Journal of Cellular Biochemistry 104:2165–2173 (2008)

Primary Human Keratinocytes Externalize Stratifin Protein Via Exosomes

Claudia Chavez-Muñoz, Jennifer Morse, Ruhangiz Kilani, and Aziz Ghahary*

Department of Surgery, BC Professional Burn and Wound Healing Research Lab, The University of British Columbia, Vancouver, British Columbia, Canada

Abstract Although, stratifin (SFN) is externalized by keratinocytes and stimulates the expression of matrix metalloproteinase-1 (MMP-1) in fibroblasts, its mechanism of externalization is not known. Here, we hypothesize that keratinocytes have a capacity to release stratifin through externalization of exosomes. To test this hypothesis, exosomes were purified from human keratinocyte conditioned medium (KCM) and analyzed for the presence of SFN by Western blot analysis using lysosomal-associated membrane protein 2 (LAMP-2) and heat shock cognate 70 (hsc70) as exosomal markers. The results showed the presence of SFN in keratinocyte lysate, concentrated KCM and exosomes, but not in concentrated unconditioned medium. Transmission electron microscopic examination revealed the presence of unique ''saucer-like'' structures characteristic of exosomes whose diameters were <100 nm. Similar to the recombinant SFN, the exosomes associated proteins stimulated MMP-1 expression in fibroblasts. Depletion of the exosomes markedly reduced this MMP-1 stimulatory effect. To further statistically confirm these findings, fibroblasts were treated with three different exosome preparations and the finding showed more than 7.4-fold increase in the level of MMP-1 in the treated cells. Furthermore, we found that approximately 1% of the total proteins contained in exosomes correspond to SFN. In conclusion, this study is the first report showing that keratinocytes have the capacity to produce exosomes through which some intracellular proteins such as SFN, with MMP-1 stimulating activity for fibroblasts, is externalized into keratinocyte microenvironment. J. Cell. Biochem. 104: 2165–2173, 2008.

Key words: SFN; stratafin; MMP-1; matrix metalloproteinase-1; LAMP-2; lysosomal-associated membrane protein 2; TEM; transmission electron microscopy; KCM; keratinocyte conditioned medium; KSFM; keratinocyte serum free medium; DMEM; Dulbecco's modified eagle's medium; FBS; fetal bovine serum

Stratifin (SFN) is a member of the 14-3-3 family proteins which are conserved regulatory molecules expressed in all eukaryotic cells. They have been involved in a wide range of vital regulatory processes such as mitogenic signal transduction, apoptotic cell death, and cell cycle control [Fu et al., 2000]. They are composed of seven isoforms in mammals (β, ε, γ, η, σ, τ, ζ). Recently, releasable form of 14-3-3σ, also known as stratifin, was found to have a potent matrix metalloproteinase 1 (MMP-1) stimulatory effect on dermal fibroblasts [Ghahary et al., 2004]. Although these proteins are primarily intracellular, there have been some reports indicating the presence of these proteins extracellularly such as those isoforms found in cerebrospinal fluid (CSF) of patients with certain neurological diseases [Moore, 1967; Wilker and Yaffe, 2004]. Several reports have demonstrated that cell lysis is not the preliminary route [Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988; Ghahary et al., 2004]. The isoform compositions of degenerating neurons in Creutzfeldt-Jakob disease do not reflect those of the CSF suggesting that the increased presence of 14-3-3 proteins in CSF is not merely the result of neuronal cell lysis [Wilker and Yaffe, 2004]. Furthermore, lactate dehydrogenase (LDH), a strictly cytosolic protein, was not detected in

Claudia Chavez-Muñoz and Jennifer Morse contributed equally to this work.

The authors state no conflict of interest.

Grant sponsor: Canadian Institute of Health Research; Grant number: CIHR-MOP-13387.

*Correspondence to: Aziz Ghahary, PhD, 351-2660 Oak Street, Jack Bell Research Centre, Vancouver, BC, Canada V6H 3Z6. E-mail: aghahary@interchange.ubc.ca

Received 30 October 2007; Accepted 3 March 2008

DOI 10.1002/jcb.21774

keratinocyte-conditioned medium containing stratifin and that confirmed the release of these proteins was not the result of keratinocyte lysis [Ghahary et al., 2004]. Secretion of these proteins is also unlikely as these proteins lack of conventional signal peptides [Nickel, 2003]. Several other proteins such as, Hsp70 [Lancaster and Febbraio, 2005], IL-1 β [Andree et al., 1992; Corradi et al., 1995], FGF-2 and galectins [Nickel, 2003], are also released by non-classical (ER/Golgi independent) mechanisms.

ER/Golgi-independent secretion was discovered in the early 1990s and since then, at least four different pathways have been identified. One of the most common mechanisms is exosome-dependent secretion [Nickel, 2003]. Exosomes are singled membrane vesicles of endocytotic origin. These intralumenal vesicles are generated during endosome maturation by inward budding of the limiting membrane, a process during which a small portion of cytosol is trapped into the vesicle [Caby et al., 2005]. Exosomes secretion has first been described for reticulocytes during their differentiation [Harding et al., 1983; Pan et al., 1985]. Later, other hematopoietic cells including B lymphocytes [Raposo et al., 1996], dendritic cells [Thery et al., 1999], T lymphocytes [Peters et al., 1990; Blanchard et al., 2002] and mast cells [Raposo et al., 1997; Skokos et al., 2001] have been shown to release exosomes. Further, the results of several other studies showed that non-hematopoietic cells such as intestinal epithelial cells [Van Niel et al., 2001], neuroglial cells [Fevrier et al., 2004], and tumor cells [Wolfers et al., 2001] also have the ability to release exosomes. There are some specific characteristics related to exosomes that distinguish them from other vesicles that originate from different cellular locations such as plasma membrane [Théry et al., 2002]. Their size ranges from 50 to 90 nm in diameter and following a negative staining they display a unique "saucer-like" morphology (flattened sphere that is limited by a lipid bi-layer) by electron microscopy. Biochemical and proteomic analysis on exosomes purified from the supernatants of several cells revealed the presence of common proteins [Raposo et al., 1996; Thery et al., 1999, 2001; Van Niel et al., 2001; Wubbolts et al., 2003]. Depending on the cells examined, all or some of the following cytosolic proteins such as, hsp70, hsc73, hsp90, Tsg 101, several annexins, Rab proteins, cytoskeletal proteins (actin, tubulin) and milk-fat globule (MFG)-E8 (or lactadherin) have been identified to be associated with exosomes. Membrane-bound proteins, such as, tetraspanin (CD9, CD63, CD81, CD82) and MHC class I and LAMP-2 molecules were also detected in exosomal proteins. Another specific feature of exosomes is that they float on sucrose gradient, and their density ranges from 1.13 to 1.22 g/ml. Contaminating material, such as protein aggregates or nucleosomal fragments are separated readily from exosomes by flotation on sucrose gradients [Thery et al., 2001; Théry et al., 2002]. Altogether, this specific composition distinguishes this population of vesicles from other vesicles or shed membranes. Exosomes are not the only type of secreted membrane. Larger membrane structures can be purified from supernantants of prostate cells (prostasome like granules), platelets, and activated netrophils (called ectosomes). Such vesicles can be observed by confocal microscopy and are greater than 100nm in diameter. As such, they cannot be considered as exosomes.

Although, we have previously demonstrated easy detectable levels of stratifin in keratinocyte conditioned medium, the mechanism by which this protein is externalized from keratinocytes is unknown. Here, we found that keratinocytes have the capacity to release some intracellular proteins such as stratifin via the externalization of exosomes into conditioned medium. In fact, a systematic proteomic analysis of exosomes obtained from dendritic cell conditioned medium revealed that three 14-3-3 isoforms (η, γ, and γ/δ) are within a large group of proteins identified in exosomes [Thery et al., 2001]. In order to explore this mechanism in keratinocytes, we conducted a series of experiments to test our working hypothesis that primary human keratinocytes release exosomes through which some associated cytosolic proteins such as stratifin is released into conditioned medium. The findings of these series of experiments, therefore, showed that: (1) keratinocytes have capacity to produce and release exosomes and (2) exosomes contain stratifin with MMP-1 stimulatory activity in fibroblasts. This finding was then confirmed by depleting the exosomes from keratinocyte conditioned medium and showing a marked reduction in expression of MMP-1.

MATERIALS AND METHODS

Antibodies

Antibody raised in rabbit against human stratifin was generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland), and monoclonal anti-human stratifin was obtained from MEDICOR (Montreal, QB, Canada). Additionally, monoclonal anti-human LAMP-2 (CD170B) antibody was obtained from BD Pharmingen (San Diego, CA), mouse anti-human MMP-1 monoclonal antibody from R&B systems (Minneapolis, MN) and mouse anti-human β-actin monoclonal antibody from SIGMA (Saint Louis, MO). Horseradish peroxidase conjugated secondary antibodies against mouse and rabbit IgG were obtained from BioRad Lab. (Hercules, CA).

Cell Cultures

After informed consent, foreskin samples were obtained from patients undergoing elective circumcision. Samples were colleted individually in Keratinocyte Serum Free Medium (KSFM) (GIBCO, Grand Island, NY), and washed several times in sterile PBS supplemented with antibiotic–antimycotic preparation (100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (GIBCO). Skin was dissected free of fat and cut into small pieces 1 cm in diameter and incubated in Dispase enzyme (25 μg/ml in KSFM) (GIBCO) at 37°C for 2 h in order to separate epidermis from dermis. Epidermis was incubated for 4 min in trypsin at 37°C fingervortexing every minute. Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) with 10% Fetal Bovine Serum (FBS) was used to stop trypsin reaction. Centrifugation at 800 rpm at 4°C for 8 min allowed us to obtain keratinocytes that were seeded into 25 cm^2 flasks and cultured with 5 ml of KSFM with growth supplement (GIBCO) and antibiotic–antimycotic preparation (GIBCO). The dermal layer was minced into small pieces ~0.5 mm in diameter and distributed into 60 mm × 15 mm Petri dishes. Fibroblasts migrated from these minced pieces and were cultured with 10 ml of DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic–antimycotic preparation (GIBCO). Keratinocytes up to passage 4 and fibroblasts at passage 3–6 were used in all experiments conducted in this study.

Exosome Purification

Exosomes were purified from the supernatant of 2-day-old human keratinocyte cells (~5×10^7 cells) cultured in KSFM with growth supplement (GIBCO) at 37°C. To purify exosomes, the mini-scale exosome purification protocol described by Lamparski et al. [2002] was used. Briefly, 100–150 ml of keratinocyte conditioned medium was cleared by centrifugation at 6,000*g* at 4°C for 10 min in order to remove cell debris. The precleared medium was then concentrated to a volume of 1–2 ml using a 100 kDa MWCO Centricon Plus-20 filter capsule (Millipore, Billerica, MA). The sample was then transferred to a 5 ml ultracentrifuge tube and then underlayed with 300 μl of 30% sucrose-deuterium oxide (D_2O). The sample was ultracentrifuged at 100,000*g* for 1h at 4°C. Three hundred fifty microliters of the cushion of sucrose from the bottom of the tubes was collected and diluted in 15 ml of PBS. The purified exosomes were then washed and concentrated by centrifugation at 1,000*g* for 10–25 min using 100kDa Millipore Ultrafree-15 capsule filter (Millipore). Fresh exosomes were used in subsequent TEM imaging. The remaining fraction was stored at −20°C until used for Western blot analysis.

Transmission Electron Microscopy

Exosomal preparation (5 μl) was fixed with 4% paraformaldehyde and deposited onto formvar-coated carbon EM grids (Canemco Inc., St. Laurent, Quebec) and incubated at room temperature for 10 min. Excess moisture was removed and the grids were negatively stained with 1% uranyl acetate for 15 s. Excess stain was removed and the grids were air dried before viewing with a Hitachi H7600 Transmission electron microscope (TEM) with a side mount AMT Advantage (1 mega-pixel) CCD camera (Hamamatsu ORCA) (UBC Bioimaging Facility, Vancouver, BC, Canada).

Treatment of Fibroblast With Purified Keratinocyte Exosomes and Conditioned Medium

Dermal fibroblasts were seeded in 6-well plates (2×10^5 cells per well) and treated in duplicate with either KCM, exosome depleted KCM or with purified keratinocyte exosomes. After 24 h of treatment, cells were lysed and the protein concentration was determined by Bradford assay. Western blot was used to assess

MMP-1 levels using β-actin as control for equal protein loading.

Western Blotting

Human keratinocytes and dermal fibroblasts were lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.025% NaN3, 1% Triton-X100, 0.5% IGEPAL CA-630 and 1:100 protease inhibitor) for 1h at 4°C, then nuclei and cell debris were removed by centrifugation. Total proteins of the cell lysate were quantified by Bradford assay and subjected to SDS–PAGE analysis on either a 12% or 10% of acrylamide gel to visualize either stratifin or MMP-1 proteins, respectively. The gels were electrotransferred onto polyvinylidine difloride membranes (Millipore). Non-specific proteins on membranes were blocked with 5% skim milk powder in TBS containing 0.05% Tween 20 for 90 min at room temperature. Immunoblotting was performed using rabbit anti-stratifin antibody (1:1,000 dilution) Dr. Atiken's and commercially available, anti-human β-actin (1:25,000), LAMP-2 (1:1,000 dilution), and MMP-1 monoclonal antibodies (1:250 dilution). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,500 dilution). Immunoreactive proteins were then visualized using Western blotting luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To show the specificity of the polyclonal anti-human stratifin used, the experiments related to those blots shown in Figures 2–4 were repeated by using a monoclonal anti-human stratifin antibody purchased from MEDICOR, (Montreal, QB, Canada) (1:1,000 dilution).

Quantitative Analysis of the Exosome Associated Stratifin

The quantity of the exosome associated stratifin was evaluated by purifying keratinocyte exosomes and comparing the level of stratifin in 10 μg of total exosomal proteins with that of various concentrations (0, 0.06, 0.12, 0.25, 0.5, and 1.0 μg/lane) of recombinant stratifin loaded in a 12% acrylamide gel using Western blot analysis ($n = 3$). The densities of the signals were then compared by densitometry and their values were calculated and quantified using a linear regression model.

RESULTS

Human Keratinocytes Release Exosomes Into Conditioned Medium

To examine whether keratinocytes release exosomes, 100–150 ml of 48 h conditioned medium was collected, concentrated and the presence of exosomes was examined using the exosome purification method developed by Lamparski et al. [2002] (described in Materials and Methods Section). To examine the presence and visualize the ultrastructure of the purified keratinocyte exosomes, fresh samples were obtained and subjected to analysis by a transmission electron microscopy (TEM). TEM images shown in Figure 1 identified many clusters of small vesicles under 100 nm in diameter with the unique "saucer-like" shape that are salient features of exosomes. The morphology of these exosomes were very similar to those first demonstrated by Pan et al. [1985]

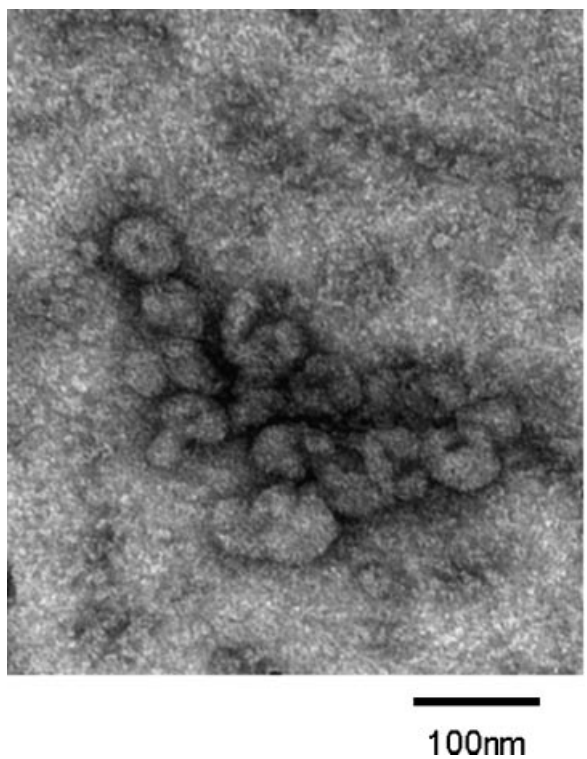

Fig. 1. TEM observation of membrane particles (exosomes) purified from keratinocyte conditioned medium. Purified exosomes were incubated briefly on formvar-coated carbon grids and excess moisture removed. The exosomes were negatively stained using 1% uranyl acetate to visualize ultrastructure immediately before examination by TEM. Cup-shaped structures 50–100 nm in diameter were identified as being exosomes.

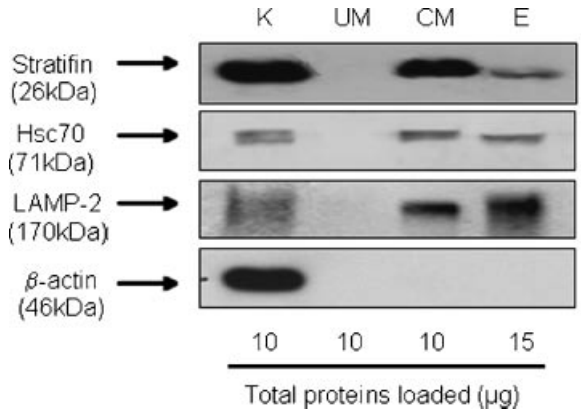

Fig. 2. Western blot analysis of stratifin in exosomes. To purify exosomes, keratinocyte conditioned medium was collected, concentrated and exosomes were separated based on their unique size and density by ultracentrifugation using a sucrose-D_2O cushion. The exosome fraction was collected, rinsed once with PBS and concentrated. Fifteen micrograms of the total proteins associated with isolated exosomes (lane E) were analyzed by Western blot analysis using rabbit anti-human stratifin, mouse anti-human LAMP-2, and monoclonal anti-human hsc70. Ten micrograms of total proteins from either keratinocyte cell lysate (lane K), keratinocyte conditioned medium (lane CM) were used as positive controls; while, the same amount of total protein of non-conditioned medium (lane UM) served as negative control. The same blots were also probed with β-actin antibody to show that detection of stratifin in KCM is not due to cell lysis.

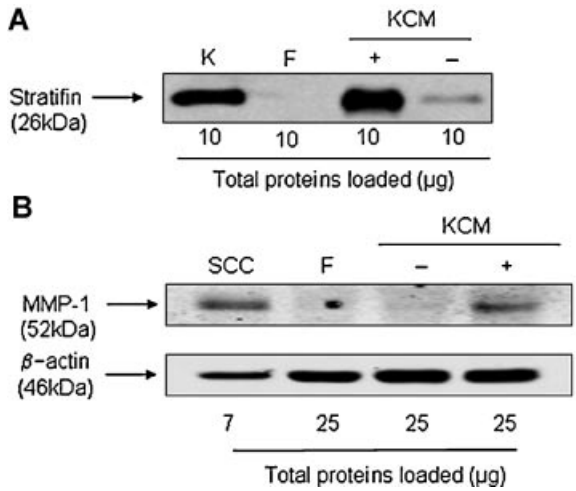

Fig. 3. Exosome depleted keratinocyte-conditioned medium lacks SFN protein and MMP-1 activity for fibroblasts. **A**: Keratinocyte-conditioned medium was collected and a 10 ml aliquot was set aside and concentrated. The rest was used for exosome preparation. After exosome purification and filtration, the retentate and filtrate were separately collected and used as exosome enriched (KCM+) and depleted (KCM−) conditioned media, respectively. Western blot analysis was then used to evaluate the level of stratifin in 10 μg of total proteins from exosome enriched KCM (KCM+) and exosome depleted KCM (lane KCM−), keratinocyte lysate (K, positive) and fibroblasts (F, negative) were used as controls. The procedure of Western blot analysis was the same as that described in the Materials and Methods. **B**: To evaluate the stimulatory effect of exosome associated stratifin in fibroblasts, Western blot analysis was used to detect the level of MMP-1 in fibroblasts treated with either nothing (lane F), exosome enriched (KCM+) or depleted (KCM−) conditioned medium using 7 μg of total squamous cell carcinoma lysate (lane SCC) as positive control for detection of MMP-1.

in sheep reticulocytes, and then by Raposo et al. [1996] in human B cells.

Purified Exosomes From Keratinocyte Conditioned Medium Contain Stratifin

The level of exosome associated stratifin was evaluated by Western blot analysis (Fig. 2). The intensity of stratifin band (lane E) in keratinocyte exosomes was then compared with that present in either human keratinocyte lysate (lane K), concentrated keratinocyte conditioned medium (lane CM) or concentrated keratinocyte un-conditioned medium (lane UM). The blot related to the same samples was then evaluated for exosomal markers such as lysosomal-associated membrane protein 2 (LAMP-2) and heat shock cognate 70 (hsc70) previously used by other investigators [Thery et al., 2001]. The results showed the presence of stratifin in keratinocyte exosomes (lane E), concentrated KCM (lane CM), but not in concentrated unconditioned culture medium (lane UM). Further evaluation of the same samples revealed that a cytosolic protein, β-actin, was only detectable in keratinocyte cell lysate (lane K). This finding indicates that the presence of exosome associated stratifin is unlikely to be due to cellular disruption.

Exosome Associated Stratifin Stimulates the Expression of MMP-1 in Fibroblasts

To examine whether the presence of exosome associated stratifin in keratinocyte conditioned medium is responsible for its MMP-1 stimulatory effect in fibroblasts, the effect of the exosome depleted and un-depleted KCM on expression of MMP-1 in fibroblasts was evaluated (Fig. 3). Prior to achieve this, the level of stratifin in these conditioned media were evaluated to be sure that stratifin level is reduced or depleted from the KCM. As shown in Figure 3A, the finding of this experiment revealed the presence of stratifin in KCM containing exosomes (lane KCM+) in a greater amount compared to KCM depleted of exosomes (lane KCM−). The levels of stratifin in

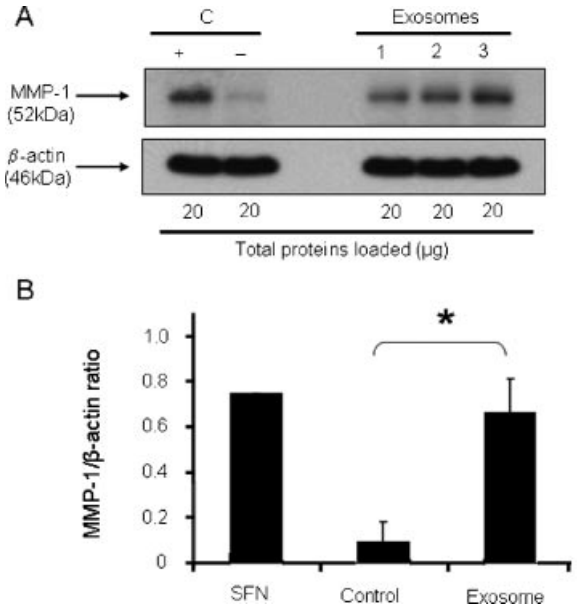

Fig. 4. Quantitative analysis of the MMP-1 stimulatory effect of exosome associated stratifin in fibroblasts. **A**: Fibroblasts were either treated with nothing (C−), 5 μg of recombinant stratifin (C+) or 15 μg of total exosomal proteins prepared from each of the three different strains of keratinocytes (exosome, lanes 1, 2, and 3). Fibroblasts were then harvested, lysed and 20 μg of total proteins extracted from treated and untreated fibroblasts was used to evaluate the level of MMP-1 protein by Western blot analysis using a 10% acrylamide gel. The level of β-actin as a loading control was also evaluated. **B**: For quantitative analysis, the signals for MMP-1 and β-actin were quantified by densitometry and the mean ± SD of MMP-1/β-actin ratio was calculated for three independent experiment and depicted in panel B. * indicates the significant difference ($P < 0.05$) in the level of MMP-1/β-actin ratio between exosome treated and untreated fibroblasts.

keratinocyte and fibroblast lysate were also evaluated and used as positive (lane K) and negative control (lane F), respectively. Confirming that stratifin can be reduced from KCM by exosomal depletion, the MMP-1 stimulatory effect of the same samples in 24 hr treated and untreated fibroblasts were then evaluated using Western blot analysis (Fig. 3B). The results showed that KCM stimulates the expression of MMP-1 protein in treated fibroblasts (KCM+) compared to, either untreated fibroblasts (lane F) or fibroblasts treated with exosomes depleted KCM (KCM−). In parallel, squamous cell carcinoma lysate (7 μg/lane) was run and evaluated as a positive control for MMP-1 expression (lane SCC). The finding of this experiment suggested that exosome associated stratifin, at least in part, is responsible for MMP-1 stimulatory effect in dermal fibroblasts. For further confirmation and statistical evaluation, dermal fibroblasts were treated with keratinocyte purified exosomes (15 μg of total proteins) prepared from three different strains of human keratinocytes (Fig. 4A). The levels of MMP-1 in fibroblasts treated with recombinant stratifin (5 μg/ml) and untreated fibroblasts were also evaluated in parallel and used as positive (C+) and negative (C−) controls, respectively. The results of Western blot analysis clearly showed that fibroblasts treated with purified keratinocyte exosomes (lanes 1–3) expressed 7.4 times more MMP-1 protein compared to that of untreated fibroblasts (0.66 ± 0.15 vs. 0.09 ± 0.09, $n = 3$, $P < 0.05$) (Fig. 4B). This finding further confirmed that exosomal stratifin released from keratinocytes is biologically active and can significantly stimulate the production of MMP-1 protein in dermal fibroblasts. To estimate the amount of stratifin in total exosomal proteins in an obtained keratinocyte exosome preparation, we conducted Western blot analysis ($n = 3$) in which we loaded and compared the stratifin signal of 10μg of total protein of keratinocyte exosomes with those of a serial dilutions of the recombinant stratifin ranging from 0 to 1 μg/sample (0, 0.06, 0.12, 0.25, 0.5, and 1 μg/sample). When the intensities of corresponding bands were quantified by densitometry we created a standard curve generating a linear regression model ($y = 10634x + 301.42$ with $r^2 = 0.9954$) in order to quantify the amount of stratifin protein in our exosome sample. The results showed that approximately 1% of the total exosomal proteins is related to stratifin (0.1 μg of stratifin in 10 μg of total exosomal proteins loaded) (Fig. 5).

DISCUSSION

Stratifin was found to be secreted by epidermal keratinocytes [Katz and Taichman, 1999], but no physiological function was assigned. We previously identified stratifin, also named as keratinocyte-derived anti-fibrogenic factor (KDAF), in keratinocyte-conditioned medium [Ghahary et al., 2004]. The mechanism by which this protein is released into the extracellular environment is not known. In this study, we used the exosome purification method developed by Lamparski et al. [2002] and showed that the vesicles purified from primary human keratinocyte conditioned medium were in fact

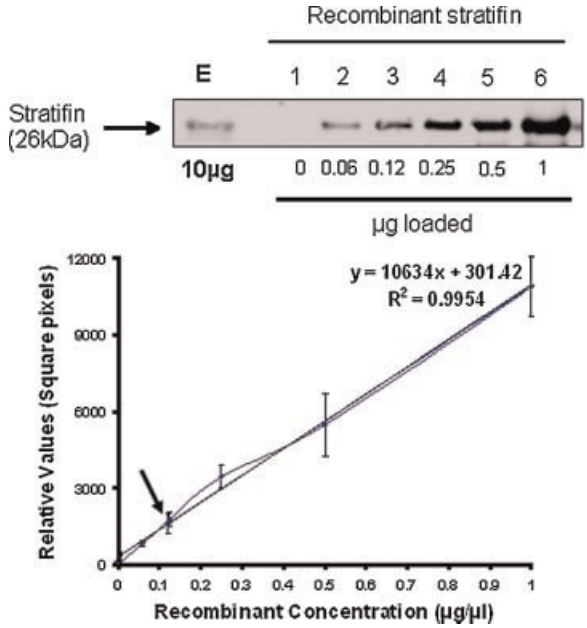

Fig. 5. Estimation of the percentage of stratifin in total keratinocyte exosomal proteins. Ten micrograms of total proteins from keratinocyte exosomes (lane E) was run along with different concentrations of recombinant stratifin (from 0, 0.06, 0.12, 0.25, 0.5 to 1 µg/lane) (n = 3). The intensity of corresponding signals were then quantified by densitometry and compared. A linear regression model was generated ($y = 10634x + 301.42$ with $r^2 = 0.9954$) in order to quantify the amount of stratifin protein in keratinocyte exosomes sample. The results show that approximately 1% of all exosomal proteins correspond to stratifin (0.1 µg of stratifin in 10 µg of total exosomal proteins loaded).

exosomes. There are several reasons for this claim. Firstly, consistent with previous TEM exosome images [Harding et al., 1983; Pan et al., 1985; Johnstone et al., 1987; Raposo et al., 1996], the ultrastructure examination of the exosomal preparation by TEM showed small vesicles less than 100 nm in diameter with the unique "saucer-like" shape, which are characteristic features of exosomes. Secondly, the result of the Western blot analysis of the exosomal fraction not only identified stratifin, it contained LAMP-2 and hsc70 which are two well established exosomal markers. Thirdly, preliminary result of a proteomic analysis of the purified keratinocytes exosomes revealed the presence of stratifin and other exosomal related common proteins such as cytoskeletal, cytosolic, annexins, Rab, tetraspanins and MHC class I proteins (data not shown). Further, the method established by Lamparski et al. [2002] used to purified exosomes from KCM consists of several steps that exclude other non-exosomal structures and cell debris. The first centrifugation procedure excludes cell debris and subsequent use of filters with large pore size (100 kDa) during concentration stages of exosome preparation allow free stratifin to pass into the elute, while exosomes associated stratifin retains in the filtrate. Finally, the ultracentrifugation with a sucrose gradient allows only vesicles with exosome characteristics (density and size) to be purified and that excludes other types of vesicles such as apoptotic blebs [Thery et al., 2001].

Upon characterization of the purified keratinocyte exosomes, we then evaluated the level of exosome associated stratifin along with exosomal markers in the same exosomal preparation using specific antibody raised against human stratifin. Although, there is ~60% of homology amongst all seven 14-3-3 isoforms, stratifin (14-3-3 sigma) is the only isoform that shows less than 43% homology with other isoforms. Further, according to the previous report by Aitken's group [Martin et al., 1993], this antibody was raised against acetylated N-terminal peptide of the non-homologous region of the stratifin. Using this antibody, we were able to easily detect the level of stratifin in KCM isolated exosomes using keratinocyte lysate as a positive control.

Although, the level of keratinocyte releasable exosome associated stratifin was easily detectable by Western blot analysis, its value was less than that found in the keratinocyte conditioned medium. For this reason and for a better comparison, we had to load 1.5 times more of total exosomal proteins than that of concentrated KCM (15 µg/lane vs. 10 µg/lane of total concentrated protein in exosomes and KCM, respectively). In theory, if exosome externalization is the only mechanism by which stratifin is released, one expects to see a higher stratifin/total protein ratio in purified exosomes than that of KCM derived from the same numbers of cells or protein content. However, this was not the case in this study. This is because, as stated in the introduction, in addition to exosome externalization, there are other pathways of non-classical externalization of proteins through which stratifin can also be released into conditioned medium. In fact, the result of the experiment on exosomal depletion shown in Figure 3A confirms this theory. Having said that, our finding revealed that at least 1/10 of the stratifin present in KCM is released by the mechanism of exosomal externalization. It

remains to be seen what other mechanism(s) is involved in stratifin release other than exosomal externalization. Further more, we wanted to know what percentage of stratifin was associated with exosomal proteins, and we found that approximately 1% of the total exosomal proteins correspond to stratifin. Considering this, the increase on the expression of MMP-1 protein in dermal fibroblasts might be due to other proteins associated with exosomes including other 14-3-3 isoforms. In fact, our group recently published data showing that not only stratifin increases MMP-1 protein expression in dermal fibroblasts, but also recombinant 14-3-3 η [Kilani et al., 2007] and 14-3-3 β isoforms (data not published).

According to the previous published study [Théry et al., 2002], 14-3-3 isoforms were identified to be inside exosomes externalized from dendritic cells. Although, we speculate that this might also be the case for the keratinocyte exosome associated stratifin, a systematic study is needed to confirm this point. Considering this, one may ask how intra-exosomal stratifin can stimulate the expression of MMP-1 in fibroblast. There are two possibilities to explain this issue. The first possibility is that stratifin is released from exosomes sometime after their externalization and interacts directly with fibroblasts and stimulates MMP-1 expression. The second possibility is that stratifin containing exosomes are taken up by the fibroblasts and by an unknown mechanism this would result in an increase in MMP-1 expression in fibroblasts. The results of the previous reports are in favor of the first theory. This is because our previous published paper [Lam et al., 2005] showed that recombinant stratifin directly stimulates MMP-1 mRNA expression in fibroblasts through p38 mitogen-activated protein kinase (MAPK). This finding was supported by showing that SB203580 (specific inhibitor of p38MAPK activity) inhibits the MMP-1 stimulatory effect of stratifin in fibroblasts. We further showed that treatment of dermal fibroblast with SFN resulted in a rapid and transient up-regulation of c-jun and c-fos mRNA levels. This finding reveals that the only way that stratifin can interact with the fibroblasts and stimulate MMP-1 is by its release due to disruption of exosomes sometimes after their externalization from keratinocytes. In fact, according to our experience, the overnight keratinocyte conditioned medium used to evaluate the exosomal shape (Fig. 1) by TEM did not show any intact exosomal structures until we have used fresh KCM instead. This finding indicates that exosomes are fragile and have tendency to burst upon their release. However, a systematic study is needed to confirm this finding.

In conclusion, by purification and characterization of the exosomes from keratinocyte conditioned medium, we provided evidence that keratinocytes have the capacity to externalize exosomes in which stratifin is associated with. Further, the finding shows that exosome associated stratifin has a potent MMP-1 stimulatory effect for fibroblasts and this effect can be abrogated by depletion of exosomes from KCM. Thus, the findings of this study open a new insight into the biological role of exosome associated proteins such as stratifin in keratinocytes/fibroblast interaction.

ACKNOWLEDGMENTS

This research was supported by the Canadian Institute of Health Research and BC Professional Firefighter's Fund. We are grateful to Dr. A. Aitken (School of Biomedical and Clinical Sciences, University of Edinburg, Scotland) for his generous gift of stratifin antibody, also to G. Martens (BioImaging Facility at The University of British Columbia) for his assistance in transmission electronmicroscopy. Special thanks to coworkers Dr. A. Medina and A. Ghaffari for their guidance and support.

REFERENCES

Andree HA, Stuart MC, Hermens WT, Reutelingsperger CP, Hemker HC, Frederik PM, Willems GM. 1992. Clustering of lipid-bound annexin V may explain its anticoagulant effect. J Biol Chem 267:17907–17912.

Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, Hivroz C. 2002. TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/{zeta} Complex. J Immunol 168:3235–3241.

Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. 2005. Exosomal-like vesicles are present in human blood plasma. Int Immunol 17:879–887.

Corradi A, Franzi AT, Rubartelli A. 1995. Synthesis and secretion of interleukin-1[alpha] and intedeukin-1 receptor antagonist during differentiation of cultured keratinocytes. Exp Cell Res 217:355–362.

Decker T, Lohmann-Matthes M. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Meth 115:61–69.

Fevrier B, Vilette D, Archer F. 2004. Cells release prions in association with exosomes. Proc Natl Acad Sci 21:21.

Fu H, Subramanian RR, Masters SC. 2000. 14-3-3 proteins: Structure, function, and regulation. Annu Rev Pharmacol Toxicol 40:617.

Ghahary A, Karimi-Busheri F, Marcoux Y, Li Y, Tredget EE, Kilani RT, Li L, Zheng J, Karami A, Keller BO, Weinfeld M. 2004. Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. J Invest Dermatol 122:1188–1197.

Harding C, Heuser J, Stahl P. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 97:329–339.

Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 262:9412–9420.

Katz AB, Taichman LB. 1999. A partial catalog of proteins secreted by epidermal keratinocytes in culture. J Invest Dermatol 112:818–821.

Kilani R, Maksymowych W, Aitken A, Boire G, St-Pierre Y, Li Y, Ghahary A. 2007. Detection of high levels of 2 specific isoforms of 14-3-3 proteins in synovial fluid from patients with joint inflammation. J Rheumatol 34:1650–1657.

Korzeniewski C, Callewaert D. 1983. An enzyme-release assay for natural cytotoxicity. J Immunol Meth 64:313–320.

Lam E, Kilani RT, Li Y. Tredget EE, Ghahary A, 2005. Stratifin-induced matrix metalloproteinase-1 in fibroblasts is mediated by c-fos and p38 mitogen-activated protein kinase activation. J Invest Dermatol 125:230–238.

Lamparski HG, Metha-Damani A, Yao J-Y, Patel S, Hsu D-H, Ruegg C, Le Pecq J-B. 2002. Production and characterization of clinical grade exosomes derived from dendritic cells. J Immunol Meth 270:211–226.

Lancaster GI, Febbraio MA. 2005. Exosome-dependent trafficking of HSP70: A novel secretory pathway for cellular stress proteins. J Biol Chem 280:23349–23355.

Martin H, Patel Y, Jones D, Howell S, Robinson K. Aitken A. 1993. Antibodies against the major brain isoforms of 14-3-3 protein. An antibody specific for the N-acetylated amino-terminus of a protein. FEBS Lett 331:296–303.

Moore B. 1967. Specific acidic proteins of the nervous system. Englewood Cliffs, New Jersey: Prentice Hall.

Nickel W. 2003. The mystery of nonclassical protein secretion. A. current view on cargo proteins and potential export routes. Eur J Biochem 270:2109–2119.

Pan BT, Teng K, Wu C, Adam M, Johnstone RM. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol 101:942–948.

Peters P, Geuze H, van der Donk H, Borst J. 1990. A new model for lethal hit delivery by cytotoxic T lymphocytes. Immunol Today 11:28.

Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. 1996. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 183:1161–1172.

Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. 1997. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol Biol Cell 8:2631–2645.

Skokos D, Le Panse S, Villa I. 2001. Mast-cell dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. J Immunol 166:868.

Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S. 1999. Molecular characterization of dendritic cell-derived exosomes: Selective accumulation of the heat shock protein hsc73. J Cell Biol 147:599–610.

Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S. 2001. Proteomic analysis of dendritic cell-derived exosomes: A secreted subcellular compartment distinct from apoptotic vesicles. J Immunol 166:7309–7318.

Théry C, Zitvogel L, Amigorena S. 2002. Exosomes: Composition, biogenesis and function. Nat Rev Immunol 2:569.

Van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M. 2001. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology 121:337–349.

Wilker E, Yaffe M. 2004. 14-3-3 proteins—A focus on cancer and human disease. J Mol Cell Cardiol 37:633–642.

Wolfers J, Lozier A, Raposo G. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med 7:297.

Wubbolts R, Leckie RS, Veenhuizen PTM, Schwarzmann G, Mobius W, Hoernschemeyer J, Slot J-W, Geuze HJ, Stoorvogel W. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem 278:10963–10972.