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ARTICLE

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Covalent immobilization of recombinant fusion proteins with hAGT for single molecule force spectroscopy

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Abstract A genetically modified form of the human DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (hAGT) was used to immobilize different recombinant hAGT fusion proteins covalently and selectively on gold and glass surfaces. Fusion proteins of hAGT with Glutathione S-Transferase and with tandem repeats of Titin Ig-domains, were produced and anchored via amino-polyethylene glycol benzylguanine. Anchoring was characterized and quantified with surface plasmon resonance, atomic force microscope and fluorescence measurements. Individual fusion proteins were unfolded by single molecule force spectroscopy corroborating the selectivity of the covalent attachment.

Keywords Molecular recognition · SPR · AFM · Suicide coupler · hAGT · SNAP-tag

Abbreviations hAGT: O^6-alkylguanine-DNA-alkyltransferase · GST: Glutathione S-Transferase · PEG: Polyethylene glycol · BG: Benzylguanine · SPR: Surface plasmon resonance · AFM: Atomic force microscope · EDC: 1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride · NHS: *N*-hydroxy succinimide · GST: Glutathione S-transferase · CMC: Carboxymethylcellulose

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Introduction

Various areas of modern biotechnology are in great demand for strategies and protocols to attach recombinant proteins permanently, selectively and in a defined manner to solid surfaces. Especially in the field of single molecule biophysics the need for such techniques is emerging. For a broad range of conventional binding studies at surfaces, physisorption of, e.g. a capture antibody in an ELISA, is sufficient as long as the spontaneous off-rate is slower than the one of the target molecule, a covalent attachment is essential for the rapidly growing number of experiments, where forces are measured between molecules. In such experiments the weakest of the bonds in series ruptures first and it must not be the attachment. However, since the force required to rupture a bio-molecular complex is not directly correlated to the binding energy, physisorption, although thermally stable is in many cases insufficient. Various strategies for a covalent attachment have therefore been investigated and established, most of them based on covalently binding the protein of choice either via amines or the thiol group of a cysteine. Besides the limited lifetime due to hydrolysis, the low selectivity and the limited yield of these coupling reactions motivate the search for alternative strategies. Here, we investigated the possibility of using fusion proteins with a mutant of O^6-alkylguanine-DNA-alkyltransferase (hAGT) also known as SNAP-tag in combination with its substrate polythylene glycol (PEG)–benzylguanine (BG) as a promising strategy for the covalent and directed attachment of proteins for single molecule force spectroscopy.

The natural role of hAGT is the repair of alkylation damage of the DNA at the O^6-position of guanine in a unique, stoichiometric reaction (Daniels and Tainer 2000). Since hAGT also accepts free O^6-benzylguanine as a substrate it is possible to inactivate hAGT irreversibly with this small molecule (Pegg et al. 1993). Interestingly, oligonucleotides containing derivatives of O^6-benzylguanine with substituted benzyl rings are also accepted as

substrates of hAGT (Damoiseaux et al. 2001). As a consequence, various derivatives of BG were used to label hAGT fusion proteins with small molecules in vivo (Keppler et al. 2003). A BG–PEG-amino derivative, covalently attached to carboxy dextran gold surfaces (Biacore) via EDC/NHS chemistry, was used in a previous study to immobilize GST-hAGT fusion proteins on these BG activated slides (Kindermann et al. 2003).

Here we used the same BG–PEG-amino derivative as an anchor (Fig. 1) and verified the immobilization of Gluthathione S-Transferase (GST)–hAGT fusion proteins on gold surfaces. In the next step, Titin–GFP–hAGT fusion proteins (Fig. 2) were anchored on gold and glass surfaces and were investigated with surface plasmon resonance (SPR), fluorescence and single molecule measurements.

Materials and methods

If not stated otherwise, all chemicals used for the functionalization of surfaces were of analytical standard and

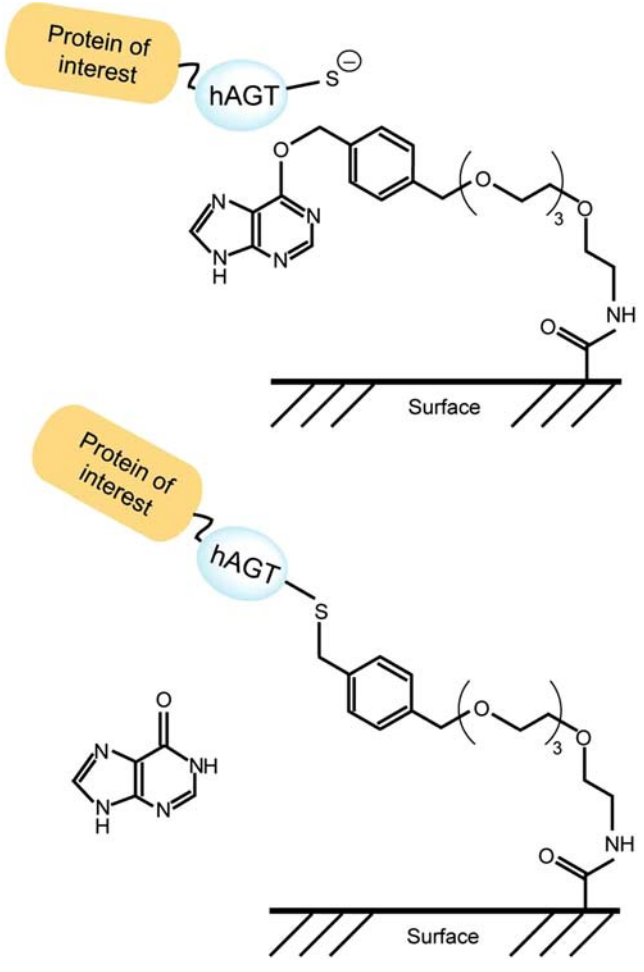

Fig. 1 Immobilization principle of hAGT fusion proteins. The BG–PEG-amino derivative is attached to carboxylized gold and glass surfaces via EDC/NHS chemistry. The hAGT protein accepts BG as a substrate and connects itself to the surface

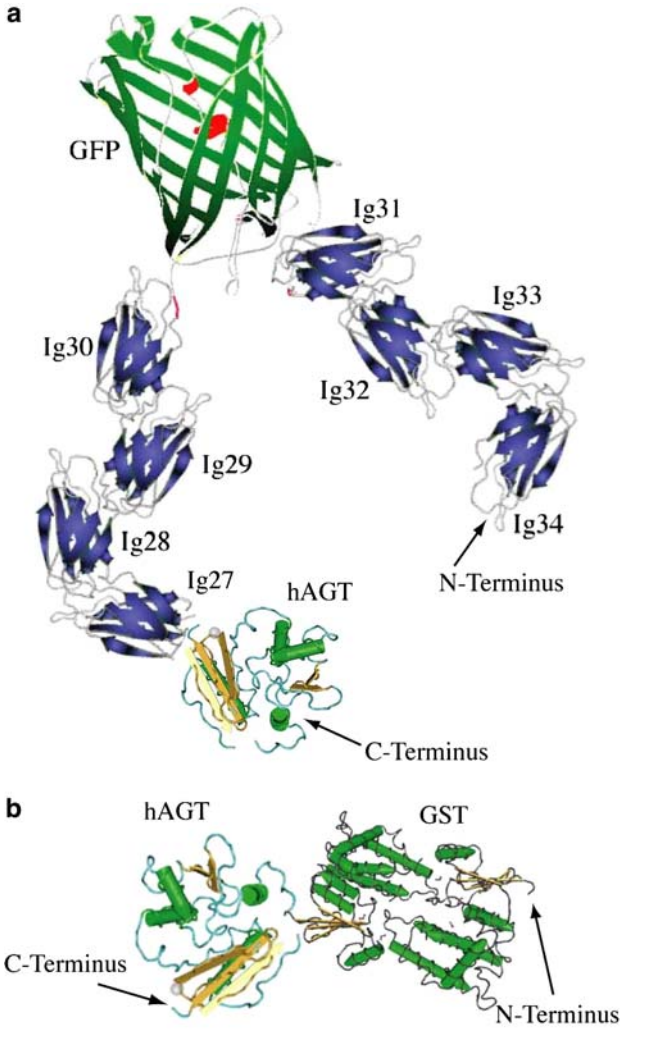

Fig. 2 O^6-alkylguanine-DNA-alkyltransferase fusion proteins used for the immobilization experiments. **a** Titin–GFP–hAGT fusion protein. Molecular weight about 100 kDa. GFP serves as a fluorescence marker, the eight Ig-domains as molecular rulers for unfolding forces and segment lengths. **b** GST–hAGT fusion protein. Molecular weight about 45 kDa

purchased by Sigma (Taufkirchen, Germany). PBS (10 mM PBS, 150 mM NaCl, pH 7.4) and MES (10 mM MES, 150 mM NaCl, pH 6.3) were used as buffer solutions. For the hAGT fusion proteins, we used a genetically modified form of the wild type form of hAGT, that has a 20-fold increased activity against BG (Juillerat et al. 2003). In addition, the DNA binding site was mutated (Gendreizig et al. 2003) and cysteine 62 was exchanged to alanine (unpublished data). The DNA sequence of this hAGT mutant was C-terminally fused to the sequences of GST and Titin–GFP using standard molecular biology protocols. The recombinant proteins were expressed in *E. coli*. The GST–hAGT fusion protein was expressed following the protocol in Kindermann et al. (2003) and purified with a GST affinity column following the instructions of the affinity medium (Amersham Biosciences, Freiburg, Germany). Purified GST–hAGT fusion proteins were stored in PBS or MES

at 4°C. The Titin-GFP–hAGT fusion protein was expressed exactly as described for Titin–GFP fusion proteins (Dietz and Rief 2004b). The Titin–GFP–hAGT fusion proteins were immobilized without prior purification. Therefore BG functionalized slides were incubated directly with crude extracts of *E. coli* cells expressing this fusion protein.

Surface plasmon resonance measurements

To investigate the binding of hAGT fusion proteins on gold-slides, we used a homebuilt multi-channel SPR device that consists of several commercially available SPR-sensor chips (Neuert et al. 2004). Spreeta Evaluation Module software (version 5.21) was used to analyse the SPR curves. All SPR experiments were performed at constant room temperature with thoroughly degassed PBS or MES buffer solutions at a constant flow-rate of 0.03 ml/min.

Cover slides were evaporated at a pressure of 1–2×10^{-6} mbar with 1 nm chrome/nickel (GoodFellow, GB) as adhesive layer and 50 nm high-purity gold (purity degree: 99.99%, Leybold Optics, Germany). Afterwards, the slides were incubated with cysteamine (20 mM) for 12 h to obtain a cysteamine monolayer. Carboxymethylcellulose (CMC) was bound to these amino groups using standard EDC protocols to obtain carboxylized gold surfaces. In addition to CMC surfaces, sulphur–PEG–COOH ($M = 20$ kd) (Rapp Polymere GmbH, Tübigen, Germany) gold coated surfaces were prepared. For this purpose S–PEG–COOH molecules were solved in H_2O (3 mM). The COOH groups of that polymers were activated in solution with EDC (100 mM) and *N*-hydroxysuccinimide (NHS) (50 mM). Those activated polymers were incubated with BG (5 mM) for 12 h. All non-reacted COOH groups were quenched with ethanolamine (1 M) for 30 min. Gold coated cover slides were incubated with the BG activated PEG for 4 h. The control sample was treated identically except for the BG activation, which was omitted.

These gold slides were optically coupled to the SPR sensors using index matching oil (518 C, Zeiss, Germany).

Fluorescence binding measurements

To verify the specific anchoring of Titin–GFP–hAGT fusion proteins using the auto-fluorescence properties of GFP, Titin–GFP–hAGT proteins were immobilised on aldehyde-functionalised glass slides (Quantifoil Micro Tools GmbH, Germany). The aldehyde groups were oxidised with potassium permanganate to carboxyl groups. After that, spots of BG (3 mM) were attached to these groups using standard EDC/NHS protocols. All non-reacted NHS groups were blocked with 1 M ethanolamine. Following this, the Titin–GFP–hAGT fusion proteins were coupled to this surface by incubating the whole slide with the crude extract of hAGT-expressing *E. coli* cells. After an incubation time of 45 min all unbound proteins from the cell extract were removed by extensive washing with PBS.

A fluorescence-scanner (LS100, Tecan, Austria) was used to determine the amount of bound fusion proteins. GFP was excited with a 488 nm laser and the emitted light was filtered with a 500–570 nm band-pass filter. The spatial resolution was 20 μm. Mean fluorescence as well as background intensity was determined by using NIH IMAGE software (National Institutes of Health, Bethesda).

Single molecule force spectroscopy

All single molecule force measurements were performed with a custom-built atomic force microscope (AFM) (Oesterhelt et al. 1999). Cantilevers were calibrated in solution using the equipartition theorem (Butt and Jaschke 1995; Florin et al. 1995). This method provides a resolution, in force, of roughly 10%. Two types of gold-coated cantilevers (Bio-Levers, Olympus, Japan) with spring constants and resonance frequencies of 30 pN/nm and 8.5 kHz or 6 pN/nm and 1.5 kHz, respectively, were used. The force curves of the Titin–GFP–hAGT construct were collected at pulling speeds ranging around 300 nm/s. All experiments were conducted at room temperature in PBS buffer.

Titin–GFP–hAGT fusion proteins were immobilised on BG activated aldehyde-functionalised glass slides as described before (see fluorescence binding measurements).

Results and discussion

Binding studies with surface plasmon resonance

In the beginning we describe an experiment on CMC functionalized cover slides. The CMC layer in channel 1 was activated with BG (Covalys Biosciences AG, Switzerland) using standard EDC/NHS protocols. As a control for specific immobilisation of the fusion proteins in channel 2 no BG, but also EDC/NHS was added. The attachment of the BG–PEG-amino derivative causes an increase of layer thickness of about 6 Å in channel 1 (Fig. 3). After blocking all non-reacted NHS groups from both channels with 1 M ethanolamine, each channel was incubated with GST–hAGT fusion protein. The sensor response of channel 1 (BG activated) was about four times higher than the response of channel 2 (non-activated with BG) (Fig. 4).

The SPR measurements show that a protein layer of the same thickness as a GST–hAGT monolayer is bound only to the BG activated surface. The result of this immobilisation experiment is in good accordance with literature values (Kindermann et al. 2003).

With the anchoring protocol established for GST, in the second experiment we now immobilised a Titin–GFP–hAGT fusion protein on a S–PEG–COOH coated

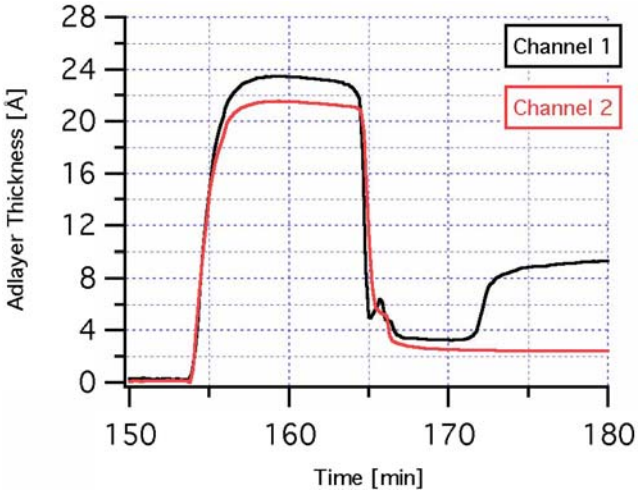

Fig. 3 Surface plasmon resonance signal of the BG-PEG-amino anchor. Carboxylized gold surfaces were activated with EDC/NHS ($t = 154$ min). After a short washing step with H_2O (Millipore, Germany) ($t = 164$ min) the BG-PEG-amino derivative was anchored to the surface of channel 1. The immobilization of the BG anchor causes an increase in layer thickness of about 6 Å

gold surface. The PEG of channel 1 was activated with BG and the PEG of channel 2 was not. First the surfaces were equilibrated in MES buffer. After 10 min the surfaces of both channels were incubated with crude cell extract of Titin–GFP–hAGT expressing bacteria resuspended in MES buffer. We observed a sizable increase (about 12 Å) in the measured adlayer thickness, which we attribute to the high density of the cell content (Fig. 5). The thickness of the surface in channel 2 slightly decreased in time to drop to nearly zero after

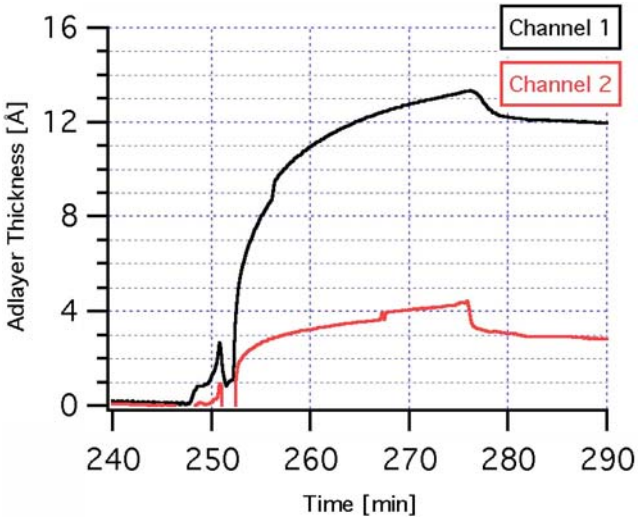

Fig. 4 Surface plasmon resonance signal of GST–hAGT fusions proteins. Surface of channel 1 was activated with BG. Surface of channel 2 was not activated with BG. At time $t = 248$ min the surfaces of both channels were incubated with GST–hAGT fusion proteins. After an incubation time of about 30 min all unbound proteins were washed away with PBS buffer ($t = 276$ min). The SPR response of channel 1 was about four times higher than that of channel 2

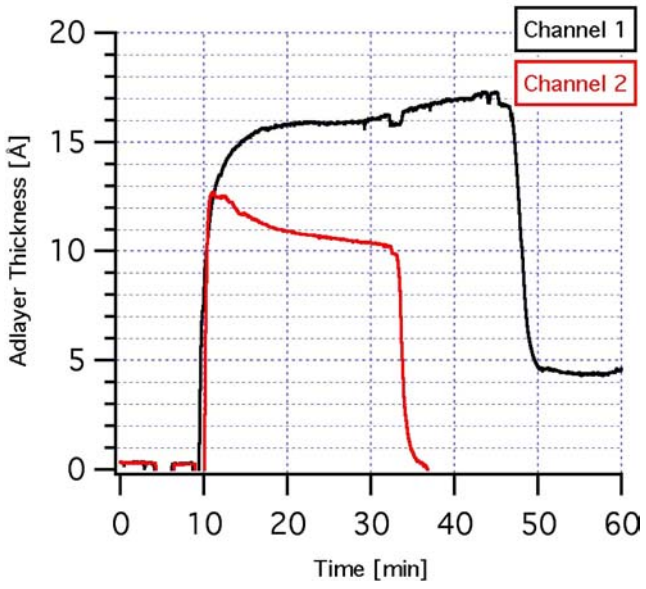

Fig. 5 Surface plasmon resonance signal of Titin–GFP–hAGT fusion proteins. The surface of channel 1 was activated with BG. The surface of channel 2; Å was not activated with BG. At time $t = 10$ min the surfaces of both channels were incubated with crude cell extract of Titin–GFP–hAGT expression bacteria. After an incubation time of about 35 min all unbound proteins were washed away with MES buffer ($t = 45$ min, channel 1; $t = 35$ min channel 2). On the surface of channel 2 no protein was bound whereas in channel 1 a film thickness of 4.5 Å remained

extensive rinsing with MES buffer. The thickness in channel 1, however, slightly increased with time. After rinsing, a final thickness increase of 4.5 Å remained.

Verification of anchoring with fluorescence measurements

The specific binding of Titin–GFP–hAGT fusion proteins on BG coated surfaces was verified by spotting BG on EDC/NHS activated slides. Titin–GFP–hAGT fusion proteins were anchored to these slides as described above. The amount of bound fusion proteins was detected by fluorescence measurements. The result is shown in Fig. 6.

The result of the fluorescence-binding assay clearly shows that Titin–GFP–hAGT fusion proteins are only bound to BG activated spots of the glass slide. It also proves the high selectivity of this immobilisation technique since the anchoring was carried out with crude cell lysate.

Single molecule force spectroscopy on anchored proteins

The selectivity of the attachment of hAGT fusion proteins was also investigated by single molecule force spectroscopy. This method is complementary to SPR and fluorescence measurements. Single proteins anchored between surface and AFM cantilever tip can be

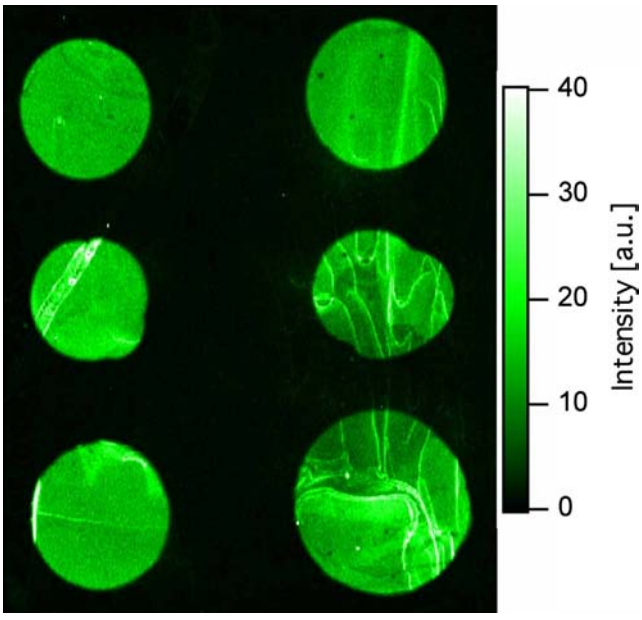

Fig. 6 Fluorescence signal of immobilized Titin–GFP–hAGT fusion proteins. The whole area was activated with EDC/NHS and six spots of BG were coupled to this surface. After blocking all non-reacted NHS groups with 1 M ethanolamine the whole area was incubated with Titin–GFP–hAGT fusion proteins. The fluorescence signal between BG activated areas to non-activated areas was typically 17:1

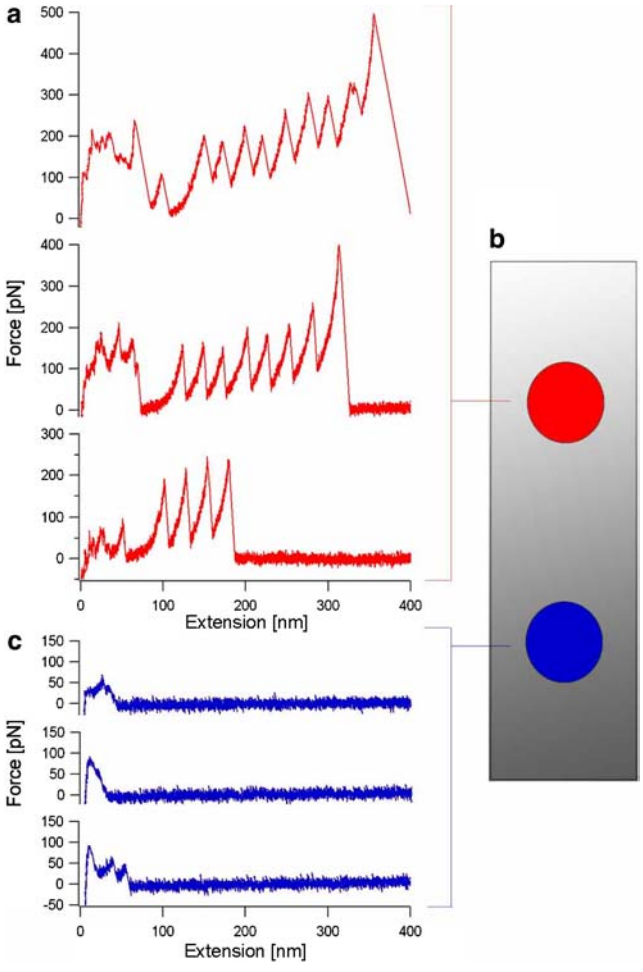

Fig. 7 Atomic force microscope experiments on immobilized hAGT–Titin fusion proteins. The upper spot of the slide was activated with the BG anchor while the lower spot was not activated (**b**). Both spots were incubated with Titin–GFP–hAGT fusion proteins. Before the AFM experiments, all unbound proteins were washed away with PBS buffer. **a** Typical force-extension traces collected at the BG activated spot. **c** Typical force-extension traces collected at the non-activated spot

identified via their specific mechanical unfolding pattern (Rief et al. 1997). Recently, the mechanical unfolding of single Titin–GFP proteins (lacking the hAGT domain) has been investigated and their specific mechanical unfolding pattern has been identified (Dietz and Rief 2004b). Those experiments were performed with unspecific adsorbed proteins. Here we anchored hAGT–Titin–GFP fusion proteins with BG on a glass slide in a site-directed manner. For this purpose, one spot (upper spot Fig. 7b) on the glass slide was activated with BG while the other spot was not activated. Both spots were incubated for 45 min with *E. coli* crude extract and afterwards extensively rinsed with PBS buffer to remove all unbound molecules.

Figure 7a shows typical force-extension traces collected at the BG activated spot. They exhibit the typical saw-tooth pattern due to sequential domain unfolding in single Titin and Titin–GFP molecules as described before (Dietz and Rief 2004b; Rief et al. 1997). At extensions below 100 nm all traces exhibit complicated force patterns, which are most probably due to multiple molecule interactions. Then, at higher extensions the force gradually increases according to polypeptide elasticity until one of the contained Titin domains unfolds. This leads to a quasi-instantaneous increase in the contour length of the polypeptide and the force drops rapidly. Then, subsequent stretching of the lengthened molecule takes place until the next Titin domain unfolds. These unfolding events are equidistant since the Titin domains are identical in size. Then ultimately, the whole molecule ruptures from the cantilever (reflected by the last force peak in each trace) and the force drops to zero.

Titin domains exhibit a much higher unfolding force than GFP and thus GFP unfolding always occurs at small extensions. Therefore, this unfolding event will be often masked by non-specific interactions (Dietz and Rief 2004a). This can also be seen in our data—especially in the two topmost traces in Fig. 7a. There we note at least seven Titin domain unfolding events and therefore, also expect a detected GFP unfolding event. However, this event is obviously masked by the multiple molecule interactions below 100 nm extension. The same will most probably be true for the hAGT domain contained in the investigated molecules. In our data we could not find clear indication for an additional event reflecting the unfolding of the hAGT domain. This also supports the notion that hAGT loses partly its structural integrity when it binds to its target BG (Daniels et al.

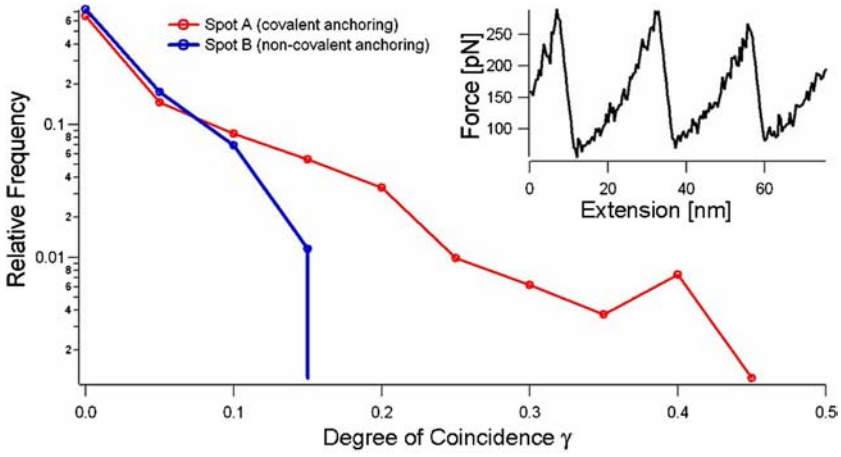

Fig. 8 Results of the search for Titin domain unfolding events on the activated and on the non-activated spot with a pattern recognition algorithm. We searched all force-distance curves from both areas for the typical Titin fingerprint (inset). It is clearly visible that at the BG activated spot the frequency of partial ($\gamma > 0.2$) and good matching ($\gamma > 0.35$) with the three Titin domain-unfolding pattern is by far higher than at the non-activated spot

2000). The mechanical contribution of the amino-polyethylene glycol linker to the force-extension curves should be negligible, since the PEG linker consists of only three monomers. It will be an important task for the future to further characterise the mechanical properties of this enzyme.

However, the traces in Fig. 7a clearly demonstrate on the single molecule level the successful anchoring of the Titin–GFP–hAGT molecules. In contrast, the traces collected at spot B (Fig. 7c) exhibit only unspecific low-force interaction patterns, which cannot be attributed to the unfolding of modules contained in the Titin–GFP–hAGT molecule.

To compare quantitatively the yield of force-extension traces exhibiting Titin unfolding patterns collected on both spots, we performed an analysis based on pattern recognition techniques as described in Dietz and Rief (2004a). This method involves first definition of a test pattern, then identification of the best matching section with the test pattern in each force trace and finally calculation of a degree of coincidence γ with the pattern as defined in equation 10 in Dietz and Rief (2004a). As a test pattern we chose a section of a measured single molecule force-extension trace exhibiting three Titin domain-unfolding events (Fig. 8, inset). The graph shows the distribution of the degrees of coincidence with the given pattern as they have been assigned to each force trace contained in the data sets collected at the BG activated spot and at the non-activated spot. It is clearly visible that at the BG activated spot the frequency of partial ($\gamma > 0.2$) and good matching ($\gamma > 0.35$) with the three Titin domain-unfolding pattern is by far higher than at the non-activated spot. This testifies again that proteins containing Titin domains are selectively immobilised only on the BG activated spot.

We therefore conclude that the anchoring is indeed performed via the hAGT–BG coupling mechanism. However, from our single molecule experiments we cannot infer directly if the binding is covalent since the forces at which the molecules rupture from the cantilever are compromised by the fact that the connection between the stretched molecules and the cantilever was still unspecific. It will be necessary to anchor single proteins selectively and specifically on both the substrate and cantilever. Then, from the rupture forces one would be able to infer if the nature of the binding is covalent, since rupture forces should then reach into the nN regime (Grandbois et al. 1999).

Conclusion

Our study clearly shows that anchoring of fusion proteins via hAGT to BG activated surfaces is a suitable technique for single molecule force spectroscopy. The results show that the hAGT in the fusion acts as an anchor for the coupling and that it does not influence the unfolding behaviour of the molecule of interest. This technique offers several advantages: the first one lies in the gentle coupling procedure (in particular no drying required). There is no need for any (chemical) modification on the protein of interest making it possible to investigate the protein under native conditions. The possibility to use different functionalized surfaces (here CMC and S–PEG–COOH coated surfaces) is another advantage especially in terms of investigations with the AFM. The highly specific, self-searching coupling mechanism, which relies on biological recognition, allows the implementation of patterning experiments; hAGT will direct the protein of interest to the desired positions and anchor it on the surface covalently. Furthermore, time-consuming purification steps could be avoided and proteins can be coupled directly from crude cell extract onto the BG coated surfaces. Due to the high fidelity of this coupling method, covalent attachment of recombinant proteins out of single cells expressing hAGT fusion proteins should be possible.

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