similarity of kinetic behavior between the isolated SH2- and the full-length Grb2-protein, as analyzed with the conformational change model, suggest that in the full-length protein the conformational changes upon binding to the SH2 domain are mainly restricted to this domain. For both proteins, the same value for k_{conf} is found. This is in line with the Grb2 crystal structure in which the SH2 binding site is opposite to that of the two SH3 domains [11]. Short peptide ligands for the SH2 domain bind in a β-turn conformation, making contacts with other parts of the protein unlikely. From HDX experiments, the decrease on protein dynamics for each of the two proteins upon binding to the Shc peptide is also comparable. Comparing HDX of the isolated domain to the full-length protein, similar degree of hydrogen protection upon binding was measured in both cases. A slightly higher number of protons (3 to 4) seemed to be protected in bound Grb2 as compared to the bound SH2 domain, and it would be very appealing to argue that this difference might come from an extended protein dynamics reduction further out the SH2 domain. However, this difference is small and falls close to the mass deviation error (± 2). When looking at the Grb2 structure, such an SH2 exceeding effect on the dynamics is feasible. The NMR structure of ligand-loaded Grb2 in solution shows that the linker regions between the SH2 domain and the SH3 domains are flexible [10]. It might be possible that by binding to the SH2 domain, the flexibility of these linker regions decreases somewhat, and that the position of both SH3 domains is more fixated upon binding to SH2.

This is the first report giving evidence for a structural change upon binding of a phosphotyrosine ligand to an SH2 domain based on a kinetic study. Moreover, our HDX results demonstrate a significant reduction in the dynamics of the Grb2 SH2 protein and possibly even more in the full-length Grb2 protein. This change in flexibility is relevant for the thermodynamics of phospholigand binding to Grb2, as it implies unfavorable binding entropy. It has been reported that binding to the SH2 domain does not influence the affinity of the Grb2 SH3 domains for proline-rich sequences [34,35]. Nevertheless, in the latter studies, only monovalent binding proline-rich peptides were investigated, while for high-affinity binding, tandem proline-rich ligands are needed, binding to both SH3 domains simultaneously [36]. Chook et al. [37] found that Grb2-mSos1 complex binds phosphopeptides with higher affinity than Grb2 alone, which indicates that binding to the SH2 and SH3 domains of Grb2 might not be independent of each

other. To learn more about the role of flexibility in the functionality of the Grb2 protein, we intend to study conformational changes and flexibility upon binding of tandem proline-rich ligands.

4. Materials and methods

4.1. Proteins expression and purification

4.1.1. Grb2 SH2 domain

The cDNA corresponding to residues 54 to 164 of mouse Grb2, amplified by PCR and cloned into the pGEX-KG vector [38] was a generous gift from Dr. Andrey Shaw, Washington University, St Louis, MI. The recombinant protein was expressed as a soluble N-terminal glutathione-S-transferase (GST) fusion protein with a cleavage sequence for thrombin in *Escherichia coli* strain DH5α. Cultures grown in Luria Broth medium supplemented with 50 μg/ml ampicillin were induced with isopropyl β-D-1-thiogalacto-pyranoside for 3 h at 37 °C. The bacterial pellet was resuspended in ice-cold lysis buffer (1% Triton, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 50 mM Tris–HCl [pH 8.0], Complete Mini Cocktail [Roche Diagnostics], 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol), frozen at −80 °C, defrosted and subsequently lysed for 30 min at 4 °C in the presence of lysozyme. The supernatant collected after centrifugation at 75,000 × *g* for 1 h at 4 °C was diluted four times in PBS buffer and loaded onto a GSTrap column (Amersham Pharmacia Biotech). According to prescription of the manufacturer, the GST-fusion protein was cleaved on-column using thrombin. The purity of the eluted Grb2 SH2 protein was assessed on SDS-PAGE and the concentration determined by micro-BCA assay (Bio-Rad).

A fraction of the Grb2 SH2 protein was obtained as a metastable dimer [18]. The monomer was isolated from the dimer by size exclusion chromatography using Superdex 75 (Amersham Pharmacia Biotech) in HBS buffer (pH 7.4). The protein concentration was determined using a molar extinction coefficient of 15,220 at 280 nm. Experiments were performed with the monomeric form of the SH2 domain.

4.1.2. Full-length Grb2 protein

The full-length Grb2 protein was expressed and purified as described in Guilloteau et al. [39]. Briefly, the complete Grb2 cDNA sequence was subcloned into the vector pGEX-TT (derived from pGEX-2T, Amersham Pharmacia Biotech) [39]. The protein was expressed as a fusion with GST and purified by affinity chromatography on glutathione agarose. Grb2 was eluted from the glutathione agarose beads by cleavage with thrombin. The protein was further purified on a Mono Q ion exchange chromatography column, where Grb2 monomer was eluted with 0.125 M NaCl. Experiments were performed with the monomeric form of Grb2.

4.2. Peptide synthesis

A peptide derived from the Shc adaptor protein (position Y427 in hShc) Ac-PSpYVNVQN-NH_2, referred to as Shc peptide (1041 a.m.u.), was synthesized on solid phase using standard ABI FastMoc 0.25 mmol protocols on Rink amide resin. The fmoc-peptide building blocks (Advanced Chemtech, Louisville, KY) were doubly coupled to prevent sequence deletions. The completed peptide was acetylated and cleaved from the resin with a mixture of 2.5% ethanedithiol, 2.5% water, and 95% trifluoroacetic acid, which simultaneously removed protecting groups. After precipitation in ice-cold methyl-*tert*-butyl ether, the crude product was purified by reversed-phase HPLC and the identity of the peptide was confirmed by mass spectrometry.

For coupling to the SPR sensor chip, the Shc peptide was extended at the N-terminus with 6-aminohexanoic acid (Ahx-PSpYVNVQN-NH_2). This peptide was not acetylated at the N-terminal side of the Ahx-group.

4.3. SPR experiments and kinetic analysis

For kinetic analysis, independent SPR measurements of either Grb2 SH2 or full-length Grb2 protein binding to immobilized Ahx-PSpYVNVQN-NH_2 in HEPES buffer pH 7.4 were performed with a two-channel IBIS II instrument (IBIS Technologies, Enschede, The Netherlands). The Shc peptide elongated with a 6-aminohexanoic acid spacer was covalently coupled to a CM5 sensor chip (BIAcore AB, Uppsala, Sweden) using EDC/NHS chemistry. Two millimolars of the Ahx-Shc peptide was allowed to react for 10 min. Running buffer in all SPR experiments was HEPES buffer pH 7.4. Between measurements, the chip was regenerated with 0.2% SDS in 50 mM HCl. Kinetic analysis of the association phase was performed using the program CLAMP developed by Morton and Myszka [13]. CLAMP fits the data to models based on a hypothetical mechanism by numerical integration of the rate equations. The program is freely available (http://www.cores.utah.edu/interaction/index.htm). Global analyses were performed by fitting several experimental curves simultaneously, i.e., the fit parameters apply to all the curves. The quality of the fit is indicated by the residual standard deviation, the RSSq value. The residual standard deviation is calculated as the square root of the sum, over all points, of the square of the measured data minus simulated data at the conclusion of the fit divided by the number of points minus the number of parameters that were fit, i.e., $\sqrt{(\mathrm{Sum}(\mathrm{meas}-\mathrm{sim})^2/(\mathrm{num\ point}-\mathrm{num\ param}))}$.

In the transport limited model, the value of k_{tr} (rate of transport of the analyte from the bulk compartment to the sensor surface compartment) obtained from CLAMP has the units m° s^{-1} M^{-1} (m° from shift in SPR angle). This can be converted in m s^{-1} by converting m° to mol m^{-2} and M^{-1} to m^3 mol^{-1}. One m° shift in SPR angle corresponds to 1/120 ng $mm^{-2} = 10^{-3}/120$ g m^{-2}. Introducing the

molecular weight (MW) of the analyte, 1 m° corresponds to $10^{-3}/(120 \times MW)$ mol m^{-2}. Further, 1 $M^{-1} = 10^{-3}$ m^3 mol^{-1}. The conversion factor for Grb2 SH2 (MW 14.7 kDa) is 5.67×10^{-13}, and that for the full-length Grb2 protein (25.3 kDa) is 3.39×10^{-13} to obtain k_{tr} in m s^{-1}.

The k_{off} rates have been assayed in separate experiments. After saturation of binding with a high protein concentration, the sample rate was increased to 5 data points per second and immediately after this, 1 mM Shc peptide was manually added into the cuvette. From control experiment with a nonbinding peptide, it appeared that injection of 1 mM of peptide caused a bulk effect on the SPR signal, which took about 1 s to come to equilibrium. Therefore, the data points for the first second were not included in the calculation of k_{off}. The remaining data was subjected to a monophasic exponential fit, in accordance with first-order decay kinetics. For the bimolecular model, K_D is calculated by k_{off}/k_{on}. For the model including conformational change (Eq. (3)), the apparent binding constant K_D is given by Eq. (5) [40]:

$$K_D = \frac{k_{off}}{k_{on}} \frac{1}{1 + k_{conf}/k_{-conf}} \quad (5)$$

4.4. H/D exchange mass spectrometry

Mass spectrometric measurements were performed on an LCT time-of-flight (ToF) mass spectrometer (Micromass, UK) equipped with Z-electrospray. Samples (100 μM) of either free protein, or protein bound to the Shc peptide (see above) (protein mixed with a three times peptide excess), were diluted 20 times with 65 mM ammonium acetate D_2O (Sigma, The Netherlands) (neutral pD). That would give a maximum of 95% deuterium intake. Protein solutions, typically 100 μl, were infused into the LCT mass spectrometer at 2 μl/min using a Harvard syringe pump. The ionization chamber of the mass spectrometer was flushed with D_2O for a few minutes before analysis. Electrospray was obtained with approximately 2500 V as capillary voltage. Data collection of 2 s over an *m*/*z* range between 300 and 4000 *m*/*z* was used. Special attention was to be paid to mass calibrating the instrument. CsI was used as mass reference compound at a concentration of 2 mg/ml in a 50% isopropanol solution. When analyzing the noncovalent protein–peptide complex by ESI-MS, typical spectra would show exclusively the multiply charged ions corresponding to the molecular mass of the protein–peptide complex. To analyze the deuterium incorporation in the protein only, high cone voltages between 75 and 150 V were used to disrupt the protein–peptide complex, obtaining the ions corresponding to the protein. The same cone voltage of 75–150 V was applied when spraying the free protein to keep equal experimental conditions. The total number of exchangeable hydrogens in either of the proteins was measured as follows: proteins were incubated in a water bath at 50 °C (structure destabilizing) for 15 min in 95% D_2O. Next, the protein masses were analyzed by ESI-MS and compared with the expected number of exchangeable hydrogens calculated from their sequences (see Results). The combined data from several charge states of the protein in each spectrum were used to calculate the molecular mass.

To determine the molecular masses of each of the proteins, 5 μM protein solutions in 50% acetonitrile/1% formic acid were analyzed. The molecular mass of the isolated SH2 domain was 14721 (± 1) a.m.u. and that of the full-length Grb2 was 25318 (± 2) a.m.u.

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