Xeroderma Pigmentosum Group C Protein Possesses a High Affinity Binding Site to Human Centrin 2 and Calmodulin*

Received for publication, March 12, 2003, and in revised form, July 29, 2003
Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.M302546200

Aurel Popescu§, Simona Mirone§, Yves Blouquit, Patricia Duchambron, Petya Christova¶, and Constantin T. Craescu

From INSERM U350 and Institut Curie-Recherche, Centre Universitaire, Batiments 110-112, 91405 Orsay, France

Human centrin 2 (HsCen2), a member of the EF-hand superfamily of Ca\(^{2+}\)-binding proteins, is commonly associated with centrosome-related structures. The protein is organized in two domains, each containing two EF-hand motifs, but only the C-terminal half exhibits Ca\(^{2+}\) sensor properties. A significant fraction of HsCen2 is localized in the nucleus, where it was recently found associated with the xeroderma pigmentosum group C protein (XPC), a component of the nuclear excision repair pathway. Analysis of the XPC sequence (940 residues), using a calmodulin target recognition software, enabled us to predict two putative binding sites. The binding properties of the two corresponding peptides were investigated by isothermal titration calorimetry. Only one of the peptides (P1-XPC) interacts strongly (\(K_a = 2.2 \times 10^8 \text{m}^{-1}\), stoichiometry 1:1) with HsCen2 in a Ca\(^{2+}\)-dependent manner. This peptide also binds, with a similar affinity (\(K_a = 1.1 \times 10^8 \text{m}^{-1}\)) to a C-terminal construct of HsCen2, indicating that the interaction with the integral protein is mainly the result of the contribution of the C-terminal half. The second peptide (P2-XPC) failed to show any detectable binding either to HsCen2 or to its C-terminal lobe. The two peptides interact with different affinities and mechanisms with calmodulin. Circular dichroism and nuclear magnetic resonance were used to structurally characterize the complex formed by the C-terminal domain of HsCen2 with P1-XPC.

Centrin (also called caltractin) is a Ca\(^{2+}\)-binding protein highly conserved in diverse evolutionary lineages, including algal, higher plant, invertebrate, and mammalian cells (1, 2). It is an acidic protein of 19.5 kDa belonging to the highly conserved superfamily of Ca\(^{2+}\)-binding proteins, is commonly associated with centrosome-related structures. The protein is organized in two domains, each containing two putative Ca\(^{2+}\)-binding EF-hand motifs. In humans, three centrin isoforms (HsCen1 to HsCen3) have been identified so far (3–5) with variable sequence and different tissue and cell distributions. HsCen1 and HsCen2 are highly similar to each other (sequence identity 84%) and to the algae centrin (68 and 71%, respectively), whereas HsCen3, discovered lately (5), has a more distant sequence; it shows only 54% identity with both HsCen1 and HsCen2, and is slightly closer to Cdc31, the centrin equivalent in yeast (6). A large part of the sequence diversity among the centrins occurs within the first 20 residues of the N-terminal domain, that have no counterpart in the “standard” Ca\(^{2+}\)-binding protein, CaM.

The centrins are usually found in association with the microtubule organizing centers (centrosomes in animal cells, and spindle pole bodies in yeast) that are cytoplasmic organelles encountered in almost all eukaryotic cells, with an important role in microtubule structural and temporal organization (1, 2). HsCen2 is ubiquitously expressed but was first discovered in the distal lumen of centrioles, where its presence is required for normal centriole duplication during the cell cycle (6). A large fraction of the cellular centrin is not permanently associated with the centrosome (7), but fractionates with the cytoplasm and nuclei in human cells. The precise function of these pools is not well understood and constitutes a subject of intense investigation. For instance, the presence of HsCen2 in the nuclear fractions is thought to play a role in coordinating the nuclear and cytoplasmic events during the division cycles. Recently, studies conducted in the Hanaoka group (8) shed a new light on the possible role of nuclear HsCen2 fraction in the nucleotide excision repair (NER) process. Nuclear excision repair is a major pathway for recognition and removal of bulky DNA lesions such as the UV photoproducts, or carcinogen adducts. Its dysfunction produces severe disorders in humans such as xeroderma pigmentosum, a hereditary disease characterized by a high photosensitivity and a large incidence of sunlight-induced cancer. One of the molecular component involved in several xeroderma pigmentosum forms is the XPC complex, a heterodimer composed of the XPC gene product (XPC) and HR23B, the human homologue of yeast Rad23 B. XPC complex plays a key role in the initial phase of NER and is involved in the recognition of the DNA damage. NER is of great importance for the maintenance of the genomic integrity, but the molecular mechanism of the NER pathway involving damage recognition, excision, gap-filling, and ligation steps has not been elucidated (9).
According to the recent work of Araki et al. (8), the XPC protein can contain an additional component, HsCen2, which, together with HR23B, stabilizes in a cooperative manner XPC, and thus stimulates the NER activity in vitro. This observation opens a new field of investigation of the centrin cellular functions, with the possibility that centrin may fill the gap between the DNA nuclear repair process and the functions of the mitotic spindle apparatus.

Recent biochemistry and biophysical studies performed in our and other laboratories have provided a wealth of physicochemical data on the structure and Ca\(^{2+}\) binding properties of centri
tins of various origins. From this, in agreement with the sequence alignment analysis, it appears that the integral centri
tins are composed of two independent domains, each containing two putative EF-hand motifs (10, 11). In contrast to the Chlamydomonas centrin (11), which is able to bind four Ca\(^{2+}\) ions with significant affinity, HsCen2 exhibits one strong and one weak binding site, localized in the C-terminal domain (10, 12). Structural NMR studies provided evidence that the C-terminal domain of HsCen2 is conformationally sensitive to Ca\(^{2+}\) binding, and folds into an open conformation with a large exposed hydrophobic surface (10). The capacity of both proteins to bind amphiphilic peptides, primarily mediated by the C-terminal domain (10, 11), strongly supports the hypothesis of a Ca\(^{2+}\)-dependent regulatory role of centri
tins. The next step in this direction is the search for specific molecular targets and exploration of conformational, energetic, and functional aspects of the corresponding intermolecular interactions.

In this work, we focused on a sequence-based identification of the XPC binding site to HsCen2 and on the thermodynamic and structural characterization of the intermolecular interactions. ITC experiments showed that one of the two predicted binding peptides derived from XPC binds with high affinity to HsCen2, the isolated HsCen2 C-terminal domain, and CaM. In all cases, the peptide binding is Ca\(^{2+}\)-dependent, but stoichiometry and molecular mechanism seem to be different for centrin and CaM. CD and preliminary NMR experiments enabled us to characterize some structural aspects of the complex between one XPC peptide and the C-terminal domain of HsCen2.

### EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—Recombinant proteins HsCen2 and SC-HsCen2 (Thr\(^{204}\)-Tyr\(^{172}\)) were overexpressed in Escherichia coli and purified as described previously (10, 12). For \(^{25}N\)-labeled samples, we used a culture medium (M9) containing \(^{25}N\)NH\(_4\)Cl (1.5 g/liter) as the sole source of nitrogen, and the induction step with isopropyl-1-thio-\(\beta\)-\(d\)-galactopyranoside (0.1 mM) was prolonged to 18 h.

**Target Peptides**—Two peptides from the human XPC protein, encompassing the sequences Asn\(^{847}\)-Arg\(^{865}\) (P1-XPC) and Pro\(^{703}\)-Ala\(^{720}\) (P2-XPC) were purchased from Biofidal (Vaulx en Velin, France). Purity was greater than 95%, as assessed by high pressure liquid chromatography analysis.

**CD Spectroscopy**—CD experiments were performed on a Jasco 715 CD spectrometer equipped with a Peltier temperature control unit. Far-UV spectra were recorded between 195 and 250 nm at 20 °C using 1-mm quartz cells. Spectra were collected as an average of four scans, with a scan speed of 200 nm/min and a response time of 1 s. Samples (30 \(\mu\)M) were dissolved in Tris-HCl buffer (10 mM) containing 100 mM NaCl and 2 mM Ca\(_{\text{Cl}}\). Temperature denaturation curves were obtained between 20 and 95 °C, with a temperature increasing rate of 1 °C/min.

**Isothermal Titration Calorimetry**—Thermodynamic parameters of molecular interactions between human centrin or calmodulin and the target peptides at 30 °C were investigated by ITC using a MicroCal MCS instrument (MicroCal Inc., Northampton, MA). The proteins and peptides were equilibrated in the same buffer containing 20 mM Tris (or BisTris), pH 6.5, 100 mM NaCl, and Ca\(^{2+}\) (2 mM) or EDTA (5 mM). In a standard experiment, the protein (7–20 \(\mu\)M) in the 1.337-ml calorimeter cell was titrated by the peptide (generally 10 times more concentrated) by \(\sim\)30 successive automatic injections of 7–10 \(\mu\)l each. The first injection of 2–3 \(\mu\)l was ignored in the final data analysis. Integration of the peaks corresponding to each injection and correction for the base line were done using Origin-based software provided by the manufacturer.

Fitting of the data to various interaction models results in the stoichiometry (\(n\)), equilibrium binding constant (\(K_b\)), and enthalpy of complex formation (\(\Delta H\)). The reported thermodynamic parameters represent an average of at least two experiments. Usually, control experiments, consisting of injecting peptide solutions into the buffer, were performed to evaluate the heat of dilution.

**NMR Spectroscopy**—NMR samples (0.7–1.2 mM) were obtained by dissolving the lyophilized protein in deuterated Tris-HCl buffer (20 mM, pH 6.5) containing 100 mM NaCl and 5 mM CaCl\(_2\). NMR spectra were recorded on a Varian Unity 500 NMR spectrometer equipped with a triple resonance probe and a Z-field gradient, at 308 K. Homonuclear and heteronuclear (\(^{1}H^{-}/^{13}C\)HMQC) two-dimensional spectra (HMQC) were performed using standard pulse sequences (13, 14). Spectra analysis was carried out using Felix software (Accelrys, San Diego, CA).

### RESULTS

**Prediction of the Centrin Binding Site of XPC Protein**—The available structural and functional data consistently suggest that HsCen2 is a sensor protein, capable to translate a Ca\(^{2+}\)-cellular signal into an activation/inhibition of a target molecule (10). CaM, the prototype of the Ca\(^{2+}\)-sensor proteins, has a large and diverse number of known target proteins, analysis of which revealed some molecular characteristics of the CaM-binding sites, including \(\alpha\)-helix propensity and the basic amphi
diphilic character (15–17). Recently, a web-based data base containing the known CaM target sequences and their properties, as well as sequence analysis tools permitting to predict putative CaM binding sites within a given protein sequence, has been developed (17). Assuming that CaM and HsCen2 binding sites may share common molecular features, we proceeded to an analysis of the XPC sequence using these tools. Sequence comparison failed to find any region in the XPC protein sharing significant sequence homology with known CaM binding sites but did predict two sequences with a good probability as putative CaM binding sites. The two peptides (P1-XPC, Asn\(^{847}\)-Arg\(^{865}\); and P2-XPC, Pro\(^{703}\)-Ala\(^{720}\)), situated in the last quarter of the XPC sequence (Fig. 1A), were associ
ted with the moderate to excellent score of probability (9 and 6 for P1-XPC and P2-XPC, respectively). Sequence comparison with classical CaM binding motifs suggests that the peptides belong to the 1–14 class, including the recognition sites of myosin light chain kinase, CaM kinase IV, calcineurin A, and human death-associated kinase I. P1-XPC is predicted to be the best binding sequence with a pattern of bulky hydrophobic side chains at positions 1, 5, 8, and 14 and a good propensity to form an amphiphilic \(\alpha\)-helix (Fig. 1A). It is worth noting that P1-
XPC together with Cdc31 binding peptide Kar1 include nega
tively charged side chains, whereas the CaM-binding peptides are generally positively charged (16).

**Binding to HsCen2 and Its C-terminal Domain**—Binding of the P1-XPC and P2-XPC peptides was studied using the ITC method with the protein solution in the cell and the peptides in the syringe. As illustrated in the thermograms of Fig. 2, suc
cessive injections of the peptides are accompanied by exother
mic heat pulses for which the integral decreases to a stable base line. In the presence of saturating Ca\(^{2+}\) concentrations, the data analysis indicates that the integral protein HsCen2 binds the P1-XPC peptide with a stoichiometry of 1:1 and a high affinity \(K_d = 2.2 \times 10^{4} \text{M}^{-1}\) (\(K_d = 4.5 \text{M}\)). The large reaction enthalpy of approximately \(-27\) kcal/mol largely over
times the entropic component to the free energy of interaction (Table 1). The short C-terminal domain SC-HsCen2 binds the peptide with a similar stoichiometry, affinity, and binding enthalpy, strongly suggesting that it represents the major interac
ting domain. Indeed, the free energy of P1-XPC binding to SC-HsCen2 represents 97% of the binding energy of the intact protein. In the absence of Ca\(^{2+}\), both the integral protein and
plexes of P1-XPC with HsCen2 and SC-HsCen2, the negative binding enthalpy increases significantly when the temperature varies between 20 and 34 °C. The heat capacity, calculated as the correlation coefficient between $\Delta H$ and temperature, has large negative values ($-995 \pm 78$ and $-366 \pm 49$ cal/K mol for HsCen2 and SC-HsCen2, respectively) that correspond to the upper range of values observed for CaM/target peptides (18, 19). It was noted (19) that, among the CaM binding peptides, the heat capacity changes were more negative for those exhibiting a primary interaction with the C-terminal protein domain and having a bulk aromatic side chain in the peptide N-side, as is the case for P1-XPC. Empirical observations in a number of cases pointed to the existence of a linear correlation between the interaction-induced heat capacity changes and the accompanying decrease in the water-accessible surface area (20, 21). The corresponding dehydration results in a positive entropic contribution, essentially caused by the released water molecules. Therefore, the negative heat capacity changes observed in this work should be associated with a major contribution of aliphatic and aromatic side chains to the total decrease of the accessible surface area upon complex formation (22). This is in agreement with the large hydrophobic surface exposed by the C-terminal domain of HsCen2 in the presence of Ca$^{2+}$ ions (10) and is consistent with a largely apolar protein/peptide interface. An additional factor that may account for the large negative values of the heat capacity changes observed upon P1-XPC complex formation is the significant conformational changes of both partners, as was suggested for many protein/DNA interactions (23).

We also performed titration calorimetric experiments to measure the interaction between HsCen2 and melittin, a natural peptide extracted from the bee venom often used as a CaM-binding peptide. In the presence of Ca$^{2+}$, the binding is weaker than for P1-XPC ($K_0 = 1.6 \times 10^7$ M$^{-1}$) but is still driven by a negative enthalpy change ($\Delta H = -8$ kcal/mol, at 30 °C).

**Binding to Calmodulin**—The two XPC peptides used in the present work have been selected based on the CaM target consensus sequence, and the hypothesis that proteins from the EF-hand superfamily may share similar targets. On the other hand, CaM and centrins could be observed in the same cellular regions (24–26), where proteins possessing CaM-binding motifs (27, 28) were identified. This raised the question of the possible promiscuity in recognition and binding of the molecular targets by the two EF-hand proteins, and motivated us to investigate the binding of P1-XPC and P2-XPC to CaM in the same physicochemical conditions.

In the presence of Ca$^{2+}$, the thermogram corresponding to the titration of CaM by P1-XPC shows a complex pattern, which could not be fitted using a single-site model (Fig. 4), suggesting that CaM exhibits two binding sites with distinct affinities. When the peptide to protein concentration ratio ($R$) is changed from 5.4 to 13.5, the thermogram becomes progressively dominated by the low affinity binding contribution, and the corresponding binding isotherm can be fitted confidently to a single-site model (Fig. 4). This allows us to obtain an estimation of the thermodynamic parameters characterizing the weak binding site: $K_0 = 5.9 \times 10^5$ M$^{-1}$ and $\Delta H = -8.3$ kcal/mol. Although the thermograms for lower peptide to protein ratios could not be modeled confidently, we assume that the affinity of the strong binding site is of the order of $10^9$ M$^{-1}$. P1-XPC binding to CaM is also Ca$^{2+}$-sensitive, because in the absence of the divalent cations, a single low affinity binding site ($K_0 = 7 \times 10^7$ M$^{-1}$) was observed (Fig. 4).

In contrast to HsCen2, the P2-XPC peptide also shows a significant binding to CaM. The binding isotherm could be fitted to a single-binding site model (Fig. 3), with a significant affinity ($K_0 = 1.9 \times 10^7$ M$^{-1}$) largely accounted for by the SC-HsCen2 domain are still able to bind exothermally the peptide, but with a considerably decreased affinity (28 and 17 times, respectively) (Fig. 2 and Table I).

In the case of P2-XPC, the heat rate is very small and remains roughly constant during the titration (Fig. 3), failing to show the typical transition phase observed for interacting systems. This observation indicates the absence of binding, or a very weak binding ($K_0 < 10^3$ M$^{-1}$), that cannot be detected in the present conditions. Therefore, in agreement with the knowledge-based prediction, HsCen2/Ca$^{2+}$ binds preferentially to the C-terminal domain, exhibits a strong binding to one of the two putative CaM-binding sites in the sequence of the XPC protein. The approximate localization of the regions involved in binding to HR23B, DNA, and TFIIH complex is indicated by the blue bars. The sequence of the two studied peptides is shown in comparison with other CaM-binding fragments and the Kar1 site, that was found to bind yeast and Chlamydomonas centrin, respectively.

**Analysis of the free energy components** (Table I) shows that the decrease in binding energy in the absence of Ca$^{2+}$ is the result of an increase of the unfavorable entropic contribution, only partially compensated by the increase in enthalpy. This trend is characteristic both for the intact protein and the short C-terminal construct, and it may be related to the fact that, in the absence of Ca$^{2+}$, the C-terminal domain represents an ensemble of highly disordered conformations (10), stabilization of which upon complex formation gives a negative entropic contribution.

Measurement of the temperature dependence of the binding enthalpy allowed us to evaluate the heat capacity changes ($\Delta C_p$) caused by protein/peptide interactions. For the complex of P1-XPC with HsCen2 and SC-HsCen2, the negative binding enthalpy increases significantly when the temperature varies between 20 and 34 °C. The heat capacity, calculated as the correlation coefficient between $\Delta H$ and temperature, has large negative values ($-995 \pm 78$ and $-366 \pm 49$ cal/K mol for HsCen2 and SC-HsCen2, respectively) that correspond to the upper range of values observed for CaM/target peptides (18, 19). It was noted (19) that, among the CaM binding peptides, the heat capacity changes were more negative for those exhibiting a primary interaction with the C-terminal protein domain and having a bulk aromatic side chain in the peptide N-side, as is the case for P1-XPC. Empirical observations in a number of cases pointed to the existence of a linear correlation between the interaction-induced heat capacity changes and the accompanying decrease in the water-accessible surface area (20, 21). The corresponding dehydration results in a positive entropic contribution, essentially caused by the released water molecules. Therefore, the negative heat capacity changes observed in this work should be associated with a major contribution of aliphatic and aromatic side chains to the total decrease of the accessible surface area upon complex formation (22). This is in agreement with the large hydrophobic surface exposed by the C-terminal domain of HsCen2 in the presence of Ca$^{2+}$ ions (10) and is consistent with a largely apolar protein/peptide interface. An additional factor that may account for the large negative values of the heat capacity changes observed upon P1-XPC complex formation is the significant conformational changes of both partners, as was suggested for many protein/DNA interactions (23).

We also performed titration calorimetric experiments to measure the interaction between HsCen2 and melittin, a natural peptide extracted from the bee venom often used as a CaM-binding peptide. In the presence of Ca$^{2+}$, the binding is weaker than for P1-XPC ($K_0 = 1.6 \times 10^7$ M$^{-1}$) but is still driven by a negative enthalpy change ($\Delta H = -8$ kcal/mol, at 30 °C).

**Binding to Calmodulin**—The two XPC peptides used in the present work have been selected based on the CaM target consensus sequence, and the hypothesis that proteins from the EF-hand superfamily may share similar targets. On the other hand, CaM and centrins could be observed in the same cellular regions (24–26), where proteins possessing CaM-binding motifs (27, 28) were identified. This raised the question of the possible promiscuity in recognition and binding of the molecular targets by the two EF-hand proteins, and motivated us to investigate the binding of P1-XPC and P2-XPC to CaM in the same physicochemical conditions.

In the presence of Ca$^{2+}$, the thermogram corresponding to the titration of CaM by P1-XPC shows a complex pattern, which could not be fitted using a single-site model (Fig. 4), suggesting that CaM exhibits two binding sites with distinct affinities. When the peptide to protein concentration ratio ($R$) is changed from 5.4 to 13.5, the thermogram becomes progressively dominated by the low affinity binding contribution, and the corresponding binding isotherm can be fitted confidently to a single-site model (Fig. 4). This allows us to obtain an estimation of the thermodynamic parameters characterizing the weak binding site: $K_0 = 5.9 \times 10^5$ M$^{-1}$ and $\Delta H = -8.3$ kcal/mol. Although the thermograms for lower peptide to protein ratios could not be modeled confidently, we assume that the affinity of the strong binding site is of the order of $10^9$ M$^{-1}$. P1-XPC binding to CaM is also Ca$^{2+}$-sensitive, because in the absence of the divalent cations, a single low affinity binding site ($K_0 = 7 \times 10^7$ M$^{-1}$) was observed (Fig. 4).

In contrast to HsCen2, the P2-XPC peptide also shows a significant binding to CaM. The binding isotherm could be fitted to a single-binding site model (Fig. 3), with a significant affinity ($K_0 = 1.9 \times 10^7$ M$^{-1}$) largely accounted for by the
enthalpy contribution ($\Delta H = -10.6 \text{ kcal/mol}$). In the absence of Ca$^{2+}$, the binding of P2-XPC is undetectable in the present experimental conditions.

Conformational Changes and Structural Stability Induced by the Complex Formation—CD and NMR spectroscopy were used as primary investigation tools to characterize the confor-
natorial properties of the HsCen/P1-XPC interaction. Fig. 5 illustrates the far-UV CD experiments on the complex formation of HsCen2 and its C-terminal domain with the P1-XPC in the presence of Ca\(^{2+}\). The peptide alone exhibits a CD spectrum characteristic for a highly disordered structure, as is generally the case for linear polypeptides of this size. In contrast, the Ca\(^{2+}\)-bound integral protein and SC-HsCen2 domain exhibit a CD spectrum typical for a well folded protein with a major \(\alpha\)-helical content. The spectral characteristics of the helical secondary structure are the negative bands at 222 and 207 nm and the positive band at 195 nm (29).

Adding of P1-XPC, at a 1:1 molar ratio, to SC-HsCen2/Ca\(^{2+}\) induces a considerable enhancement of the CD signal (by \(50\%\)) with a rough conservation of the relative intensities of different bands (Fig. 5A). A moderate increase of the negative ellipticity (on the order of 10–15\%) could be eventually associated to the conformational rearrangements of the protein, as was suggested in the case of Ca\(^{2+}\) binding to the apo N-terminal domain of troponin C (30, 31). However, structural studies on complexes of EF-hand domains with peptide targets showed that the protein domains undergo only small tertiary changes (32). Therefore, the large variation of the negative band intensity at 222 nm, the most characteristic for the \(\alpha\) structure, suggests that a fragment of the peptide undergoes a random coil-to-helix structural transition upon binding to the protein, bringing a significant contribution to the \(\alpha\)-helix CD band. This conclusion is corroborated by preliminary NMR studies of the SC-HsCen2/P1-XPC complex.

In the case of the integral protein (Fig. 5B), addition of the peptide induces a similar (but slightly larger) increase in the spectrum intensity, probably the result of a larger \(\alpha\)-helix content in the protein stabilized by the complex formation. Doubling the peptide-to-protein ratio (2:1) is not accompanied by significant CD changes, in agreement with the ITC-observed 1:1 stoichiometry.

The thermal denaturation profile of the Ca\(^{2+}\)-bound SC-HsCen2, studied by monitoring the CD signal at 222 nm, shows that \(60\%\) of the \(\alpha\)-helical structure unfolds progressively, starting from 20 °C (Fig. 5C). The single smooth transition extending over more than 40 °C, with the midpoint temperature of 74 °C, is characteristic for loosely organized structures (33). The temperature-induced process is totally reversible, as the CD spectrum recorded after a rapid restoration of the initial conditions is indistinguishable from the reference spectrum. The complex of SC-HsCen2/Ca\(^{2+}\) with P1-XPC shows a much steeper temperature dependence and an increase in transition temperature by 16 °C \(T_m = 90 \, ^\circ\text{C}\), reflecting a more stable structure.

The Ca\(^{2+}\)-bound integral protein (Fig. 5D) unfolds reversibly in two well distinguished steps (midpoint temperatures \(-47\) and \(-82 \, ^\circ\text{C}\)), corroborating the results of the chemical denaturation experiments (10). This observation supports the idea that the two EF-hand domains behave like independent folding units, as was also demonstrated for Chlamydomonas centrin (11). Comparison with the denaturation curve of SC-HsCen2 may suggest that the N-terminal domain, apparently unable to bind Ca\(^{2+}\) ions, is less stable and unfolds first. Complex formation keeps the modular unfolding behavior and increases significantly the thermal stability. It is interesting to note that the denaturation mid-temperature of the first step, presumably involving the N-terminal domain is up-shifted by about 20 °C.

Information on the structural and dynamic properties of the complex formed by SC-HsCen2/Ca\(^{2+}\) and P1-XPC was obtained from NMR experiments. Fig. 6 compares the two-dimensional [\(^{15}\text{N}-\text{H}\)]HSQC spectrum of SC-HsCen2 with those of the complex formed with melittin and P1-XPC. With the exception of those connected by horizontal lines, each cross-peak represents a protein backbone NH group and is situated at the crossing point of the corresponding proton (horizontal scale) and \(^{15}\text{N}\) (vertical scale) resonance frequencies. The Ca\(^{2+}\)-bound SC-HsCen2 alone gives a well dispersed spectrum that contains more than expected peaks of unequal intensity. Adding melittin to the protein solution changes considerably the spectrum and reduces the number of observable cross-peaks (\(-55\) peaks), less than the expected number (\(78\) peaks) for this protein construct. The observed signals are globally broader than in the spectrum of the unbound protein. A careful analysis, at a lower contour level, revealed a peak subset with strong line broadening, near the detection limit of the spectrometer. Finally, the complex with P1-XPC exhibits a distinct HSQC spectrum, containing the appropriate number of cross-peaks and a more regular line shape. Nevertheless, the peaks undergoing a large line broadening in the complex SC-HsCen2/melittin are still broadened in the SC-HsCen2/P1-XPC complex, albeit at a smaller scale. These findings indicate that, in agreement with the relative binding affinities of the two peptides, the protein/peptide interaction results in well organized, unique, and stable structures, which can be amenable to a more detailed structural investigation.

**DISCUSSION**

**HsCen2 as a Ca\(^{2+}\) Sensor**—The sequence similarity with calmodulin and the ability to bind Ca\(^{2+}\) ions have suggested that centrins possess a two-domain structural organization and may play a regulatory role in one or several Ca\(^{2+}\)-triggered processes. Previous studies on Chlamydomonas centrin (1) or HsCen2 (10) confirmed the modular organization and demonstrated the functional autonomy of the two domains in Ca\(^{2+}\) binding. Additionally, the Ca\(^{2+}\) sensitivity of the global conformation for centrins from several organisms were shown by CD

### Table I

| Protein          | Ligand  | Ca\(^{2+}\) | \(K_a\) (±error) | \(\Delta G\) (cal/mol) | \(\Delta H\) (±error) | \(T_m\) (°C) |
|------------------|---------|-------------|------------------|------------------------|----------------------|--------------|
| HsCen2           | P1-XPC  | +           | 2.2 (0.4)        | -11.6                  | -27.2 (0.2)          | -15.6        |
| HsCen2           | P1-XPC  | +           | 0.08 (0.01)      | -9.6                   | -35.8 (0.6)          | -26.2        |
| HsCen2           | P2-XPC  | +           | NB\(^a\)         | -10.0                  | -8.2 (0.01)          | +1.2         |
| HsCen2           | Melittin| +           | 0.16 (0.02)      | -11.2                  | -29.1 (0.1)          | -17.9        |
| SC-HsCen2        | P1-XPC  | +           | 0.07 (0.01)      | -9.5                   | -31.5 (0.1)          | -22.0        |
| SC-HsCen2        | P2-XPC  | +           | NB\(^a\)         | -10.1                  | -10.6 (0.1)          | -0.5         |
| SC-HsCen2        | P1-XPC  | +           | 0.0059 (0.0007)  | -8.0                   | -8.32 (0.03)         | -0.32        |
| CaM              | P1-XPC  | -           | 0.007 (0.001)    | -8.0                   | -6.4 (0.2)           | +1.6         |
| CaM              | P2-XPC  | +           | 0.19 (0.02)      | -10.1                  | -10.6 (0.1)          | -0.5         |
| CaM              | P2-XPC  | -           | NB\(^a\)         | -10.1                  | -10.6 (0.1)          | -0.5         |

\(^a\) NB, no binding observed in the present conditions.
or NMR (10, 11) experiments. The results obtained in the present work, showing a Ca\(^{2+}\)-dependent strong interaction with an amphiphilic peptide derived from the XPC protein, provide additional evidence for the Ca\(^{2+}\) sensor behavior of HsCen2.

The first experimental data on potential centrin targets were obtained from genetic (35) and biochemical (36) experiments in yeast, suggesting that Cdc31 may physically interact with the protein Kar1, the two proteins being localized in the spindle pole body. Indeed, a 19-residue peptide derived from the Kar1 sequence was shown to bind, in a Ca\(^{2+}\)-dependent manner and a stoichiometry close to 1:1, to centrins of various origins (Scherffelia dubia, Chlamydomonas, yeast, human) (11, 37), but not to yeast CaM (37). The reported binding constant of Kar1 (Fig. 1A) peptide to HsCen2 (~3 × 10^6 M\(^{-1}\)) (37) is ~2 orders of magnitude lower than for the peptide P1-XPC studied in the present work. It was suggested that the acidic charges in the binding sequence of Fig. 1A, mainly those in the N-terminal site, may explain the decreased affinity for both centrin and CaM.

The affinity of P1-XPC for HsCen2 is among the highest observed for CaM and its target proteins or peptides, usually exhibiting binding constants between 10^6 and 10^{11} M\(^{-1}\) (15, 19, 38–40). The Ca\(^{2+}\) dependence of P1-XPC binding to SC-HsCen2 and HsCen2 is reflected in the decrease of the binding constant upon removal of the metal ions by a factor of 17 and 28, respectively. In the case of CaM/target complexes, the Ca\(^{2+}\) dependence varies from very strong (smooth muscle myosin light chain kinase (Ref. 18) or NOS-I (Ref. 40)) to moderate or very weak (38, 41).

**Fig. 3. Titration of CaM and HsCen2 by P2-XPC.** Figure shows ITC experiments on the binding of P2-XPC to CaM and HsCen2. Note the difference in scale of the heat rate between the first panel and the next two panels.
Our results strongly suggest that binding of P1-XPC to HsCen2 is mainly determined by the C-terminal domain of the protein, which contributes more than 95% of the total binding energy. The lack of (or a very small) contribution from the N-terminal half may be related to its low Ca\(^{2+}\) affinity and to its presumed closed tertiary conformation. Indeed, there is experimental evidence that apo domains of EF-hand proteins expose only a small hydrophobic surface, being less favorable to an efficient target binding (42). The relative roles of the two lobes in HsCen2 are in agreement with the structure-function relationships established for the yeast centrin (43), showing that mutations in the central and C-terminal parts of the Cdc31 sequence are associated with a defective protein localization and G2/M arrest, whereas the N-terminal modifications induce no observable defects. Nevertheless, binding of P1-XPC to HsCen2 appears to affect also the N-terminal domain, as suggested by the significant thermal stability increase (20 °C) of the N-terminal domain. This observation is corroborated by the calorimetric measurements, showing that the heat capacity of binding is almost 3 times greater for the integral protein relative to its C-terminal domain. Although not essentially involved in the recognition and initial binding of the peptide, the N-terminal domain may move closer to the C-terminal domain, hiding a significant amount of the solvent-exposed surface. Nevertheless, understanding of the detailed role of the N-terminal domain requires further investigation.

HsCen2 binds one P1-XPC per molecule, as is the case of the majority of CaM/target peptide complexes (19, 38). In contrast, the preponderant binding role of the C-terminal half in HsCen2 is less usual (44) and suggests that the complex conformation is different from that described for MLCK, calmodulin kinase I, or calmodulin kinase kinase peptides bound to CaM (32), where both domains participate in wrapping the peptide. On the other hand, CaM appears to bind two copies of P1-XPC with affinities differing by 2 orders of magnitude, leading to the hypothesis of a distinct complex conformation. In this sense, it is worth mentioning that recent crystallographic structures of CaM in complex with larger targets revealed new types of interactions, where each CaM lobe binds to a distinct target fragment (45, 46).

The free energy of the HsCen2/P1-XPC complex formation is dominated by the negative enthalpy component, suggesting...
that the van der Waals, hydrogen bonding, and electrostatic interactions play the central role in peptide recognition. The unfavorable contribution of the entropy ($\Delta S < 0$) to the complex formation is the result of at least two main components. The first is related to the peptide change from a random coil conformation in the free state to an $\alpha$-helix secondary structure upon binding (Fig. 5) that corresponds to a decrease of the degree of freedom of its movements. The second component accounts for the decrease in structural disorder of the protein, including both the backbone and the side-chain dynamics, in the presence of the peptide.

**Fig. 5. CD analysis.** Far-UV CD spectra of P1-XPC (30 $\mu$M), HsCen2 (30 $\mu$M) and SC-HsCen2 (32 $\mu$M) in 10 mM Tris buffer, pH 6.70, 100 mM NaCl, 2 mM CaCl$_2$ at 22°C. The effect of peptide addition to the SC-HsCen2 (1:1 ratio) is shown in panel A and that of peptide addition to the integral protein (1:1 and 2:1 peptide-to-protein ratio in red and magenta, respectively) is shown in panel B. The effect of peptide binding upon thermal denaturation curves are shown in panels C and D for SC-HsCen2 and HsCen2, respectively. Denaturation was observed as the variation of the CD signal at 222 nm as a function of temperature.

**Fig. 6. NMR experiments.** [$^{15}$N-H]HSQC spectra at 500 MHz of SC-HsCen2 and its equimolar complexes with melittin (MEL) and P1-XPC. Samples (1–1.5 mM) are in 20 mM Tris ($d_{11}$) buffer, pH 6.5, 100 mM NaCl, 10 mM CaCl$_2$, and the spectra were recorded at 308 K. Horizontal lines connect the peak pairs belonging to the same amino groups from Asn and Gln side chains.
agreement with the enhanced thermal stability of the complexes, observed in CD experiments (Fig. 5). In the present case, these two negative entropy changes are larger than the usually large entropy increase caused by the partial dehydration of the interacting surfaces and the associated enhanced movement freedom of water molecules. It is expected that, in real interacting systems, including HsCen2 and XPC, where the target binding site is already α-helical, an important negative component is canceled and the entropy contribution may become more favorable for the interaction.

Nevertheless, this thermodynamic analysis is incomplete, and its predictive capacity should be taken with some caution. The published data show indeed that peptide binding to CaM or apoCaM can be both enthalpically and entropically driven processes (18). Although the majority of the studied peptides interact exothermically, melittin or fragments from cNOS and phosphodiesterase bind to CaM with a positive enthalpy (19, 47), driven by an entropic factor. The variability in energetic balance may also depend on the Ca²⁺-binding protein. For instance, in the case of melittin, our calorimetric experiments revealed a negative enthalpy change upon binding to HsCen2, in opposition with the endothermic character of the binding to CaM (19). This observation points to a distinct behavior between HsCen2 and CaM in their target recognition capacity that may be physiologically relevant for the discrimination between the specific signaling pathways of the two proteins.

**Implications for the NER Process**—Although the centrinss were initially identified in the microtubule organizing centers, the centrosomal fraction of human centrins represents less than 10% of the total cellular pool, a significant amount being localized in the nucleus (7). No functional information about this last fraction was available until the recent discovery (8) that HsCen2 could associate with the XPC/HR23B complex that intervenes in the first step of the genome-wide NER process. HsCen2 did not show any measurable influence on the efficiency of the XPC-dependent NER activity in vitro, but seems to increase the thermal stability at higher temperatures (>40 °C) and assembly efficiency of the XPC complex. Our present results reinforce the previous observations by the identification of a short XPC fragment that is able to bind strongly both to HsCen2 and CaM. This binding site is situated within the C-terminal end (constituting 10% of the sequence) of the nuclear protein, showing a high sequence conservation among various homologues from yeast to humans. In particular the Trp residue, placed in the N-terminal part of the binding site, but also the other residues forming the consensus hydrophobic and basic pattern, are highly conserved (Fig. 1C). This observation supports the biological relevance to the P1-XPC sequence tested in the present work and suggests that the mode of interaction HsCen2/XPC in human cells could be extended to the corresponding molecular pairs in other eukaryotic cells. For instance, Rad4, the S. cerevisiae counterpart of XPC, may interact with Cdc31 protein, which plays the role of the human centrin in yeast.

The physicochemical and structural properties of XPC protein are presently not known, precluding a coherent molecular explanation of its functional mechanism. However, recent experimental results (63) suggested that the last 124 residues, including the P1-XPC fragment studied here, are critically involved in the recruitment of the large transcription factor complex TFIIH (Fig. 1A) that is necessary to unwind the damaged DNA double helix. The functional importance of this C-terminal domain is further supported by the analysis of the XPC gene in xeroderma pigmentosum patients (64). In the majority of studied cases, presenting a rather homogeneous phenotype, the identified causative mutations produce a truncated protein of various lengths, but always lacking the TFIIH binding region. These observations emphasize the critical functional role of the P1-containing structural domain of XPC and suggest a possible interference in the binding of HsCen2 and the transcription factor to the same structural domain.

By its implication in the nuclear DNA repair process, centrin may constitute a functional link between the chromosome duplication and the centrosome control of the mitotic spindles. Existence of this type of correlation was previously suggested by genetic studies in yeast showing that deletion of Rad23 and DSK2 (very similar to Rad23) provokes a lethal phenotype produced by an inefficient spindle pole body duplication (65). Elucidation of the HsCen2 role in the NER system and the eventual correlation with its centrosomal function need further investigation. Utilization of peptides as models for the protein/protein interactions has obvious limitations: other fragments of the target protein may participate to the contact interface or the access binding site could be hindered in the native threedimensional structure of the integral target. In addition, the mechanism of the physiological response consecutive to the interaction could hardly be revealed by using the simplified model (50). Expression of a larger XPC construct, including the P1-XPC peptide, and the investigation of its physicochemical, biochemical, and functional properties are currently in progress in our laboratory.
