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The conserved His8 of the Moloney murine leukemia virus Env SU subunit directs the activity of the SU–TM disulphide bond isomerase

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Abstract

Murine leukemia virus (MLV) fusion is controlled by isomerization of the disulphide bond between the receptor-binding surface (SU) and fusion-active transmembrane subunits of the Env-complex. The bond is in SU linked to a CXXC motif. This carries a free thiol that upon receptor binding can be activated (ionized) to attack the disulphide and rearrange it into a disulphide isomer within the motif. To find out whether His8 in the conserved SPHQ sequence of Env directs thiol activation, we analyzed its ionization in MLV vectors with wtEnv and Env with His8 deleted or substituted for Tyr or Arg, which partially or completely arrests fusion. The ionization was monitored by following the pH effect on isomerization *in vitro* by Ca^{2+} depletion or *in vivo* by receptor binding. We found that wtEnv isomerized optimally at slightly basic pH whereas the partially active mutant required higher and the inactive mutants still higher pH. This suggests that His8 directs the ionization of the CXXC thiol.

Keywords: Moloney murine leukemia virus; Receptor binding; Membrane fusion; Env activation; Disulphide isomerization; CXXC-motif; Thiol ionization; Fusion from without

Introduction

The membrane fusion proteins of retroviruses are composed of three copies of a two-subunit protein (Env) (Hunter, 1997). One of the subunits transverses the viral envelope (TM, transmembrane) and the other has a peripheral location (SU, surface). The membrane fusion activity is loaded into the TM subunit and it is controlled by the associated SU subunit. When SU binds to the virus receptor on the cell surface, it is displaced and TM activated (Moore et al., 1990). In some cases, efficient activation requires low pH in addition to receptor binding (Mothes et al., 2000). According to the prevailing model the membrane fusion process is linked to refolding of TM into a more stable form. This involves relocation of its fusion peptide into the lipid bilayer of the target membrane and a jack-knife-like back folding of the α-helical core of TM from a prehairpin to a hairpin conformation. As a result, the fusion peptide-bound cell membrane and the transmembrane peptide-bound viral envelope will be forced together for fusion. The model is supported by the bundle of 6 α-helices found in crystals of TM ectodomain fragments, the inhibition of membrane fusion by peptides corresponding to the α-helical TM core, in particular its C-terminal part, and by fusion inhibition by lipid molecules incompatible with concavely curved membranes (Weissenhorn et al., 1997; Chan et al., 1997; Melikyan et al., 2000; Wild et al., 1994; Jiang et al., 1993; Kobe et al., 1999; Jinno et al., 1999; Netter et al., 2004; Hernandez et al., 1997; Smith et al., 2004; Wallin et al., 2005a).

According to this model, it is the interaction between the two subunits that controls the activation of Env. In this respect, it is notable that apparently almost all retrovirus but lentivirus carry an intersubunit disulphide bond in Env. The Cys residue in TM that participates in the bond is the last residue of a conserved CX_6CC motif. This is flanking, on the C-terminal side, the region of the TM chain that is used for chain reversal during TM refolding (Kobe et al., 1999). Therefore, the bond seems to be optimally located for prevention of premature TM back folding. The problem how fusion can be triggered despite the intersubunit disulphide bond appears to have found two kinds of solutions. One is to take up the retrovirus into an endosome and use low pH in addition to receptor binding. At these conditions, it seems to be possible to back fold TM even in the presence of the intersubunit disulphide bond. This solution is

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used by the α-retroviruses (e.g., avian leukosis virus, ALV) (Mothes et al., 2000). The second solution is to combine the SU Cys residue of the intersubunit disulphide, with a disulphide bond isomerization-active motif, CXXC (Pinter et al., 1997; Wallin et al., 2004, 2005a, 2005b). In this motif, the other Cys residue has a free thiol, which can be induced by receptor binding to attack the intersubunit disulphide bond and cause rearrangement of the latter into a disulphide bond isomer within the motif. The non-covalently associated SU will now be easily dissociated from TM, which becomes activated for fusion (Wallin et al., 2005a). The latter solution is used by the γ-retroviruses (e.g., murine leukemia virus, MLV) and probably also by the δ-retroviruses (e.g., human T-cell leukemia virus, HTLV) and most β-retroviruses as they all share the same CXXC-linked intersubunit disulphide bond arrangement.

The SU is composed of a receptor-binding N-terminal domain, RBD, and a C-terminal domain with the isomerization motif (Fass et al., 1997; Davey et al., 1997). The two domains are linked together by a proline rich polypeptide region (PPR) (Kayman et al., 1999; Lavillette et al., 1998). In order for the CXXC thiol to become activated for isomerization of the SU–TM disulphide, it must be deprotonated. Therefore, it is reasonable to assume that receptor binding via the RBD induces changes in the locale of the CXXC thiol, which activates the latter by reducing its pK_a value. What these changes are and how they are brought about are mostly unknown. Earlier studies have shown that receptor-induced activation involves changes in the interaction between the RBD and the SU C-terminal domain (Barnett and Cunningham, 2001). The triggering interaction engages a conserved disulphide bonded loop in the SU C-terminal domain downstream of the isomerization motif (Lavillette et al., 2001). Another study has demonstrated that receptor binding releases isomerization suppressing Ca^{2+} ion(s) from Env (Wallin et al., 2004). Furthermore, the triggering of Env involves initially exposure of the CXXC thiol for modification by alkylation. This arrests the conformational transition of Env in an intermediate stage and prevents fusion (Wallin et al., 2005b). Characteristic for the intermediate is that it exposes the TM as a prehairpin for externally added TM peptides (Wallin et al., 2006). The intersubunit disulphide of the intermediate also becomes susceptible for external reduction with DTT, which rescues Env activity. Thus, the following order of activation steps can tentatively be defined for the Mo-MLV Env: receptor-induced alteration of RBD structure, release of Ca^{2+} ion(s), establishment of a new interaction between RBD and the SU C-terminal domain, formation of the CXXC thiol-alkylatable intermediate, isomerization of the intersubunit disulphide, SU release and TM hairpin formation. The fusion peptide might be inserted into the target membrane when the alkylatable intermediate is formed. Interestingly, a Moloney MLV (Mo-MLV) Env mutant with a deletion of His8 in the conserved SPHQ sequence of SU has been described (Bae et al., 1997). The His8 deletion mutant binds normally to its receptor but cannot complete the membrane fusion process and is therefore non-infectious (Bae et al., 1997; Lavillette et al., 2000; Barnett and Cunningham, 2001; Zavorotinskaya et al., 2004). However, the infectivity of the receptor-bound mutant virus can be rescued by providing the N-terminal receptor-binding domain (RBD) of wt SU *in trans* (Lavillette et al., 2000). Here we have related the His8 function to the Env activation steps. In particular, we have studied whether the His8 could act as an inducer for isomerization. We monitored the ionization properties of the CXXC thiol in wt Env and His8 deletion and His8 substitution mutants of Env. We found that the ionization properties of the CXXC thiol in the mutants changed in a way that was consistent with His8 acting as an isomerization inducer.

Results

High pH facilitates isomerization in vitro

Previously, we showed that receptor-triggered isomerization of the SU–TM disulphide bond is mediated by removal of a stabilizing Ca^{2+} ion from Env (Wallin et al., 2004). Consequently, it was possible to isomerize the intersubunit disulphide bond in free virus by Ca^{2+} depletion. We used this *in vitro* assay to follow the pH sensitivity of the intersubunit disulphide bond isomerization reaction in Mo-MLV Env. It should reflect the ionization, i.e., the activation properties of the CXXC thiol in the SU subunit of Env. Samples of [^{35}S]Cys-labeled Mo-MLV in MOV-3 cell supernatants were taken up in TN pH 6.6–8.6, with or without 1.8 mM Ca^{2+}, by ultrafiltration at 4 °C and then subjected to incubation at 37 °C for 5 h. The samples were lysed and the viral proteins captured by immunoprecipitation for non-reducing SDS–PAGE. As NP-40 lysis buffer is a potent inductor of isomerization, we added NEM to 20 mM at the end of the incubation period. In this way, it was possible specifically to follow the Ca^{2+}-depletion-induced isomerization at the different pH values. As a control, we used a virus sample that was kept on ice in the presence of NEM. Analysis of the control showed the disulphide bonded SU–TM complex and the capsid protein (CA) of the virus as well as free SU, which also had been released from the virus producing cells (Fig. 1A, lane 2) (Wallin et al., 2004). When the samples were incubated at increasing pH values starting from pH 6.6, we observed increasing isomerization of the intersubunit disulphide bond of the SU–TM complexes (Fig. 1A, lanes 3–13). This was seen as a decrease in the amount of SU–TM complexes, an increase in the amount of free SU and the appearance of free TM. The degree of isomerization at the different pH values has been quantified in Fig. 1C. This shows that half maximal isomerization is achieved at pH 7.4–7.6. When incubations were done in the presence of 1.8 mM Ca^{2+} the SU–TM complexes remained essentially stable up to pH 7.6 after which a slight isomerization was apparent (Fig. 1B and C). We conclude that the Ca^{2+}-depletion-induced SU–TM disulphide bond isomerization starts to occur at neutral pH (pH 7) and is enhanced at higher pH. This pH sensitivity profile implies that the pK_a of the CXXC thiol has been reduced to a value close to neutral by a Ca^{2+}-depletion-induced structural change in Env.

The kinetics of the Ca^{2+}-depletion-induced SU–TM disulphide bond isomerization reaction was studied at pH 7, 7.5, 8 and 8.5 using incubation times from 30 min to 5 h. We found that at pH 7 there was only insignificant isomerization, at 7.5