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Surface Translocation of Pactolus Is Induced by Cell Activation and Death, but Is Not Required for Neutrophil Migration and Function

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Pactolus is a cell surface protein expressed by murine neutrophils. Pactolus is similar to the β integrins, except it lacks a functional metal ion-dependent adhesion site domain and is expressed without an α-chain partner. The majority of the Pactolus protein is held within the cell in dense granules in a highly glycosylated form. This intracellular form of Pactolus can be released to the cell surface following inflammatory activation or ligation of Pactolus on the cell surface. In addition, intracellular Pactolus translocates to the neutrophil surface following induction of apoptosis. Neutrophil activation studies suggest that Pactolus does not serve as an activating or phagocytic receptor for the neutrophil. To further define the function of Pactolus, a Pactolus-null mouse was generated. Pactolus-deficient animals mature appropriately and possess normal numbers of neutrophils, display appropriate migration into sites of inflammation, and combat introduced infections efficiently. These data suggest that Pactolus does not function as a neutrophil phagocytic or adhesion receptor, but may instead serve as a sugar-bearing ligand for lectin recognition by other cells. The Journal of Immunology, 2003, 171: 6795–6806.

Pactolus is a β integrin-like protein found on mature and immature murine neutrophils (1). It exhibits close homology, in the extracellular region, to the β2 and β3 integrin subunits (2). However, unlike the β2 integrin subunit, Pactolus is missing the metal ion-dependent adhesion site domain that is important for ligand binding and subunit interactions (3–7). All integrins are heterodimers consisting of α and β subunits. An α-chain partner for Pactolus has not been found, suggesting that Pactolus is expressed as a monomer on the cell surface. The predicted size of the full length Pactolus peptide is 81,000 M₀; however, extensive glycosyl modifications provide for a final mature protein of ~130,000 M₀ (1).

The pactolus gene produces two distinct transcripts: one that predicts a truncated form, and a second that produces the transmembrane protein. The truncated form appears to be rapidly degraded upon translation (if, indeed, it is translated), whereas the transmembrane form has a long half-life (>12 h). The neutrophil is the major, if not exclusive, mature cell that transcribes the pactolus gene and produces the protein. Two distinct alleles of pactolus exist: that of the C57BL/6 animal specify production for only the transmembrane form, whereas those of BALB/c, C3H/HeJ, and 129/Sv produce both the full-length and truncated transcripts (1).

Stimulation of neutrophils with PMA or Pactolus antiserum can induce an immediate increase in Pactolus surface expression independent of protein synthesis. This suggests that Pactolus is stored in an intracellular compartment and, upon stimulation, is exocytosed to the surface of the neutrophil. This type of response is similar to that of other neutrophil activation receptors, such as human CR1 and human/mouse Mac-1, both of which serve as neutrophil phagocytic receptors (8–10).

Neutrophils play a critical role during an inflammatory response, because they are the first major cell type recruited to a site of infection (for review, see Ref. 11). When an infection occurs, neutrophils migrate from the blood to the tissue site. This process involves activation of both the endothelial cell layer as well as the circulating neutrophils, relying upon the selectins and integrins (and their respective ligands) for extravasations (12). The selectins are instrumental for the tethering and rolling of the neutrophils on the endothelial cells, whereas the integrins are involved in tight adherence and extravasations into the tissue matrix (13). The neutrophils then follow a gradient of cytokines, chemokines, and bacterial/viral products to the site of infection. When neutrophils arrive at the focus of infection, they are in a primed state and can follow several options to clear the infection. They can degranulate, releasing proteases, cytokines, and superoxides, creating an environment inhospitable for the infective agent. The primed neutrophil can also phagocytose the pathogen, usually making use of complement and Ab deposited on the pathogen surface as opsonins for the relevant neutrophil receptors (14). Many such recruited and activated neutrophils are in excess and consequently undergo apoptosis and are removed from the site via phagocytosis by macrophages (15). This cleanup step can make use of a number of receptor/ligand interactions between the neutrophil and macrophage (some well-characterized, some not) (16–18), removing both apoptotic and necrotic cells (19).

Our previous analyses of Pactolus suggested that it may function as an activating receptor for neutrophils, perhaps aiding in cell migration and/or phagocytosis of pathogens. It was with this model in mind that we analyzed the expression of Pactolus by migrating neutrophils and the cellular responses to Pactolus ligation. Surprisingly we found that Pactolus did not appear to be involved in...
any neutrophil function, including migration, cross-linking activation, or phagocytosis. In addition, Pactolus-deficient animals demonstrated normal neutrophil functions, including migration and response to bacterial infections. These data leave open the possibility that Pactolus instead may serve as a ligand to mark dead and dying neutrophils for elimination.

Materials and Methods

Mice and cells

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute, National Institutes of Health (Bethesda, MD), and 129svs mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Murine neutrophils were obtained either from the bone marrow or by lavage after peritoneal cavity recruitment. Neutrophils were stimulated with 100 ng/ml PMA, 100 μM MLMP, or 5 μM leukotriene B₄ (LTB₄; Sigma-Aldrich, St. Louis, MO). Cells were stimulated with Bac Pac (Pactolus polyclonal Ab) spectrum, was used to discriminate permeable, 7-AAD-positive cells in the

Biosciences, Mountain View, CA) was used to identify external phosphatase activity and Multianalyst software (Bio-Rad, Hercules, CA). See below for greater detail for Western blot analysis of the Pactolus protein.


cell lines.

Bone marrow neutrophils were isolated following the protocol previously described (20). Briefly, mouse marrow tissue was flushed from murine femurs with cold PBS. The RBC were lysed by treating the marrow with ACK lysing buffer (0.15 M NH₄ Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA) for 5 min on ice. After 2 washes, the cells were resuspended in PBS and layered onto a Percoll (Amersham Pharmacia Biotech, Arlington Heights, IL) gradient consisting of equal volumes of the different densities of 1.080 g/ml (lower layer) and 1.065 g/ml (upper layer). The gradients were then centrifuged at 1000 × g for 20 min at 4°C. Cells were visible as a pellet (most mature neutrophils) and at the interface of the two densities (less mature neutrophils). Cells were removed and centrifuged in PBS to remove the Percoll and were ready for use. Unless specified, all gradient dilutions were made following the protocol from Amersham Pharmacia Biotech using 0.15 M NaCl. Neutrophils were obtained from the peritoneal cavity by recruitment. Mice were injected with either 1 ml of a 1% solution of soybean oil or 0.5 ml of a 3% thiglycolate solution into the peritoneal cavity. After 4 hr a peritoneal lavage was performed. The lavage fluid consisted of primarily neutrophils (21) and was confirmed by cytoospin staining.

FACS analysis

Mouse bone marrow cells and peritoneal neutrophils were isolated. RBC were lysed as described previously. Some 1 × 10⁶ cells were used for each staining reaction. Cells were stimulated with agonists (PMA, MLMP, and LTB₄) for 20 min at 37°C. The rabbit anti-Bac Pac (1) was detected using an FITC-conjugated goat anti-rabbit Ig Ab (Cappel Laboratories, West Chester, PA). The other Abs used to FACS was Mac-1 (clone M1/70; BD Pharmingen, San Diego, CA) and L-selectin (clone MEL-14; eBioscience, San Diego, CA). Annexin V protein directly conjugated to PE (BD Biosciences, Mountain View, CA) was used to identify external phosphatidylserine, the result of membrane inversion. The dye 7-aminocanthomycin D (7AAD) (BD Biosciences), which is detected in the far red FL3 spectrum, was used to discriminate permeable, 7-AAD-positive cells in the later stages of apoptosis and death, from those that still possess intact membranes (alive or early in apoptosis) that are not permeable to the dye (7-AAD-negative).

Surface iodination

Oyster glycogen and thiglycolate neutrophils were isolated from the peritoneal cavity. Cells were surface iodinated using IODO beads (Pierce, Rockford, IL), following the recommended protocol. Some 1 × 10⁶ cells were labeled per reaction with 1 μCi of Na¹²⁵I/10⁶ cells.

Granule fractionation, marker assay, and Western blot analysis

Granule fractionation was performed using the protocol previously described (22). Neutrophils (3 × 10⁶) were isolated as described and resuspended in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM ATPNa₂, 3.5 mM MgCl₂, and 10 mM piperazine N,N’-bis[ethanesulfonic acid], pH 7.2) containing 0.5 μM PMSF (Sigma-Aldrich). A two-layer Percoll gradient was set up according to the protocol. Cells were nitrogen cavitated for 5 min at 380 psi using a nitrogen bomb (Parr Instruments, Moline, IL). The cavitated was then centrifuged to remove whole cells and nuclei, and was layered onto a two-layer Percoll gradient. Granules were centrifuged at 37,000 × g for 30 min at 4°C. Fractions were collected from the bottom of the tube. Percoll was removed from the fractions by centrifugation at 100,000 × g for 1.5 hr. Each fraction was then assayed for specific granule markers: myeloperoxidase (MPO; azurophilic), gelatinase B (MMP-9), alkaline phosphatase (AP; secretory vesicles), and Pactolus. Fractions were sonicated for 5 min, freeze-thawed twice, then assayed for MPO in a 4/1 dilution of assay buffer to fraction volume (0.2 mg/ml o-dianisidine and 158 μM H₂O₂ in 50 mM KPO₄, pH 6.0) for 5 min. Reactions were performed in triplicate, and absorbance was recorded at 460 nm. AP was assayed following the protocol provided with a phosphatase substrate kit (Pierce). Gelatinase and Pactolus were assayed by Western blot. Fraction samples were separated by SDS-PAGE, blotted to nylon membranes, and probed with Abs against gelatinase (Chemicon, Temecula, CA) or Pactolus (the Bac Pac antiserum) (1). Gelatinase was quantified by densitometer analysis of Western blot images and Multianalyst software (Bio-Rad, Hercules, CA).

Induced apoptosis and necrosis

Bone marrow cells were isolated from C57BL/6 mice. Apoptosis was induced by placing the bone marrow samples into a six-well (Ultra Low Attachment Plate; Costar, Cambridge, MA) dish and irradiated with 300-μW UV transilluminator for 10 min, followed by 4-h incubation at 37°C in the presence of 5% CO₂ incubator. Necrosis was induced by taking the same bone marrow sample and incubating at 55°C for 15 min, followed by a 3-h culture at 37°C in a 5% CO₂ incubator.

Generation and analysis of a Pactolus-deficient mouse

The targeting construct was produced by subcloning two EcoRI fragments of the pactolus gene (8.4 and 6.7 kb) into pSK vector (Stratagene, San Diego, CA) from λ phage containing pieces of pactolus (2, 23). The BgIII site of exon 5 in the 8.4-κb subclone was changed to a ClaI site by cutting with BgIII and inserting a double-stranded oligo containing the ClaI site. The K373lox A plasmid that contained the neomycin gene under the control of the polymerase II promoter was cut with ClaI, and the neomycin fragment was gel-purified, then inserted in the reverse orientation into the new ClaI site of exon 5. The new plasmid containing 15.1 kb of the pactolus gene interrupted by the neomycin gene was linearized by Sall and Sfil, then ligation into the thymidine kinase (TK1-TK2) vector Xhol and XbaI sites. The resulting plasmid was linearized with Nof for transfection into the embryonic stem (ES) cell line.

ES cell lines were transfected with the linear pactolus gene-targeting construct, then selected for G418 resistance and gancyclovir sensitivity. DNA was isolated from the ES cells and analyzed by Southern blot after digestion with EcoRI (5’ probe) or Sxl (3’ probe). The probes for Southern blots were made by PCR of the either the λ phage DNA (for the 5’ probe, exon 1, primers 1047 and 860) or pactolus cDNA (for the 3’ probe, exon 12, primers 1008 and 1231). An ES cell line derived from the 129sv strain containing one copy of the pactolus-targeted allele, 2e86, (as evidenced by Southern blot analysis with both the 5’ and 3’ probes) wasiendoed into blas-tocysts and implanted in pseudopregnant female mice. One male chimera offspring (85% chimera) was mated to C57BL/6 female mice and was successful for germline transmission of the disrupted gene. The F₂ generation, heterozygous for the modified pactolus allele (+/−), was mated to generate the F₃ population.

Tail DNA isolation and genotyping

Tail clips (1 cm) were added to 1 ml of tail digestion buffer (0.1 M NaCl, 0.02 M EDTA, 1% SDS, and 0.01 M Tris, pH 8) with 0.2 mg of proteinase K and incubated at 55°C overnight. After the debris settled, 500 μl of supernatant was transferred to a new tube, followed by phenol and chloroform extraction and ethanol precipitation. Nine nanograms of DNA was used per PCR reaction with the genotype primers (1 3’ primer, 506, and 1052, respectively) 1 Genotyping PCR reactions used 68°C annealing, 5-s elongation, and 28 cycles. Primers 863f and 1028r made the neomycin/exon 5 product (130 bp).

DNA isolation, cDNA synthesis, and PCR

Cells were isolated from murine bone marrow with PBS, and total RNA was extracted (24). cDNA synthesis reactions were previously described (25). The PCR reaction were prepared as previously described (25). The 95°C denaturing, 72°C elongation, 1 s for annealing, and denaturing times

° Abbreviations used in this paper: LTB₄, leukotriene B₄; AP, alkaline phosphatase; MMP-9, gelatinase B, MPO; myeloperoxidase, PTXN, peroxis toxin; SCR, stem cell factor; 7-AAD, 7-aminocanthomycin D; ES, embryonic stem; TK, thymidine kinase.
were kept constant, whereas the annealing temperature, elongation time, number of reaction cycles, and amount of DNA (200 ng for cDNA and 9 ng for genomic DNA) per reaction varied with the experiment. The actin PCR (product size, 135 bp) required 60°C annealing, 5-s elongation, and 15 cycles, whereas the pactolus PCR required 60°C annealing, 4-s elongation, and 25 cycles. The pactolus PCR product with primers 1001f and 990r (which span exons 3–4, before the neomycin) was 223 bp, that with primers 803f and 828r (exons 4–6, over neomycin insertion) was 215 bp, and that with primers 1010f and 1004r (exons 6–8, after the neomycin insertion) was 207 bp. The primers were as follows (sequence listed 5’ to 3’): actin control for equivalent cDNA: primer 62, GTACAATGCCAT GTTCAAT; primer 339, CTCCATCCTGTCGCGCTTCT; Southern probe PCR primers: primer 1047f, TCCCAAGCACTGCGCTCCTTG; primer 863f, TTTCGCTTCAGGCGCCCTT; primer 1008r, TGGCCAT CATATCTCTCA; primer 1231f, TCGCCCGTTACCCTGGA; and primer 1052f, CGCTCGATGTTCAGCCCAAGC; primer 1028r, CGAT

primer 1047f, TCCCAAGCAGCTGCCCTTCTG; primer 339, CTCCATCGTGGGCCGCTCTAG; Southern genotyping primers: primer 1001f, CCGCTCGAGACAGCTGCCGGCCTGACCTGGAG; and primer 1010f, CCGAAGCGATGTCGAGCCACCTG; primer 1004r, CGGCTCGAGTTCAGCCCAAGC; primer 1028r, CGAT

Materials and Methods

Neutrophils were isolated from C57BL/6 bone marrow (3 × 10⁸ cells) and disrupted by nitrogen cavitation, and the supernatant was centrifuged over a discontinuous Percoll gradient. The gradient was then fractionated and centrifuged over another discontinuous Percoll gradient (22). The latter finding suggested that interaction of Pactolus with its putative ligand should enhance cell surface expression by translocating the intracellular Pactolus to the cell surface. Using the anti-Pactolus Ab for cell immunofluorescence, we have also found that the majority of Pactolus protein in an unactivated neutrophil is held within intracellular granules (data not shown). To follow up on these data, we first examined the intracellular localization of Pactolus and then evaluated whether neutrophil inflammatory migration resulted in translocation of the intracellular stores of Pactolus to the cell surface.

Neutrophils were isolated from the bone marrow of C57BL/6 mice using a Percoll (Amersham Pharmacia Biotech) centrifugation method for neutrophil isolation. Such cells were then used for granule fractionation (20, 22). The literature conditions described were for human neutrophils; however, similar banding patterns were obtained from mouse bone marrow neutrophils. Isolated neutrophils were placed in relaxation buffer and nitrogen-cavitated using a nitrogen bomb (Parr Instruments). After cavitation, the supernatant was collected and centrifuged over another discontinuous Percoll gradient (22). The gradient was then fractionated and

**FIGURE 1.** Granule localization of intracellular Pactolus. Neutrophils were isolated from C57BL/6 bone marrow (3 × 10⁸ cells) and disrupted by nitrogen cavitation, and the supernatant was centrifuged over a discontinuous Percoll gradient. The gradient was then fractionated from the bottom and assayed for MPO, MMP-9, AP (top plot), and Pactolus (bottom gel), as described in Materials and Methods. The two predominant forms of Pactolus, shown by Western blot, are indicated (130,000 and 98,000 Mr).
were injected into the peritoneal cavity of C57BL/6 mice. After
the newly recruited peritoneal cavity neutrophils.
activated levels of Pactolus protein should be present on the surface of
does induce translocation of the intracellular stores) (1), then ele-
numbers within the same time frame. We reasoned that if neutro-
compound recruits neutrophils into the peritoneal cavity in similar
into the peritoneal cavity of a naive C57BL/6 mouse (21). Each
stimuli: injection of either 1% oyster glycogen or 3% thioglycolate
To accomplish this we used two different neutrophil-recruiting

Translocation of intracellular Pactolus during neutrophil migration

We next chose to investigate whether the recruitment of neutrophils into a site of inflammation would translocate this intracellular Pactolus to the cell surface, and whether both forms of the protein (130,000 and 98,000 Mₙ) would be expressed on the cell surface. To accomplish this we used two different neutrophil-recruiting stimuli: injection of either 1% oyster glycogen or 3% thioglycolate into the peritoneal cavity of a naive C57BL/6 mouse (21). Each compound recruits neutrophils into the peritoneal cavity in similar numbers within the same time frame. We reasoned that if neutrophil recruitment used Pactolus ligation (and ligation of Pactolus does induce translocation of the intracellular stores) (1), then elevated levels of Pactolus protein should be present on the surface of the newly recruited peritoneal cavity neutrophils.

Sterile solutions of 1% oyster glycogen or 3% thioglycolate were injected into the peritoneal cavity of C57BL/6 mice. After 4 h, peritoneal lavages were performed, and cells were incubated with or without PMA for 20 min at 37°C in 5% CO₂, and analyzed by FACS (Fig. 3A). We have previously shown that PMA promotes the release of intracellular Pactolus to the cell surface. Those cells recruited with oyster glycogen expressed low levels of cell surface Pactolus, in that treatment with PMA resulted in translocation of the intracellular protein to the cell surface. Alternatively, those neutrophils recruited with thioglycolate already expressed Pactolus at the maximum level, in that treatment with PMA had no effect. These recruited cells, with or without PMA, were labeled with Na¹²⁵I, and the Pactolus proteins were identified by immunoprecipitation. As shown (Fig. 3B), only the 130,000 Mₙ form of Pactolus was found on the cell surface. There were virtually identical quantities of the protein on the surface of the control or PMA-treated, thioglycolate-recruited cells, whereas the oyster glycogen-recruited cells required PMA-mediated translocation for maximal Pactolus expression. As a control, the level of Mac-1 staining was equal in both the oyster glycogen and thioglycolate-recruited neutrophils (and could not be increased with PMA treatment), suggesting the Mac-1 intracellular stores were fully translocated to the cell surface during neutrophil migration (data not shown).

These data suggest that Pactolus ligation does not occur during neutrophil extravasation into the peritoneal cavity, because if the protein did engage its cognate receptor, we would have expected to

![FIGURE 2](image-url) The heavily modified form of Pactolus is indicative of mature neutrophils. Mouse bone marrow (C57BL/6) was fractionated in a Percoll gradient as described in Materials and Methods. Cells from the top of the gradient (T), the middle interface (M), and the pellet at the bottom of the tube (B) were lysed, and equivalent protein extracts were analyzed by immunoprecipitation using anti-Pactolus mAb, followed by Western blot using anti-Pactolus Bac Pac Ab (Pactolus) or preimmune antisem (control), as detailed in Materials and Methods. Molecular mass markers are marked on left.

![FIGURE 3](image-url) Cell surface expression of Pactolus after neutrophil recruitment. A, Neutrophils were recruited into the peritoneal cavity of C57BL/6 mice using either oyster glycogen (left panel) or thioglycolate (right panel). Neutrophils were recruited for 4 h, and a peritoneal lavage was performed. Neutrophils were then nonstimulated (solid line) or stimulated with PMA (100 ng/ml; dotted line) for 20 min at 37°C. Cells were then stained for Pactolus and analyzed by FACS. B, Cells treated as described above were surface-labeled with Na¹²⁵I, lysed in RIPA, and directly immunoprecipitated with the polyclonal Bac Pac Ab. Only the ~130,000 Mₙ form of Pactolus was detected.
observe high levels of Pactolus on the surface of the oyster glycogen-recruited neutrophils. Additionally, the translocation of Pactolus to the cell surface appears to preferential involve the denser Pactolus-containing granules (comigrating with MPO), because the cell surface expression of the 98,000 M₉ form in the lighter granules is undetectable on the surface of the recruited and/or PMA-treated cells. These and our previous data (1) strongly support the contention that the 98,000 M₉ form of Pactolus matures to the 130,000 M₉ form by remodeling the sugar backbone and adding additional sialic acid residues. It is the 130,000 M₉ form of Pactolus that we would anticipate is recognized in a receptor/ligand interaction.

**Agonists involved in the exocytosis of Pactolus**

The preceding data suggested that the recruitment of neutrophils into the peritoneal cavity did not use Pactolus as a migration receptor, because the cells recruited with oyster glycogen expressed basal levels of Pactolus after recruitment. However, the cells recruited with thioglycolate did express high levels of Pactolus, suggesting that these two agents are not identical in their effects in the animal. During the course of an immune response, chemokines and other inflammatory products are released to enhance the recruitment and activation of neutrophils and macrophages (26). If Pactolus expression was not elevated by the process of recruitment, would it be increased via neutrophil activation? Incubation of bone marrow neutrophils in culture with thioglycolate did not alter the cell surface expression level of Pactolus (data not shown), suggesting the translocation of Pactolus on the recruited cells was not due to neutrophil/thioglycolate interactions. To test whether a product released by a cell other than the neutrophil was responsible for the Pactolus translocation, a peritoneal wash of an animal previously treated with thioglycolate was incubated with bone marrow cells (~40% of which are neutrophil precursors and synthesize Pactolus). Such treated cells demonstrated an increase in Pactolus staining and a decrease in L-selectin staining (compared with the naive wash control; Fig. 4A). These data suggested a soluble product in the irritated peritoneal cavity was responsible for the translocation of Pactolus.

Two cell types in the peritoneal cavity that could be responsible for this soluble factor are the mast cell and macrophage. To test whether the products derived from freshly degranulated mast cells could induce the translocation of Pactolus, bone marrow cells were incubated with supernatants derived from degranulated mast cells and analyzed for the expression of surface markers (Fig. 4B). The mast cell supernatant did induce the maturing neutrophils in the marrow to translocate their internal stores of Mac-1 integrin and shed L-selectin; however, the expression of Pactolus was unchanged.

**FIGURE 4.** Translocation of Pactolus by macrophage supernatant and LTB₄. A, Peritoneal lavage was obtained from naive mice or from mice previously treated with thioglycolate for 24 h and was incubated with total bone marrow cells (C57BL/6) for 60 min. Cells were then washed and stained with the anti-Pactolus Bac Pac Ab (left panel) or an Ab specific for L-selectin (right panel). The dotted line (−) represents supernatant from the naive animals, whereas the solid line (+) represents supernatant obtained from the thioglycolate-treated animal. B, Animals were treated with oyster glycogen, and total cells were recovered by lavage. One of these mixed cell populations was left untreated (−), whereas the other (+) was treated with anti-DNP IgE (50 μg total; Sigma-Aldrich) for 20 min, followed by DNP-BSA (1 mg; Molecular Probes) for 100 min at 37°C to induce mast cell degranulation and cell responses. Both cell populations were then washed and stained for Pactolus expression (left panel), Mac-1 (middle panel), or L-selectin (right panel). C, Neutrophils were isolated from bone marrow, stimulated with fMLP (100 μM) or LTB₄ (5 μM) for 60 min, and analyzed for an increase of Pactolus (left panel), Mac-1 (middle panel), or L-selectin (right panel) expression by FACS analysis. D, Neutrophils were isolated from the peritoneal cavity (recruited by oyster glycogen) stimulated with fMLP (100 μM) or LTB₄ (5 μM) for 60 min, and analyzed for an increase in Pactolus (left panel), Mac-1 (middle panel), or L-selectin (right panel) expression by FACS analysis.
Thus, granule components of the mast cell do not induce Pactolus translocation.

Both mast cells and macrophages produce a variety of products after activation that are highly stimulatory for the inflammatory process (27). Therefore, total bone marrow cells were treated with a number of different compounds, and the expression of Pactolus was compared with that of PMA-treated controls. One of the most potent inflammatory products found that translocated intracellular Pactolus to the surface was LTB4. LTB4 stimulates neutrophil chemotaxis, enhances cell-cell contacts, and stimulates neutrophils to become activated, leading to degranulation and the release of enzymes, mediators, and superoxides (28). Mast cells synthesize and release LTB4 after activation/degranulation. Bone marrow was isolated from a C57BL/6 mouse and stimulated with LTB4 or fMLP. Pactolus, Mac-1, and L-selectin were then analyzed by FACS (Fig. 4C). LTB4 was able to increase Pactolus movement to the surface of the neutrophil. fMLP had a minor effect on Pactolus expression of such cells. Mac-1 was also increased on the surface after stimulation with LTB4 and fMLP, similar to Pactolus. L-selectin expression was unchanged in the presence of both compounds.

The same experiment as that described above was then performed upon mature neutrophils that had been recruited into the peritoneal cavity with oyster glycogen (Fig. 4D). Treatment of such cells with LTB4 and fMLP had a minor effect on Pactolus expression of a number of different compounds, and the expression of Pactolus was compared with that of PMA-treated controls. One of the most potent inflammatory products found that translocated intracellular Pactolus to the surface was LTB4. LTB4 stimulates neutrophil chemotaxis, enhances cell-cell contacts, and stimulates neutrophils to become activated, leading to degranulation and the release of enzymes, mediators, and superoxides (28). Mast cells synthesize and release LTB4 after activation/degranulation. Bone marrow was isolated from a C57BL/6 mouse and stimulated with LTB4 or fMLP. Pactolus, Mac-1, and L-selectin were then analyzed by FACS (Fig. 4C). LTB4 was able to increase Pactolus movement to the surface of the neutrophil. fMLP had a minor effect on Pactolus expression of such cells. Mac-1 was also increased on the surface after stimulation with LTB4 and fMLP, similar to Pactolus. L-selectin expression was unchanged in the presence of both compounds.

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staining was elevated to the level seen with PMA-mediated translocation (Fig. 5B, bottom left). At higher levels of staurosporine treatment, Pactolus did not translocate. PMA activation of these staurosporine-treated cells demonstrated an equal level of surface Pactolus staining with the naive cells treated with PMA with or without 0.1 µm staurosporine. Cells treated with higher concentrations of staurosporine were insensitive to the PMA-mediated stimulation (Fig. 5B, lower right). As controls, the surface levels of L-selectin were unaffected by treatment with staurosporine alone, but were increasingly protected, after PMA activation, with the higher level of staurosporine (Fig. 5B, top). These data indicate that at low concentrations of staurosporine, the enzyme(s) critical for maintaining Pactolus within the cell is inhibited, allowing the protein to translocate. Oddly, this inhibition is not observed with the higher concentrations, suggesting that multiple pathways of varying sensitivity to staurosporine are blocked, effectively inhibiting the movement of the Pactolus protein to the cell surface.

Translocation of Pactolus to the cell surface after apoptosis

The translocation of Pactolus protein to the cell surface after treatment with staurosporine could be due to the direct inhibition of kinase activities and/or the induction of apoptosis of the treated cells. To test the latter hypothesis, we used a standard apoptosis-inducing protocol, UV light, followed by a 3-h incubation in culture. For this assay we used total bone marrow cells, ~50% of which are Pactolus-expressing granulocyte precursors. As a contrast, we induced cell necrosis by incubating cells at 55°C for 15 min. Cells were also cultured at 37°C for 3 h in a 5% CO₂ incubator as a control. After apoptosis/necrosis induction, cells were stained with either preimmune serum or anti-Pactolus antiserum (Bac Pac), followed by an FITC-labeled anti-rabbit anti-serum (FL1) and were counterstained with PE-labeled annexin V (FL2) to test for the presence of phosphatidylserine on the cell membrane (indicative of the membrane inversion observed on cells in the early stages of apoptosis). Cells were also treated with the dye 7AAD, which discriminates between intact, nonpermeable cells and those that take up the dye due to membrane leakage. This dye is detected in FL3.

UV-treated and control cells were first split into 7AAD-negative and -positive quadrants (Fig. 6, A, F, and K; the cells treated for necrosis had no significant population of 7AAD-negative cells). These two populations were then analyzed for Pactolus expression and annexin V binding. The control cells (Fig. 6, B–E) showed a low level of Pactolus expression indicative of the majority of the protein still residing within the cells. In contrast, the UV-treated cells (Fig. 6, G–J) demonstrated an elevated level of Pactolus expression on those Pactolus-expressing cells, suggesting such treatment leads to translocation of the intracellular protein to the cell surface. In both the 7AAD-positive and -negative UV-treated samples, the highest level of Pactolus translocation was coincident with the highest level of annexin V binding, providing for a direct correlation between the induction of apoptosis and the release of intracellular Pactolus. The elevated levels of Pactolus on the surface of the UV-treated cells that were 7AAD-positive (contrast G and H) also suggests that cells undergoing postapoptotic necrosis did not shed the Pactolus protein. This Pactolus translocation was also evident by those cells induced to undergo necrosis directly (Fig. 6, L and M). These data suggest that Pactolus can be induced to translocate to the cell surface during either necrotic or apoptotic neutrophil death, and that after such cell death, the Pactolus protein is not shed, but remains associated with the cell.

Is Pactolus a signaling/phagocytic receptor?

The extracellular sequence of Pactolus is very similar to that of the β integrin subunits. This sequence conservation, however, is not maintained in the cytoplasmic residues, suggesting that potential cytoplasmic signaling via Pactolus would occur via different routes than those used by integrins. The Pactolus cytoplasmic sequence has several potential phosphorylation sites. A number of different agonists were used (PMA, Bac Pac antiserum, and pervanadate) in a variety of time courses using bone marrow cells or neutrophils recruited into the peritoneal cavity with thioglycolate to induce phosphorylation of neutrophil proteins, including Pactolus. Pactolus protein was immunoprecipitated with monoclonal anti-Pactolus Ab, and Western blots were performed using Abs specific for phosphotyrosine, phosphoserine, and phosphotheorine. To confirm that we immunoprecipitated Pactolus, the membranes were stripped and blotted with polyclonal Bac Pac Ab. Under all activation conditions tested we were unable to demonstrate the phosphorylation of Pactolus (data not shown). Although an increase in total cellular protein phosphorylation was observed after PMA and

\[ \text{FIGURE 6.} \text{ Apoptosis induces the translocation of intracellular Pactolus to the cell surface. Mouse bone marrow (C57BL/6) was incubated for 3 h in culture (A–E), treated with a 302-nm UV transilluminator for 10 min, followed by 3-h incubation in culture (F–J), or incubated at 55°C for 15 min until trypan blue uptake indicated compromise of the membrane integrity (K–M). Cells were incubated with the dye 7AAD (FL3; A, F, and K) to discriminate between dead and live cells and were counterstained with preimmune or anti-Pactolus antiserum (FITC; FL1) and annexin V-PE (FL2). Cells positive for 7AAD staining (B, C, G, and H) and negative for 7AAD staining (D, E, I, and J) were gated and analyzed separately for annexin V and Pactolus expression. The samples induced to undergo necrosis (K–M) were not gated based upon 7AAD staining, because virtually all of the cells were 7AAD-positive.} \]

\[ \text{Is Pactolus a signaling/phagocytic receptor?} \]
FIGURE 7. Generation of a *pactolus* null mouse. A, Diagram of the *pactolus* gene (23). The neomycin cassette was inserted into the BglII site in exon 5. The two genomic phage subclones of the *pactolus* gene that were used to make the *pactolus*-targeting construct are shown (8.4- and 6.7-kb fragments). B, The wild-type *pactolus* allele is aligned to the *pactolus*-targeting construct, and the expected recombination is shown (targeted allele). If the ES cell line DNA had recombined with the *pactolus*-targeting construct, the size of the genomic DNA fragments after SstI restriction enzyme digestion would decrease by 0.7 kb, whereas the EcoRI fragment 5′ of the insertion would decrease by 1 kb. Similarly, the size of the EcoRI genomic fragment generated by homologous recombination and probed with the 5′ probe would decrease by ~2 kb. The probes used for Southern blot analysis of the ES cell lines were outside the region of homology used in the targeting construct and are shown on the targeted allele. The 3′ probe detected the SstI digestion difference, whereas the 5′ probe detected the EcoRI digest difference (EcoRI sites denoted E). The fragment sizes are not drawn to perfect scale. C, Southern blot of the SstI digest of the ES cell lines hybridized with the 3′ probe. The *first lane* is an ES cell line that was recombination-negative, the *second lane* was recombination-positive, and the *third lane* was mouse liver DNA control. The targeted ES cell line, 2e#6 (shown in the *second lane*), was used to make the *pactolus*-null mouse. Germline transmission was achieved with an animal demonstrating 85% chimera characteristics. D, Southern blot analysis of the
pervanadate treatment, no change was evident after Pactolus ligase (data not shown).

The high degree of homology of Pactolus to the β3 integrin subunit (CD18) raised the possibility that Pactolus, like the CD18 integrin subunit as part of the Mac-1 complex, may be a phagocytic receptor. We used a phagocytic assay with Abs against different neutrophil proteins linked to NeutrAvidin-labeled fluorescent microspheres (Molecular Probes, Eugene, OR). These spheres, which possess avidin, were coated with biotinylated preimmune antiserum, Bac Pac antiserum, or anti-CD18 Abs. Neutrophils were recruited into the peritoneal cavity with 3% thioglycolate and isolated by lavage. Cells were then incubated with the beads for 30 min at 37°C and analyzed by fluorescent microscopy. Two hundred neutrophils were counted blindly, and the phagocytic index was determined. Latex beads coated with preimmune and Bac Pac were only minimally phagocytosed, whereas the CD18-coated beads were very effectively internalized (data not shown). An analogous experiment was conducted using fixed Staphylococcus aureus (Pansorbin) charged with Abs to Pactolus; they too were not phagocytosed compared with the complement-coated controls (data not shown). These data suggest that Pactolus on its own does not serve as a phagocytic receptor on the surface of neutrophils.

Creation and analysis of a Pactolus-deficient animal

To more fully explore the role that Pactolus may have in neutrophil functions in the animal, we generated a pactolus-null animal. A pactolus-targeting construct was prepared for homologous disruption of the endogenous gene as described in Materials and Methods (23). The neomycin gene cassette was inserted into exon 5 of the gene (Fig. 7, A and B). Two probes, one 5′ and the other 3′, were generated to test for correct homologous recombination. Both these probes were just outside the genomic sequence of the targeting vector (shown by the two cross-hatched lines in Fig. 7). Homologous recombination into 129/sv ES cells yielded a 12-kb SstI digest fragment compared with the wild-type 12.7-kb SstI fragment with a 3′ probe in a Southern blot (Fig. 7C). The ES cells were also tested by EcoRI digestion and a 5′ probe to confirm appropriate recombination (Fig. 7D). As expected, the vector-disrupted EcoRI fragment detected with the 5′ probe was ~2 kb shorter than the wild-type fragment. Approximately 50% of the ES cell lines analyzed contained the targeted allele. One such cell (2e#6) was then used with C57BL/6 blastocysts to obtain a chimeric animal demonstrating ~85% coat color associated with the ES lineage. This animal was used for germline transmission of the disrupted gene.

A PCR-based assay was developed (Fig. 7E) to easily discriminate among the various genotypes generated in such matings. Transmission rates for the first 95 offspring from such heterozygous matings were 30 (+/+), 49 (+/−), and 16 (−/−). Litter sizes in wild-type and pactolus null matings were similar. Therefore, it appears that the pactolus-null mutation does not affect the viability or the fertility of the mice.

The homozygous pactolus-targeted mice were analyzed for pactolus gene products (Fig. 8A). Bone marrow mRNA isolated from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) pactolus-targeted mice were used for RT-PCR. Three sets of pactolus primers were used: one set was before the neomycin insert (sequences from exons 3–4), another set flanked the neomycin insert (sequences from exons 5–6), and the third set was after the neomycin gene (sequences from exons 6–8). As controls for pactolus expression, mRNA from Pactolus-negative mucosal mast cells (derived with IL-3 from bone marrow) and Pactolus-positive immature connective tissue-like mast cells (derived with stem cell factor) were used (2). Animals that possessed two targeted pactolus alleles clearly did not produce transcripts 3′ of the neomycin insertion and would thus be unable to create a membrane-bound form of the protein. Such animals were also analyzed for Pactolus protein production by immunoprecipitation/Western blot analysis (Fig. 8B) and flow cytometry (Fig. 8C). The Pactolus protein was immunoprecipitated from bone marrow cells with anti-Pactolus mAb and subsequently identified via Western blot using a polyclonal anti-Pactolus sera (Bac Pac) (1). The Pactolus protein can be found in two forms, depending on the amount of glycosylation, in total bone marrow samples. These two forms of Pactolus were detected in the wild-type mice (+/+), whereas no Pactolus protein was detected in the pactolus (−/−) mice. The lower m.w. bands seen in the gel are probably Pactolus breakdown products generated by neutrophil proteases released during the experimental procedures. FACS analysis (Fig. 8C) using polyclonal anti-Pactolus Bac Pac antiserum did not show any staining of PMA-treated bone marrow cells obtained from the deficient animal (−/−), whereas the control total marrow cells from C57BL/6 and 129/sv mice, also treated with PMA, demonstrated the expected level of Pactolus-expressing cells (+/+).

Gross anatomical analysis of the Pactolus-deficient animals (liver, bone marrow, lung, small intestine, and spleen) did not show any difference between these animals and the wild-type littermates (data not shown). We investigated whether the Pactolus deletion could inhibit neutrophil development or the release of mature neutrophils into the periphery; the percentages of B cell, neutrophils, and other progenitor cells were equivalent between such mice. Cytospin analysis of total bone marrow cells indicated that Pactolus-deficient animals possessed both immature and mature neutrophils in identical ratios to those found in wild-type animals. Similar analyses were extended by isolating peripheral blood leukocytes from wild-type (+/+) and pactolus-null (−/−) mice, followed by their subset analysis by flow cytometry. Again, there was not a remarkable difference in blood leukocyte populations between the wild-type and deficient animals. These data vary from those obtained from the β3 integrin-deficient animals, which demonstrated elevated neutrophils counts in the blood (33, 34).

Two experiments were conducted to determine whether Pactolus-deficient neutrophils demonstrated a loss of migration efficiency. Migration of neutrophils into the skin was evaluated by treating mouse ears with 2% dinitrochlorobenzene for 5 h, followed by histological analysis (35). Neutrophils were detected in the ears of Pactolus-deficient animals and demonstrated similar recruitment kinetics as those observed with treated wild-type ears (data not shown). Recruitment of neutrophils into the peritoneal cavity of three ES cell lines hybridized with the 5′ probe. The first two lines are recombination-negative and only possess the genomic 11-kb EcoRI fragment. The third line, 2e#6, shows both the native genomic fragment as well as the recombined product of 9 kb. E. Genomic DNA was isolated from mouse tails and the neomycin insertion in the targeted allele detected by PCR using the three primers shown. Primers 1 and 2 produced the exon 5 product with the wild-type allele and no product from the modified allele. Primers 2 and 3 produced the neomycin/exon5 (neo) product with the targeted allele DNA. Examples of PCR with DNA from a homozygous pactolus-targeted mouse (−/−), a wild-type pactolus allele (+/+) and a heterozygous mouse (+/−) are shown. Primers 1, 2, and 3 are primers 863, 1028, and 1052, respectively.
FIGURE 8. Analysis of pactolus expression in the deficient animals. A, RT-PCR for pactolus transcripts. Bone marrow mRNA was isolated from wild type (+/+), heterozygous (+/−), and two pactolus-null (−/−) mice. RT-PCR was achieved using primers derived from the pactolus sequence before neomycin insertion (from exons 3–4), over the neomycin gene (from exons 5–6), and after the neomycin insertion (from exons 6–8). The pactolus-negative mucosal mast cells and the pactolus-positive connective tissue mast cells are also shown as controls. Actin primers were used for equivalent loading. B, Immunoprecipitation (IP)/Western analysis for Pactolus. Bone marrow cells were isolated from wild-type (+/+) and pactolus-null (−/−) mice, lysed, and immunoprecipitated with either a Pactolus mAb or an isotype control. The precipitated protein was detected by Western blot with the Pactolus polyclonal rabbit antiserum (postimmune serum). The two predominant forms of high molecular mass Pactolus (130,000 M                                                           ) and low molecular mass Pactolus (98,000 M                                                           ) are shown. C, FACS analysis of PMA-treated fresh bone marrow obtained from the Pactolus-deficient animal (−/−; left panel), a C57BL/6 mouse (middle panel), and a 129/sv animal (right panel) using the rabbit polyclonal anti-Pactolus Bac Pac antiserum (solid line) and the preimmune serum (dotted line).

Discussion
Since the pactolus gene product was first described, it has been a puzzle to define its function. The high level of homology shared between Pactolus and the β integrin subunits led to the assumption that Pactolus might share in some of the adhesive and cell-signaling properties of the integrins. However, the lack of a functional metal ion-dependent adhesion site domain, divergent cytoplasmic domain, and lack of a paired α-chain have undermined that proposal. Previous studies of Pactolus have highlighted two findings. First, the majority of the protein is found within the cell and can only translocate to the surface after cell activation. Second, the cell specificity of expression of Pactolus is tightly controlled and is only evident in the mature/maturing neutrophil. In fact, expression constructs for Pactolus cannot be transfected into a number of bone marrow-derived cell lines, including B, T, and macrophage cell lines (S. Garrison and J. H. Weis, unpublished observations), suggesting that granule-targeting sequences within the Pactolus protein may not allow for this protein to be produced in most other cell types.

The focus of this research has been to expand upon the earlier findings, to try to define neutrophil responses to Pactolus ligation, and to examine the biological effects of an animal lacking Pactolus. The first step in Pactolus function appears to be translocation of the bulk of the protein from an intracellular granule to the cell surface. Although such a translocation can be accomplished by cross-linking the protein directly, we have now shown that activation of cells with inflammatory stimuli such as LTB4 can also induce Pactolus protein expression. Translocation of granule constituents after inflammatory stimuli is not a new finding; a number
of neutrophil receptors, including human CR1 and Mac-1, are shuttled to the cell surface after activation (36–38).

The specific granules to which Pactolus is assigned is difficult to determine, but does not appear to be one of the well-characterized granules. Cross-linking Pactolus does translocate the protein to the surface, but does not provide for the release of many of the other granule compartments, such as MPO (1). Thus, Pactolus and MPO, although demonstrating a similar density in the Percoll gradient, are present in distinct granules. The recruitment of neutrophils into the peritoneal cavity with oxygen glycosylation does translocate the bulk of the β2 integrin to the cell surface while leaving the majority of the Pactolus protein intracellular. These data suggest that Pactolus is not involved in the process of neutrophil migration to sites of inflammatory stress and does not reside in the same secretory vesicles/granules as the β2 integrins. Finally, the extensive glycosylation of Pactolus that occurs within the neutrophil suggests that this protein follows an unusual maturation pathway.

We have investigated possible signaling pathways and neutrophil functions that could use cell surface-translocated Pactolus. Such cells, once recruited to a site of infection, take on a number of functions to control an infection, including granule release, superoxide production, and phagocytosis of opsonin-tagged foreign particles. As described, we have been unable to ascertain any cell responses that occur after ligation of the Pactolus protein. No increase in granule release after Pactolus ligation has been noted nor responses that occur after ligation of the Pactolus protein. No increases in granule release after Pactolus ligation has been noted nor has any cellular activation response, including calcium flux (data not shown) and total protein (and Pactolus-specific) phosphorylation. Although it is difficult to rule out all possible cellular responses to Pactolus ligation, the summation of these analyses has generated the conclusion that Pactolus does not function as either an activating or a phagocytic receptor of the neutrophil.

We have also shown that mice lacking Pactolus do not possess any obvious neutrophil developmental phenotype or sensitivity to infections. The immunological literature is rife with examples of the absence of an obvious phenotype with the deletion of a protein for which there are other redundant, functional analogues. Only when single-cell behavior is analyzed or when multiple null mutations are bred into a single animal is a phenotype detected. The same has been shown for Pactolus, in that the null animals appear normal, but the in vitro macrophage response to purified neutrophils lacking Pactolus is not.

The fate of a neutrophil recruited into a site of infection depends upon whether the cell becomes necrotic, releasing cellular fragments into the connective tissue matrix, or undergoes apoptosis. For the latter, the cell membrane remains intact, maintaining potentially harmful granule constituents within the cell. As we have shown, the Pactolus protein is also translocated to the surface when neutrophils are induced to undergo apoptosis. If neutrophils fail to be cleared, chronic inflammation can result (39). A number of murine/human receptors have been described for the macrophage uptake of apoptotic neutrophil. One such set is the αβ integrin expressed on the macrophage in a complex with CD36 and potentially CD14 (16). This complex can bind thrombospondin, which then binds to an ill-defined ligand on the surface of the apoptotic cell (16, 17). Another set of macrophage receptors potentially recognizes phosphatidylserine on the surface of the apoptotic cell (17). The potential receptors to mediate this binding include the scavenger receptors SRA-I/II, CD36, and macrosialin/CD68 (18, 40), all of which are known to bind apoptotic cells; however, these receptors may also recognize ligands other than phosphatidylserine on the surface of the neutrophil. An additional potential ligand/receptor interaction involves a macrophage lectin whose binding to apoptotic cells is blocked by N-acetylgalactosamine (41). Finally, CD31 homodimers have been implicated as signals for the uptake of neutrophils by macrophages (42). It appears that a combination of these receptors leads to the appropriate clearance of apoptotic neutrophils in the human. Interestingly, if several of these receptors are blocked, phagocytosis of the neutrophils decreases, but is not completely inhibited (19). Pactolus may represent an inducible ligand on the neutrophil that mediates uptake by macrophages. Such an “eat me” tag (43) would most likely use the extensive sialic acid modification present on Pactolus to mediate recognition by a cognate lectin on the surface of a macrophage. Whether such specific binding occurs and how its abrogation might alter the resolution of an inflammatory response are currently under investigation.

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References


