Functional Characterization of Pactolus, a β-Integrin-like Protein Preferentially Expressed by Neutrophils*

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Murine Pactolus is a β-integrin-like molecule expressed exclusively on the surface of granulocytes. Cell surface expression of Pactolus is dramatically increased following activation of bone marrow neutrophils with known agonists, and cross-linking of cell surface Pactolus, suggesting the bulk of the protein is in intracellular stores. The mature protein is found in two forms depending upon the extent of N-linked glycosylation. There is no evidence to suggest that Pactolus requires an associated α chain for expression. In some mouse strains, a truncated form of the protein is predicted based upon alternative splicing: this form, however, is unstable and rapidly degraded after synthesis. Differences in the quantities of these Pactolus mRNA isoforms have defined two alleles. BALB/c and C3H/HeJ mice possess allele B and preferentially express the truncated, unstable product, whereas C57Bl/6 mice possess allele A and only produce the membrane-bound form. Sequence analysis has shown the difference between these two alleles is due to a single base pair difference at the splice acceptor site for the truncated product. The increased expression of the membrane form of Pactolus by granulocytes of C57Bl/6 mice suggests a compensatory adhesion function that is reduced in cells from the low producing strains.

Pactolus coding sequences were first isolated in a differential display screen of mouse marrow differentiated into cell subtypes in the presence of stem cell factor or interleukin-3 (1). Pactolus was expressed in those cells maintained in stem cell factor, but lost when cells were grown in interleukin-3. In the mature animal, the tissue of highest expression was the bone marrow.

The Pactolus gene encodes, via alternative splicing, two distinct isoforms of products that share homology with the β-integrins, primarily β2 and β7. The full-length form, dubbed Pac A, resides on the membrane as a class I type glycoprotein (1). The truncated form, Pac B, lacks transmembrane and cytoplasmic sequences and, if stable, is predicted to be a soluble and/or secreted form. These differing transcripts are due to an alternative splicing event within exon 13 of the gene. A third form of Pactolus transcript, Pac C, which also predicts a truncated protein, is also due to alternative splicing near this exon; however, it represents only a minor constituent of Pactolus transcripts in the mature animal (2).

Pactolus has been referred to as β-integrin-like due to the lack of conservation within the metal ion-dependent adhesion site (MIDAS)1 domain that is maintained in the integrin subunits (3). Pactolus has a point mutation in the critical DXXSXS motif required for a functional MIDAS domain and has an apparent deletion of 28 amino acids immediately carboxy-terminal to this site (1). Mutations within the MIDAS domains of other integrin subunits abrogates heterodimer formation and ligand recognition (4–8). Immunoprecipitation analyses of Pactolus have not identified an α partner chain even when such experiments were performed under conditions that maintained other α/β-integrin pairs (1). The high degree of sequence homology between Pactolus and the β-integrins is evident in the extracellular domains; however, the cytoplasmic region of the Pactolus protein does not possess similar sequences (9). Exon/intron mapping of the Pactolus gene suggests Pactolus and β3-integrin arose from a common precursor even though they now reside on different mouse chromosomes (2).

In this article we report our investigations pertaining to the genetics and biology of the production of the truncated and full-length Pactolus products. Using a variety of anti-Pactolus antisera, we demonstrate that the maturing and mature neutrophil is the major cell type that expresses Pactolus. We show that the full-length Pactolus product is stably expressed on the cell surface and undergoes extensive N-linked glycosylation, whereas the truncated Pactolus product is unstable and rapidly degraded immediately after synthesis. Cross-linking of cell surface Pactolus leads to neutrophil activation and the rapid mobilization of intracellular stores of Pactolus to the cell surface. Finally, a number of mouse strains have been analyzed and two distinct Pactolus alleles have been found. These alleles appear to be directly responsible for the quantities of Pactolus transcript (and protein) isoforms found in selected strains; these differences are due to single nucleotide alteration in a splice acceptor site of the truncated product.

EXPERIMENTAL PROCEDURES

Mice, Tissues, and Cells—Inbred mice (BALB/c, C57Bl/6, C3H/HeJ) and outbred mice (Swiss NIH) were obtained from NCI, National Institutes of Health (Bethesda, MD). Tissues were isolated from adults 1

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1 The abbreviations used are: MIDAS, metal ion-dependent adhesion site; PMF, phosphor myristate acetate; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; Endo H, endoglycosidase H; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; PE, phycoerythrin; HRP, horseradish peroxidase; RIPA, radiimmune precipitation buffer.

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(greater than 10 weeks of age) or immature animals (10 days).

Bone marrow cells were obtained by flushing femurs with RPMI without fetal calf serum. Activation of purified bone marrow cells was done using 20 ng/ml PMA for FACS analysis and 100 ng/ml PMA for degranulation assays. Cells were activated with the Bac Pac antisemum at a 1:500 dilution of serum (or the immunglobulin sample buffer). Cells were transfected with the Baculolus expression plasmid and BaculoGold, the linear recombinant substrate to generate a stable line of cells producing the recombinant baculovirus. Virus supernatants were used to infect 1.8 × 10^6 cells. Supernatants were collected 6 days after infection. After overnight dialysis against PBS, pH 6.0, the supernatants were brought up to 20 mM imidazole. They were then incubated for 60 min with 2 ml of nickel-nitrotriacetic acid slurry (Qiagen). After the incubation, the mixture was bound to a column, washed two times with 10 ml of wash solution (1× PBS, pH 6.0, and 20 mM imidazole), and proteins were eluted in 1-ml aliquots using elution buffer (1× PBS, pH 6.0, and 500 mM imidazole).

Two rabbits were injected with the purified Bac Pac protein (5 µg/animal). Although both animals responded to the immunization, the highest titer antisemum from one of the animals was used for experimentation purposes. The antisemum was defined to be specific for Pactolus based upon its FACS, Western blot, and immunoprecipitation analyses (detailed in the present report).

Lewis rats were immunized with the Pactolus recombinant protein. Rat sera were tested until Pactolus-specific antibodies were detected. Splenic cells were associated from the spleen, washed, and counted. Cells (2.8 × 10^6) were incubated with equal amounts of SP2/0 cells. Spleocytes and SP2/0 cells were fused over 1 min using 30% polyethylene glycol. Cells were then centrifuged and plated in complete media (RPMI 1640, 10% penicillin/streptomycin, 10% fetal calf serum) and 10 M M NaCl, 25 mM Tris, pH 7.5, 1 mM EDTA, 0.1% SDS, 1% sodium deoxychylate, 1% Nonidet P-40, 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and complete TM (Mini protease inhibitor mixture tablet; Roche Molecular Biochemicals) for 12 min. Cell lysates were collected and precleared by incubating with pre-immune rabbit serum and 10 mg of protein A-Sepharose overnight at 4 °C. The lysates were then precleared two times with 12.5 mg of protein A-Sepharose. The supernatants were then incubated with either polyclonal rabbit antisemum against the Bac Pac extracellular protein or the cytoplasmic tail sequence. The protein antibody complex was then absorbed with protein A-Sepharose for 30 min at 4 °C. After absorption the Sepharose beads were washed five times: one with 1× NaCl and RIPA, two times with RIPA buffer, and twice with a final wash solution (50 mM NaCl, 25 mM Tris, pH 7.5, 1 mM EDTA, 0.1% SDS, 1% sodium deoxychylate, 1% Nonidet P-40, 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and complete TM) for 20 min. Gel was then dried and placed overnight on Kodak X-Omat AR film.

Glycosylation Analysis—Deglycosylation enzymes were purchased from Glyko (Novato, CA) unless otherwise stated: NANase III, specific for α-2,3,6,8,9-N-acetylluralaminic acid; sialidase, specific for α-2,3,6,8,9-N-acetylluralaminic acid; N-glycanase, specific for β-asparylol glycoyls; endo-β-N-acetylglucosaminidase (endo-N); and endoglycosidase H (Endo H; Roche Molecular Biochemicals), cleavage between the GlcNAc residues of the chitobiose unit of N-glycans linked to asparagine.

The glycoproteins were first immunoprecipitated with the B10 Pac- toclus monoclonal antibody, and the precipitates were incubated with the appropriate enzyme using standard protocols and buffers supplied by Glyko. Endo H cleavage was performed by first placing the pellet in a 2× sample buffer (40% methanol and 10% acetic acid) or non-reducing (40% methanol and 10% acetic acid) for 15 min, and Amplify (Amersham Life Sciences) for 20 min. Gel was then dried and placed overnight on Kodak X-Omat AR film.

Reverse Transcriptase-PCR—Total RNA was isolated using CsCl guanidine (10). RNA was resuspended in water and quantified by NanoDrop (U. S. Biochemical Corp) was added, and 5 µl was resolved in a 6% acrylamide sequencing gel for autoradiography. Gene-specific primers were designed with the aid of the Primer Express program (Applied Biosystems). PCR was conducted on cDNA solutions containing 200 ng of cDNA, 70 pmol amounts of each primer, 0.72 unit of Ampli-Taq DNA polymerase (Life Technologies, Inc.), 0.8 µl dNTP, 1× Taq buffer (50 mM Tris, pH 8.3, 3.3 mM MgCl2, 20 mM KCI, and 500 mM bovine serum albumin), and 2.5 µl of 31PdCTP. Samples were loaded into capillary tubes and incubated in an air thermocycler (Idaho Technology, Idaho Falls, ID) for denaturing at 94 °C for 1 s, for annealing at 60 °C for 1 s, and for extension at 72 °C for 3 s (11). This cycle was repeated 14 times for amplification of β-actin transcripts and 23 times for amplification of Pactolus transcripts. After amplification, 10 µl of each sample was run on a 1.5% agarose gel in TAE buffer, 4 h after incubation was completed. The reaction was incubated at 37 °C for 1 h. 2 µg of template DNA was incubated with 55 µl of rabbit reticulocyte lysate ( Idaho Technology, Idaho Falls, ID) for denaturing at 94 °C for 1 s, for annealing at
neutrophils were obtained by injection of 1 ml of a 1% solution of oyster glycogen into the peritoneal cavity of mice, followed by aspiration of the cavity 16 h later. Cells were collected, red blood cells lysed, and the resulting cells analyzed by FACS.

The rabbit anti-Bac Pac was detected using a FITC-conjugated goat anti-rabbit Ig antibody (Cappel). Antibodies specific for Gr-1 (clone RB6-8C5), B220 (clone RA3–81), and Mac-1 (clone M1/70) were obtained from the University of Utah Stem Cell core facility and were directly conjugated with phycoerythrin (PE).

RESULTS

**Cell Surface Expression of Pactolus**—We previously documented the expression of the full-length, transmembrane form of Pactolus on adult bone marrow cells using antisera generated against the cytoplasmic region (anti-CT) (1). To generate Pactolus-specific antisera, the extracellular portion of the full-length form (excluding the signal sequence, transmembrane, and cytoplasmic domains of Pactolus) was inserted into the Baculovirus vector pAcGP67B. The Baculovirus-derived Pactolus protein (termed Bac Pac) was used to generate a polyclonal rabbit antiserum (Bac Pac antiserum) (Fig. 1A). This antiserum can detect the Pactolus gene product by immunoprecipitation, Western blot, and FACS analysis (see below). The Baculovirus-derived Pactolus protein was also used to create a monoclonal antibody (see below).

The anti-Pactolus Bac Pac polyclonal antiserum was tested for recognition of the Pactolus protein using in vitro transcription and translation. The open reading frame of the Pac A transcript was placed in a T7 promoter expression plasmid. The coding sequence was truncated by digestion of the plasmid with single cutting enzymes Smal (which would be predicted to encode a truncated protein of M, 62,000) and HpaI (which would be expected to encode a truncated protein of M, 41,000) (Fig. 1B). The full-length Pactolus peptide sequence encodes a protein of M, 81,000. Translation and immunoprecipitation of these truncated proteins indicated that the polyclonal Bac Pac antiserum recognizes epitopes present in the HpaI- and Smal-generated products. Therefore the Bac Pac antiserum should be functional for the detection of both the Pac A (full-length) and Pac B (truncated) protein products.

The Bac Pac antiserum was then tested for its ability to recognize Pactolus expressed on the surface of cells. Previously we had determined bone marrow samples expressed the highest level of Pactolus transcripts (1). Therefore, we obtained bone marrow from 10-day-old and 10-week-old C57BL/6, C3H/HeJ, and BALB/c mice and analyzed by FACS using Bac Pac antiserum with pre-immune serum as control. As shown in Fig. 2A, the Bac Pac antiserum specifically stained ~50% of the bone marrow cells from the adult and juvenile C57BL/6 mouse (M1) marrow. The marrow from the C3H/HeJ and BALB/c mice demonstrated a slight positive shift regardless of age.

The depressed levels of Pactolus protein on the surface of the BALB/c and C3H/HeJ bone marrow cells, compared with the C57BL/6 cells, suggested either 1) there are fewer Pactolus transcripts in the C3H/HeJ and BALB/c cells or 2) the production of the spliced isoform of Pactolus to produce the full-length, transmembrane form of the protein is decreased in the BALB/c and C3H/HeJ cells compared with those of the C57BL/6 animal. cDNA was prepared from RNA isolated from total bone marrow cells obtained from these mice and analyzed, via semiquantitative reverse transcriptase-PCR, for either total Pactolus transcripts or those forms that, through alternative splicing, produce either the full-length, transmembrane protein or the truncated form. For the former assessment oligonucleotides were used that span a region of the gene present in all Pactolus transcripts, and for the latter oligonucleotides that span the alternatively spliced junction were used. The C57BL/6 sample appeared to possess only slightly more total Pactolus transcripts than the BALB/c samples (Fig. 2B). In the C57BL/6 sample, the only Pactolus transcript was that encoding the transmembrane, Pac A form whereas the BALB/c cells were highly enriched for transcripts encoding the Pac B, truncated...
form. Since the truncated form of Pactolus does not encode a transmembrane-spanning region for cell surface localization, this protein must be degraded, stored within the cell, or secreted (see below). This difference in transcript profile between the C57BL/6 and the other strains could easily account for the difference in cell surface staining intensity for Pactolus.

Pactolus Is Expressed by Maturing Neutrophils in the Marrow and Mature Neutrophils in the Periphery—Major populations of cells in the marrow include B cell and granulocyte lineages that can be distinguished with monoclonal antibodies specific for B220 and Gr-1, respectively (12, 13). In order to determine the lineage of cells that express Pactolus, bone marrow cells from C57BL/6, C3H/HeJ, and BALB/c mice were analyzed by two-color FACS analysis with anti-Gr-1, anti-B220, and anti-Mac-1 (14). Shown in Fig. 3A, cells from the C57BL/6 strain that expressed Pactolus also possessed the Gr-1 marker and did not express B220. Pactolus-positive cells also did not stain with CD19 (15) (data not shown). These results indicated that cells of the granulocytic, but not lymphocytic, lineage express Pactolus. Mac-1 expression indicated that there is a subset of cells within the marrow that stain brightly for both Mac-1 and Pactolus, another that only stains for Mac-1, and a third subset that is not recognized by either antibody. These data also demonstrate that the Bac Pac antiserum does not cross react with the ß2-integrin subunit, which is part of the Mac-1 complex. The analysis of bone marrow cells from the C3H/HeJ and BALB/c strains demonstrated only slight staining of Pactolus on the Gr-1 subset, as expected from the transcript data described above.

Bone marrow was further analyzed by FACS Vantage cell sorting using the Bac Pac antiserum to fractionate the marrow (Fig. 3B). Two populations (positive and negative for Pactolus staining) were collected, spun onto glass coverslips, and analyzed by Wright stain. The cells found within the negative population were heterogeneous but included a large percentage of immature B lymphocytes. The majority of cells in this population stained with B220 (see Fig. 3A). Alternatively, the Pactolus-positive subset was highly enriched for cells of the neutrophil lineage. Cells representing each stage of neutrophil development were apparent in this stain.
Gr-1-positive cells in the bone marrow express Pactolus. A key question was whether Pactolus is preferentially expressed in the bone marrow or if Gr-1-positive cells (primarily neutrophils) still express Pactolus once they enter the periphery of the animal. The anti-Pactolus Bac Pac antiserum was used with two-color staining, using anti-Gr-1, to analyze total splenocytes from C57BL/6 animals. As shown in Fig. 4A, only a small percentage of cells in the spleen stain brightly with Gr-1 (R2). Of these, virtually all possess Pactolus (R3). Again, these data demonstrate that the Bac Pac antiserum does not react with the β2-integrin, which is expressed on splenic T and B cells.

To test for Pactolus expression by mature neutrophils in the periphery, oyster glycogen was injected into the peritoneal cavity to recruit neutrophils (16). After 16 h, aspirated cells from the peritoneal cavity are highly enriched for neutrophils. We performed such a strategy using C57BL/6, BALB/c, and C3H/HeJ mice and analyzed the resulting cells for the co-expression of Pactolus and Gr-1 (Fig. 4B). In the C57BL/6 sample, all of the peritoneal cells that stained for Gr-1 also expressed Pactolus. As expected, the same subset of Gr-1-positive cells in the BALB/c and C3H/HeJ stained only minimally for Pactolus. These data indicate that the primary cell of the mouse that expresses Pactolus is the maturing and mature neutrophil, and that the mouse strain differences in Pactolus cell surface expression identified for the bone marrow granulocytes were recapitulated in peripheral neutrophils.

Characterization of the Pactolus Proteins—The Pactolus gene encodes two primary transcripts that utilize alternative splicing at exon 13 to generate the full-length form, Pac A, or a truncated form, Pac B (1, 2). The Pac B transcript predicts a primary protein of Mₚ 55,000, whereas the full-length form predicts a primary protein of Mₚ 81,000. The reactivity of the Bac Pac antiserum with the in vitro transcription/translation products suggests it should detect both of these Pactolus products. Mouse bone marrow cells from C57BL/6 mice were labeled with [³⁵S]Met and chased with cold methionine for different periods of time. Samples were immunoprecipitated with pre-immune serum, the Bac Pac antiserum, and the antiserum raised against the Pactolus cytoplasmic tail sequence (1). Both the cell pellet and the supernatant were analyzed for Pactolus protein products (Fig. 5A). At the 0 chase time (left panel), the Mₚ 98,000 mature Pactolus product was observed with the Bac Pac and cytoplasmic region antisera. After the 6-h label, a second diffuse band of Mₚ/110,000 was evident in addition to the Mₚ 98,000 protein, suggesting that the smaller protein had been further modified during the time course of the labeling. We have never been able to fully "chase" the Mₚ 98,000 form into the Mₚ 130,000 form, suggesting that a portion of the Pactolus product does not bear this increased modification. The Mₚ 98,000 form is also the size of the full-length Pactolus protein expressed on the surface of Chinese hamster ovary cells transfected with an expression construct encoding the full-length Pactolus product (data not shown).

The same type of analysis was carried out with bone marrow...
cells obtained from BALB/c mice. Cells were labeled with \[^{35}\text{S}]\text{Met}\) and chased with cold methionine for 0 and 6 h. Lysates and cell supernatants were immunoprecipitated with the Bac Pac and pre-immune antisera. As shown in Fig. 5B, neither the full-length or truncated Pactolus product was evident. Less than 10% of the Pactolus transcripts from the BALB/c cells encode the full-length Pac A product and since we can only barely detect Pactolus expression on the surface of BALB/c bone marrow neutrophils by FACS analysis, it is not surprising that we do not readily observe the $M_r 98,000$ protein. However, the truncated Pac B product should be evident in either the supernatant as a secreted product or within a storage compartment of the BALB/c cells. Since the level of total Pactolus transcripts between the BALB/c and C57BL/6 is very similar, and the Bac Pac antisera does recognize epitopes within the predicted Pac B product, our inability to observe this protein in the cells or their supernatant suggests it is rapidly degraded soon after synthesis. We have increased and decreased the labeling time, widely varied the chase times, and increased the quantity of \[^{35}\text{S}]\text{Met}\) used to label the BALB/c marrow cells; however, we have been unable to detect the Pac B product within the cell or its supernatant (data not shown).

To further characterize the full-length Pac A product, we generated a monoclonal antibody against the extracellular domains of Pactolus contained within the Bac Pac Baculovirus protein (see Fig. 1). As shown in Fig. 5C, the anti-Pactolus B10 monoclonal recognized both the $M_r 98,000$ and 130,000 forms of the Pac A protein product by immunoprecipitation from \[^{35}\text{S}]\text{Met}\)-labeled C57BL/6 marrow cells. In Fig. 5D, this same monoclonal antibody was used to immunoprecipitate bone marrow lysates from BALB/c and C57BL/6 cells, which, after resolution through SDS-PAGE, were analyzed by Western blot using the Bac Pac antisera. This analysis more accurately demonstrates, compared with the pulse-chase experiments, the actual steady state ratio of the $M_r 98,000$ to the $M_r 130,000$ form of the Pac A protein in these cells. We did not identify the Pac A product in the BALB/c sample in this experiment, which again may be due its low level of expression compared with the C57BL/6 cells.

The $M_r 130,000$ protein identified with the Bac Pac and monoclonal antiserum appears to be a posttranslationally modified version of the Pac A $M_r 98,000$ protein and not an associated $\alpha$ chain or another chaperone-type protein since 1) the immunoprecipitations were done under conditions of high stringency (which would disassociate virtually all integrin $\alpha/\beta$ pairs), and 2) the $M_r 98,000$ and 130,000 proteins share common epitopes. However, to ensure these proteins are not somehow linked via a covalent bond and thus maintain association during the immunoprecipitation process, bone marrow cells from the C57BL/6 animal were labeled and chased for 6 h and immunoprecipitated under reducing and non-reducing conditions (Fig. 5E). If the Pactolus protein was associated with another chain via a disulfide linkage, a larger molecular weight complex of about 240,000 in the non-reduced sample would be expected. The profile pattern of the two bands is very similar in both gels, although the apparent molecular weight of the two forms is altered in the non-reduced gel, suggesting Pactolus possesses intrachain disulfide bridges. These data continue to support our conclusion that Pactolus does not require the formation of a heterodimer with another protein to be expressed on the cell surface.

Analysis of Post-translational Modifications of Pactolus—The molecular weight of the primary peptide of the Pac A product is $\sim 81,000$. We have shown that two “mature” forms of the protein are produced by the GR1+ cells of the marrow: a distinct product of $M_r 98,000$ and a heterogeneous product of about $M_r 130,000$. There are 12 potential N-glycosylation sites, numerous potential O-glycosylation sites (serine or threonine), and seven potential proteoglycan side chain addition sites in the extracellular Pactolus sequence. In order to discern the nature of the post-translational modifications of Pactolus, we analyzed the Pactolus protein products of C57BL/6 bone marrow cells with enzymes that remove specific modifications. The Pactolus proteins were immunoprecipitated with the B10 Pactolus monoclonal antibody, digested with the appropriate enzyme, resolved by SDS-PAGE, and detected by Western blot with the Bac Pac antibody. Digestion with enzymes specific for proteoglycan side chains had no effect of the apparent molecular weight of the Pactolus products (data not shown). Digestion with Endo H, which cleaves partially modified N-glycosylation residues, demonstrated a clear loss of the $M_r 98,000$ form of the protein to its primary size of about $M_r 81,000$ (Fig. 6). However, the $M_r 130,000$ diffuse product was unchanged, suggesting that either it was a heavily modified N-glycosylation product or it possessed alternative modifications. We then performed a similar digestion with N-glycanase, which is effective at cleaving all N-linked glycosylations regardless of any additional side chain modifications. As shown in Fig. 6, treatment with N-
Glycanase completely shifted both the $M_r$ 98,000 and 130,000 forms to the primary peptide size, indicating virtually all of the posttranslational modifications seen for Pactolus are through N-glycosylation site(s). Finally, digestion with sialidase’s NANase III and sialidase partially decreased the size of the $M_r$ 130,000 form but left the $M_r$ 98,000 form unchanged. (Fig. 6). NANase III is specific for $\alpha$2–3,6,8,9 N-acetyleneuraminic acid, whereas sialidase is specific for $\alpha$2–3,6,8 N-acetyleneuraminic acid.

Neutrophil Activation Leads to an Increase of Pactolus Expression—Previously we had shown that cell surface biotinylation and immunoprecipitation of Pactolus from bone marrow cells identified a single protein of $M_r$ 98,000 (1). However, our pulse-chase and Western blot data have shown the presence of
the heavily N-glycosylated, $M_r 130,000$ form produced by these cells. These data suggest that the $M_r 130,000$ form may reside within the unactivated cell and could be mobilized to the cell surface upon cellular activation. A number of proteins such as human CR1 and Mac-1 are produced by neutrophils and held within secretory vesicles (17). After cellular activation, these pre-formed proteins are rapidly mobilized to the cell surface to aid in cell trafficking and phagocytosis. We therefore examined mouse bone marrow cells for their ability to mobilize Pactolus to the cell surface following activation with PMA. PMA is a broad inducer of cell activation through, in part, activation of protein kinase C (18). PMA promotes neutrophil activation and release of both granule and secretory vesicle contents (17). When bone marrow cells were treated with PMA, there was a demonstrable increase (~2–3-fold increase) in Pactolus staining on a portion of the cells (the GR1+ subset) from the BALB/c sample, and a very definable increase in staining (~10-fold) with the same subset from the C57BL/6 cells (Fig. 7A). Since activation with PMA was for only 30 min, it suggests that internal stores of the Pac A form were rapidly mobilized to the cell surface and the increase in staining was not due to de novo synthesis of protein. These data were confirmed by immunoprecipitation analyses using biotin-labeled cell surface extracts from PMA-activated cells (Fig. 7B). C57BL/6 marrow cells were labeled with biotin before and after activation and immunoprecipitated with streptavidin/Sepharose and analyzed by Western blot with the Bac Pac antibody (left panel) or immunoprecipitated with the anti-cytoplasmic tail Pactolus antisera and analyzed by Western blot with avidin-HRP (right panel). Both experiments demonstrated that activation with PMA results in the rapid extracellular localization of the heavily glycosylated, $M_r 130,000$ form of Pactolus.

If Pactolus is playing a role in neutrophil adhesion and/or homing, then it would be expected that the intracellular stores of the protein could be mobilized to the cell surface in the absence of degranulation, as has been been shown previously to occur with human CR1 and other membrane proteins (17). One such stimulus for Pactolus mobilization might be cross-linking of the Pactolus protein constitutively expressed on the surface of the maturing/mature neutrophil. Accordingly, bone marrow cells from C57BL/6 mice were treated with a subsaturating 1:500 dilution of the polyclonal anti-Pactolus Bac Pac antibody, or with the pre-immune control, incubated for various lengths of time, fix in 0.25% paraformaldehyde, and stained with a 1:100 dilution of the pre-immune serum or Bac Pac antisemur (Fig. 8A). As shown, the Bac Pac antisemur induced the cell surface expression of Pactolus. Activation of such cells with the Bac Pac antisemur for 5 min (light solid line) or 15 min (bold solid line) demonstrated significant increased staining. Compared with the non-activated cells (dense dotted line), the increase in Pactolus staining was comparable to that seen for PMA treated cells of such cells. The increase in FACS staining intensity was simply not additive, in that similar staining of cells fixed in 0.25% paraformaldehyde prior to activation with the 1:500 dilution of the Bac Pac antisemur and followed with the 1:100 dilution of the pre-immune or Bac Pac serum demonstrated only a minimal increase in Pactolus staining (data not shown). Cells activated for 30 min demonstrated the same level of induced Pactolus staining as that seen for the 15-min sample (data not shown).
The preceding data suggested that the binding of Pactolus leads to the release of a select subset of Pactolus-containing secretory vesicles in that no increase of staining of the Mac 1 integrin (which is also present in some secretory vesicles) was evident by these cells following Pactolus-mediated activation (Fig. 8B). C57BL/6 neutrophils were either treated with pre-immune serum or the Bac Pac antiserum (left panel) and stained for Mac 1 cell surface expression. No increase was detected, yet PMA treatment of such cells did demonstrate a release of pre-formed Mac 1 to the cell surface. Conversely, treatment of the C57BL/6 neutrophils with the Mac 1 antibody did not demonstrate an increase in Pactolus staining (right panel) although PMA treatment did.

Although cross-linking of external Pactolus could induce the
heightened expression of additional cell surface Pactolus while leaving the cell surface expression of Mac 1 unaffected, it was not clear if a similar induction could result in the release of the major granule contents. Accordingly C57BL/6 bone marrow cells were labeled with [35S]Met and incubated for 30 min with PMA or a 1:500 dilution of the Bac Pac antibody. Cell supernatants were analyzed for the release of granule contents. Antibodies specific for major granule constituents such as lactoferrin and myeloperoxidase of murine neutrophils are not available; those specific for the human proteins do not detect the mouse products (data not shown). However, as shown in Fig. 8C, the PMA-induced release of granule contents into the cell supernatant is evident without antibody detection; the release of the same components is absent following neutrophil activation with the anti-Pactolus Bac Pac antibody. Cell supernatants were analyzed for the release of granule contents. Antibodies specific for major granule constituents such as lactoferrin and myeloperoxidase of murine neutrophils are not available; those specific for the human proteins do not detect the mouse products (data not shown). However, as shown in Fig. 8C, the PMA-induced release of granule contents into the cell supernatant is evident without antibody detection; the release of the same components is absent following neutrophil activation with the anti-Pactolus Bac Pac antibody. Thus, neutrophil activation by Pactolus cross-linking does not result in the release of the major granule contents and instead appears to target a subset of the secretory vesicles.

**Strain-dependent Differences in Alternative Splicing of Pactolus Transcripts Define Two Distinct Alleles**—Previously we have shown that GR1+ bone marrow cells and peripheral neutrophils from the C57BL/6 animal express much higher levels of Pactolus on the cell surface than do the BALB/c and C3H/HeJ strains, and this can be attributed to strain-specific differences in the quantity of transcripts encoding the membrane-bound Pac A and the truncated Pac B forms of the protein (see Fig. 2). When F1 mice of C57BL/6 and BALB/c (and C57BL/6 and C3H/HeJ) matings were analyzed for cell surface Pactolus expression, the same percentage of cells, compared with the C57BL/6 animal, were positive, but to only about half the staining intensity of the inbred C57BL/6 animal (data not shown). These data suggested that the differential production of the Pactolus isoforms is co-dominant and may be due to differences in the Pactolus alleles between the C57BL/6 mice and the BALB/c and C3H/HeJ strains.

In order to map this trait in an unbiased fashion, we utilized a polymorphic microsatellite probe that discriminates between BALB/c and C57BL/6 genotypes. This probe maps to the end of murine chromosome 16, which is where the Pactolus gene resides (2). If the difference in Pactolus products is indeed due to different Pactolus alleles, then this genetic polymorphism should be linked to the phenotype.

F2 intercross animals generated from F1 (C57BL/6 × BALB/c) animals were analyzed for Pac A and Pac B transcripts as well as genotypes with the polymorphic PCR probe. As shown in Fig. 9A, of 6 offspring analyzed, animals 1 and 2 demonstrated a transcript profile identical to that of the parental C57BL/6 animal, animals 3 and 6 showed a profile...
identical to the BALB/c parent, and animals 4 and 5 possessed transcript profiles similar to the F1 progeny. Genotyping showed the genetic component predicted from the transcript profile if the Pactolus gene alone (or another gene very tightly linked) was responsible for the transcript differences (Fig. 9B). A total of 14 F2 animals were analyzed using this procedure (Fig. 9C). Of those, there was no discrepancy between the genotype predicted from the Pactolus transcript phenotype, and the actual determined genotype.

**Figure 10. Sequence analysis of two distinct Pactolus alleles.** Figure shows the sequence of the alternatively spliced region of the Pactolus gene derived from BALB/c (top sequence) and C57BL/6 (bottom sequence). The splice donor sites are denoted by the underlined GT. The splice acceptor sites are denoted by the underlined AG except for the alteration within the C57BL/6 sequence, which is an underlined GG. The derived amino acid sequences are noted including the altered amino acid between the two sequences, which is also underlined. Exonic sequences are boxed, the intronic sequence of intron 12 is condensed between exons 12 and 13. Spliced isoforms are denoted by the heavy, medium, or dotted lines to denote predominant, limited, or absent transcript forms, respectively.

Sequencing Analysis of the Alternative Splice Region of the Pactolus Gene from C57BL/6 and BALB/c Mice—To determine if the difference in quantity and type of spliced Pactolus gene products could be directly linked to a Pactolus gene polymorphism, the sequences of exons 11, 12, and 13 plus those of introns 11 and 12 were determined from DNA obtained from C57BL/6 and BALB/c animals (Fig. 10). The two strain sequences at that site were identical except for a single A → G difference between the BALB/c to C57BL/6 sequence at the second splice acceptor site in exon 13 (underlined). When this splice acceptor site in the BALB/c sequence is utilized, the resulting product (Pac B) is the truncated form of Pactolus due to an alteration in the reading frame and introduction of stop codons. Splice acceptor sites are consistently AG nucleotides preceded by a T/C-rich region (19); both such acceptor sites in the BALB/c sequence fit the consensus. The sequence does not provide any obvious clues as to why the Pac B acceptor site is used more frequently over that of the Pac A acceptor site in the BALB/c cells but does indicate that the acceptor site for the Pac B product in the C57BL/6 sequence would not be functional. Thus, virtually all of the C57BL/6 Pactolus transcripts would be forced to use the Pac A acceptor site, a prediction that is evident from the data. This sequence change will also alter a single amino acid between the two sequences (S for BALB/c but G for C57BL/6) at that site.

**DISCUSSION**

The first description of Pactolus indicated that the gene is primarily expressed in the bone marrow of the mature animal, and that the gene encodes two predominant forms (1). One of these encodes a cell surface-bound form, Pac A; the other form predicts a truncated product, Pac B, possessing the majority of the extracellular sequences but lacking the transmembrane and cytoplasmic domains. We observed that the Pactolus gene and product was structurally similar to the β-integrins, but was distinct in that it did not appear to form a heterodimer with an α-integrin subunit. In this study we have addressed key aspects of the biochemistry, cell biology, and genetics of the Pactolus gene and gene products.

The neutrophil is the major cell type of the mouse that expresses Pactolus. FACS staining and sorting analysis have shown that the immature and mature neutrophils within the bone marrow and periphery express the Pactolus products. Although the Pactolus gene produces transcripts encoding both a truncated and full-length form of the protein via alternative splicing, only the full-length, Pac A form is stable; the truncated form is apparently rapidly degraded after synthesis. We have observed strain differences in the expression of Pactolus by maturing and mature neutrophils (the C57BL/6 strain predominantly expresses the full-length, Pac A form, whereas out-bred mice and C3H/HeJ and BALB/c strains primarily express the truncated, Pac B form). Using polymorphic genotype mapping markers (20–22), we demonstrated that the isoform phenotype of the C57BL/6 and BALB/c strains did map to the Pactolus gene (or was closely linked). The promoter strength of these two alleles appears to be very similar because there is not a significant level of difference in the amount of total Pactolus transcripts between the three strains. Sequence analysis demonstrated an alteration within exon 13 that appears to define the phenotype of the alleles. Although the C57BL/6 strain expresses the highest levels of the Pac A form, clearly the other strains (personified by BALB/c) express the cell surface form after neutrophil activation. The development of cell surface staining monoclonal should enable us to more fully define the level of cell surface expression of Pactolus on the surface of unactivated, BALB/c neutrophils. One intriguing question left open is whether or not cell activation or maturation can modulate the choice of the splice acceptor site with the BALB/c allele, thus altering the ratio of the truncated to full-length transcripts. The fact that two such sites do lay in tandem within the exon and our conclusion that the truncated product is unstable suggests such a modulatory effect may be observed.

The data presented in this and our previous report (1) suggest that the full-length Pac A protein is present in the neutrophils in two forms and at two sites. The M, 98,000 form...
appears to be constitutively expressed on the cell surface, whereas the M, 130,000 form appears to reside within secretory vesicles inside the cell waiting for cell activation to allow for cell surface expression. Of the three most probable post-translational modifications available to the Pactolus protein (N-linked, O-linked glycosylation, and the addition of proteoglycan side chains) the addition of N-linked glycosylation residues appears to be responsible for most if not all of the increase in molecular weight compared with the primary peptide sequence. We do not fully understand the nature of these extensive modifications; additional sugar labeling, digestion, and lectin binding experiments will be required to fully elucidate their structures and constituents.

The finding that increased cell surface expression of Pactolus can be accomplished by translocating intracellular stores of the protein places Pactolus in that class of proteins that includes Mac-1, human CR1, CD14, and others (17, 23–26) that are found in the secretory vesicles of neutrophils. It is not clear how diverse secretory vesicle are with regards to membrane receptor components; however, it is noteworthy that we observe increased levels of Pactolus in the absence of increased quantities of Mac-1 (data not shown), suggesting that they may reside in different sets of such vesicles.

The release of the intracellular stores of Pactolus by cross-linking cell surface Pactolus suggests that Pactolus participates in neutrophil function in the absence of degranulation. One such role may be in the trafficking of neutrophils into the sites of infections. Migration of circulating neutrophils from the blood into the tissue requires molecules to first slow the neutrophil down (rolling via the selectin/selectin-binding proteins) and then bind it tightly to the endothelial surface to facilitate diapedesis (primarily via integrin/ligand interactions). Pactolus could obviously participate in either of these steps. A previous study by Ramos and colleagues utilized a blocking monoclonal antibody against murine E-selectin (which is expressed by the endothelial cells) to inhibit neutrophil migration. Interestingly, they demonstrated a virtual block of migration of neutrophils in BALB/c mice following antibody treatment, but could not demonstrate any effect on the migration of the same cells in a C57BL/6 animal. The causative difference between the responses of these two strains was not determined in this study. However, since the C57BL/6 animals express much higher levels of Pactolus on their neutrophils than is seen on BALB/c cells neutrophils, Pactolus may be complementing the loss of the E-selectin interactions allowing for functional migration of the C57BL/6 neutrophils in the presence of the anti-E-selectin antibody.

If Pactolus is participating in a selectin-like function, then it could reasonably be selectin-like, or selectin ligand-like. The selectins possess an N-terminal C-type lectin domain, which Pactolus does not. Alternatively, the selectin ligands are notable for possessing the sialy1 Lewis X carbohydrate modification; however, this modification is based upon a core 2 branch of the serine/threonine-linked oligosaccharides (O-glycans), which Pactolus does not appear to possess. However, there is a recent report suggesting that such modifications may also be N-linked and thus resistant to digestion with O-sialoglycoproteinase (27). Therefore, defining the full structure of the heavily modified Pactolus protein and using this knowledge to help identify its ligand(s) are two crucial steps that must be accomplished to fully elucidate the function of Pactolus.

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