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Bone-resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains

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SUMMARY

Bone-resorbing multinucleate osteoclasts exhibit a ruffled border membrane apposing the bone and a basal membrane contacting the circulation. A junctional complex called the sealing zone separates these two membrane domains, but the defined nature of these membrane domains has remained obscure. We now show, using enveloped viral glycoproteins and lectins as tools, that osteoclasts exhibit a novel membrane domain in the basal surface when they are polarized for resorption. Influenza haemagglutinin, which is apically targeted in epithelial cells, is targeted to a restricted area at the top of the basal surface, while vesicular stomatitis virus G-protein which is basolaterally targeted in epithelia, occupies the rest of the basal surface. Neither of these viral glycoproteins is gathered to the ruffled border nor sealing zone area, but they share in a specific way the basal surface. To show that the division of basal membrane into two different domains also occurs in non-infected cells, we have analyzed the distribution of receptors for these viruses and binding sites of some lectins. Both of these methods show that also some endogenous proteins are located in different domains in the basal surface in active osteoclasts. We also show that these two different membrane domains can be distinquished in scanning electron microscopy level due to the villus appearance of the central basal domain.

Key words: Osteoclast, Polarity, Membrane domain, Ruffled border, Virus, Influenza virus, Vesicular stomatitis virus, WGA lectin, Protein targeting, Bone resorption, Bone

INTRODUCTION

The osteoclast is a multinucleate bone-resorbing cell which is formed through fusion of bone marrow-derived mononuclear precursor cells. Its main function is to degrade mineralized bone matrix produced by osteoblasts (Vaes, 1988). During bone resorption, osteoclasts first attach to the bone surface by forming a tight contact, a so-called sealing zone, between the cell membrane and mineralized matrix (Holtrop and King, 1977). The pre-resorptive stages of attachment of osteoclasts are clearly vitronectin receptor mediated but the molecular characteristics of tight sealing are still unknown (Davies et al., 1989; Horton et al., 1991; Lakkakorpi et al., 1991, 1993; Väänänen and Horton, 1995). Osteoclasts undergo a profound reorganization of cytoskeletal elements during activation to resorption (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1991).

Cytoskeletal reorganization and tight attachment to the bone surface are connected to marked changes in the organization of various plasma membrane domains. The sealing zone divides the plasma membrane of the resorbing osteoclast into different domains, enclosing the ruffled border membrane, the actual resorbing organ, inside. Thus a closed compartment, bordered by the sealing zone, is formed between the ruffled border membrane and bone matrix. The rest of the osteoclast cell membrane, the basal membrane domain, remains outside the sealing zone, facing the bone marrow and extracellular fluid. Osteoclasts secrete protons and a large variety of proteolytic enzymes into resorption lacunae to dissolve minerals and degrade collagenous matrix (Baron et al., 1985; Vaes, 1988; Blair et al., 1989; Väänänen et al., 1990).

Osteoclasts isolated from neonatal animals can be cultured on bone slices and they resorb bone, offering an excellent model for functional studies in vitro (Boyde et al., 1984; Chambers et al., 1984). Osteoclast cultures reveal cells in different phases of the resorption cycle with various kinds of cytoskeletal organization and polarization (Lakkakorpi and Väänänen, 1991). These changes in the actin cytoskeleton can be used to distinguish resorbing osteoclasts from non-resorbing osteoclasts. Actin forms a clearly visible ring structure in attached, resorbing osteoclasts in the sealing zone area, while in non-resorbing cells, it is dispersed in the cytoplasm (Lakkakorpi and Väänänen, 1995).

The ruffled border membrane is enriched in vacuolar-type proton pumps in active osteoclasts (Blair et al., 1989; Väänänen et al., 1990) and appears as a very convoluted lining of the bone matrix. The enrichment of acid-secreting organelles on the ruffled border membrane has brought about the idea that the ruffled border could be a counterpart of the apical domain of acid-secreting epithelial cells such as gastric parietal or kidney intercalated epithelial cells. On the other hand, the ruffled border membrane has been shown to contain

lysosomal membrane proteins and it has been suggested to be the functional equivalent of a secondary lysosome (Baron et al., 1988).

Several enveloped viruses have been used to study cellular polarity in epithelial cells, such as in MDCK (Madin-Darby canine kidney) cells, and in neurons. It has been shown that the G-protein of vesicular stomatitis virus (VSV) is sorted only to the basolateral membrane domain in epithelia (Rodriquez-Boulan and Sabatini, 1978; Rodriquez-Boulan and Pendergast, 1980; Pfeiffer et al., 1985), and to the membrane of dendrites and the soma in neurons after synaptogenesis has occurred (Dotti and Simons, 1990). At the same stages haemagglutinin of influenza virus is sorted to the apical or axonal domain, respectively (Dotti and Simons, 1990; Kobayashi, 1992).

To explore the polarity features and nature of the various membrane domains in osteoclasts, we have used these viral glycoproteins as tools to investigate protein targeting in non-resorbing and resorbing osteoclasts. We observed that in non-resorbing osteoclasts these glycoproteins were evenly distributed through the plasma membrane, but in resorbing, polarized osteoclasts, they were targeted to two different domains on the basal surface, but not to the ruffled border membrane or the sealing zone area.

MATERIALS AND METHODS

Cells and viruses

Osteoclasts were isolated from the long bones of 2-day old rat pups as described earlier (Lakkakorpi and Väänänen, 1991) and cultured for 2 days on bovine bone chips. Briefly, osteoclasts were mechanically harvested from the endosteal surface of long bones by curetting with a scalpel blade. Cells were allowed to attach to 120-140 μm thick bovine bone slices for 15 minutes and the chips were rinsed three times in fresh culture medium to remove non-attached cells. Osteoclasts were then cultured in Dulbecco's modified Eagle's medium buffered with 20 mM Hepes and containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, sodium bicarbonate (0.84 g/l), penicillin (100 i.u./ml), and streptomycin (100 μg/ml), pH 7.0, at 37°C and 5% CO_2/95% air.

The cultures were infected with either VSV wild type (Indiana serotype) (Metsikkö et al., 1992) at 5 pfu/cell or with influenza virus (WSN) (Martin and Helenius, 1991) at 5 pfu/cell at 37°C. The viruses were allowed to adhere to the cell surface at 37°C for 1 hour in culture medium as described earlier but containing only 0.5% fetal calf serum. The excess of viruses was then washed away and the cells were moved back to medium containing 10% FCS. Cells infected with VSV were grown at 37°C for 3 hours. Influenza virus-infected cells were propagated for 5 hours at 37°C. In some experiments 40 μM cycloheximide was present during the last hour of the incubation to prevent further synthesis of proteins. The cells were fixed with paraformaldehyde (3%, 10 minutes) containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at 20°C. For virus receptor stainings, viruses were allowed to adhere to the cell surface for 1 hour at 0°C, cells were washed in PBS and fixed in 3% PFA for 15 minutes at 0°C. In these experiments, Sendai virus was used in addition to VSV and influenza viruses. Sendai virus and antibodies against Sendai fusion protein were a kind gift from Dr Robert Rydbeck, Sweden (Örvell and Grandien, 1982). For lectin stainings, cells were cultured for 48 hours and fixed in 3% PFA for 10 minutes at 20°C.

Immunofluorescence procedures

VSV G-protein was located using rabbit polyclonal antibodies (Metsikkö et al., 1992). Influenza virus glycoproteins were located using polyclonal antibodies specific for haemagglutinin. These antibodies and WSN influenza viruses were a kind gift from Dr Ari Helenius, Yale University School of Medicine (Martin and Helenius, 1991). The antibodies were pre-adsorbed with non-infected osteoclast cultures. Rhodamine-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulins (Dakopatts A/S, Glostrup, Denmark) were used as secondary antibodies at a dilution of 1:100. F-actin was

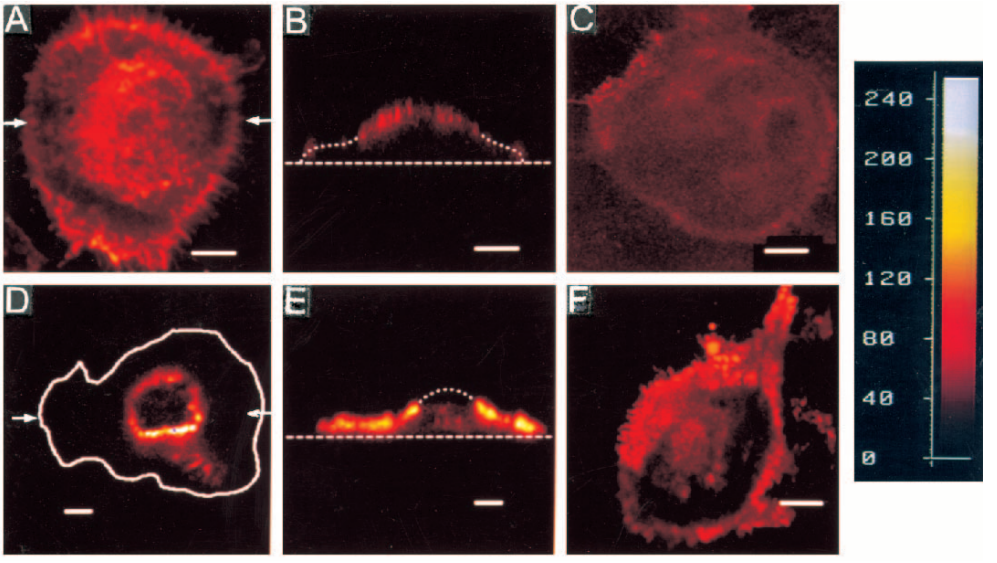

Fig. 1. Localization of basolateral and apical viral marker proteins at the surface of bone-resorbing osteoclasts. (A,B) Localization of influenza virus haemagglutinin on the basal surface of an active osteoclast. (A) An extended focus image of non-permeabilized osteoclast reconstructed from the original horizontal confocal microscopy sections by adding them together. This covers the whole basal surface showing the novel cap-like domain at the top of the cell and a narrow staining pattern at the most peripheral parts of the cell. (B) Vertical section of the same cell showing the lateral view of the basal surface. The influenza haemagglutinin staining covers only the central area of the basal surface and a small belt-like area at the most peripheral parts of the cell. Dashed lines indicate bone surface and cell membranes. (C) A single horizontal section of a permeabilized cell at the level of the ruffled border shows weak staining for influenza glycoproteins. Cells were treated with cycloheximide for 60 minutes just before fixation to diminish intracellular staining. (D,E) Distribution of VSV wild-type (Indiana serotype) G protein at the basal surface. (D) A single horizontal section from the upper part of the basal surface indicating a blank area in VSV G-protein distribution at the top of the osteoclast. (E) Vertical section of the same cell. VSV G-protein is located on the lower parts of the basal surface leaving the top of the cell unstained. The border between the stained and blank area is sharp and clearly different from the diffuse area seen with influenza virus glycoprotein. Dashed lines indicate bone surface and cell membranes. (F) A single section at the ruffled border level showing that this membrane area lacks VSV G-protein. The staining seen at this level is concentrated at the basal surface outside the sealing zone area. Bars, 10 μm.

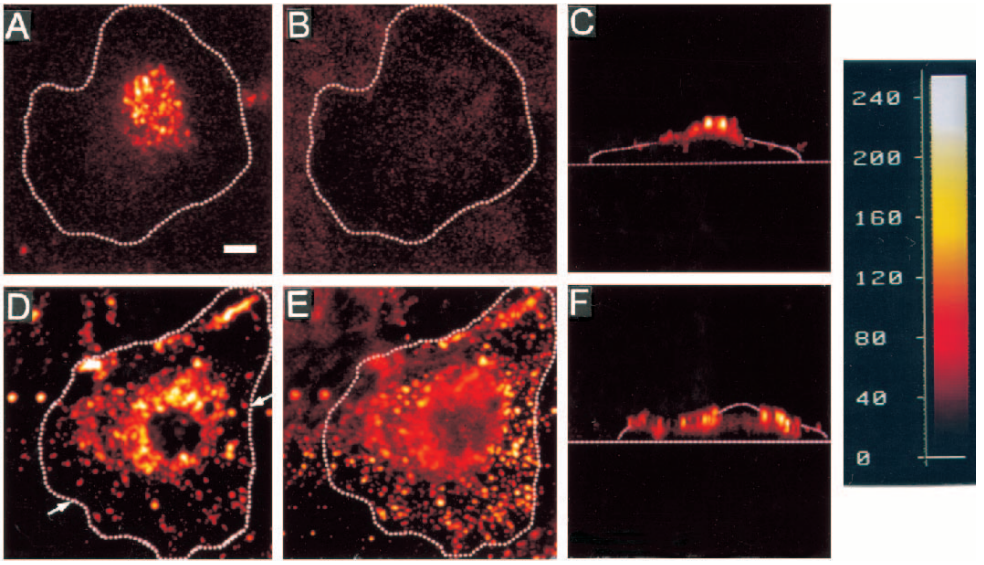

Fig. 2. Virus receptors in osteoclasts. (A,B,C) The distribution of Sendai virus receptors. (A) A single confocal section from the top of the cell showing that Sendai virus receptors are located at the cap-like domain in the basal membrane. (B) A single section from the level of the bone surface. There is no staining at the outer cell border facing the bone. (C) A lateral view of the same cell showing that the Sendai virus receptors are located only at the upper and central parts of the basal membrane. (D,E,F) VSV receptors in an osteoclast. (D) The uppermost staining pattern leaves the top of the cell blank. (E) At the bone surface level there is also staining in the peripheral parts of the basal membrane. (F) Vertical section of the same cell, showing that the central area of the basal surface does not have receptors for VSV. Bar, 10 μm.

stained with fluorescein-labelled phalloidin (Molecular Probes Inc.), at 5 units/ml. Bone slices were mounted in Mowiol (Hoechst) in phosphate-buffered saline containing 2.5% 1.4-diazobicyclo-[2,2,2]-octane (Sigma Chemical Co.). TRITC-conjugated WGA-lectin and TRITC-conjugated PNA lectin were purchased from Sigma, St Louis, USA. Fixed cells were incubated with WGA-lectin at 1:50 in PBS for 15 minutes or with PNA-lectin at 1:30 in PBS for 30 minutes at 20°C and rinsed thoroughly.

The samples were analyzed using laser scanning confocal microscopy consisting of a Wild Aristoplan fluorescence microscope and Leica Lasertechnik GmbH 1.05 software (Heidelberg, Germany) equipped with a multiline 750 mW air-cooled Omnichrome argon-crypton laser (Chino, California, USA), providing 488 and 568 nm wavelengths. Cells were viewed with 40× and 63× objectives using a 256×256 image format. The photographs were printed using a color video printer (Sony UP-5000P, Japan) coupled to the computer of the confocal microscope system.

Scanning electron microscopy

Cells were cultured on bovine bone slices as described above and fixed first in 3% paraformaldehyde for 10 minutes at 20°C and then in 2.5% glutaraldehyde in PBS for 2 hours, at 20°C. Samples were dehydrated in an ascending ethanol series, and after the critical point drying gold sputtered and examined using a JEOL JSM-6300F field emission scanning electron microscopy system.

RESULTS

VSV G-protein and influenza haemagglutinin are both targeted to the basal surface in bone resorbing osteoclasts, but they have their own domains

Rat osteoclasts, cultured on bone or on glass, supported in vitro both VSV and influenza virus infections. In permeabilized cells, both influenza and VSV viral glycoproteins were seen in immunofluorescense staining throughout the protein synthesis pathway. Infected cells appeared normal in morphology and maintained their attachment to bone within the time limits used in this study (VSV 1+3 hours, influenza 1+5 hours). Cytopathic effects of viral infection were seen when VSV infection was continued for 1+7 hours or influenza infection for 1+10 hours or more.

Haemagglutinin of influenza virus was found at the top of the basal surface in a novel membrane domain which varied in size and shape but was less than half of the basal surface area (Fig. 1A,B). The staining pattern appeared thick in confocal microscopy suggesting that this domain area at the basal surface can be convoluted. Staining was most intense at the central area of the cap-like domain, diminishing step-wise towards the outer border; 75% of osteoclasts showed the presence of central basal membrane domain, while in 25% of osteoclasts haemagglutinin was evenly distributed on the basal surface (n=60). In addition, influenza glycoproteins were found in a narrow belt-like pattern at the most peripheral parts of the basal surface facing the bone. This staining did not, however, reach the sealing zone area. In permeabilized cells, weak staining at the periphery of the ruffled border was seen with influenza haemagglutinin antibodies (Fig. 1C), but no gathering of these glycoproteins at the ruffled border area was seen.

In non-permeabilized osteoclasts, VSV G-protein was found on the basal surface. However, it was unevenly distributed

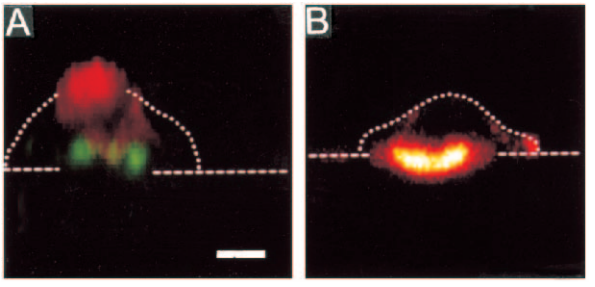

Fig. 3. Localization of wheat germ agglutinin (WGA)-lectin receptors at the central area of the basal surface of an osteoclast. (A) Lateral projection reconstructed from the original horizontal sections indicates localization of WGA-lectin at the central area of the basal surface (red color). Green color shows a cross section of the actin ring made visible with FITC-coupled phalloidin. Intensities less than 15% of the maximum were cut off to fade the cytoplasmic staining. (B) The ruffled border area in a permeabilized osteoclasts was made visible with peanut agglutinin (PNA)-lectin (Takagi et al., 1988). Dashed lines indicate bone surface and cell membranes.

(Fig. 1D,E). In 70% (n=50) of osteoclasts cultured on bone slices, G-protein was seen in the lower parts of the basal surface, leaving the uppermost part of the cell blank (Fig. 1D,E). The border between the stained and blank area was sharp and the staining was most intense just outside the blank area. In 30% of osteoclasts VSV G-protein was evenly distributed on the whole basal surface. In permeabilized cells no staining was seen at the ruffled border area (Fig. 1F). Cycloheximide resulted in G-protein moving from the cell interior to the surface, but the distribution pattern remained similar, leaving the ruffled border and top of the basal surface empty.

Virus receptors and lectins show similar polarity features in non-infected osteoclasts

We next analyzed non-infected osteoclasts in order to demonstrate the observed membrane domains using some endogenous proteins as markers. Virus suspensions were allowed to adhere to cell surface for 60 minutes at 0°C, and cells were washed and fixed immediately at 0°C. Both Sendai (Fig. 2A,C) and influenza virus (data not shown) binding revealed the new cap-like membrane domain at the basal surface, while VSV adhered in small dots over the basal surface leaving only the top of the cell blank (Fig. 2D,F). These findings reinforced the idea that some endogenous proteins show similar polarity features and membrane domains to those seen with viral glycoproteins. Hence, we began to search for lectins which would show the new partition of the basal membrane. We found that rhodamine labelled wheat germ agglutinin (WGA)-lectin specifically stained the uppermost cap-like area on the basal surface of active osteoclasts (Fig. 3A), indicating that only this part of the basal membrane contains binding sites for WGA-lectin. In permeabilized cells, WGA staining was also seen inside the cell from the ruffled border area to the top of the basal surface. We also observed labeling of the ruffled border membrane with PNA-lectin as shown earlier (Takagi et al., 1988) (Fig. 3B).

The phase of the resorption cycle in each osteoclast studied was determined using phalloidin to stain actin filaments. Actin forms a ring structure at the sealing zone area only in actively resorbing cells (Lakkakorpi and Väänänen, 1991). Interestingly, the division of basal membrane into two different membrane domains was only observed in actively resorbing osteoclasts.

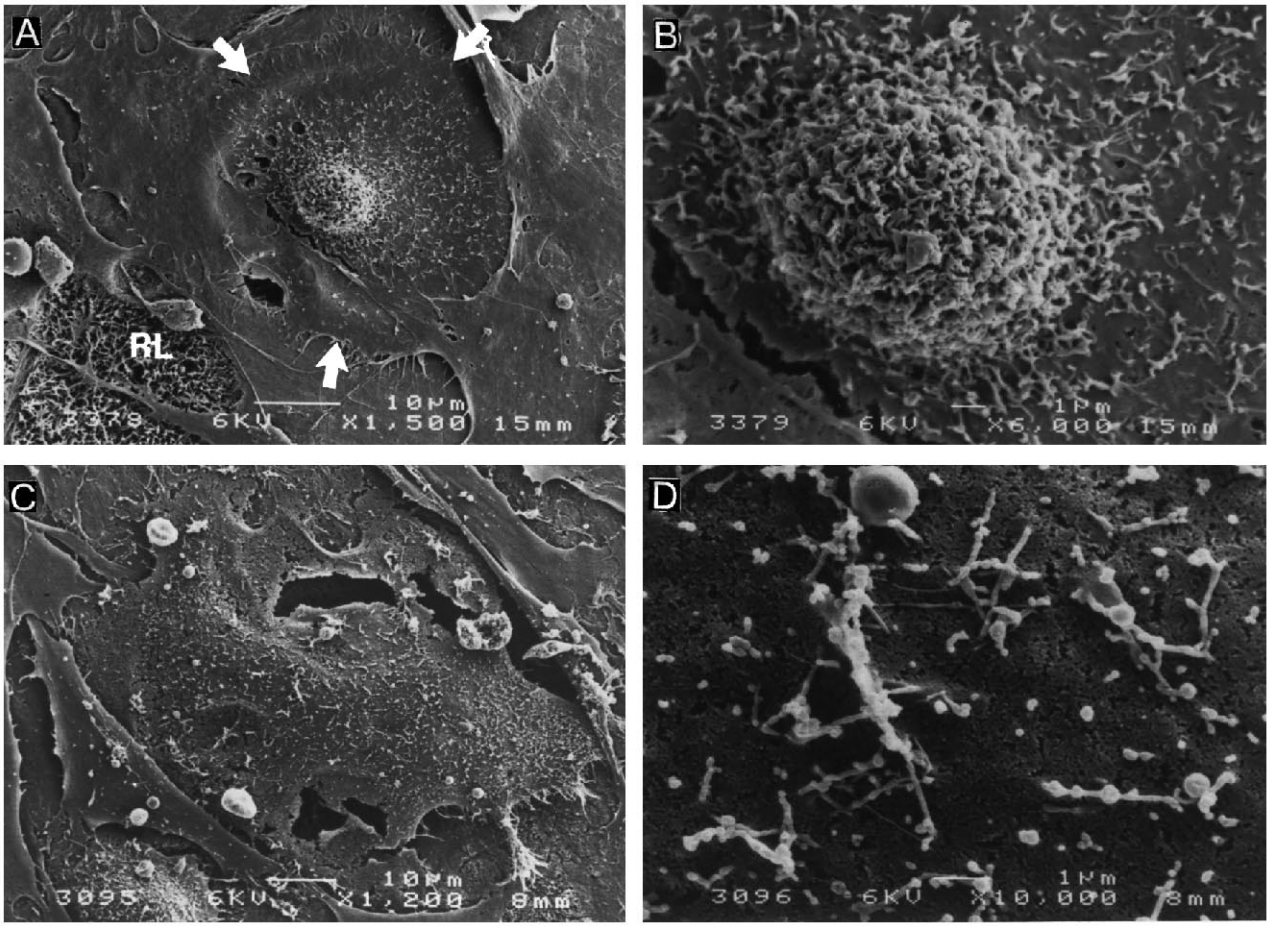

Fig. 4. (A) Scanning electron microscopy of an osteoclast cultured on a bovine bone slice. Osteoclast shows villus-like processes and small outvaginations concentrated in a limited area of the basal surface. This is the most abundant type of osteoclast in cultures. The area of membrane folding varies from cell to cell and the peripheral parts of the basal membrane appear smooth. The division of the basal membrane in this way very much resembles that seen with influenza haemagglutinin. Arrows indicate cell boundaries. In the lower left corner the irregular surface of former resorption lacuna is seen (RL). (B) A detail of the new basal membrane domain showing numerous villi. (C,D) An osteoclast showing few processes evenly distibuted over the basal surface. No specialized basal membrane domains are seen. Bars, 10 µm.

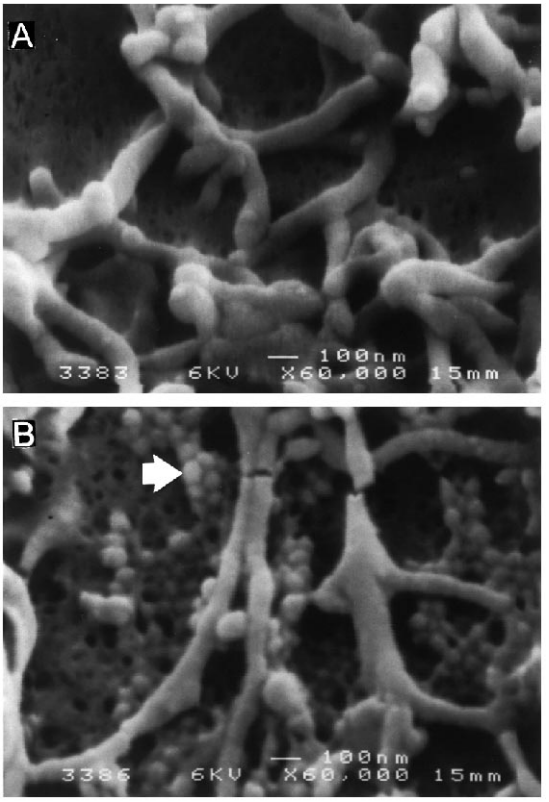

Fig. 5. Budding of influenza virus in osteoclasts. (A) A detailed FESEM image from the villus area of a non-infected osteoclast. The mebrane between villi appears smooth. (B) In infected osteoclasts the villus area shows numerous budding particles (arrow) similar to influenza viruses in size and shape. Bars 10 μm.

Scanning electron microscopy reveals morphological differences between these two basal membrane domains

Because the novel basal membrane domain was seen in immunofluorescence both in infected and non-infected osteoclasts, we began to search for morphological differences in the basal membrane using scanning electron microscopy. FESEM (field emission scanning electron microscopy) studies revealed that the majority of osteoclasts cultured on bone chips showed a morphologically distinct membrane area at the basal surface. This area contained numerous villus-like processes (Fig. 4A) and was limited to the central area of the basal membrane. The border between the cap area and the periphery of the basal membrane showed step-wise changes (Fig. 4B), as seen also with influenza haemagglutinin. The cell membrane in the periphery of the osteoclasts was mainly smooth. There were also some osteoclasts which showed a uniform distribution of villus-like processes all over the basal surface (Fig. 4C), or no processes at all. In these cells, the processes were much lower in density and were not concentrated at any part of the membrane (Fig. 4D). In addition to these two main types there was a spectrum of cells representing various intermediate stages in number, localization and density of villus-like processes. In order to study the relationship between this villus membrane area seen in FESEM, and influenza haemagglutinin positive area seen in confocal microscopy, we studied the budding of influenza viruses in osteoclasts. In non-infected cells the cell membrane at the villus area appeared smooth between villi (Fig. 5A), but in influenza infected cells it showed numerous particles budding from the membrane (Fig. 5B). These particles corresponded to influenza viruses in size and shape and they appeared only in infected cells. Few particles were also seen at the most peripheral parts of the cell, but there was no budding in the smooth membrane area (data not shown). The fact that the influenza haemagglutinin positive area appeared thick in confocal microscopy is in accordance with these morphological observations showing that influenza viruses bud from the villus-containing area. It must be emphasized, however, that at this stage it is not possible to separate resorbing and non-resorbing cells in FESEM, so we cannot conclude if this distinct morphological feature of cellular processes is directly connected to resorptive activity.

DISCUSSION

These results show that active, resorbing osteoclasts possess four different membrane domains, namely the ruffled border, sealing zone and two different membrane areas at the circulation-contacting basal membrane (Fig. 6). The fact that influenza virus haemagglutinin was not targeted to the ruffled border area suggests that this membrane domain in osteoclasts is not a direct counterpart of the apical domain in epithelial cells.

On the basis of our results it appears that the ruffled border, although it is exposed to the extracellular space, is a specialized secreting membrane domain with features not typical of either apical or basolateral plasma membranes. This observation is in agreement with those of a previous study showing some lysosomal features of ruffled border membranes (Baron et al., 1985).

The sealing zone area by which an osteoclast is attached to bone surface did not contain viral glycoproteins and it did not bind the lectins we used in this study. At present the molecular composition of the sealing zone membrane is very poorly known and further studies are needed to characterize it (for review see Väänänen and Horton, 1995).

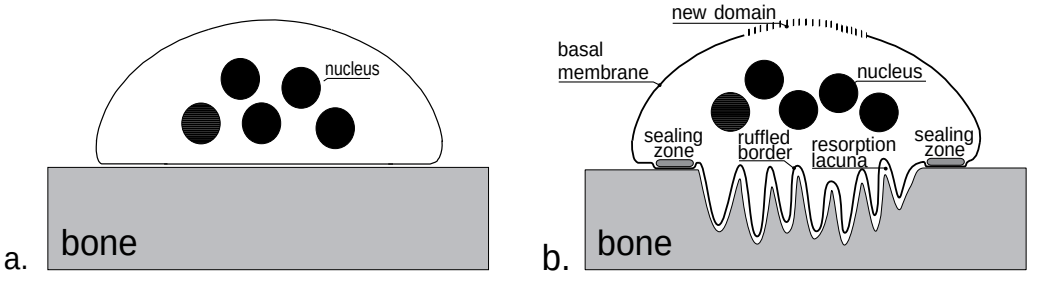

Fig. 6. Schematic drawing showing different membrane domains in non-resorbing and resorbing osteoclasts. (a) Non-resorbing, non-polarised osteoclast; (b) resorbing, polarised osteoclast.

The basal surface of resorbing osteoclasts showed two different membrane domains for viral glycoproteins. VSV G-protein, which is targeted basolaterally in epithelial cells, occupied the lower parts of the basal membrane, while influenza haemagglutinin was targeted to the top. So the basal membrane of resorbing osteoclasts is divided in two different domains although it has been thought to be homogeneous in shape and protein composition. In non-resorbing osteoclasts, cultured either on glass or bone, viral glycoproteins did not show evidence of these separate membrane domains.

Distribution of WGA-lectin staining and, in particular, the sorting of viral glycoproteins, suggest that in resorbing osteoclasts there is a specific membrane area with features of an apical domain in the middle of the basal membrane, without cell-cell or cell-extracellular matrix contacts. It remains to be seen if any other type of structural barrier, as in neurons (Dotti and Simons, 1990; Kobayashi, 1992), exists between this domain and the rest of the basal membrane, or if this novel domain is functional, maintained by a continuous flow of intracellular membrane vesicles to and from this specific area.

Together, these data show that the new basal membrane domain first seen with viral glycoproteins also exists in non-infected cells, and that it is variable in size and shape depending on the phase of resorption cycle. This kind of membrane domain differs from those seen in other cells. Further studies are needed to elucidate the nature of the border between the different basal membrane domains in osteoclasts. Barriers to diffusion such as in epithelial cells do not seem plausible, since no junctional complexes exist in osteoclasts. In sperm cells and in neurons a barrier to lateral diffusion of membrane proteins exists in single cells (Cowan et al., 1987; Angelides et al., 1988; Kobayashi, 1992). If a similar barrier is present in osteoclasts remains to be seen, but it should be noted that the boundaries of this novel membrane domain seem to be much more dynamic in nature than those described in neurons and other cells. It is also unlikely that the new membrane domain which we observed in resorbing osteoclasts is equivalent to apical microdomains (Lisanti and Rodriques-Boulan, 1990; Powell et al., 1991; Brown and Rose, 1992; Skibbens et al., 1989) or basal subdomains described earlier (Drenckhahn et al., 1985; Koob et al., 1987).

This data also shows that the polarity features and membrane domains in osteoclasts are dynamic in nature. The polarity features in epithelial cells are formed during development of the epithelium and are maintained continuously (Simons and van Meer, 1988; Rodriquez-Boulan and Nelson 1989). Polarity in neurons is also constant. Once formed during synaptogenesis, the polarity is maintained, revealing two different plasma membrane domains with distinct features. These features of stable polarity have made epithelial cells in particular a good model for studying vesicle traffic and protein sorting in detail.

Our results show that in polarized osteoclasts basal membrane is divided in two morphologically and functionally different membrane domains. The central area of the basal membrane resembles the apical plasma membrane domain on the basis of targeting of influenza haemagglutinin. This, and the fact that this specialized central domain is not seen in non-resorbing cells, raises the question of whether this new membrane domain has some specific function related to the resorption itself. We have earlier shown that some bone matrix glycoproteins bind WGA-lectin strongly (Selander et al., 1994). One possible explanation for the accumulation of WGA-lectin on the central basal membrane area in resorbing cells could be a transcytotic transport of degraded bone matrix material from the ruffled border area to this new membrane domain. Further studies are needed to find out whether our hypothesis of a transcytotic route is valid or not.

Our results further show that in osteoclasts, polarity and new membrane domains are formed and changed several times during the resorption process and this can be followed easily in routine culture conditions. This suggests that osteoclasts can be a model to study the mechanisms of formation and maintenance of different plasma membrane domains as well as vesicle trafficking.

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