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A lipid modified ubiquitin is packaged into particles of several enveloped viruses

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Abstract An anti-ubiquitin cross-reactive protein which migrates more slowly (6.5 kDa) by SDS-PAGE than ubiquitin was identified in African swine fever virus particles. This protein was extracted into the detergent phase in Triton X-114 phase separations, showing that it is hydrophobic, and was radiolabelled with both [^{3}H]palmitic acid and [^{32}P]orthophosphate. This indicates that the protein has a similar structure to the membrane associated phosphatidyl ubiquitin described in baculovirus particles. A similar molecule was found in vaccinia virus and herpes simplex virus particles, suggesting that it may be a component of uninfected cell membranes, which is incorporated into membrane layers in virions during morphogenesis.

Key words: Ubiquitin; Envelope virus

1. Introduction

Ubiquitin is a 76 amino acid protein found in abundant quantities in all eukaryotic cells. It is covalently coupled in a multistep process, by enzymes of the ubiquitin conjugation system, to Lys residues in substrate proteins. Multi-ubiquitinated proteins generated by this process are targeted for degradation by the proteasome (see [9,15] for reviews).

Although the ubiquitin system was thought to be involved only in the degradation of cytosolic proteins, recent reports have demonstrated ubiquitination of membrane proteins. Misfolded endoplasmic reticulum (ER) proteins, such as mutant forms of the cystic fibrosis transmembrane conductance regulator [32], a mutant form of the yeast carboxypeptidase ysc Y [11] and the yeast sec61p complex [2], are translocated out of the ER, conjugated to ubiquitin and degraded by the proteasome.

Immunogold electron microscopy shows gold particles corresponding to ubiquitin-protein conjugates in the endosome-lysosome system of normal cells [22], as well as in similar organelles in Epstein-Barr virus transformed lymphoblastic cells [19] and in prion encephalopathies [20]. One reason for ubiquitin-protein conjugates in the endosome-lysosome systems is that a number of cell surface receptors are ubiquitinated on their cytoplasmic tails. For example, the growth hormone receptor is ubiquitinated following ligand binding and this signals internalisation and degradation within the endosomal/lysosomal system [21,31]. Similarly ubiquitination of the yeast cell surface α-factor exporter occurs at the cell surface and is required for Ste6p entry into the vacuole [18]. Ubiquitination of the Ste2p pheromone receptor is obligatory for internalisation into the vacuole [10].

Ubiquitin was thought to be exclusively a cytosolic protein until a report described a modified form which was extracted into the detergent phase by Triton X-114 (TX114) and was present in the insect virus, baculovirus, particles [8]. This modification was shown to be a lipid consisting of a phosphatidyl group containing two palmitate molecules linked via a phosphate group to an internal Ser or Thr residue on ubiquitin. This modified ubiquitin was localised on the cytosolic face of the baculovirus outer membrane [8].

Neither a lipid modified form of ubiquitin nor this type of protein modification had previously been described. However, its discovery in baculovirus particles suggested that it may be baculovirus or insect cell specific and that the enzymes required for the modification are encoded by the baculovirus genome or are only present in insect cells.

Here we report that a similar modified form of ubiquitin is packaged into purified virions from several virus families which contain lipid membranes. The discovery of this molecule in virions which replicate in a range of different mammalian cells suggests the modified ubiquitin is synthesised by the host cell and may have a function in the physiology of uninfected cells. Possible roles for a lipid-modified, membrane-associated ubiquitin in ubiquitination of membrane proteins are discussed.

2. Materials and methods

2.1. Cells and viruses

Vero and BSC40 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum. Sf9 insect cells were grown in Grace's insect cell medium. The African swine fever virus (ASFV) BA71V isolate [7] was grown in Vero cells, the vaccinio virus VV WR strain in BSC40 cells and the baculovirus AcNMPV in Sf9 cells. ASFV particles were purified from tissue culture cell supernatants by Percoll gradient centrifugation [5], vaccinia virus particles were purified from cell extracts by caesium chloride centrifugation [14] and baculovirus particles by sucrose gradient centrifugation [8]. Purified herpes simplex virus particles and L particles were a gift from Dr Frazer Rixon, Institute of Virology, Glasgow.

2.2. Radiolabelling of ASFV particles with [^{3}H]palmitic acid and [^{32}P]orthophosphate

Vero cells in five 175 cm^2 flasks were incubated for 12 h in DMEM medium containing 50 μCi/ml of [^{32}P]orthophosphate (NEN) supplemented with 2% foetal calf serum or 50 μCi/ml of [^{3}H]palmitic acid (NEN) supplemented with delipidated foetal calf serum. Radioactive medium was removed and cells were infected with ASFV at a multiplicity of infection of 10 for 72 h. Radiolabelled ASFV particles were purified from the extracellular medium by Percoll gradient centrifugation.

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2.3. TX114 extractions

TX114 extractions were carried out as described [3]. Proteins from aqueous and detergent phases were either precipitated with acetone and redissolved in SDS-PAGE sample buffer or diluted 200-fold in 0.5 M NaCl, 20 mM Tris pH 7.5, 2 mM EDTA and immunoprecipitated with anti-ubiquitin sera.

2.4. Immunoprecipitations and Western blotting

A cocktail of anti-ubiquitin sera (mouse monoclonals IG2E2 and ID8, a gift from Dr L. Guarino, a mouse monoclonal against human ubiquitin (Novacastra), and rabbit anti-ubiquitin sera (Dakopatts)) was used to immunoprecipitate ubiquitin from radiolabelled virion proteins. Protein G Sepharose was used to pellet immunoprecipitates and these were washed in 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.02% NP40. Non-radioactive proteins were blotted onto Hybond C membrane (Amersham) and blots were probed with rabbit anti-ubiquitin sera (1/50). Bound antibodies were detected using horseradish peroxidase (HRP) conjugated goat anti-rabbit sera followed by enhanced chemiluminescence.

3. Results

3.1. A ubiquitin related protein of 6.5 kDa is packaged into ASFV particles

We have previously identified a number of anti-ubiquitin cross-reactive proteins present in purified ASFV particles. One of these migrated with similar mobility to purified ubiquitin [13]. To further characterise this molecule, we analysed, by SDS-PAGE, ASFV proteins from purified virions and purified ubiquitin (Sigma). Blots were probed with anti-ubiquitin sera and bound antibodies were detected using HRP conjugated secondary antibodies and enhanced chemiluminescence. This showed that the smallest anti-ubiquitin cross-reactive protein present in ASFV particles (Fig. 1B, lane 2) migrated more slowly (~6.5 kDa) than purified ubiquitin (Fig. 1B, lane 1) (~5 kDa), suggesting that it is a modified form of ubiquitin such as phosphatidyl ubiquitin found in baculovirus particles [8], a ubiquitin related protein with a slightly larger molecular weight than ubiquitin, or ubiquitin conjugated to a small peptide.

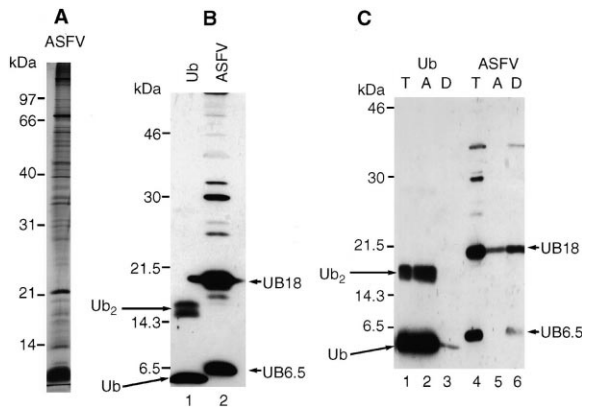

Fig. 1. A 6.5 kDa anti-ubiquitin immunoreactive protein present in ASFV particles is extracted into the TX114 detergent phase. ASFV particles were purified from tissue culture supernatants by Percoll gradient centrifugation [5]. Virion proteins separated by 12.5% SDS-PAGE and stained with silver are shown in A. B shows purified ubiquitin (25 ng, Sigma, lane 1) and ASFV virion proteins (5 μg, lane 2) separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes and probed with anti-ubiquitin antisera. Bound antibodies were detected with HRP conjugated secondary antibodies and enhanced chemiluminescence (Amersham). C: Purified ubiquitin (50 ng, lanes 1–3) and ASFV particles (10 μg, lanes 4–6) were treated with 1% TX114 then separated into aqueous and detergent phases [3]. Proteins were precipitated with acetone and redissolved in SDS-PAGE sample buffer. Lanes 1 and 4 show ubiquitin and ASFV proteins which had not been treated with TX114. Lanes 2 and 5 show protein extracted into the aqueous phase from purified ubiquitin (lane 2) and ASFV proteins (lane 5). Lanes 3 and 6 show proteins extracted into the detergent phase from purified ubiquitin (lane 3) and ASFV proteins (lane 6). Mono- and dimeric forms of ubiquitin are labelled Ub and Ub2, UB6.5 and UB18 molecules referred to in the text are labelled. The positions of molecular weight markers run in parallel are indicated.

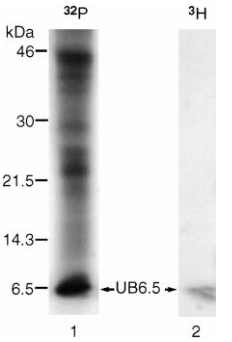

Fig. 2. Immunoprecipitation of ASFV virion proteins labelled with [^{3}H]palmitic acid or [^{32}P]orthophosphate with anti-ubiquitin sera. Vero cells in 10×175 cm^2 flasks were incubated for 12 h in medium containing either [^{32}P]orthophosphate (50 μCi/ml, lane 1) or [^{3}H]palmitic acid (50 μCi/ml, lane 2). Cells were then infected with the BA71V ASFV isolate at a multiplicity of infection of 10 and after 72 h virus was purified from the extracellular medium by Percoll gradient centrifugation [4]. Purified labelled virions were solubilised in 1% TX114 then separated into aqueous and detergent phases [3]. Proteins in the detergent phase were diluted 40-fold then immunoprecipitated with a cocktail which included four different anti-ubiquitin antisera. Immunoprecipitates were separated by 15% SDS-PAGE and labelled proteins detected by autoradiography (lane 1) or fluorography (lane 2). The position of molecular weight markers run in parallel is shown.

3.2. The ubiquitin related protein in ASFV particles is hydrophobic and contains phosphate and palmitate groups

The phosphatidyl ubiquitin found in baculovirus particles is extracted into the detergent phase by TX114 phase separation because of the lipid modification [8]. To determine if the 6.5 kDa molecule present in ASFV particles might also be a lipid modified form of ubiquitin, we lysed purified ASFV particles in 1% TX114 and separated the proteins into detergent and aqueous phases [3]. Proteins were separated by SDS-PAGE, transferred to membranes and probed with anti-ubiquitin antibodies. Purified ubiquitin was analysed in parallel (Fig. 1C). This showed that the 6.5 kDa protein from ASFV virions was extracted into the detergent phase (Fig. 1C, lane 6) whereas purified ubiquitin was present in the aqueous phase (Fig. 1C, lane 2). Thus, the 6.5 kDa protein present in ASFV particles may be a lipid modified form of ubiquitin similar to that found in baculovirus particles. Multimers of ubiquitin are found in cells and are also present in the purified ubiquitin preparation. In Fig. 1C, lanes 1 and 2, di-ubiquitin is present as a prominent band migrating with a mobility of about 16 kDa (Ub2, Fig. 1). An anti-ubiquitin cross-reactive protein of about 18 kDa was detected in extracts from ASFV virion proteins. This may be a di-ubiquitin form of the 6.5 kDa lipid modified protein. Consistent with this, the 18 kDa protein was largely extracted into the TX114 detergent phase (Fig. 1C, lanes 4–6). To quantify the amount of the 6.5 kDa protein

present in ASFV particles, dilutions of known amounts of ASFV proteins and of purified ubiquitin were separated by SDS-PAGE and blotted. Blots were probed with anti-ubiquitin sera and signals obtained compared by densitometry. This showed that 1 μg of purified ASFV virions contained 25–50 ng of the 6.5 kDa molecule (data not shown) corresponding to 2.5–5% of the total virion protein.

To further characterise the 6.5 kDa ubiquitin-like molecule, we radioactively labelled ASFV virions with either [^{32}P]orthophosphate or [^{3}H]palmitic acid. Vero cells were incubated for 12 h in the presence of [^{32}P]orthophosphate or [^{3}H]palmitic acid then infected with the BA71V strain of ASFV. After 72 h virus was purified from the extracellular medium [5]. Purified labelled virion proteins were lysed in 1% TX114 for 1 h and proteins extracted into the detergent phase were diluted 40-fold to reduce the concentration of detergent, then immunoprecipitated with a cocktail of anti-ubiquitin antisera. Immunoprecipitated proteins were separated by SDS-PAGE and detected by autoradiography or fluorography (Fig. 2). Proteins of 6.5 kDa were immunoprecipitated with anti-ubiquitin sera from the TX114 detergent phase of both the [^{32}P]orthophosphate and [^{3}H]palmitic acid labelled virion proteins (Fig. 2, lanes 1 and 2) showing that the 6.5 kDa detergent extractable ASFV anti-ubiquitin cross-reactive protein contains both phosphate and palmitate groups. This is consistent with it being the same structure as the phosphatidyl ubiquitin previously described in baculovirus particles purified from infected insect cells [8].

3.3. *A 6.5 kDa ubiquitin related molecule is present in purified vaccinia virus and herpes simplex virus particles*

We decided to analyse a number of other viruses which contain lipid membranes to determine if a similar modified form of ubiquitin may also be incorporated into these virions.

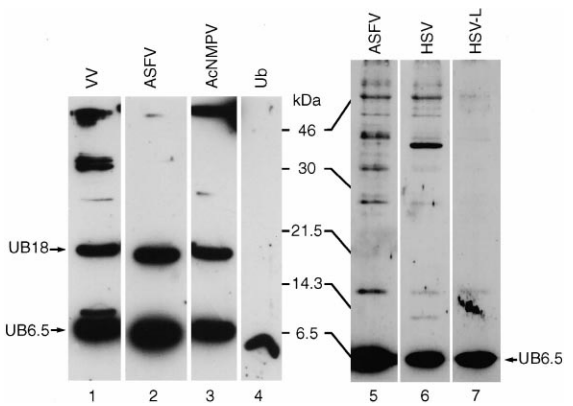

Fig. 3. A 6.5 kDa, TX114 detergent extractable, anti-ubiquitin reactive protein is present in purified vaccinia virus and herpes simplex virus virions. Purified vaccinia virus (intracellular mature form, VV, 10 μg, lane 1), ASFV (10 μg, lanes 2 and 5), baculovirus (AcNMPV, 10 μg, lane 3), herpes simplex virus (HSV, 10 μg, lane 6) and HSV-light particles (HSV-L, 10 μg, lane 7) were solubilised in 1% TX114 then proteins separated into aqueous and detergent phases. Proteins were precipitated with acetone from the detergent phase. These detergent extracted virion proteins and purified ubiquitin (25 ng) were separated by 15% SDS-PAGE, blotted onto nitrocellulose and reacted with anti-ubiquitin antisera (Dakopatts). Bound antibodies were detected using horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence. The position of molecular weight markers run in parallel is shown.

Purified vaccinia virus (VV) virions (a gift from Dr Sue Jacobs), herpes simplex virus (HSV) virions and HSV-light particles (a gift from Dr Frazer Rixon, Institute of Virology, Glasgow), baculovirus particles (AcNMPV) and ASFV particles [5,8] were lysed in TX114. The proteins extracted into the detergent phase were separated by SDS-PAGE, blotted onto membranes and probed with anti-ubiquitin sera. An anti-ubiquitin cross-reactive band of 6.5 kDa was detected in purified VV and HSV preparations as well as in ASFV and baculovirus particles (Fig. 3). This suggests that a lipid modified form of ubiquitin similar to that present in ASFV and baculovirus is also present in VV and HSV particles. The amount of the UB18 protein recovered varies between different virus preparations (compare for example Fig. 3, lanes 1–3 with 5–7).

4. Discussion

Several pieces of evidence suggest that the 6.5 kDa anti-ubiquitin cross-reactive protein present in ASFV particles is the same structure as the phosphatidyl ubiquitin previously described only in baculovirus particles. Properties shared with phosphatidyl ubiquitin include extraction into the TX114 detergent phase, incorporation of [^{3}H]palmitic acid and [^{32}P]orthophosphate, same mobility on SDS-PAGE and cross-reactivity with anti-ubiquitin sera. Together these data strongly suggest the molecules are the same structure. It is not likely that the 6.5 kDa protein in ASFV particles is a ubiquitin-related molecule which has a slightly larger molecular weight than ubiquitin. Ubiquitin-related proteins characterised to date include SUMO which is 101 amino acids long and UCRP which migrates at 15 kDa on SDS-PAGE [17]. However, none of the ubiquitin related molecules characterised to date is hydrophobic, nor do they contain palmitate or phosphate groups. Also, although they are structurally related, they have relatively low amino acid identity compared to ubiquitin and do not cross-react with anti-ubiquitin sera.

Our data show that the same type of modified ubiquitin molecule is packaged into other virus particles which contain lipid membranes including VV, a poxvirus and HSV.

These viruses are all from different families and although all have large DNA genomes, it seems unlikely that all encode enzymes required for the synthesis of a lipid modified ubiquitin. Complete genome sequences are available for each of these viruses [1,16,23,33] and there are no obvious encoded enzymes in common which might be involved in lipid modification of ubiquitin. It is more likely that the lipid modified ubiquitin is a host cell molecule which is present in cell membranes and is packaged into the envelope of virus particles during virion morphogenesis. ASFV, VV, HSV and baculovirus all have different mechanisms of morphogenesis and incorporate host membranes derived from different cellular compartments into their virions. Thus, VV incorporates membranes from the intermediate compartment and from the trans-Golgi network [28,29]. HSV particles are thought to acquire their envelopes by budding through the ER-inner nuclear membranes [25,30], although a recent report suggests that HSV particles lose this nuclear membrane and gain their envelope from the trans-Golgi network [4]. ASFV incorporates membranes from the endoplasmic reticulum and plasma membrane [6,27] and baculovirus particles acquire their envelopes from the plasma membrane [26]. The implication is that

if the lipid modified ubiquitin incorporated into virus particles is from host cells, this must be present in several different membrane compartments. Phospholipids are synthesised in the ER and transported from there to other membrane compartments in cells. Thus, the presence of lipid modified ubiquitin in virus particles which derive their membranes from several different intracellular compartments, is consistent with synthesis of this molecule in the ER and transport from there to other compartments.

A lipid modified ubiquitin has not been reported in uninfected cells. There may be several reasons for this. First, it may represent a small proportion of the total ubiquitin and might only be detected if purified away from the bulk of cellular ubiquitin by, for example, purifying the membrane fraction. Secondly, it may be susceptible to degradation by phospholipases and therefore difficult to isolate. It may be easier to detect in purified virions because it is concentrated in these particles and protected from degradation by cellular enzymes.

4.1. Possible functions of a membrane associated, lipid modified form of ubiquitin

One implication from our results is that a phosphatidyl modified ubiquitin may have a role in normal cell physiology. As discussed in the introduction, ubiquitin has a role in targeting misfolded ER proteins for degradation by the proteasome and in internalisation of membrane receptors. A likely role for a membrane attached ubiquitin is as a source of ubiquitin for conjugation to membrane proteins. Another possible function for a membrane anchored ubiquitin is as a novel mechanism for membrane attachment of proteins. Since enzymes (de-ubiquitinating enzymes or DUBS) which remove ubiquitin from proteins exist [17,24], this type of membrane attachment could be reversible. The modified ubiquitin incorporated into virus particles may either have a specific function during virus replication or it may merely be incorporated as a consequence of being in cell membranes that are incorporated in the virus structure. Possible roles during virus replication are as a source of ubiquitin for conjugation to host or virion proteins so that they are targeted for degradation. This may, for example, be required as part of the virion uncoating process or to activate gene transcription. Interestingly, ASFV encodes a ubiquitin conjugating enzyme (UBCv) which is also packaged into virus particles [12]. This suggests that UBCv may conjugate ubiquitin to either virus or host proteins early during the virus replication cycle. Possibly the modified ubiquitin in virus particles is used for this purpose.

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