BIOCHEMICAL PHARMACOLOGY 76 (2008) 1620–1627

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Modelling and molecular dynamics of the interaction between the E3 ubiquitin ligase Itch and the E2 UbcH7

Domenico Raimondo [a,1], *Alejandro Giorgetti* [b,1], *Francesca Bernassola* [c,d], *Gerry Melino* [c,e], *Anna Tramontano* [a,f,*]

[a] *Department of Biochemical Sciences "Rossi-Fanelli", University of Rome "La Sapienza", P.le Aldo Moro 5, Rome, Italy*
[b] *Scientific and Technologic Department, University of Verona, Strada Le Grazie 15, 37134 Verona, Italy*
[c] *IDI-IRCCS Biochemistry Laboratory, Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy*
[d] *IEO c/o IFOM-IEO Campus, Via Adamello 16, 20139 Milan, Italy*
[e] *Medical Research Council, Toxicology Unit, Hodgkin Building, Leicester University, Lancaster Road, P.O. Box 138, Leicester LE1 9HN, UK*
[f] *Pasteur Institute–Cenci Bolognetti Foundation, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy*

ARTICLE INFO

Article history:
Received 15 June 2008
Accepted 26 August 2008

Keywords:
E3 ubiquitin ligases
HECT domain
UbcH7

ABSTRACT

Itch, a member of the E6AP carboxy terminus (HECT) domain-containing family of ubiquitin E3 ligases, acts in concert with the ubiquitin activating enzyme (E1) and the ubiquitin conjugating enzyme (E2) to catalyze ubiquitylation of protein targets. This sub-family of E3s shares a 350 residue C-terminal HECT domain having a strictly conserved catalytic Cys, and recruiting its cognate ubiquitin-loaded E2. HECT domains possess intrinsic enzymatic activity, by accepting ubiquitin from an E2, forming a ubiquitin thiolester intermediate, and directly catalyzing ubiquitylation of the target protein. Several hypotheses have been proposed for the biochemical mechanism underlying the structural relationship of the HECT-E2 association and subsequent ubiquitin transfer. Nonetheless, a detailed characterization of the process is still missing. In this work, we have used molecular dynamic simulations, free energy calculations, protein modelling techniques and normal modes analysis to get a deeper characterization of the static and dynamical properties of this interaction mechanism. We hypothesize a correlated slow-frequency motion that involves two different hinge regions of the HECT domain. The identification of the amino acid residues responsible for the HECT-E2 interaction, and for the dynamical properties of the ubiquitin transfer process, may be of relevant interest for pharmacological and therapeutical purposes.

* *Corresponding author at*: Department of Biochemical Sciences "Rossi-Fanelli", University of Rome "La Sapienza", P.le Aldo Moro 5, Rome, Italy. Tel.: +39 06 49910556; fax: +39 06 4440062.
E-mail addresses: anna.tramontano@gmail.com, anna.tramontano@uniroma1.it (A. Tramontano).

[1] These authors have equally contributed to this work.

Abbreviations: E1, E1 ubiquitin activating enzyme; E2, E2 conjugating enzyme; E3, E3 protein ubiquitin ligase; HECT, homologous to E6AP carboxy terminus; MD, molecular dynamics.

0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.08.026

1. Introduction

Ubiquitin ligases (E3s) act in concert with the ubiquitin activating enzyme (E1) and the ubiquitin conjugating enzyme (E2) to catalyze ubiquitylation of protein targets [1–7]. The ubiquitylation enzymatic cascade begins when the C-terminal carboxylate of ubiquitin binds an active site Cys of E1, thus forming a thiolester bond. Ubiquitin is then transferred to the catalytic Cys of E2s, via a similar thiolester linkage. In the final step, an E3 recruits ubiquitin-loaded E2, recognizes specific substrates and facilitates or directly catalyzes ubiquitin transfer to a Lys residue of the target protein.

Itch is a member of the HECT (homologous to the E6-associated protein carboxy terminus) domain-containing family of E3s [8]. With a few exceptions, the HECT-type E3s contain an N-terminal Ca^{2+}-dependent phospholipid-binding C2 domain, followed by multiple protein–protein interaction WW domains, and a C-terminal catalytic HECT domain [9,10]. While the C2 domain mediates protein localization to intracellular membranes, the WW modules recruit substrate molecules by recognizing Pro-rich regions, most commonly the PPXY consensus sequence or a phospho-Ser/phospho-Thr followed by a proline residue. The HECT domain is an approximately 350 amino acid module that coordinates the recognition of its cognate E2 and provides the intrinsic enzymatic E3 activity [9].

Structural studies based on the crystal structure of the HECT domain of the WWP1 E3 [11] revealed that it is formed by two distinct domains, an N-terminal lobe containing the E2 binding site and a C-terminal lobe accommodating the active site Cys residue. These two lobes are connected by a hinge region that provides conformational flexibility to the HECT domain N- and C-terminal halves, and regulates the enzymatic activity of the E3.

Additional mechanisms regulating the catalytic properties of the HECT E3s are based on the establishment of intramolecular interactions implying the HECT domain [12,13]. Itch catalytic activation is negatively regulated through the association of the HECT domain with the central region, including the WW motifs [12]. The conformational change required to destabilize the self-inhibitory intramolecular interactions is induced by Jun N-terminal kinase1 (JNK1)-mediated Ser/Thr phosphorylation [14]. Itch phosphorylation simultaneously allows substrate recruitment and catalytic activation by releasing the active site Cys residue. Another example is provided by the ability of a portion of the HECT module containing the catalytic Cys to associate with the C2 domain. This intramolecular interaction inhibits the E3 ubiquitylating activity by interfering with ubiquitin thiolester formation [13].

In *in vitro* ubiquitylation assays a recombinant purified Itch catalyzes its own ubiquitylation as well as modification of protein substrates in the presence of E1 and the E2 UbcH7 [12,15]. Conversely, an Itch mutant, in which the highly critical Cys residue in the HECT domain is mutated to Ala is catalytically inactive (Bernassola and Melino, personal communication). These findings indicate that an intact HECT domain of Itch in conjunction with the E2 UbcH7 are required for Itch catalytic activity.

The hinge region is critical for the juxtaposition of the E2 and E3 catalytic Cys residues during ubiquitin transfer. A conformational change altering the relative orientation of the two lobes is needed to allow the transthiolation reaction to take place, because the distance between the E2 and E3 Cys residues observed in the available experimental structures is far too long to permit ubiquitin transfer. In an attempt to understand the degree and precise localization of the required conformational change, Verdecia et al. [11] have manually modified the dihedral angles of specific residues of the hinge loop (Met 804, Gln 805 and Glu 806). The selection of these latter residues was based on structural considerations only, and shown to reduce the distance between the HECT's catalytic Cys and a modelled thiolester bond [16] between ubiquitin and E2's catalytic cysteine, to about 5 Å. Even taking into account the size of the ubiquitin molecule, the distance between the catalytic Cys residues should be of about 2.5–3.0 Å for the transfer to take place [11]. Clearly a more complex dynamic behaviour of the hinge region, most likely not limited to only a few amino acids, has to be invoked for explaining the catalytic mechanism of the enzyme and this necessarily requires an investigation of the molecular forces responsible for the dynamic behaviour of the proteins involved.

The investigation of the dynamic properties of molecular systems are not easily accessible by experimental methods, especially for protein complexes of the size of HECT–UbcH7, therefore they can only be investigated computationally. Although much care has to be taken in interpreting results of simulations, these are extremely important tools to formulate useful hypothesis. In this work, we used molecular dynamics (MD) simulations, free energy calculations and protein modelling techniques to map the interacting surfaces among Itch HECT domain and UbcH7, and to study HECT–UbcH7–ubiquitin transient thiolester complex in order to clarify ubiquitin molecule transfer to a target protein.

2. Materials and methods

2.1. *HECT domain modelling*

The HECT domain of Itch was modelled by homology. A BLAST [17] search for homologous proteins of known structure was carried on the non-redundant protein sequence database and the protein of known structure (WWP1 HECT domain E3 ligase; PDB accession code: 1ND7) with the lowest *E*-value was selected as template. The sequence identity between the HECT domain and the template is about 80%. The human HECT domain was modelled using the automated mode of the SWISS-MODEL tool [18]. The relative position of the Itch and UbcH7 was modelled using as template the structure of an E6AP–UbcH7 complex (PDB code: 1C4Z), by optimal superposition of the main chain of the modelled HECT domain structure to the main chain of E6AP protein present in the X-ray solved complex. In order to investigate the transfer of the ubiquitin molecule from the E2 protein to the E3-ligase protein, we modelled HECT–UbcH7–ubiquitin complex in a putative catalytic conformation.

The ubiquitin moiety is present in the structure of Ubc1–ubiquitin thiolester complex [16] (PDB code: 1FXT) in which Ubc1 structure is similar to UbcH7. Therefore, using the program VMD [19], we superposed the main chain coordinates of the Ubc1 to the main chain of UbcH7 protein transferring

the ubiquitin coordinates into the Itch–UbcH7 model. The rmsd for this superposition was 2.0 Å. The structure of Ubc1 was subsequently removed. The relaxation of the entire system was performed by a 200 steps of steepest descent energy minimization using GROMOS96 force field [20].

2.2. Molecular dynamics simulation

The MD simulations were performed using the NAMD program [21]. The system was solvated using VMD software [19] adding 9200 water molecules. Periodic boundary conditions were used. The added waters were such that the distance between the protein and its image protein in the adjacent unit cell was about 12 Å. The system electroneutrality was granted by the addition of four sodium ions. The CHARMM22 [22] and TIP3P [23] force fields were used for the protein and the water, respectively. The time step was set to 2 fs. The electrostatic interactions were treated using the Ewald particle mesh methods [24,25]. The simulation was run at constant temperature (300 K) and pressure (1 atm).

The simulation in water of the docked system was carried out in three steps: (1) water minimization through 1000 steps of conjugate gradients; (2) minimization of the entire system through 1000 additional steps of conjugate gradients and (3) MD simulation of the entire system. The system was equilibrated at 300 K in steps of 50 K each 50 ps. Next, a 3.5 ns molecular dynamics simulation at room pressure and at 300 K was performed.

2.3. Normal mode analysis (β-Gaussian model)

The coarse-grained model, the β-Gaussian network model [26] provides a reliable and not very computational time-consuming description (especially if compared with full atom MD simulations) of concerted large-scale rearrangements in proteins. In this approach, the concerted motions are calculated within the quasi-harmonic approximation of the free energy F around a protein native state (assumed to coincide with the crystallographic structure or with a minimized model structure). Thus, a displacement from the native state $\delta R = \{\delta r_1, \delta r_2, \ldots, \delta r_n\}$ (r_i being the displacement of Cα atom i) is associated with a free energy change $\Delta F = (1/2)\delta R^{\dagger} F \delta R$, where F is an interaction matrix derived from the knowledge of contacting Cα (or CA) and Cβ centroids in the native state and the † superscript indicates the transpose matrix. The large-scale motions of the system correspond to the eigenvectors of F with the smallest non-zero eigenvalues.

To overcome the bias due to starting from a single initial configuration, the Gaussian model was calculated in four different systems: (1) the original model, (2) the configuration proposed by Verdecia and collaborators [11], in which the ψ and ϕ angles of the amino acids belonging to the hinge loop were modified in order to reduce the distance between the Cys residues involved in the interaction, and two different snapshots of the MD simulation at 2 and 3.5 ns, respectively.

2.4. Electrostatic potential calculation

The electrostatic potential on the surface of the proteins was calculated by solving the Poisson–Boltzmann equation using the program MolMol [27].

2.5. Computational mutagenesis

The Rosetta interface computational mutagenesis protocol [28] was used to predict the change in the binding free energy ($\Delta\Delta G_{bind}$) of residue mutations capturing the van der Waals, solvation and hydrogen bond contributes of interface residues to the binding. The algorithm estimates $\Delta\Delta G$ by modelling the residue mutation in both the protein complex and the uncomplexed monomers using a backbone-dependent rotamer library and the Rosetta Dock energy function. The Rosetta interface server can be accessed at http://robetta.bakerlab.org.

3. Results

3.1. Determination of the interaction surfaces of the HECT/UbcH7 complex

As discussed in the introduction, the biochemical mechanism of ubiquitin transfer requires conformational changes affecting the relative positioning of the HECT domain of Itch and the UbcH7 E2. These can be investigated by combining several well-established computational techniques, such as MD simulations, normal mode analysis and electrostatic potential calculations.

The three-dimensional structure of the UbcH7 protein has been experimentally determined (PDB code: 1C4Z), while no structure is available for Itch. Therefore we built a comparative model of the latter using the HECT type E6AP protein coordinates (PDB code: 1FXT) as template (see Section 2).

The dihedral angles of HECT residues Met 804, Gln 805 and Glu 806 were modified to the same final values used by Verdecia et al. [11] ($\phi = 59.9$, $\psi = 45.7$; $\phi = 12.1$, $\psi = -6.1$; $\phi = -98.7$, $\psi = -3.9$, respectively). As mentioned before, after this manual modification, the distance between the sulphur atoms of the catalytic Cys residues is about 8 Å. The electrostatic surface potential of the two proteins, calculated by solving the Poisson–Boltzmann equation, is shown in Fig. 1. The interaction surfaces of the two proteins are clearly complementary from an electrostatic point of view. They include a hydrophobic central region (Fig. 1, shown in white), including Phe63, Pro97 and Ala98 for UbcH7 protein and Phe684, Val691, Met703, Val741, Leu688 for HECT domain. The hydrophobic regions are surrounded by two charged rings, one positive on the UbcH7 surface and the other negatively charged on the HECT surface, that are stabilized by several salt bridges. Tables 1 and 2 show the predicted hydrophobic and ionic interactions at the interface between Itch and UbcH7. This is consistent with what we expect for intermolecular interactions where the hydrophobic forces stabilize the protein–protein interaction and the electrostatic forces orient the molecules and confer specificity to the interaction.

3.2. Virtual Ala scanning

An approximate estimate of the individual contributions of the residues involved in the interaction can be obtained by computational methods. We used the Rosetta interface computational mutagenesis approach [28], similar in principle to the experimental Ala-scanning mutagenesis procedure, to

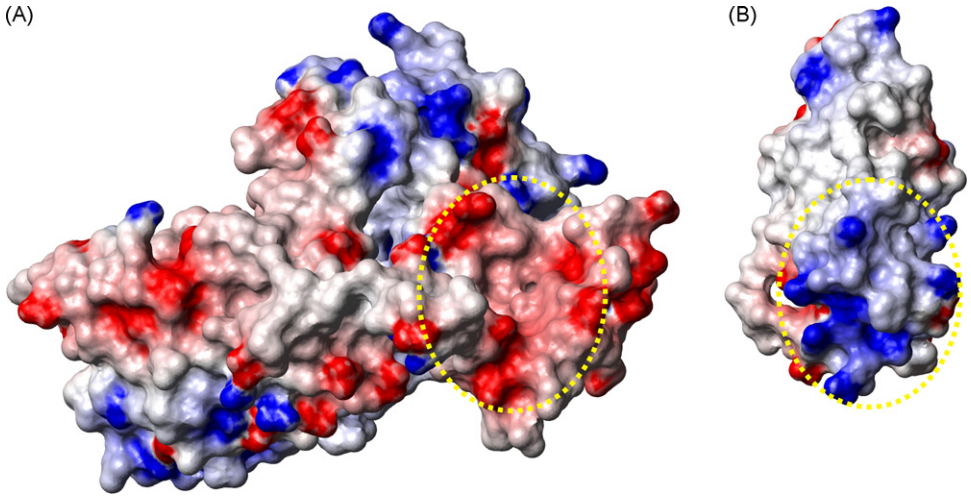

Fig. 1 – Electrostatic potential mapped on the molecular surface of the HECT domain, (A) and of the UbcH7 protein, (B). Red and blue represent negative and positive potential calculated using MOLMOL, respectively. (A) Negatively charged amino acids on the HECT surface are indicated by circles. (B) The circle highlights the UbcH7 ring of positively charged amino acids.

estimate the change in the binding free energy ($\Delta\Delta G_{bind}$) when each residue at the interface of the HECT/UbcH7 complex is mutated to Ala.

We retained the hard cut-off of $\Delta\Delta G_{bind} > 1.0$ kcal/mol to qualitatively identify hot-spot residues, defined as those essential for the interaction, since this is expected to correctly identify 80% of hotspot residue mutations [28] as judged by a test performed on a set of 19 high-resolution protein–protein complex crystal structures. A value of $\Delta\Delta G_{bind}$ for a residue between −1.0 kcal/mol and 1.0 kcal/mol is indicative of a neutral mutation, and therefore of a weak contribution of the interaction, $\Delta\Delta G_{bind} < -1.0$ kcal/mol indicates that a mutation to Ala can potentially increase binding affinity; values above 1.0 kcal/mol are predictive of important residues, whose mutation would lead to affinity decrease. We found that two HECT domain residues (Phe175 and Trp181) show a positive $\Delta\Delta G_{bind}$ (1.19 kcal/mol and 1.03 kcal/mol, respectively) and one UbcH7 residue (Phe63) resulted in an even larger positive $\Delta\Delta G_{bind}$ value (2.14 kcal/mol). These results suggest that these two HECT domain residues (Phe175 and Trp181) could be crucial for HECT/UbcH7 complex binding, and therefore that hydrophobic interactions are the larger contributors to the affinity of the complex. All other residues at the interface have $\Delta\Delta G_{bind}$ values not larger than 0.6 kcal/mol, suggesting that their mutation to Ala might have no functional consequences on the HECT/UbcH7 interaction.

Table 1 – Inter-protein hydrophobic interactions within 5 Å

Position	Residue	Chain	Position	Residue	Chain
684	Phe	A	63	Phe	D
688	Leu	A	63	Phe	D
680	Trp	A	97	Pro	D
680	Trp	A	98	Ala	D
691	Val	A	63	Phe	D
703	Met	A	63	Phe	D

Chains A and D indicate amino acids belonging to HECT domain and UbcH7 protein, respectively.

Table 2 – Inter-protein ionic interactions within 6 Å

Position	Residue	Chain	Position	Residue	Chain
681	Asp	A	64	Lys	D
702	Glu	A	5	Arg	D
702	Glu	A	9	Lys	D
700	Asp	A	6	Arg	D
700	Asp	A	6	Lys	D

Chains A and D indicate amino acids belonging to HECT domain and UbcH7 protein, respectively.

3.3. *MD simulation*

The time evolution of the conformational change was investigated by performing 3.5 ns of molecular dynamics simulation. The MD simulation was already equilibrated after 2 ns (data not shown). The salt bridges between the amino acids belonging to the charged rings shown in Table 2 were followed during the entire MD simulation in order to assess the stability of each ionic interaction. The results are shown in Fig. 2, where it can be clearly seen that the interactions are maintained during the entire MD simulation. Although the initially proposed salt bridges are conserved and reinforced during the MD simulation, the distance between the residues Asp700 (on HECT) and Lys9 (in UbcH7) become larger after 2 ns (about 6 Å), indicating a weaker interaction. The distance did not return to its initial value throughout the simulation. This supports the hypothesis that this salt bridge is not crucial for the stabilization of the HECT/UbcH7 interaction. Conversely, the interaction between the two residues Asp681 (HECT) and Lys64 (UbcH7) is stabilized during the MD simulation and their distance becomes even closer after 2 ns, when the system has converged.

In order to analyse the long-term dynamical properties of the system, we examined the normal modes of the complex,

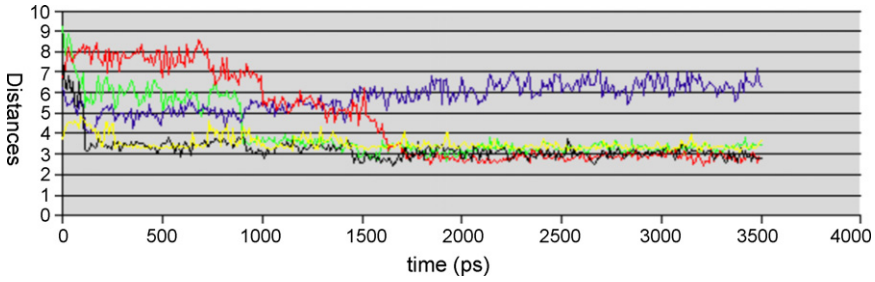

Fig. 2 – Distances (in A) between the amino acids forming salt bridges during the 3.5 ns MD simulation (for details see Table 2).

that is the vibrational modes at low frequencies. It is known that the analysis of normal modes requires the evaluation of the system evolution through tens of nanosecond, which is an unaffordable task in terms of computing time. To overcome this difficulty, we used the beta-gm method that implements a coarse-grained model to describe the dynamics of the protein. The normal mode analysis, in particular the projection of the eigenvalues along the principal eigenvector direction, allowed us to find two different hinge regions around which the lower frequency movements occur.

The first hinge region comprises the so called ''hinge loop region'' that extends from Met785 to Gln786, as already proposed by Verdecia and collaborators [11]. The second hinge region is very close to the N-lobe binding region and can be thought as an imaginary axis joining Gly673 and Gly749 alpha carbon atoms that we named the ''N-lobe axis'' (see Fig. 3). This hinge axis, divides the N-lobe domain into two different regions, a central region (blue in Fig. 3) and the interaction region (yellow in Fig. 3). These two regions of the HECT domain are connected by two loop regions that are likely to be responsible for the observed flexibility, which corresponds to a rotation of the interaction region around the N-lobe axis, shown by the green arrow in Fig. 3. Furthermore, we found that, upon rotation around the hinge loop region and the N-lobe axis, i.e., following a combination of the two principal slow-frequency motions, the distance between the catalytic Cys residue sulphur atoms, reached 3.5 Å, lower than the previously reported distances by Verdecia et al. [11], and close enough to account for the ubiquitylation process. Similar results were obtained by calculating the normal modes using as starting structure the HECT–UbcH7 complex model without changing the dihedral angles as proposed by Verdecia et al.

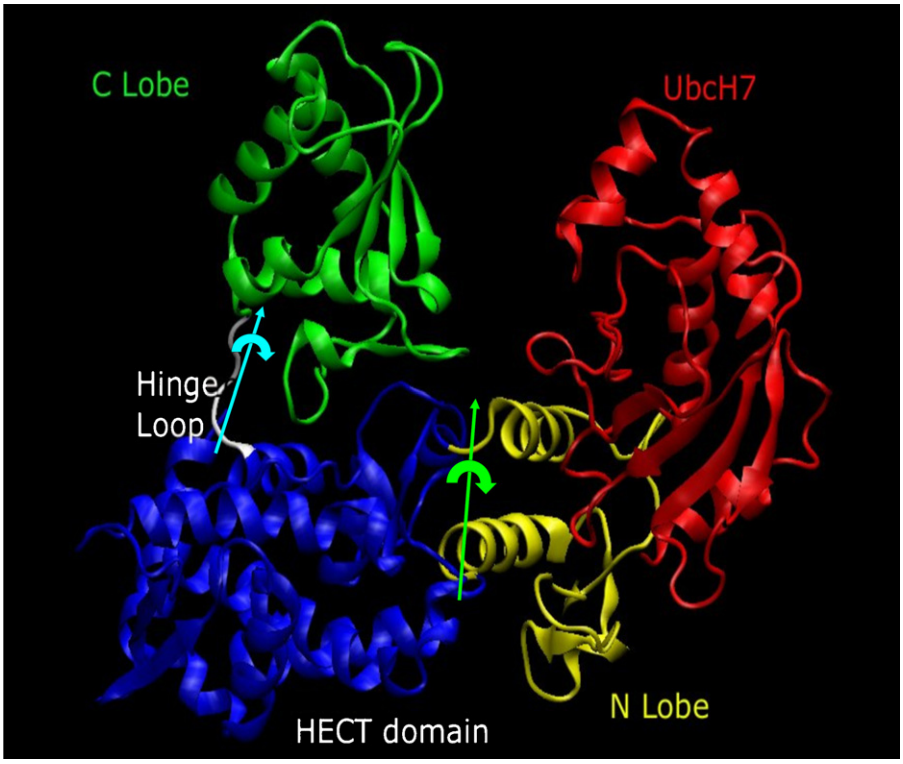

Fig. 3 – HECT/UbcH7 complex model, with the indication of the interaction partners and their domains (red, UbcH7; green, HECT C-lobe; yellow, the interaction sub-domain of the HECT N-lobe and blue, HECT central sub-domain. The proposed hinge regions, "hinge loop region" (white) and the "N-lobe axis", are shown as light-blue and green arrows, respectively.

[11] and using two snapshots of the MD simulation (data not shown).

Our analysis of the dynamic behaviour of the system highlighted the occurrence of a variety of conformational changes by the HECT domain (E3) not limited to three residues forming the hinge loop as previously hypothesized, but consisting of two relevant slow-frequency movements: (a) the rotation of the C-lobe around the hinge loop and (b) the rotation of the N-lobe around the N-lobe axis. In other words, the conformational changes involve the C-lobe region as previously stated [11], but this flexibility is part of a more complex mechanism.

4. Discussion

Our analysis of HECT–UbcH7 complex leads to a model of the ternary complex HECT–UbcH7–ubiquitin in its putative catalytic form that explains the arrangement that leads to their recognition and to the transfer of the ubiquitin molecule from the E2 to the targeted substrate. *In vitro* the thiolester intermediate has a high conversion rate [29] precluding its three-dimensional determination by either NMR spectroscopy or X-ray crystallography; therefore we built a model of the intermediate taking advantage of the structure of Ubc1–ubiquitin thiolester complex [16] (PDB code 1FXT; see Section 2). In this model the distances between the HECT catalytic Cys and the UbcH7–ubiquitin thiolester bond is 2.8 Å (Fig. 4), a value near the optimal distance for catalytic activity [11], while the distance between UbcH7 and HECT catalytic Cys is 3.5 Å. Our model provides a realistic picture of the structural relationships between the E2-ubiquitin thiolester intermediate and the E3 protein.

The computational analysis of protein structure and dynamics is a useful tool for studying phenomena that cannot be easily investigated experimentally. Because our understanding of the protein sequence, structure and flexibility relationships is neither completely accurate or complete, much care has to be taken in interpreting the results and the analysis is only valid when supported by experimental data. In this study we used state of the art methodologies to gain access to the dynamics of ubiquitin transfer between Itch and UbcH7. Our findings are rather convincing since, although no constraint was introduced in the simulations, their results produce a very realistic model that can account for the proposed catalytic mechanism.

The discovery of the ubiquitin-mediated protein system (UPS) degradation was achieved thanks to the pioneering work of Aaron Ciechanover, Avram Hershko and Irwin Rose, the 2004 Nobel Laureates in Chemistry (http://www.nobelprize.org © The Nobel Foundation 2004) [2–7], who developed an unprecedented concept: a 2.5 million Dalton protease, three tagging enzymes and a large protein tag able to specifically degrade proteins. With the concept came the idea to use this novel pathway for therapy.

Inhibitors of the proteasome have been developed in the last years [1]. Alfred L. Goldberg, a pioneer in the field of proteasome research, co-founded MyoGenetics (subsequently absorbed by ProScript and later by Millenium) with the original aim of developing proteasome inhibitors to slow the progression of muscle wasting diseases. He recognized that inhibiting the proteasome was an excellent way to study its own function. His team identified MG132 (MG standing for MyoGenetics), which remains the most widely used proteasome inhibitor in basic research, and PS-341 (PS standing for ProScript; also known as Bortezomib or Velcade®, Millennium Pharmaceuticals, Inc.) that has been approved for the treatment of multiple myeloma (Phase III clinical trial), and has demonstrated efficacy against other cancers. The original group of enzymologists (led by Ross Stein), chemists (led by Julian Adams) and cell biologists (led by Vito Palombella) at MyoGenetics led to the development of

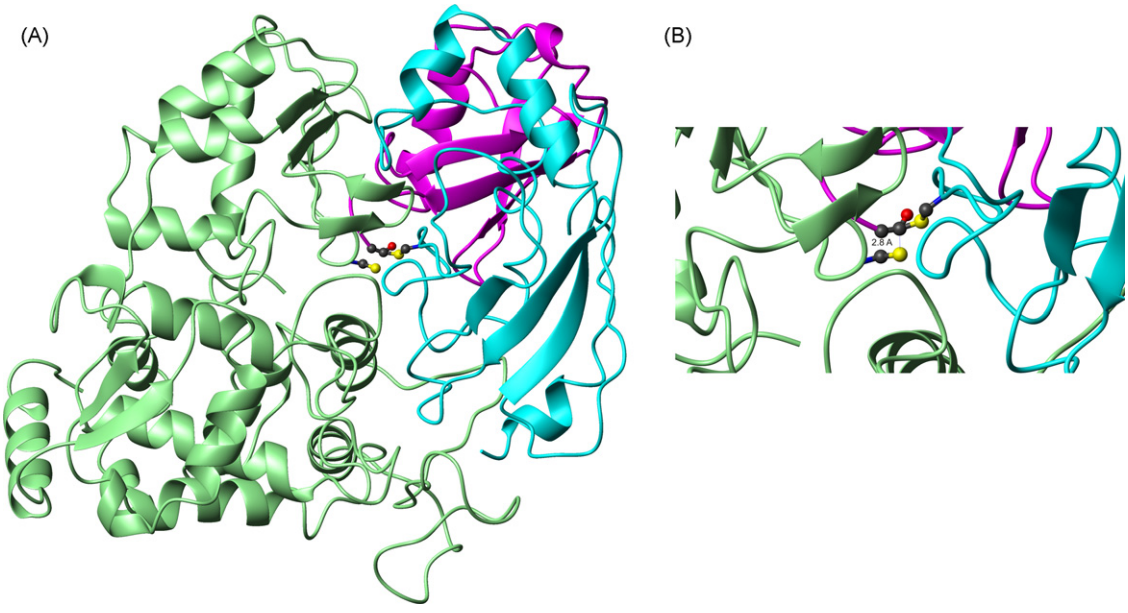

Fig. 4 – HECT–UbcH7–Ubiquitin ternary complex model in a putative catalytic conformation. (A) The HECT domain of Itch is shown in green; the Ubiquitin molecule in violet and UbcH7 in light blue. (B) The covalent bond that links the E2 (UbcH7) C88 thiol to the Ub C-terminal carboxylate to form the thiolester bond is shown in a ball and stick representation.

many other UPS inhibitors, such as PS-519, now also in clinical trials. A relevant survival regulator controlled by the UPS is NF-kB, where the inhibitor IkB is ubiquitylated by the E3 ligase β-TrCP and is thereby targeted for degradation. UPS inhibitors potentiate cisplatin-induced apoptosis, and revert cisplatin drug resistance by inhibiting the NER-dependent repair of cisplatin–DNA adducts via two distinct mechanisms: (i), proteasome inhibitor-induced depletion of ubiquitylated histone H2A in nucleosomes, which promotes chromatin condensation and possibly interferes with the function of DNA damage recognition and repair enzymes; (ii), proteaosome inhibitors diminish the excision repair cross-complementation group 1 (ERCC-1) response to cisplatin, perhaps secondary to changes in chromatin structure that interfere with transcription of the ERCC-1 gene. These inhibitors of the proteasome are therefore powerful inducers of apoptosis and are now undergoing evaluation in clinical trials focussed on several cancers.

The regulation of protein stability by the UPS is a critical issue for further understanding the molecular basis of tumorigenesis. Aberrant proteolysis of substrates involved in the regulation of cell cycle, gene transcription and apoptosis has been shown to contribute to carcinogenesis. As a consequence, development of reagents that specifically inhibit components of the ubiquitin system is crucial in analyzing molecules of the ubiquitylation pathways as potential molecular targets in cancer therapy.

The stringent hierarchical organization of the UPS offers a novel source of molecular targets for innovative pharmacological therapies. In fact mammals have one proteasome, while E1s, E2s and E3s show a logarithmic sequential expansion, with several hundreds of E3s described so far. Given the lack of specificity of proteasome inhibitors, in principle, manipulating more specific UPS component, such E2s or E3s should provide a more powerful therapeutic tool. Indeed, targeting specific E3s should affect a limited number of protein substrates, thus reducing unwanted side effects. In reality, the theoretical advantage of targeting E3s should be considered against its problems: the promiscuity of both enzymes and substrates, and the complicated intrinsic enzymology required to identify small molecule inhibitors of E3s. Still, drugs targeting ubiquitin ligases promise a better therapeutic ratio than proteasome inhibitors. The identification of specific inhibitors of human Mdm2 to regulate p53 in cancer has been successfully described as a paradigm of E3s inhibitors [30]. The HECT E3s family, with its intrinsic catalytic ubiquitin transfer activity (reminiscent of E2s), is easier to inhibit than RING E3s family. Importantly, the HECT-containing E3s show a clear involvement in cancer, as indicated in a recent review [8]. Itch promotes the ubiquitylation of a variety of protein targets, many of which are crucially involved in controlling cell growth, differentiation and apoptotic cell death [31]. In particular, Itch has several cancer-related substrates [reviewed in Ref. [31]], including p73 [15], p63 [32], c-jun, jun-B [14] and Notch, erbB, indicating that an Itch inhibitor should show anti-cancer properties. Indeed, at least *in vitro*, the selective inhibition of Itch is able to sensitize cells to chemotherapy [33]. This raises the intriguing possibility that Itch inhibition could prove valuable for therapeutic purposes. Studies aimed to unveil distinct structural features of single HECT E3s and/or their catalytic mechanism may facilitate the development of molecules able to inhibit specific E3s.

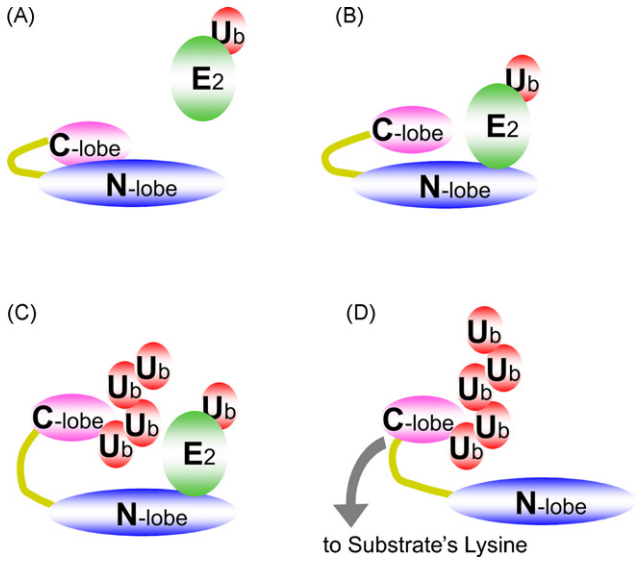

Fig. 5 – Model of the catalytic action of the HECT. Model of the interaction between the HECT domain (N- and C-lobe) and the E2, based on the structure of the E6AP–UbcH7 and the models WWP1/AIP5-UbcH5 and Itch/AIP4-UbcH7; modified from Refs. [11] and [34]. The HECT C-lobe (pink) interacts with the E2 (green) thanks to a hinge region (yellow) and specific residues (not shown) to allow the catalysis of the catalytic site [11,34]. The scheme (panels A–D) shows the transfer of ubiquitin (red) from the E2 and formation of the ubiquitin elongation (panels B–D) by the HECT domain, before the transfer to the substrate's lysine (panel D). Potential areas for drug design are, indeed, (i) the catalytic site, (ii) the hinge region, as well as (iii) the HECT-E2 and HECT-substrate interacting residues.

The data presented in this paper are relevant for the development of Itch inhibitors. Blocking Itch activity could be achieved by chemically inhibiting E2 recruitment or, alternatively, by preventing specific substrate recognition by E3s (either directly or allosterically). Fig. 5 shows a schematic model of action of Itch. Itch, following binding of an ubiquitin-charged E2, forms a thioester bond between its own active site cysteine and the ubiquitin C-terminus. Subsequently, the HECT domain catalyzes the formation of an isopeptide bond between the ε-N side chain of a lysine residue and the ubiquitin C-terminal carboxylate. Catalysis requires a rotation of the N- and C-lobes of the HECT domain on a specific polypeptide hinge-linking region, shown in yellow in Fig. 5 [11,34]. As shown in Fig. 5, potential areas for drug development on Itch are (i) the catalytic site, (ii) the hinge region and (iii) the HECT-E2 and HECT-substrate interacting residues. In fact, the experimental data reported in the present paper contribute by identifying the residues involved.

Acknowledgements

This work was partially supported by the BioSapiens project, funded by the European Commission within its FP6 Programme, under the thematic area "Life sciences, genomics

and biotechnology for health'', contract number LSHG-CT-2003-503265, by the Istituto Pasteur – Fondazione Cenci Bolognetti and by FIRB projects LIBI and ITALBIONET awarded to A.T. This work has been also supported by EU-Grant EPISTEM (LSHB-CT-019067); ''Alleanza contro il Cancro'' Grant (ACC12); the MIUR/PRIN Grant RBIP06LCA9_0023; the AIRC Grant 1338; Grant Telethon GGPO4110 and ISS ''Programma Italia-USA'' N526D5 awarded to G.M. Research described in this article was also supported in part by Philip Morris USA Inc. and Philip Morris International.

REFERENCES

[1] Melino G. Discovery of the ubiquitin proteasome system and its involvement in apoptosis. Cell Death Differ 2005;12:1155–7.
[2] Hershko A. Early work on the ubiquitin proteasome system, an interview with Avram Hershko. Cell Death Differ 2005;12:1158–61.
[3] Rose I. Early work on the ubiquitin proteasome system, an interview with Irwin Rose. Cell Death Differ 2005;12:1162–6.
[4] Ciechanover A. Early work on the ubiquitin proteasome system, an interview with Aaron Ciechanover. Cell Death Differ 2005;12:1167–77.
[5] Hershko A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. Cell Death Differ 2005;12:1191–7.
[6] Rose I. Ubiquitin at Fox Chase. Cell Death Differ 2005;12:1198–201.
[7] Ciechanover A. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Cell Death Differ 2005;12:1178–90.
[8] Bernassola F, Karim M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell 2008;14(1):10–21.
[9] Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci USA 1995;92:5249.
[10] Schwarz SE, Rosa JL, Scheffner M. Characterization of human hect domain family members and their interaction with UbcH5 and UbcH7. J Biol Chem 1998;273:12148–54.
[11] Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, et al. Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol Cell 2003;11:249–59.
[12] Gallagher E, Gao M, Liu YC, Karin M. Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. Proc Natl Acad Sci USA 2006;103:1717–22.
[13] Wiesner S, Ogunjimi AA, Wang HR, Rotin D, Sicheri F, Wrana JL, et al. Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. Cell 2007;130:651–62.
[14] Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu YC, et al. Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. Science 2004;306:215–71.
[15] Rossi M, De Laurenzi V, Munarriz E, Green DR, Liu YC, Vousden KH, et al. The ubiquitin-protein ligase Itch regulates p73 stability. Embo J 2005;24:836–48.
[16] Hamilton KS, Ellison MJ, Barber KR, Williams RS, Huzil JT, McKenna S, et al. Structure of a conjugating enzyme-ubiquitin thiolester intermediate reveals a novel role for the ubiquitin tail. Structure 2001;9:897–904.
[17] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[18] Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 1997;18:2714–23.
[19] Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol Graph 1996;14:33–8 [27–8].
[20] van Gunsteren WFB, Billeter SR, Eising AA, Hunenberger PH, Kruger P, Mark AE, et al. Biomolecular smulation: the GROMOS96 manual and user guide. Zurich/Groningen: vdf Hochschulverlag AG and der ETH Zurich and BIOMOS bv; 1996.
[21] Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, et al. Scalable molecular dynamics with NAMD. J Comput Chem 2005;26:1781–802.
[22] MacKerell AD, Bashford D, Bellott M, Dunbrack RL, Evanseck JD, Field MJ, et al. All-atom empirical potential for molecular modeling and dynamics studies of proteins. J Phys Chem B 1998;102:3586–616.
[23] William LJ, Jayaraman C, Jeffry DM, Roger WI, Michael LK. Comparison of simple potential functions for simulating liquid water. J Chem Phys 1983;79:926–35.
[24] Tom D, Darrin Y, Lee P. Particle mesh Ewald: An N [center-dot] log(N) method for Ewald sums in large systems. J Chem Phys 1993;98:10089–92.
[25] Ulrich E, Lalith P, Max LB, Tom D, Hsing L, Lee GP. A smooth particle mesh Ewald method. J Chem Phys 1995;103:8577–93.
[26] Micheletti C, Carloni P, Maritan A. Accurate and efficient description of protein vibrational dynamics: comparing molecular dynamics and Gaussian models. Proteins 2004;55:635–45.
[27] Koradi R, Billeter M, Wuthrich K. MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graph 1996;14:51–5 [29–32].
[28] Kortemme T, Baker D. A simple physical model for binding energy hot spots in protein–protein complexes. Proc Natl Acad Sci USA 2002;99:14116–21.
[29] Hodgins R, Gwozd C, Arnason T, Cummings M, Ellison MJ. The tail of a ubiquitin-conjugating enzyme redirects multi-ubiquitin chain synthesis from the lysine 48-linked configuration to a novel nonlysine-linked form. J Biol Chem 1996;271:28766–71.
[30] Yang Y, Ludwig RL, Jensen JP, Pierre SA, Medaglia MV, Davydov IV, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell 2005;7:547–59.
[31] Melino G, Gallagher E, Aqeilan RI, Knight R, Peschiaroli A, Mario Rossi M, et al. Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. Cell Death Differ 2008. doi:10.1038/cdd.2008.60.
[32] Rossi M, Aqeilan RI, Neale M, Candi E, Salomoni P, Knight RA, et al. The E3 ubiquitin ligase Itch controls the protein stability of p63. Proc Natl Acad Sci USA 2006;103:12753–8.
[33] Hansen TM, Rossi M, Roperch JP, Ansell K, Simpson K, Taylor D, et al. Itch inhibition regulates chemosensitivity in vitro. Biochem Biophys Res Commun 2007;361:33–6.
[34] Diao J, Zhang Y, Huibregtse JM, Zhou D, Chen J. Crystal structure of SopA, a Salmonella effector protein mimicking a eukaryotic ubiquitin ligase. Nat Struct Mol Biol 2008;15:65–70.