The Chemokine ESkine/CCL27 Displays Novel Modes of Intracrine and Paracrine Function

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We have previously shown that the β-chemokine ESkine/CCL27 is differentially spliced to produce two alternative forms. One is a secreted chemokine (ESkine), whereas the other (PESKY) lacks a signal peptide and is translocated to the nucleus. The role of this nuclear-targeted chemokine has not so far been defined, and it was the purpose of this study to examine this chemokine variant in more depth. To identify the region of PESKY involved in the nuclear translocation we tagged fragments with enhanced green fluorescent protein and expressed them in Chinese hamster ovary cells. We show PESKY nuclear translocation to be dependent on C-terminal residues that are shared with the signal peptide-bearing variant ESkine. Indeed we further demonstrate that ESkine can also use these C-terminal residues to enter the nucleus of cells following receptor (CCR10)-mediated internalization. To examine biological roles for PESKY we have overexpressed it in 3T3 cells. Such overexpression results in marked cytoskeletal rearrangements that are coincident with a radical reorganization of the cellular actin cytoskeleton. Microarray analyses and AB neutralization studies indicate that these changes are mediated in part by insulin-like growth factor-I. Furthermore, monolayer wounding assays indicate that PESKY expression correlates with markedly increased migratory capacity. Thus, it is our contention that nuclear PESKY and ESkine both enter the nucleus by either intracrine or paracrine mechanisms and may facilitate cellular migration by inducing actin cytoskeletal relaxation. Therefore, nuclear ESkine/PESKY represents a novel paradigm for chemokine function. The Journal of Immunology, 2002, 169: 1387–1394.

Chemokines are members of a large family of peptides that are typically characterized as proinflammatory mediators (1). The family is defined on the basis of sequence homology and on the presence of variations on a conserved cysteine motif, which allows the family to be divided into four subfamilies. The most populous subfamilies each have four conserved cysteine residues and differ in the presence or absence of a single amino acid inserted between the first two cysteines. Thus, in the CXC or α-chemokine family the first two cysteines are separated by a single variable amino acid, whereas in the CC or β-chemokine family these first two cysteines are juxtaposed. Two other subfamilies are characterized by single members, with lymphotactin being a member of the C family of chemokines and fractalkine being a member of the CX3C family. All chemokines mediate their actions through members of the seven-transmembrane family of G protein-coupled receptors with CXC, or α-chemokine receptors being referred to as CXCRs (currently 6) and CC, or β-chemokine receptors being referred to as CCRs (currently 11). There exist single C and CX3C chemokine receptors, a number of viral chemokine receptors, as well as more promiscuous receptors such as D6 and Duffy Ag receptor for chemokines (2).

While chemokines are classically regarded as proinflammatory mediators, they also exhibit a number of other well characterized functions. Thus, chemokines have roles in development (3), angiogenesis (4), and control of hematopoietic stem and progenitor cell proliferation (5), and it is likely that other noninflammatory roles will be revealed in the future. We have recently identified and characterized a novel member of the CC chemokine family that we cloned from an embryonic stem cell subtracted library (6). We have called this chemokine ESkine to denote its embryonic stem cell origin (7). ESkine is identical to proteins previously described as cutaneous T cell-attracting chemokine (CTACK)3 (8), ALP (9) and IL-1Rox locus chemokine (10) and is now systematically referred to as CCL27 in keeping with the recently adopted chemokine nomenclature system (11). Clear biological functions for ESkine/CCL27 have been difficult to demonstrate (9, 10); however, it has been shown to be a chemoattractant for cutaneous lymphocyte-associated Ag-positive T cells (8) and for a subset of CD4+ T cells (7). We and others have been involved in trying to identify the receptor for ESkine/CCL27 and, while it does not bind to CCRs1-9, it does bind to a protein-coupled receptor 2, a previously identified orphan receptor (12), which has now been renamed CCR10 to indicate its chemokine receptor binding function (13, 14). ESkine shares binding to CCR10 with another recently described CC chemokine, CCL28 (15).

Further analysis of the transcripts generated from the ESkine genomic locus indicates that, in addition to ESkine, an alternative differentially spliced transcript is generated. As a result of alternative first exon usage, this transcript, which we refer to as PESKY, lacks the classical N-terminal signal peptide seen in ESkine and has replaced this with an alternative stretch of N-terminal amino acids that is not predicted to function as a signal peptide. Indeed, we have demonstrated that this alternative spliced
variant is not secreted from producer cells but is targeted to the nucleus (7). This represents the first example of such radical differential splicing within the chemokine family and further represents the first demonstration of nuclear translocation by a chemokine. Interestingly, whereas ESkine/CCL27 has very restricted expression patterns in vivo, PESKY is widely expressed, suggesting a relatively general function for this unusual chemokine variant. This nuclear-targeted chemokine therefore potentially represents a novel paradigm for chemokine function.

In this work we report that the nuclear targeting motif in PESKY lies predominantly within the C-terminal tail of the mature ESkine protein and specifically localizes to two basic amino acid residues in this region. Furthermore, we demonstrate that mature ESkine is translocated to the nucleus following receptor-mediated internalization, suggesting that at least aspects of both PESKY and ESkine function are likely to be shared and mediated within the nucleus. Finally, we have examined the impact of PESKY overexpression in NIH3T3 cells and demonstrate that PESKY induces marked cytoskeletal changes in NIH3T3 cells with associated reorganization of the cellular actin cytoskeleton. These PESKY-expressing cells display a much-enhanced migratory competence, suggesting that a potential role for PESKY is to relax the cytoskeleton and thus facilitate cellular migration and potentially diapedesis.

Materials and Methods

Cell culture and transfection

NIH3T3 cells were cultured in DMEM/10% FBS (all media purchased from Life Technologies, Rockville, MD). L1.2/hCCR10 and L1.2/D6 cells were cultured in RPMI 1640/10% FBS as previously described (13). Chinese hamster ovary (CHO) cells were cultured in special liquid medium/10% FBS.

All transfections were performed using SuperFect (Qiagen, Crawley, West Sussex, U.K.). For expression of enhanced green fluorescent protein (EGFP) fusion constructs in CHO cells, 2 µg EGFP-chemokine constructs were used to transiently transfect semiconfluent CHO cells in chambered slides (Nunc, Naperville, IL). Stable transfectants of NIH3T3 cells were derived by transfecting semiconfluent NIH3T3 cells grown in 100-mm dishes with 10 µg of a PESKY-pcDNA3.1 construct, a macrophage-inflammatory protein (MIP)-1α body construct, or control vector. Stable transfected clones were obtained after selection in 1.5 mg/ml G418 and verified by RT-PCR and Northern blotting. Morphological changes in NIH3T3-PESKY cells were analyzed by staining with Giemsa and examined on a Zeiss Axiosvert 25 microscope (Zeiss, Oberkochen, Germany) with a Fuji digital SLR camera (Fuji, Tokyo, Japan).

Generation of EGFP-fusion constructs

Full-length PESKY (7) and all other fragments were cloned into pEGFP-C1 or pEGFP-N2 (Clontech Laboratories, Palo Alto, CA) as appropriate. PCR primers used to facilitate cloning were as follows: 1) PESKY full-length (for C-terminal fusion with EGFP), 5′-CGGGGATCCATGGTTCAAAA-3′ and 5′-CTCGTTTATGTACCTCTGAG-3′; 2) ESkine body (for N-terminal fusion with EGFP), 5′-GGATCCGACGCTGTCGTGCTTGAATGGTTTTGATTCTTAGGT-3′; 5′-PESKY-specific N terminus (for C-terminal fusion with EGFP), 5′-CGGGGATCCCATGTCCTCAAA-3′ and 5′-GGAGGTCGTAGCTAGGACCCTGGGT-3′; 4) ESkine N terminus (for N-terminal fusion with EGFP), 5′-GGATCCGACGCTGTCGTGCTTGAATGGTTTTGATTCTTAGGT-3′; 5′-PESKY-specific C terminus (for N-terminal fusion with EGFP), 5′-GGGGGGATCCCTCAGGTGTTCAAAA-3′ and 5′-CTCGTTTATGTACCTCTGAG-3′; 5′-ESkine C terminus (for N-terminal fusion with EGFP), 5′-GGGGGGATCCCTCAGGTGTTCAAAA-3′ and 5′-CTCGTTTATGTACCTCTGAG-3′. BamHI recognition sites are underlined.

Alanine replacement of either or both of the two basic amino acid regions in the C terminus of ESkine was performed using the QuickChange Site-Directed Mutagenesis kit (Qiagen). The template for this mutagenesis was the ESkine C-terminal green fluorescent protein (GFP) construct generated using primer set 5 above.

The EGFP-tagged RANTES body cDNA was generated by PCR from a full-length murine RANTES cDNA template. The primers used were 5′-TTCGAGCTCAGCTCTGCTGCTTGTCTACTGTTCTC3′ and 5′-AACTTTGGAGATGACCTGATGGCTGAGG-3′.

Analysis of subcellular localization of GFP fusion constructs

Twenty-four hours after transfection (as outlined above) CHO cells were fixed with 3% PFA (Sigma-Aldrich, St. Louis, MO) in PBS, treated with 100 µg/ml RNase-A for 15 min, and mounted with mounting medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA). Subcellular localization of GFP fusion constructs was analyzed using a Leica SP2 confocal microscope (Leica, Milton Keynes, U.K.).

Nuclear import assays

L1.2/hCCR10 or L1.2/D6 cells from cultures in log phase were washed once in fresh medium, twice in ice-cold binding medium (BM; RPMI 1640 medium/bicarbonate but containing 10 mM HEPES [pH 7], 0.2% BSA) and resuspended at 5 × 10^6 cells/ml in BM. For analysis of ESkine internalization, 1 × 10^6 L1.2/CCL20 cells aliquots in 200 µl BM were incubated with 2 µg/ml biotinylated human ESkine (CTACK-biotin; Glyphon Sciences, San Francisco, CA) by adding 20 µl of a stock solution of 200 µg/ml biotinylated ESkine in PBS. For analysis of MIP-1α internalization, L1.2/D6 cells were used at a similar density but were incubated in the presence of 1 µg/ml biotin MIP-1α (mouse MIP-1α-biotin conjugate; R&D Systems, Minneapolis, MN). Cells were incubated for 1 h at 37°C with or without sodium azide (0.2%) or for 3 h at 4°C (for the L1.2/CCL20 cells), following which they were cytoplasm with a Cyto tek centrifuge (Cytotech, Martigny, Switzerland) at 500 rpm for 5 min. Slides were fixed in 3% PFA/PBS for 10 min, washed twice in PBS, and incubated in quench solution (50 mM NH₂Cl in PBS) for 20 min. After quenching, slides were incubated for 30 min in PBS-gelatin containing 0.2% fish skin gelatin and 0.05% saponin. Permeabilized cells were incubated with either a 1/100 dilution of a monoclonal anti-biotin Ab (Sigma-Aldrich) or a 1/200 dilution of extravidin biotin detection reagent (Sigma-Aldrich) for 1 h. Both dilutions were in PBS/gelatin. After staining, slides were washed four times for 5 min with PBS-gelatin and mounted with mounting medium containing PI.

Actin staining

Stably PESKY transfected NIH3T3 cells were cultured in eight-well chambered slides (Nunc). Cells in log phase (Nunclon) were used to transiently transfect semiconfluent NIH3T3 cells at 3 × 10^5 cells/ml in BM. For analysis of ESkine internalization, L1.2/hCCR10 cell aliquots in 200 µl were fixed for 10 min in 3% PFA/PBS and washed in PBS. Fixed cells were quenched in quench solution for 30 min followed by a 30-min incubation in PBS/gelatin/saponin as above. Nonspecific binding sites within the permeabilized cells were blocked by incubating in 0.1% saponin/10% PBS/PBS for 1 h. Cells were then incubated for 45 min with 1 µg/ml phalloidin-FITC (Sigma-Aldrich) in the blocking solution. After incubation slides were washed three times for 5 min in blocking solution and mounted with mounting medium containing PI. Actin staining in NIH3T3 cells was analyzed by confocal microscopy.

Anti-IGF-1 receptor Ab treatment

Vector control or PESKY transfected NIH3T3 cells were seeded at 3 × 10^5 cells/well, in two-well chambered slides (Nunc). Cells were treated with PBS alone, or with either 1 µg/ml monoclonal or 5 µg/ml polyclonal anti-human insulin-like growth factor (IGF)-1R blocking Abs (R&D Systems, Abingdon, U.K.) for 24 h. Cells were washed in PBS and then fixed for 10 min in 3% PFA. Fixed cells were then stained for actin using phalloidin-FITC, as described above. Effects on the actin cytoskeleton were assessed by capturing multiple images at random and blind comparison of the PBS- and Ab-treated cell images.

Monolayer wounding assays

Vector control or PESKY-expressing 3T3 cells were plated in 24-well dishes and grown to confluence. The monolayers were scored with a disposable pipette tip (blue) and the migration of the cells into the resulting wound followed by photographing over 24 h. The migration rates were quantified using time lapse photography with a charge-coupled device camera (Hamamatsu C4742; Hamamatsu, Middlesex, NJ). The images were captured every 30 min over a 24-hour period and analyzed using Open Lab software (Improvement Software, Coventry, U.K.).

Microarray analysis

For transcriptional profiling of control and PESKY transfected cells, mRNA was prepared from two PESKY transfected 3T3 clones and two vector control clones. Array analysis was carried out using the pooled PESKY cell RNA and the pooled vector control cell RNA and was performed as a custom service by Incyte Genomics (Palo Alto, CA) using mouse GEM microarrays.
Results

The PESKY nuclear targeting signal lies within the mature ESkine sequence

Having identified PESKY as a nuclear-targeted variant of ESkine/CCL27, we have sought to determine the structural basis for this nuclear translocation. Nuclear translocation signals (NTS) typically comprise clusters of basic amino acid residues (16) and, as shown in Fig. 1a, PESKY contains a relatively high density of basic amino acids. There are two possible explanations for the nuclear translocation of PESKY. The first is that the NTS lies within the specific PESKY sequence and that basic amino acids in this region are responsible for the carriage of PESKY to the nucleus. Therefore, in this model, the presence of the PESKY-specific N terminus is central to the nuclear translocation process. The second possibility is that the NTS resides within the mature ESkine sequence and that the major function of the PESKY-specific N-terminal sequence is to replace the signal peptide and thus subvert the secretory process, allowing PESKY to be translocated to the nucleus.

To discriminate between these two possibilities we have subdivided PESKY into the PESKY-specific sequences and the region of PESKY corresponding to the mature ESkine/CCL27 protein, which will henceforth be referred to as the ESkine body (Fig. 1a). These peptide regions have been incorporated into GFP fusion constructs and their nuclear targeting capability has been studied after transfection into, and expression in, CHO cells. Full-length PESKY is translocated to the nucleus following translation with the majority of GFP staining being seen in the nucleus and very little in the cytoplasm (Fig. 1b and Ref. 7). Analysis of the two fragments of PESKY reveal that this translocation is not mediated by basic residues within the PESKY-specific sequence that does not translocate to the nucleus, as shown by the diffuse GFP staining throughout the cells. In contrast, the ESkine body mediates extensive nuclear targeting that is indistinguishable from that seen with the full-length PESKY protein. Thus, nuclear targeting of PESKY is mediated by residues within the ESkine body and not by those in the PESKY-specific sequence.

To investigate whether this ability of mature ESkine to translocate to the nucleus is shared by other chemokines, we have also generated GFP fusions with RANTES incorporating the complete coding sequence for the mature secreted RANTES protein. Following expression in transfected CHO cells, and in contrast to the ESkine body, the RANTES body does not translocate to the nucleus and is seen throughout the cell (Fig. 1c). Similar experiments with MIP-1α also suggest that this chemokine does not translocate to the nucleus following expression as a GFP fusion protein. Thus, nuclear translocation of PESKY is driven by sequences within the mature ESkine sequence and such nuclear translocation is not a generic property of the chemokine family.

Nuclear translocation of PESKY is predominantly driven by sequences within the C terminus of ESkine

To more precisely map the NTS within the ESkine body, we have further subdivided it into the C terminus, which is particularly rich in basic amino acids and which demonstrates a high degree of evolutionary conservation of basic residues, and the remainder of the N terminus of ESkine (Fig. 1a). Again, these fragments were used to generate GFP fusion constructs that were then transfected into CHO cells to examine nuclear targeting competence. The analysis of these cells (Fig. 2a) demonstrates that the N terminus of ESkine is incapable of mediating nuclear translocation, while the C terminus did support nuclear translocation. It should be noted that while the nuclear translocation seen with the C-terminal fragment is consistent, it is not as complete as is seen with the full-length ESkine body, and some cytoplasmic fluorescence is also seen with this construct. Therefore, it may be that while the majority of nuclear translocation is driven by sequences in the C terminus of the protein, these sequences may conspire, with other basic residues in the full-length protein, to mediate more complete nuclear translocation. In addition, it may be that there are as-yet-unidentified nuclear retention signals within the N terminus of the body of ESkine that contribute to the more complete nuclear translocation seen with the full-length ESkine body. Alignment of the C-terminal peptide sequences of murine, rat, and human ESkine (Fig. 2b) identify two conserved basic regions (amino acids 18/19 and 33/34). To determine if either or both of these C-terminal regions are involved in the nuclear translocation we have neutralized these regions by alanine (A) replacement. Three mutants have been produced, one in which the first two basic amino acid residues (B) are neutralized (AABB), one in which the second two are neutralized (BBAA), and one with both regions are neutralized (AAAA). These have again been linked to GFP, and their ability to transport GFP to the nucleus has been assessed after transient transfection into CHO cells. As shown in Fig. 2c, mutant AABB shows similar nuclear localization ability to that seen with the wild-type C terminus, although on repeated experiments AABB is even less efficient at nuclear translocation than the C-terminal construct shown in Fig. 2a. In contrast, neither the BBAA nor the AAAA mutants display any nuclear translocating ability, suggesting that the second of these conserved basic regions is the major

FIGURE 1. Sequences in the body of ESkine/CCL27 mediate nuclear translocation. a, Diagrammatic representation of PESKY indicating the distribution of basic amino acid residues (marked +) and the subdivision of the molecule into regions for determination of nuclear translocation ability. b, Confocal imaging of the subcellular fate of EGFP/PESKY fusion proteins in transfected CHO cells. Green coloration is EGFP and red is PI. The individual regions listed correspond to those outlined in a, c, Confocal imaging of the subcellular fate of RANTES body/EGFP fusion proteins in transfected CHO cells. b and c are representative of at least three repeat experiments.
staining or staining-associated artifacts, we have performed these experiments at 37°C, at which temperature internalization should take place, and also at 4°C or at 37°C in the presence of sodium azide, both of which should block energy-dependent internalization, resulting in exclusively membrane-associated staining. The results of these experiments are shown in Fig. 3A and demonstrate that, as expected, at 37°C in the presence of sodium azide, or at 4°C, biotinylated ESkine remains predominantly associated with the L1.2 cell membrane. In contrast, results from CCR10-bearing L1.2 cells treated with biotinylated ESkine at 37°C show it to be effectively internalized. Additionally, while L1.2 cells display a high nuclear to cytoplasmic ratio, it is still clear from the biotin/PI merged picture that the biotin staining, and hence presumably the protein, is localized predominantly within the nucleus. As a further control we have examined the fate of biotinylated ESkine following interaction at 37°C with L1.2 cells carrying the CXCR3 receptor, which does not bind ESkine/CCL27. These cells displayed neither surface nor intracellular staining for ESkine/CCL27, confirming the requirement for CCR10 for internalization and nuclear trafficking (data not shown). To examine whether the nuclear accumulation of ESkine following receptor-mediated internalization is seen with other internalized chemokines, we have examined the subcellular fate of biotinylated MIP-1α following binding to L1.2 cells expressing the high-affinity D6 receptor (18). This receptor, in contrast to CCR10, does not display a classical signaling response to ligand binding. However, it is effectively internalized contributor to nuclear translocation in ESkine and PESKY. Interestingly, AAAA not only did not display any nuclear translocation competence, but it was consistently excluded from the nucleus. We have seen this pattern with other non-nuclear translocating chemokines (data not shown), and while the nature of these cytoplasmic foci of staining remains obscure it is our assumption that these represent insoluble aggregates of the proteins that are too large to freely diffuse into the nucleus.

**Mature ESkine is translocated to the nucleus following receptor-mediated internalization**

Given that the NTS for PESKY resides within the mature ESkine sequence it is possible that mature secreted ESkine may be able to interact with receptor-bearing target cells and be translocated to the nucleus following receptor-mediated internalization. Indeed, while this would be an unprecedented finding within the chemokine family, there are precedents from a number of other growth factor families such as the fibroblast growth factors (17), which are translocated to the nucleus following receptor-mediated internalization. To examine possible receptor-mediated internalization and subsequent nuclear translocation of mature ESkine/CCL27, we have studied the fate of this protein following interaction with its receptor (CCR10) expressed on L1.2 cells (13). This approach has capitalized on the availability of biotinylated ESkine/CCL27, which was therefore applied to CCR10-bearing L1.2 cells as described (see Materials and Methods) and the subcellular fate of the biotin ESkine/CCL27 was examined. To control for nonspecific

**FIGURE 2.** Sequences in the C terminus of ESkine mediate nuclear translocation. *a*, Confocal imaging of the subcellular fate of the ESkine N and C termini (defined as outlined in Fig. 1a). Again green is GFP and red is PI. *b*, Alignment of the C-terminal peptide stretches of murine, rat, and human ESkine. Basic residues are in red. *c*, Confocal imaging of the subcellular fates of the C-terminal basic amino acid mutants. A, Alanine; B, basic amino acids. Replacements are of amino acids 18/19 and 33/34 in B. *a* and *c* are representative of at least three repeat experiments.

**FIGURE 3.** ESkine is translocated to the nucleus following receptor-mediated internalization. *a*, Imaging of the internalization of biotinylated ESkine into L1.2 cells stably expressing CCR10. The left panels (green) record biotinylated ESkine protein. The middle panels record PI staining, and the right panels represent the merging of the green and red signals. Conditions used are indicated to the left of each row. *b*, Imaging of the internalization of biotinylated MIP-1α into L1.2 cells expressing D6 in the presence or absence of azide at 37°C. Note the perinuclear location of the biotin staining in the cells treated in the absence of azide. Data are representative of at least three repeat experiments.
PESKY induces morphological changes and actin cytoskeletal reorganization in 3T3 cells

Given the expression of PESKY in the majority of murine tissues (Ref. 7 and data not shown) we have reasoned that it is likely to have a role that is evident in many diverse cell types. The likely widespread effects of this protein we regarded it as appropriate to study its biological function in NIH3T3 fibroblasts, for which numerous cellular and biochemical parameters can be measured. Therefore, we have generated NIH3T3 cell clones stably expressing this protein. During repeated attempts it has proven difficult to obtain large numbers of stable transfected clones and, typically, those clones that are obtained express only low levels of PESKY transcripts (Fig. 4a), although PESKY protein is detectable in the nucleus of these cells using anti-CCL27 Abs (Fig. 4b). Simple morphological examination of all isolated clones of PESKY-expressing NIH3T3 (seven clones studied to date) in comparison to vector control NIH3T3 cells revealed marked cytoskeletal abnormalities in the transfectants (Fig. 4c) with the extent of cytoskeletal abnormalities correlating directly with the expression levels of PESKY in the individual clones. The PESKY-expressing cells typically display shrunken nuclei and sparse cytoplasm with numerous filopodia extending from the cells. Morphologically, these PESKY-expressing cells resemble Ras-transformed fibroblasts (19); however, these cells do not appear to be transformed, as they do not display any capacity for anchorage-independent growth and show no alterations in sensitivity to serum withdrawal (data not shown). In fact, although these cells are morphologically quite different from the vector control cells, they display an identical proliferative rate, doubling approximately every 24 h during log phase growth. There is no evidence of excessive cell death among these transfectants, and they can be passaged to high numbers without obvious loss of viability. These striking morphological alterations have been seen in all PESKY transfected clones studied (six from two separate stable transfections).

Typically, marked cytoskeletal alterations such as those seen in the PESKY-expressing fibroblasts result from a rearrangement of components of the cellular actin cytoskeleton (20). This actin cytoskeleton, which is represented in the form of stress fibers of aggregated actin in fibroblasts, can be visualized using phalloidin. Thus, to attempt to determine the role of actin rearrangements in the cytoskeletal abnormalities in PESKY transfectants, we have examined the actin cytoskeleton in these cells. As can be seen from Fig. 4d, phalloidin staining of the vector control NIH3T3 cells reveals a typically dense arrangement of actin stress fibers. In contrast, examination of the PESKY transfected NIH-3T3 cells reveals a striking reorganization of the actin cytoskeleton with a marked absence of actin stress fibers in comparison to the vector control cells. This absence of stress fiber associated actin staining is coincident with the emergence of numerous densely stained filopodia emanating from the PESKY transfected cells and additionally with an increase in membrane-associated filamentous actin. As with the gross morphological observations, the extent of the reorganization of the actin cytoskeleton correlates well with the levels of PESKY expression and has been seen in all the different transfected clones examined. Such morphological and actin cytoskeletal rearrangements are not seen with 3T3 cells expressing the MIP-1α body (data not shown), suggesting that these alterations do not represent generic responses to expression of a chemokine within 3T3 cells. Thus, these data show that nuclear-targeted PESKY induces profound cytoskeletal changes in NIH3T3 cells resulting predominantly from a radical realignment of the cellular actin cytoskeleton.

FIGURE 4. PESKY mediates marked cytoskeletal rearrangements in transfected 3T3 cells. a. PCR analysis of PESKY expression in vector control and PESKY transfected NIH 3T3 cells. b. Nuclear staining of PESKY in transfected cells using anti-CCL27 Abs. c. Morphological examination of vector control and PESKY transfected 3T3 cells. d. Phalloidin visualization of actin stress fibers in vector control and PESKY transfected NIH3T3 cells. Data are representative of at least three repeat experiments.
The cytoskeletal effects of PESKY are mediated in part by IGF-1

To examine the molecular basis for the cytoskeletal effects of PESKY expression in NIH3T3 cells, we compared the transcriptomes of vector control and PESKY-expressing cells using gene array technology. Preliminary gene array analysis reveals surprisingly few differences in gene expression between these two cellular populations (data not shown). It has been difficult to identify a role for the majority of these genes in the induction and maintenance of the PESKY-related phenotype. However, one of the most highly overexpressed genes in the PESKY-expressing cells is IGF-1 (5.4-fold higher expression in the PESKY cells compared to control cells), and IGF-1 is interesting in this context because treatment of NIH3T3 cells or other cells with this growth factor induces cytoskeletal alterations similar to those seen in the PESKY transfected cells (21). Furthermore, PCR analysis has confirmed elevated expression of IGF-1 in a panel of PESKY transfectants compared to vector control cells (Fig. 5a).

To attempt to examine a role for IGF-1 as an intermediate in the mechanism of action of PESKY, we have used blocking Abs to the IGF-1R and have studied the impact of these Abs on the actin cytoskeleton. As shown in Fig. 5b, anti-IGF-1R Abs have no discernable effects on the actin cytoskeleton in vector control NIH3T3 cells. However, as also shown in Fig. 5b, treatment of the PESKY-expressing NIH3T3 cells with this Ab induces significant reformation of actin stress fibers, indicating at least a partial role for IGF-1 as an intermediate in the cytoskeletal effects of PESKY. PESKY transfected NIH-3T3 cells display higher motility than control NIH-3T3 cells

Because reorganization of the cytoskeleton is typically associated with cellular migration (22), with stress fibers being regarded as antimigratory structures, we have examined the migratory potential of the PESKY transfected cells. To do this we have performed monolayer wounding assays. This assay involves growing the adherent NIH3T3 cells to confluence and then wounding the monolayer by scoring it with a disposable pipette tip. Wild-type NIH3T3 cells will migrate into the space created over 48 h (23), and the effect of expressed genes on this motility can be measured by photographing the cells throughout the migratory process. As shown in Fig. 6, while it took 8 h to see the initial signs of migration in the control NIH3T3 cells, the PESKY transfected cells displayed initial migration at 4 h, which was marked by 8 h and which had significantly closed the gap generated by the wounding by 24 h. Even by 24 h the migration seen with the control NIH3T3 cells was, at best, equivalent to that seen with the PESKY transfected cells at 4 h. We have also used time lapse photography to quantify the increase in motility in two additional PESKY transfected clones of 3T3 cells. The data collected indicate that these clones migrate at rates that are respectively 1.6- and 3.2-fold faster than the control 3T3 cells. Thus, these data demonstrate that PESKY mediates an increase in motility of transfected NIH3T3 cells, which apparently correlates with a rearrangement of the cellular actin cytoskeleton.

FIGURE 5. Anti-IGF-1R Abs partially reverse the cytoskeletal effects of PESKY. a, PCR analysis of the expression of IGF-1 in vector control and PESKY-expressing NIH3T3 cells. b, Visualization of the actin stress fiber content of vector control cells or PESKY transfected cells treated with PBS or blocking anti-IGF-1R Abs for 24 h. Cells were viewed at a magnification of ×40. Data are representative of three repeat experiments.
chemokines (connective tissue-activating peptide-III and growth-factor-related oncogene-α) in the nucleus of leukocytes (24, 25). Furthermore, a number of β-chemokines such as secondary lymphoid tissue chemokine, MIP-3β, and CCL28 (which also binds to CCR10) have high densities of basic amino acids at their C termini and may also be candidate nuclear localizing chemokines. Indeed, our preliminary results indicate that a number of other β-chemokines will translocate to the nucleus as GFP fusion proteins (our unpublished data), indicating that nuclear localization of chemokines may be a more widespread phenomenon than previously suspected.

With a view to examining the possible roles for nuclear-targeted ESkine/PESKY, we have generated stable NIH3T3 PESKY transfectants. These cells revealed marked cytoskeletal alterations compared to wild-type or vector control NIH3T3 cells. We have also demonstrated that a radical reorganization of the cellular actin cytoskeleton underlies the morphological alterations and that this apparent relaxation of the fibroblast cytoskeleton is associated with an enhanced migratory potential as revealed in the monolayer wounding assays. It appears from the Ab studies reported in Fig. 5b that IGF-1 is involved in the effects of PESKY. It is important to emphasize that the Ab-treated PESKY cells do not display a complete reversal of phenotype. However, the increase in stress fiber formation seen in the Ab-treated cells is marked, indicating that IGF-1 has a significant role to play in mediating the effects of PESKY. Intriguingly, a recent study has reported further interactions between IGF-1 and the chemokine family with IGF-1 being capable of inducing expression of chemokines (26). One other overexpressed gene in the PESKY-expressing 3T3 cells is the chemokine C10 and thus it is possible that IGF-1 may be an intermediate step in a more complex network of cytoskeletal regulators induced by PESKY.

Discussion

ESkine/CCL27 exhibits radical differential splicing to generate two variants, one of which bears a signal peptide and is secreted from producer cells (ESkine/CTACK/ALP/IL-11Rα locus chemokine) and the other (PESKY) which does not have a signal peptide but has replaced this with a nonsignal peptide competent stretch of amino acids. This nonsecreted isoform displays strong nuclear localization tendencies following expression in a range of cell types. In this work we have analyzed the nuclear targeting ability of PESKY in more detail and demonstrate that the NTS resides within the main body of mature ESkine, predominantly at the C terminus. Specifically, we have identified two basic amino acid residues that appear to be primarily involved in the nuclear translocation of ESkine. Given the fact that the NTS resides in the mature ESkine sequence, we have reasoned that it is possible, as is seen with other growth factors (17), that mature ESkine may be able to enter target cells by interacting with its receptor (CCR10), after which it may be able to migrate to the nucleus where it mediates a number of its biological effects. Indeed, using CCR10 transfectants we demonstrate here that exogenous ESkine can enter CCR10-bearing cells and translocate to the nucleus following receptor-mediated internalization. Thus, at least for aspects of ESkine function, the receptor may serve more as a gateway into the cell, facilitating nuclear translocation, than as the primary mediator of biological function. However, it is important to note that ESkine/CCL27 does trigger a downstream signal following receptor binding (13, 14) and thus it may be that there are dissociable activities associated with this protein that separately require receptor signaling or receptor-mediated internalization. While we have demonstrated the above for ESkine/CCL27 and have shown that cytosolic RANTES and extracellular MIP-1α do not translocate to the nucleus, it remains possible that post-receptor nuclear translocation of chemokines may be a more widespread phenomenon. Indeed, there have been occasional reports of the detection of other chemokines (connective tissue-activating peptide-III and growth-factor-related oncogene-α) in the nucleus of leukocytes (24, 25). Furthermore, a number of β-chemokines such as secondary lymphoid tissue chemokine, MIP-3β, and CCL28 (which also binds to CCR10) have high densities of basic amino acids at their C termini and may also be candidate nuclear localizing chemokines. Indeed, our preliminary results indicate that a number of other β-chemokines will translocate to the nucleus as GFP fusion proteins (our unpublished data), indicating that nuclear localization of chemokines may be a more widespread phenomenon than previously suspected.

With a view to examining the possible roles for nuclear-targeted ESkine/PESKY, we have generated stable NIH3T3 PESKY transfectants. These cells revealed marked cytoskeletal alterations compared to wild-type or vector control NIH3T3 cells. We have also demonstrated that a radical reorganization of the cellular actin cytoskeleton underlies the morphological alterations and that this apparent relaxation of the fibroblast cytoskeleton is associated with an enhanced migratory potential as revealed in the monolayer wounding assays. It appears from the Ab studies reported in Fig. 5b that IGF-1 is involved in the effects of PESKY. It is important to emphasize that the Ab-treated PESKY cells do not display a complete reversal of phenotype. However, the increase in stress fiber formation seen in the Ab-treated cells is marked, indicating that IGF-1 has a significant role to play in mediating the effects of PESKY. Intriguingly, a recent study has reported further interactions between IGF-1 and the chemokine family with IGF-1 being capable of inducing expression of chemokines (26). One other overexpressed gene in the PESKY-expressing 3T3 cells is the chemokine C10 and thus it is possible that IGF-1 may be an intermediate step in a more complex network of cytoskeletal regulators induced by PESKY.

Interestingly, the cytoskeletal and actin rearrangements associated with PESKY expression are very similar to those seen in cells treated with other growth factors such as growth hormone (27), TGF-α (28) and, as mentioned above, IGF-1 (21). In addition, cdc42-dependent transient rearrangement of the actin cytoskeleton has been shown to be involved in the migratory response to chemokines (29–31). What do these observations tell us about the role of PESKY in cell function/migration? It appears that PESKY relaxes the cellular cytoskeleton, and previous reports indicating the negative influence of stress fibers on cell motility (32, 33) are in keeping with our observations of enhanced motility in the PESKY-expressing cells. Further analyses are now needed to identify the precise cell types expressing PESKY in different tissues and to examine the role of PESKY in regulating their motility. In the wider chemokine context, if nuclear translocation of chemokines is a more widespread phenomenon, then it is possible that the nuclear-mediated disruption of the actin cytoskeleton may be a common mechanism for facilitating cell movement and transendothelial migration. We are currently examining this possibility in the laboratory.

In summary, we have demonstrated that ESkine and PESKY are targeted to the cell nucleus by sequences within the C terminus of the mature ESkine protein and that this nuclear targeting can also take place following receptor-mediated internalization. Furthermore, the nuclear translocation of PESKY is associated with marked cytoskeletal rearrangements involving alterations to the cellular actin cytoskeleton. These rearrangements are associated with enhanced motility of the PESKY-expressing cells. These actions, which are likely to be mediated within the nucleus, reveal ESkine/CCL27 to display an unprecedented mode of action that enhances our understanding of the wider capabilities of members of the chemokine family of proteins.
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References