Published in final edited form as:

J Biomed Mater Res A. 2008 May ; 85(2): 513–529. doi:10.1002/jbm.a.31306.

LC/MS identification of 12 intracellular cytoskeletal and inflammatory proteins from monocytes adherent on surface-adsorbed fibronectin-derived peptides

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Abstract

The extent and duration of the host response determines device efficacy yet the mechanism is poorly understood. U937 pro-monocytic cells were cultured on peptide-adsorbed tissue culture polystyrene to better understand surface-modulated intracellular events. Phosphotyrosine proteins were enriched by immunoprecipitation and analyzed by nanospray HPLC-coupled tandem mass spectrometry (LC/MS). Tyrosine phosphorylated proteins were chosen based on physiological significance and previous densitometry results, which identified a set of proteins ranging from ~200 to ~23 kDa showing altered phosphorylation levels in response to various surface-adsorbed ligands and phosphorylation inhibitor AG18. While LC/MS has been used for nearly a decade, its application to the field of biomaterials is relatively novel. 12 intracellular proteins identified by nanospray LC/MS are potentially related to the host response. Eight of the 12 proteins are related to the cytoskeleton including: moesin, heat shock protein 90 β , α -tubulin, elongation factor 1 α , beta actin, vimentin, plasminogen activator inhibitor 2 and heterogeneous ribonuclear protein A2. The remaining four proteins: high mobility group box 1, caspase recruitment domain 5, glycoprotein 96 and heterogeneous nuclear ribonucleoprotein D0 modulate inflammation. The specific effect each peptide has upon modulating the phosphorylation state of these proteins cannot be determined from the current work; however 12 viable targets have been identified for further investigation into the role each plays in the surface-mediated monocyte response.

Keywords

U937; phorbol myristate acetate; RGD; PHSRN; AG18; host response; inflammation

Introduction

Macrophages play a key role in the inflammatory and immune reaction associated with biomaterials [1–4]. While the exact mechanism underlying the host inflammatory response is not currently known, the identity and conformation of adsorbed proteins on the material surface is believed to direct both the extent and duration of monocyte-biomaterial

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interaction. This interaction mediates adhesion [5,6], monocyte activation [7], fusion to form foreign body giant cells [8] and secretion of bioactive molecules [9]. Therefore the effect different peptide ligands have upon intracellular signaling and protein expression is key to resolving the mechanism of the monocyte response. The extracellular matrix protein fibronectin (FN) is of particular interest as a ligand for monocytes due to its function up regulating the pro-inflammatory cytokine interleukin 1 β (IL-1 β) [10]. The tri-peptide arginine-glycine-aspartic acid (RGD) is a known cell adhesion sequence in FN while the synergistic sequence proline-histadine-serine-arginine-asparagine (PHSRN) increases cell adhesion to RGD [11]. Because of its adhesive properties RGD is widely used in tissue engineering to promote interaction between cells and the material surface [12,13]. This interaction is mediated by adhesion and begins a signaling cascade driven by protein phosphorylation leading to altered gene transcription and protein expression [14,15]. Of the residues phosphorylated, tyrosine phosphorylation shows higher gain and tighter regulation than serine or threonine phosphorylation [16] and thus is critical for resolving the monocyte mediated inflammatory response to biomaterials.

In order to resolve the intracellular signaling events of monocytes post adhesion, we have previously probed the human monocytic cell line U937 using tissue culture poly(styrene) (TCPS) adsorbed with various protein or peptide ligands and increasing amounts of the broad spectrum phosphorylation inhibitor tyrphostin 23 (AG18). The cells were allowed to adhere for 12 or 24 hrs, lysed, immunoprecipitated for phosphorylated tyrosine proteins using monoclonal antibodies and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue dye and analyzed by densitometry. This analysis revealed a set of proteins ranging from ~200 to ~23 kilo Dalton (kDa) showing altered phosphorylation levels in response to the various surface associated ligands and varying levels of AG18 (Table 1) [17]. The previously utilized densitometry relies on band intensity to identify up or down regulation of proteins offering a relatively quick and inexpensive analysis of protein trends. However, densitometry is limited in individual protein identification. Thus more specific proteomics tools must be used to identify the proteins comprising these bands. Western blotting can be used to probe cell lysates for the presence of a specific protein [18–20], but each blot detects only a single protein and requires protein identification *a priori*. Since multiple proteins may be present at any given molecular weight in an immunoprecipitated cell lysate and *a priori* knowledge of the proteins involved in the host response is incomplete, Western blotting is not a viable analysis tool for surveying the tyrosine phosphorylated proteins present in monocytes adherent to various peptide-adsorbed TCPS surfaces.

Mass spectrometry offers a more comprehensive approach to qualitative protein identification [16, 21–23]. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the two main types of mass spectroscopy instruments used for proteomics. MALDI offers quick and sensitive detection of peptide mass; however, MALDI typically does not provide sequence data and is limited to relatively simple protein mixtures. Fourier Transform Mass Spec (FT-MS) is a variation on MALDI that does offer sequence data, but limited accessibility by individual research laboratories limits widespread application of FT-MS. High performance liquid chromatography coupled to an ESI mass spectrometer (LC/MS) allows the capture of ionized peptides in an ion trap and subsequent

dissociation by collision with an inert gas. LC/MS yields detailed sequence data but is not as sensitive as MALDI and requires more sample preparation prior. The method of protein identification utilized in the present work yields only protein identity and is not quantitative. For quantitative proteomics other methodologies such as stable-isotope labeling in cell culture (SILAC) or isotope-coded affinity tags (ICAT) should be employed [22]. In the present study, nanospray LC/MS was used to qualitatively identify proteins in the bands of interest ranging from ~200 to ~23 kDa. We hypothesized that nanospray LC/MS can be used to survey and identify critical intracellular proteins mediated by various surface adsorbed peptide ligands and varying AG18 concentrations. Thus LC/MS may identify targets for future research aimed at elucidating the mechanism underlying the post adhesion monocyte response.

Experimental Section

Materials

Human monocytic cell line CRL-1593.2/U-937 (American Type Culture Collection, ATCC) [24] were used. Reagents were obtained from the following sources: glacial acetic acid (Aldrich); isopropyl alcohol (EM Sciences); sodium orthovanadate, ammonium bicarbonate ((NH₄)HCO₃), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), formic acid (FA), hexaglycine (G₆), Amido Black stain, gelatin type A 300 bloom (porcine), tyrphostin 23 (AG18), phorbol 12-myristate 13-acetate (PMA), Kodak processing chemicals GBX Developer and GBX Fixer, Kodak Biomax ML scientific imaging film (13×18cm) (Sigma); HPLC grade acetonitrile (ACN), methanol (Fisher); trypsin (modified sequence grade) (Promega); 10-well Ready Gel[®] (Tris-HCl, 10% resolving[®], 4% stacking[®]), Coomassie Brilliant Blue (G-250), 10× Tris/Glycine/SDS buffer, 10× Tris/Glycine buffer, Tween-20 (BioRad); bicinchonic acid (BCA) protein quantification kit and standards, M-PER[®] mammalian protein extraction reagent, Halt[™] Protease Inhibitor Cocktail, PepClean[™] C-18 spin columns (Pierce); G₃RGDG and G₃PHSRNG oligopeptides were synthesized by the Biotechnology Center, University of Wisconsin-Madison at >95% purity as analyzed by HPLC and mass spec; fetal bovine serum (FBS) (HyClone); Clonetics reagent pack (trypsin/EDTA, trypsin neutralizing solution (TNS), HEPES-buffered saline solution (HBSS)) (Cambrex); 1× phosphate buffered saline solution (PBS), Rosswell Park Memorial Institute (RPMI) 1640 media (CellGro); ZipTip[®] (C₁₈, P10 size), Immobilon P polyvinylidene fluoride (PVDF) transfer membrane (0.45µm pore size) (Millipore); PY20 monoclonal anti-phosphotyrosine antibody (Santa Cruz); 4G10 anti-phosphotyrosine antibody (Upstate); immobilized phospho-tyrosine monoclonal antibody (mAb) P-Tyr-100 (#9419), p42 MAP Kinase 1 (Erk2) monoclonal antiphosphoMAPK1 antibody, Phototype-HRP Western Blot detection system (anti-mouse IgG, horse radish peroxidase (HRP)-linked Ab) (Cell Signaling).

Preparation of cell lysates and immunoprecipitation

TCPS flasks (75cm², BD Falcon) were adsorbed for 24hr at 37°C with a 1 µM pre-adsorption solution of G₆, G₃RGDG, or G₃PHSRNG peptide. The surfaces were washed once with 1× PBS and seeded with U937 cells at 1.7×10⁵ cells/cm². Cells were cultured in RPMI 1640 with 5% FBS, 50 ng/ml PMA, and 0, 20, 40, 60 or 80 µM AG18 in a 5% CO₂

humidified (~95%) incubator at 37°C where 80 μ M AG18 represents the IC₅₀ value for U937 cells. After 24hr, cells were harvested with trypsin/EDTA, lysed with M-PER[®] containing 1 $\times$ HALT[™] protease inhibitor and 1mM sodium orthovanadate to inhibit phosphatase activity. The relatively short culture time of 24 hr minimized serum protein adsorption on to the peptide-adsorbed TCPS surface enabling the effect of each peptide to be resolved. Total protein in the cell lysates was quantified using BCA. 550 μ g of protein was then immunoprecipitated (IP) overnight at 4°C using P-Tyr-100 mAb against phosphorylated tyrosine proteins. The IP beads were washed twice with M-PER, proteins were denatured using 3 $\times$ loading buffer containing 50 mM DTT and the samples were heated at ~90°C for 10 min before SDS-PAGE on Tris-HCl gels (10% resolving, 4% stacking) at 84 V for 30 min followed by 129 V for 1 hr 45 min [17]. While the use of primary cells offers clinical relevancy, there are several hurdles which must first be overcome. Human monocytic cell line U937 cells offer a relatively uniform response and proliferative nature. In addition, each sample for protein analysis requires in excess of 25 million monocytes prior to adhesion, thus the number of samples required for the analyses performed here would require a prohibitively large number of donors. The goal of working with the U937 cells is to obtain a framework that can be applied to primary cell behavior.

Western Blot

Proteins from adherent U937 cells were transferred to a PVDF membrane at 74V for 3 hr. Transfers were run on ice in 1 $\times$ Transfer buffer (25 mM Tris, 192 mM glycine, 50% v/v methanol). Protein transfers were also carried out at 96 V for 1 hr and 250 mA for 1 hr. To insure protein transfer, the PVDF membrane was stained for 1 min using Amido Black stain and destained for 30 min using 25% isopropanol and 10% acetic acid. Membranes were blocked using a 0.25% gelatin solution and exposed to a primary antibody cocktail containing PY20 and 4G10 monoclonal antibodies against phosphorylated tyrosine proteins (1:1000) overnight at 4°C in blocking solution. Membranes were exposed to anti-mouse IgG secondary antibody conjugated to HRP (1:8000) for one hr followed by exposure to LumiGLO[®] for 5 min. Chemiluminescence was captured by exposure to X-ray film for 10 min.

LC/MS

All sample preparation for LC/MS was done in a laminar flow hood using aseptic techniques. 40 different combinations of surface-adsorbed peptides and AG18 concentrations were probed in duplicate (n=2). The protocol was adapted from established sample preparation techniques developed by the Biotechnology Center, University of Wisconsin-Madison. After the immunoprecipitated cell lysates were run on SDS-PAGE, the gels were washed three times in Milli-Q water for 10 min to remove residual SDS. Approximately 5 mL of Coomassie Brilliant Blue was used to stain the gel for one hr followed by destaining in ~20 mL of Milli-Q water for 1 hr. After destaining the gels were placed on a clean glass plate and the band of interest (Table 1) was excised. The excised bands were placed into siliconized 0.65 mL Eppendorf tubes and destained with 100 mM (NH₄)HCO₃/50% methanol for 10 min with intermittent vortexing until fully destained. The bands were then dehydrated twice for 10 min in 25 mM (NH₄)HCO₃/50% ACN and dried in a vacuum centrifuge for 10 min. Gel fragments were then rehydrated in fresh 25 mM DTT

and reduced for 30 min at 56°C. The gel bands were cooled to room temperature, residual liquid was pipetted off, and fresh 55 mM IAA was added. Gel fragments were then alkylated for 30 min at room temperature in the dark. Any residual liquid was removed and Milli-Q water was added for 15 min. After equilibration with water, the gel fragments were equilibrated in 25 mM (NH₄)HCO₃ for 10 min with intermittent vortexing. Gel bands were then dehydrated twice with 25 mM (NH₄)HCO₃/50% ACN for 10 min and dried on the vacuum centrifuge for 10 min. Finally the gel fragments were rehydrated for 15 min at 4°C in trypsin (20 ng/ml) suspended in 25 mM (NH₄)HCO₃. The bands were then overlaid with a minimal amount of 25 mM (NH₄)HCO₃ to keep the gel fragments immersed and incubated for 16–20 hr at 37°C. After the digestion, the solutions were transferred to a new 0.65 mL siliconized Eppendorf tube. Digested peptides remaining in the gel fragments were extracted with 0.1% TFA in Milli-Q water for 20 min with constant vortexing (high setting). The extracted peptides were then added to the solution from the overnight digestion. Two more peptide extractions were performed using 5% TFA/70% ACN for 20 min with constant vortexing (high setting). The peptide solutions were then dried in the vacuum centrifuge and stored at –80°C. Peptides were desalted using C₁₈ ZipTips or PepClean™ C-18 spin columns. Desalting was performed according to manufacturer's protocol substituting FA for TFA to prevent ion pairing during ionization. After desalting the peptides were stored at –80°C until submission for analysis.

Samples were run on an Agilent MSD ion trap mass spectrometer coupled to an 1100 HPLC system with nano-pump flow capability. The peptides were first separated by HPLC on a Zorbax C-18sb column at 0.280 µL/min. The trap column (Zorbax C-18sb) was loaded for 15 min and eluted to the analytical column (75µ × 150mm) in a 120 minute gradient from 0.1% FA/10% ACN/water to 0.1% FA/60% ACN/water. ACN was then ramped up to 95% over the course of 10 min. The column was then re-equilibrated for 20 min with 10% ACN. The eluent from the analytical HPLC column was eluted directly into the ion trap via an 8µm glass spray needle (New Objective). The ion trap was configured to prefer doubly-charged ions and switch to MS/MS with the threshold intensity of 0.1% of the absolute maximum. The actual threshold count was 100,000 counts. Ion trapping was achieved with 75,000 ions per trap cycle with a 100 ms maximum accumulation time for each cycle. Agilent proprietary software was then used to scan out fragments of peptides trapped in each cycle. The data was converted to .mgf files using the Agilent data analysis software for the ion trap. The .mgf files were then searched against the human portion of the National Center for Biotechnology Information (NCBI) database. Mascot scores based on the Mowse algorithm were used to assess significance of the protein hits [25]. Any protein identified by a peptide with a Mascot score >40 was considered significant where the Mascot score is equal to $10 \cdot \log_{10}(P)$, where P is the absolute probability the peptide detected was a random event. Thus a low probability leads to a high Mascot score. Several immunoglobulin fragments were identified at various molecular weights. These were considered an artifact of IP enrichment for phosphotyrosine proteins and disregarded.

The raw data obtained from the LC/MS experiments then had to be refined for relevance before further analysis. Any unknown or putative (theoretical) proteins were removed from consideration. Proteins identified from U937 cells grown in suspension (i.e. PMA[–]) were identified. Table 2 exemplifies the analysis scheme for those proteins identified from PMA[–]

cells. Since only proteins expressed in adherent U937 cells are of interest, those proteins appearing in both adherent cells and cells grown in suspension were removed from further consideration. As an example, Table 3 illustrates the result of the data refining process for U937 on G₃RGDG-adsorbed TCPS surfaces for all AG18 concentrations. The significant and relevant proteins remaining after data refining were then located in the Human Protein Reference Database (hprd.org) and Expert Protein Analysis System (ExPasy). Known protein function, protein-protein interactions and whether the protein is tyrosine phosphorylated were noted. Once all proteins were identified, comparisons were then made according to previous densitometry, surface adsorbed ligand and AG18 concentration.

Results

Western Blot results

SDS-PAGE is a one-dimensional protein separation based on molecular weight. Raw cell lysates and even immunoprecipitated cell lysates may contain multiple proteins at a given molecular weight range. Western blot analysis is necessary to identify specific proteins responsible for the previously observed trends. Immunoprecipitated proteins ranging from ~250 to ~20 kDa from adherent U937 to TCPS were successfully transferred to PVDF membranes. A series of 14 distinct bands was detected between ~250 and ~20 kDa using Amido Black staining. Proteins were transferred for 3 hr at 74V on ice. Cell lysates from U937 cells stimulated with 50 ng/ml PMA and adherent to TCPS were probed for phosphorylated tyrosine proteins using monoclonal antibodies PY-20 and 4G10 (Figure 1). The most intense bands appeared between 50 and 37 kDa with two more sets of bands appearing between 100 and 75 kDa. Cell lysates from U937 treated with 0, 20, 40 or 60 μ M AG18, 50 ng/ml PMA and adherent to PBS-adsorbed surfaces were probed for the presence of tyrosine phosphorylated MAPK1 protein. Bands at ~42 kDa from U937 adherent to PBS-adsorbed TCPS with 0, 20, 40 and 60 μ M AG18 were detected correlating to the expected position of pMAPK1 (42 kDa). A second set of bands at ~30 kDa was detected in both PBS 20, 40, and 60 μ M AG18. This set of bands likely represents proteolytic degradation of the phosphorylated MAPK1 protein.

LC/MS Results

LC/MS was used to sequence and identify intracellular proteins from U937 cells adherent to G₆-, G₃RGDG- and G₃PHSRNG-adsorbed TCPS surfaces. The molecular weights of the proteins analyzed were identified previously by densitometry of Coomassie stained SDS-PAGE gels. These molecular weight ranges of proteins exhibited up or down regulated protein phosphorylation in response to the peptide-adsorbed surface and AG18 concentration. Using LC/MS to identify these proteins may provide an insight into the protein(s) linking adhesion with monocyte activation. LC/MS is a powerful tool to quickly survey a cell's global proteome but protein identification via LC/MS is limited by peptide ionization, which is impacted by numerous factors such as the presence of non-phosphorylated peptides and expression level. Thus identification of proteins from adherent U937 via LC/MS provides potential target proteins for future experiments probing the role each protein plays in the monocyte-mediated inflammatory response.

Table 1 shows a summary of culture conditions identified by previous densitometry that lead to altered protein tyrosine phosphorylation expression levels. Table 1 will serve as the basis for the following analysis. The results are analyzed starting at ~200 kDa and decreasing in molecular weight to ~23 kDa. At each molecular weight analyzed, proteins were identified from cells on one ligand-adsorbed TCPS surface and AG18 concentration and either the AG18 control (i.e. same ligand-adsorbed surface without AG18) or the ligand-adsorbed TCPS control (i.e. PBS-adsorbed TCPS at the same AG18 concentration).

~200 & ~160 kDa nanospray LC/MS results—Intracellular proteins from U937 adherent to G₃RGDG-adsorbed TCPS at ~200 kDa cultured with 20 μ M or without AG18 were analyzed first. No known proteins were identified in U937 on G₃RGDG-adsorbed TCPS with 0 or 20 μ M AG18. No known proteins were identified at ~160 kDa in cells adherent to G₃PHSRNG-adsorbed TCPS with 0 or 40 μ M AG18. This failure to detect any proteins at both ~200 and ~160 kDa could arise from several factors. Phosphorylated tyrosine proteins are estimated to comprise only 0.05% of the total proteome [26]. Thus immunoprecipitating 550 μ g of protein should yield around 275 ng of tyrosine phosphorylated proteins not including dephosphorylation by phosphatases, which were inhibited with the transition state analog sodium orthovanadate. Despite inhibition phosphatase activity cannot be fully suppressed further reducing the sample amount. Another issue is the extraction of peptides from the gel fragments. The inefficiency of extracting peptides from polyacrylamide gels could lower the amount of protein present in each band from the minimum of 8 ng for Coomassie staining to near or below the 1 ng threshold for nanospray LC/MS detection. These issues illustrate why nanospray LC/MS is useful for the qualitative identification of proteins from an enriched cell lysate but cannot be used for direct comparisons without a significant amount of starting cell lysate and quantitative procedures such as SILAC.

~100kDa nanospray LC/MS results—While no proteins were identified at ~200 and ~160 kDa, three proteins were identified from U937 on G₃RGDG-adsorbed TCPS at ~100 kDa without AG18. The three proteins were: Staphylococcal nuclease domain containing protein 1, glycoprotein 96 (Gp96, also known as tumor rejection antigen 1) and ATP-dependent RNA helicase A (DDX 9) (Table 4). Gp96 can bind to antigen, activate antigen presenting cells (APCs) [27] and stimulate antigen presentation that results in dendritic cell activation via the Toll-like Receptor 2 & 4 (TLR 2 & 4) pathways [28] in a manner similar to lipopolysaccharide (LPS) treatment. Interestingly, gp96 preferentially stimulates dendritic cell activation of cytotoxic, or killer, CD8⁺ T cells [29]. The ability of gp96 to activate APCs such as macrophages is of particular interest as it relates to the host response. However, no proteins were identified from U937 adherent to G₃RGDG-adsorbed TCPS surfaces with 80 μ M AG18.

~65/70 kDa nanospray LC/MS results—Intracellular proteins were analyzed from U937 adhered to G₃RGDG-adsorbed TCPS at ~65/70 kDa with 0, 20, 60 or 80 μ M AG18 (Table 1). Moesin (membrane organizing extension spike protein) was the only protein identified in all four AG18 concentrations (Table 5). Interleukin enhancer-binding factor 3 was identified in cells with 0 and 60 μ M AG18 but not in cells with 20 or 80 μ M AG18. In

addition to moesin, three additional proteins were identified from cells on G₃RGDG-adsorbed TCPS with 20 and 60 μ M AG18. Hsp90 is a homodimer of one of two different subunits, either α or β . Hsp90 has been observed to bind to tubulin *in vitro* and inhibit tubulin polymerization [30]. This inhibition may be important in cytoskeletal rearrangement, cell locomotion and polarization. The 78kDa gastrin-binding protein was identified only from cells with 60 μ M AG18. Only moesin was identified from cells on G₃RGDG-adsorbed TCPS with 80 μ M AG18, which is significant because 80 μ M AG18 is the IC₅₀ value of AG18 for U937 cells. Thus any proteins phosphorylated in the presence of 80 μ M AG18 can be considered vital to cell survival.

The proteins from cells on PBS-adsorbed TCPS with 20 μ M AG18 were also analyzed at ~65/70 kDa (Table 5). The only protein identified from cells on the PBS-adsorbed TCPS surface was titin with an ion score of 46, which can be tyrosine phosphorylated. However, titin is known to be expressed only in striated muscle tissue [31]. Titin is known to contain numerous Ig domains as well as FN Type III domains, which are also found in other ECM proteins to which cells adhere [32]. The Mascot scoring scheme assumes each entry in the protein database consists of random amino acid sequences leading to difficulty assigning scores for proteins containing large numbers of repeating domains such as titin. A trivially high score will result if Mascot allows one experimentally measured peptide mass to match multiple peptides in a protein containing repeating domains; however, if multiple matches are not allowed one experimentally measured peptide mass is insufficient for a significant Mascot score. Therefore titin is likely an artifact of the probability based scoring algorithm used by Mascot to identify significant hits that arise only in cases where proteins contain numerous repeating domains.

~52 kDa nanospray LC/MS results—At ~52 kDa cells on G₃PHSRNG-adsorbed TCPS samples with 40 and 60 μ M AG18 exhibited decreased protein expression compared to cells without AG18 (Table 1). Table 6 shows the ~52 kDa proteins identified from U937 on G₃PHSRNG-adsorbed TCPS with 0, 40 or 60 μ M AG18. Serum albumin and alpha tubulin were identified in cells without AG18 while nucleolin was identified from cells with 40 μ M AG18. The serum albumin protein is likely an artifact of serum-based cell culture conditions since serum albumin is the most abundant protein in serum. Two proteins, tubulin 5-beta and growth regulated nuclear 68 protein, were identified in cells with 60 μ M AG18.

~42 kDa nanospray LC/MS results—Densitometry of the ~42 kDa band revealed a decreased protein expression level from cells on G₃RGDG-adsorbed TCPS with 20 μ M AG18 versus cells without AG18 (Table 1). While six proteins were detected in cells with 0 and 20 μ M AG18 (Table 7), five other proteins were identified only in cells without AG18 and two more proteins were identified only in U937 with 20 μ M AG18.

Beta actin was identified in U937 on G₃RGDG-adsorbed TCPS cultured with 0 and 20 μ M AG18 at ~42 kDa while vimentin was only identified in cells with 20 μ M AG18 (Table 7). Both of these proteins are cytoskeletal proteins comprising actin fibers and intermediate fibers, respectively, and are thus important in monocyte adhesion to biomaterial surfaces. Interleukin enhancer binding factor 2 was also detected in U937 with 0 and 20 μ M AG18. According to Expasy interleukin enhancer binding factor 2 can interact directly with DNA-

dependent protein kinase catalytic subunit as well as form a complex with ribosomal proteins L12, L13, S16 and S25 in addition to the DNA-dependent protein kinase catalytic subunit and DNA topoisomerase II α . Four of these proteins, DNA dependent protein kinase, DNA topoisomerase II, and ribosomal proteins L12 & L13, were identified previously from U937 adherent to albumin- and fibronectin-adsorbed TCPS surfaces [33]. Eef1 α 2 was identified in U937 on G₃RGDG-adsorbed TCPS without AG18 and is known to bind both actin and mRNA [34]. Citrate synthesis and phosphoglycerate kinase are both involved in glycolysis and were identified from cells on G₃RGDG-adsorbed TCPS without AG18. The La 4.1 protein is likely a member of the 4.1/Ezrin/Radixin/Moesin family that links the cytoskeleton with mRNA. The heterogeneous ribonuclear protein D0 (hnRNP D0) binds to the promoter for complement receptor 2 protein leading to up regulation of the CR2 receptor [35]. CR2 is primarily expressed in dendritic cells as well as B cells and can amplify the B cell response to antigen [36]. However, monocyte expression of CR2 is not well documented. PMA has been found to stimulate hnRNP D0 activation via PKC signaling [37] while serum up regulates CR2 in B cells via an unknown mechanism [38]. The phagocytic role of monocytes and their need to recognize complement opsonins on a biomaterial surface make both hnRNP D0 and CR2 proteins of interest for further research. Plasminogen activator inhibitor 2 (PAI-2), which inhibits urokinase activity, was detected in cells with 20 μ M AG18 on G₃RGDG-adsorbed TCPS.

In addition to the proteins identified from cells on G₃RGDG-adsorbed TCPS, cell lysate from cells on G₆-adsorbed TCPS were also analyzed. Three proteins were found in U937 on G₆-adsorbed surfaces while no significant hits were found in cells on PBS-adsorbed surfaces with 40 μ M AG18. Eef1 α was detected in U937 on G₆-adsorbed TCPS as Eef1 α 1, which is in the same family as the aforementioned Eef1 α 2 and plays a similar role binding both the actin cytoskeleton and mRNA. PAI-2 was also identified from U937 on G₆-adsorbed TCPS with 40 μ M AG18. The third protein identified was the 48 kDa histamine receptor. Neither Expasy nor HPRD had an entry for the 48 kDa histamine receptor while the NCBI only had a partial sequence for the protein.

~23 kDa nanospray LC/MS results—~23 kDa was the smallest molecular weight analyzed in this experiment (Table 8). Proteins from U937 cells on G₆-adsorbed TCPS with 0 and 60 μ M AG18 were analyzed. No significant hits were found for U937 with 60 μ M AG18 while myeloid cell nuclear differentiation antigen (MNDA), high mobility group box 1 protein (HMG1), alkyl-N-purine-DNA glycosylase (ANPG) and caspase recruitment domain protein 5 (CARD5) were found in U937 without AG18 on G₆-adsorbed TCPS. ANPG is involved in repair of DNA in response to oxidative damage from free radicals [39]. MNDA is part of the interferon-regulated 200 (HIN-200) family that is thought to interact with proteins regulating transcription [40] and is only expressed in hematopoietic cells. Evidence also points to this family playing a role in cellular differentiation and proliferation [41] as well as apoptosis [42]. MNDA is therefore a protein of interest for further research due to its role in programmed cell death.

Proteins from cells on G₃RGDG-adsorbed TCPS with 0 and 80 μ M AG18 were also analyzed at ~23 kDa. One protein was detected in cells with 0 and 80 μ M AG18 while three other proteins were identified only in cells without AG18 and two more were detected from

U937 with 80 μ M AG18 only (Table 8). Heterogeneous nuclear ribonuclear protein A2 (hnRNP A2) is involved in numerous cellular functions involving RNA including trafficking to the cytosol, alternative splicing and packaging in addition to maintaining telomere length [43]. hnRNP A2 has recently been observed to stabilize the mRNA for collagen prolyl 4-hydroxylase, which is required for hydroxylation of proline residues in collagen [44]. Without proline hydroxylated collagen will not fold properly, and since collagen is a major component of the ECM and plays an important role in wound healing hnRNP A2 will also be considered for further analysis. However, this stabilization of collagen prolyl 4-hydroxylase was observed in human fibrosarcoma cells with iron chelation and may not be applicable to monocytes present on the surface of implanted biomaterials. Another heterogeneous ribonuclear protein, hnRNP H2, was identified in cells without AG18. The hnRNP H2 protein is known to positively regulate polyadenylation [45]. Four ribosomal proteins were also identified but because they comprise protein synthesis machinery their unique importance to inflammation is believed to be minimal.

Previous densitometry demonstrated an increased protein phosphorylation level at ~23 kDa for cells on G₃PHSRNG-adsorbed TCPS compared to those on PBS-adsorbed TCPS with 20 μ M AG18. LC/MS identified only one protein from cells on G₃PHSRNG-adsorbed TCPS with 20 μ M AG18. This protein is entered into the NCBI and ExPASy databases as similar to ribosomal protein S8. No significant identifications were recovered from cells on PBS-adsorbed surfaces with 20 μ M AG18 at ~23 kDa. Since this protein appears to be related to protein synthesis its relevance to the host response is currently believed to be minimal. Proteins were also identified from cells on G₃PHSRNG-adsorbed TCPS with 0 and 60 μ M AG18 (Table 8). hnRNP A2 and ribosomal protein L7(a) were identified from cells with 0 and 60 μ M AG18. hnRNP H2 was also identified in U937 on G₃PHSRNG without AG18. The metabotropic glutamate receptor 5B (mGluR5) was identified in cells with 60 μ M AG18; this protein is a member of the group C G-coupled protein receptor family and is predominantly found in the brain to aid in transmitting glutamate signals. The metabotropic glutamate receptor family can be divided into three groups based on the G protein coupled to the receptor. Based on this knowledge mGluR5 identification by LC/MS is likely due to extensive homology with another member of the family coupled to a different G protein that is more physiologically relevant than a receptor found only in brain tissue. Three ribosomal proteins were identified from cells with 60 μ M AG18 while one was identified from cells with 0 μ M AG18 on G₃PHSRNG-adsorbed TCPS.

Discussion

Some of the proteins identified by LC/MS warrant further investigation into the role each plays in cytoskeletal rearrangement or inflammation. A brief background of the role of each protein is discussed that may potentially reveal the role each surface associated peptide plays in directing the monocyte mediated response. Moesin was identified from cells on G₃RGDG-adsorbed TCPS surfaces at ~65/70 kDa and is a member of the Ezrin-Radixin-Moesin (ERM) family that contains a C-terminal domain that can bind F-actin [46,47]. Phosphorylation of threonine 558 (T558) in moesin interrupts the association of the N- and C-terminus activating the protein and enabling C-terminal binding to actin. The N-terminus can then bind to the cytosolic tails of various transmembrane proteins localizing it to the

plasma membrane [48,49]. Moesin is phosphorylated by PKC θ [50], which is a known target of PMA. Moesin is the most predominant ERM protein in leukocytes and appears to regulate the activity of RhoGTPases at the cytosolic side of the plasma membrane especially near activated integrins [47]. This interaction between ERM proteins such as moesin and RhoGTPases [51] enables cellular polarization, migration, the formation of an immunological synapse between a T cell and an APC [52], apoptosis [53] and phagocytosis of foreign material [54]. It is believed that dephosphorylation of ERM proteins following cytokine stimulation enables the leukocyte to polarize and migrate where needed [55]. Moesin can be tyrosine phosphorylated on Y145 [56]; however, the biological activity conferred by this post translational modification is currently unknown. Thus moesin plays a critical role in the functioning of leukocytes and may play a significant role in the host inflammatory response to implanted biomaterials.

Alpha tubulin was identified from U937 adherent to G₃PHSRNG-adsorbed TCPS at ~42 kDa without AG18 (Table 6). Alpha and beta tubulin form a 110 kDa heterodimer and further polymerize to form microtubules (MT). MTs are involved in cellular transport, force generation, cell shape and motility [57,58] and signaling via interaction with G proteins [59]. Interestingly, the spleen tyrosine kinase (Syk), found predominantly in cells of hematopoietic origin such as monocytes, is known to associate with alpha tubulin [60] and act as a kinase for alpha tubulin [61]. Syk deficient cells also show increased motility [62]. The role of tubulin phosphorylation is not clearly understood yet, but tubulin plays an important role in cellular adhesion including monocyte adhesion and activation.

Eef1 α 2 was the only peptide in this experiment directly identified by LC/MS as being tyrosine phosphorylated. Eef1 α 2 was identified at ~42 kDa in cells adherent to G₃RGDG-adsorbed TCPS without AG18 (Table 7). According to Expasy, Eef1 α is responsible for catalyzing the GTP-dependent binding of aminoacyl tRNAs to the A-site on eukaryotic ribosomes. The remaining three isoforms of Eef1 form a heterotrimer, Eef1 $\beta\gamma\delta$, that exchanges GDP for GTP on Eef1 α enabling elongation to proceed [63]. Eef1 α has been observed to crosslink the actin cytoskeleton and is known to anchor both F- and β -actin mRNA to actin [64] via the Rho mediated signaling pathway [65]. Delocalization of β -actin mRNA from cell protrusions leads to the loss of migration and cellular polarity [66]. Eef1 α and β -tubulin also bind to the serine/threonine kinase Akt, which is involved in migration and apoptosis [67]. Thus Eef1 α 2 is implicated in cell attachment and motility. Eef1 α is also a known substrate of protein kinase C (PKC) [68]. U937 cells were stimulated with PMA, which is a potent activator of PKC pathways, likely upregulating Eef1 α phosphorylation [69]. However, phosphorylation of Eef1 α 2 is still of interest because phosphorylation has been shown to increase the rate of elongation [68] in addition to its role in cell adhesion and migration. Another member of the Eef1 α family, Eef1 α 1 was identified from U937 on G₆-adsorbed TCPS with 40 μ M AG18.

PAI-2 was identified at ~42 kDa in U937 adherent to G₃RGDG- and G₆-adsorbed TCPS with 20 and 40 μ M AG18, respectively (Table 7). PAI-2 is a serine protease inhibitor that inhibits the activation of urokinase (also called the urinary plasminogen activator) regulating ECM remodeling and wound healing as well as playing a role in tumor metastasis and invasion [70]. Haile *et al.* and Varro *et al.* both found that PAI-2 significantly reduces the

migration of inflammatory cells [71,72]. Therefore the presence of PAI-2 in cells adherent to biomaterial surfaces is of interest to better understand its role in ECM remodeling as well as its effect upon recruitment and migration of inflammatory cells in response to implanted biomaterials.

Another protein of particular interest is HMG1, which was identified from cells adherent to G₆-adsorbed TCPS without AG18 at ~23 kDa. HMG1 binds DNA, aids in transcription and helps stabilize the nucleosome. HMG1 also can be released into the extracellular milieu to stimulate release of pro-inflammatory cytokines in a manner similar to lipopolysaccharide [73,74] but differing in signaling pathway that involves the TLR2 and TLR4 receptors as well as the receptor for advanced glycation end products (RAGE) [75,76]. Another protein identified from cells on G₆-adsorbed TCPS at ~23 kDa was CARD5, which is a member of the CARD/PYRIN family. CARD5 is involved in apoptosis signaling by interrupting the interaction of caspase-1 with receptor interacting protein-2 (RIP2) thereby regulating nuclear factor kappa B activation. CARD5 also appears to increase cellular processing of pro-IL-1 β [77]. Because IL-1 β is potent mediator of inflammation, CARD5 is a protein of interest that may help shed light on the mechanism linking macrophage adhesion and inflammatory activation.

Conclusions

Using LC/MS we have surveyed the tyrosine phosphoproteome and its co-immunoprecipitation products in response to peptide-adsorbed TCPS. Several adhesion and inflammatory related proteins were identified that will be investigated: moesin, which is known to couple the actin cytoskeleton to the plasma membrane particularly at the leading edge of cell migration and polarized cells; heat shock protein 90-beta, which can bind tubulin and retard polymerization; alpha- and beta-tubulin, which form part of the cellular cytoskeleton; elongation factor 1 α , which localizes mRNA to cell protrusions such as filopodia; beta-actin and vimentin, which are both cytoskeletal proteins; PAI-2, which inhibits inflammatory cell migration and plays a role in ECM remodeling; hnRNP A2, which may aid ECM remodeling through collagen folding. Of particular relevance to inflammation are HMG1, which can be secreted and stimulate release of inflammatory cytokines; CARD5, which increases cellular metabolism of pro-IL-1 β ; gp96, which aids in antigen presentation and CD8⁺ T-cell activation; and hnRNP D0 that initiates transcription of CR2 potentially up regulating macrophage recognition of complement opsonins. Future work will focus on the role these proteins play in the inflammatory response to implanted biomaterials.

Acknowledgments

This work was funded in part by NIH HL077825 & EB6613. The author would like to acknowledge Jim Brown for help with LC/MS, James Dowell for help with FT-MS and MALDI and Greg Wiepz for assistance troubleshooting Western blots.

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Fig 1.

Western Blot of tyrosine phosphorylated PMA⁺ U937 cells adherent on TCPS. 550μg of raw cell lysates were immunoprecipitated using P-Tyr100 anti-phosphotyrosine protein antibodies and run on a Tris-HCl SDS-PAGE (4% stacking, 10% resolving). Proteins were transferred for 3 hr at 74V and 4°C onto a PVDF membrane, blocked with 0.25% gelatin, and exposed to PY20 and 4G10 (1:1000) antibodies overnight. The membrane was then exposed to anti-mouse IgG HRP-conjugated antibodies (1:8000) for 1 hr and LumiGLO for 10 min. Exposure time was 10 min.

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Differential expression of various proteins in adherent U937 cells on peptide modified TCPS treated with or without AG18 based on densitometric measurements^a

Table 1

MW (kDa)	Ligand Adsorbed Surface	[AG18] (μM)	Trend	Ligand Adsorbed	[AG18] (μM)
~200	G ₃ RGDG	20	<	G ₃ RGDG	0
~160	G ₃ PHSRNG	40	>	G ₃ PHSRNG	0
~110	G ₃ RGDG	80	>	G ₃ RGDG	0
~70	G ₃ RGDG	20	<		
		60	<	G ₃ RGDG	0
		80	>		
~65	G ₃ RGDG	20	<	PBS	20
~52	G ₃ PHSRNG	40	<	G ₃ PHSRNG	0
		60			
~42	G ₃ RGDG	20	<	G ₃ RGDG	0
	G ₆	40	<	PBS	40
	G ₆	60	>	G ₆	0
~23	G ₃ RGDG	80	>	G ₃ RGDG	0
	G ₃ PHSRNG	20	>	PBS	20
	G ₃ PHSRNG	G ₆	>	G ₃ PHSRNG	0

> is up regulated

< is down regulated

^aDensitometry performed on SDS-PAGE Tris-HCl gels stained with Coomassie Brilliant Blue [17]

Table 2Raw LC/MS data for U937 cells in suspension (PMA⁻)^a

Protein ID	Protein MW	Accession Number	Peptide Identified Sequence	Mascot Score
PREDICTED: similar to myocyte nuclear factor	264,161	gi 51475870	AVSLKALPDFSNVEIK	45
			MIAPILDEIADEYQGK	69
Ig fragment	13,444	gi 42543181	IIHLTDDSFDTDLK	61
			MIAPILDEIADEYQGK	56
unnamed protein product	26,759	gi 37460	VLEGNEQFINAAK	67
transketolase	67,751	gi 37267	TSRPENAIYNNEDFQVGQAK	62
			SVPTSTVFYPSDGVATEK	46
muscle specific enolase	46,957	gi 31167	AAVPSGASTGIYEALRL	66
human elongation factor 2	95,277	gi 31108	ARFPDGLAEDIDKGEVSAR	56
CLTC protein	187,771	gi 30353925	LLYNNVSNFGR	46
			GSLGGGFSSGGFSGGSFSR	125
			GSLGGGFSSGGFSGGSFSR	121
			GSLGGGFSSGGFSGGSFSR	117
			GSLGGGFSSGGFSGGSFSR	116
			GSLGGGFSSGGFSGGSFSR	107
			GSLGGGFSSGGFSGGSFSR	104
			GSLGGGFSSGGFSGGSFSR	100
			GSLGGGFSSGGFSGGSFSR	97
			ELTTEIDNNIEQISSYK	94
unnamed protein product	59,492	gi 28317	ELTTEIDNNIEQISSYK	85
			ALEESNYELEGK	85
			ELTTEIDNNIEQISSYK	81
			ELTTEIDNNIEQISSYK	74
			LENEIQTYR	71
			GSLGGGFSSGGFSGGSFSR	70
			ELTTEIDNNIEQISSYK	70
			VLDELTLTK	65
			QSLEASLAETGR	58
			GSLGGGFSSGGFSGGSFSR	58
			GSLGGGFSSGGFSGGSFSR	48
tubulin, beta polypeptide	49,640	gi 18088719	LAVNMVPPFR	55
unnamed protein product	76,104	gi 16552091	MFCYDPSHNMWLKCVSLK	48
ATP synthase, H⁺ transporting, mitochondrial F1 complex, alpha subunit	59,772	gi 15030240	ILGADTSVDLEETGR	95
similar to ribophorin I	64,542	gi 14124942	TILPAAAQDVYYR	50
			GVVDSDELPLNISR	72
heat shock protein HSP 90-alpha (HSP 86)	84,621	gi 123678	GVVDSDELPLNISR	71
			ADLINNLGTIAK	45
C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase)	101,495	gi 115206	YVVVTGITPTPLGEGK	51

^aProteins in bold are those identified after data refining

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LC/MS data refined for significance and relevance for adherent U937 proteins on G₃RGDG-adsorbed TCPS treated with various AG18 concentrations

Table 3

Sample MW (kDa)	AG18 (μ M)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	122
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	68
					APDFVFYAPR	58
		interleukin enhancer-binding factor 3	48,742	gi 1770456	IGFPWSEIR	53
					VLGETLSVNDPPDVLDR	65
20		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	117
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	84
					ALTSELANAR	65
					APDFVFYAPR	58
					EGILNDDIYCPPETAVILLASYAVQSK	55
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	51
					IAQDLEXYGVNYSIK	60
					NFILDQTNVSAQAQR	94
					GVVDSIDLPLNISR	55
					INEPTAAAIAYGLDK	51
					TQEQLALEMAELTAR	122
60		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
70		moesin (Membrane organizing spike protein)	67,778	gi 5419633	KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
70		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
60		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
70		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
60		moesin (Membrane organizing spike protein)	67,778	gi 5419633	TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
					KTQEQLALEMAELTAR	46
					VLGETLSVNDPPDVLDR	80
					VLGETLSVNDPPDVLDR	52
					FGGNGPELLTQMVS K	53
					NFILDQTNVSAQAQR	98
					GVVDSIDLPLNISR	81
					TQEQLALEMAELTAR	122
					TQEQLALEMAELTAR	118
					KTQEQLALEMAELTAR	72
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	66

Sample MW (kDa)	AG18 (μ M)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
		heat shock 70 kDa protein 1L	70,355	gi 188492	GVVDESDLPLNISR	55
					IIINEPTAAAIAYGLDK	87
	80	moesin (Membrane organizing spike protein)	67,778	gi 5419633	APDFVFYAPR	54
					SYELPDGQVITIGNER	78
		beta actin variant	41,738	gi 62897625	DSYVGDEAQSK	74
					DLYANTVLSGGTTMYPGIADR	68
		interleukin enhancer binding factor 2	44,669	gi 532313	GYSFTTTAER	55
					VAPEEHPVLLTEAPLNPK	48
		phosphoglycerate kinase	44,586	gi 41350401	ILPTLEAVAALGNK	69
					VLQSALAAIR	68
					VLNNMEIGTSLFDEEGAK	71
					VLPGVDAISNI	47
		erbB3 binding protein EBP1	38,035	gi 4099506	TAENATSGETLEENEAGD	80
					ITSGPEEPDLYK	73
	0				SEMEVQDAELK	66
					TIIQNPTDQQK	65
					ALLQSSASR	46
					IFVGGGLSPDTPEEK	82
	42	heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0)	35,801	gi 181914	NNASTDYDLSDK	60
					GIYAYGFEKPSAIQQR	54
		ribosomal protein L3	45,440	gi 337580	IIEDQQESLNK	63
					GLVYETSVLDPDEGIR	52
		eukaryotic initiation factor 4A-II	46,365	gi 485388	LSSPAVINSR	46
					FETTKYYITIIDAPGHR	48
		La 4.1 protein	33,737	gi 34209	DLYANTVLSGGTTMYPGIADR	82
					SYELPDGQVITIGNER	79
		citrate synthase	51,673	gi 3288815	SYELPDGQVITIGNER	75
					DLYANTVLSGGTTMYPGIADR	64
		PRSS2 protein	25,671	gi 20988034	VAPEEHPVLLTEAPLNPK	57
		Eef1a2 ^d	50,438	gi 11137514		
	20	beta actin variant	41,738	gi 62897625		

Sample MW (kDa)	AG18 (μ M)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
					VAPPEHPVLLTEAPLNPK	51
					ILPTLEAVAAALGNK	77
		interleukin enhancer binding factor 2	44,669	gi 532313	ILPTLEAVAAALGNK	58
		phosphoglycerate kinase	44,586	gi 41350401	LGDVYVNDAFGTAHR	46
					ITSGPEPDLK	83
		erbB3 binding protein EBP1	38,035	gi 4099506	ITSGPEPDLK	52
					IFVGGGLSPDTPEEK	74
		heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0)	35,801	gi 181914	IFVGGGLSPDTPEEK	74
					AHLMEIQVNGGTVAEK	47
		ribosomal protein L3	46,091	gi 14250148	ILLAELEQLK	68
					ILLAELEQLK	49
		plasminogen activator inhibitor type 2 precursor	46,597	gi 189563	GSYPDAILQAQAADK	64

^aTyrosine phosphorylated

Table 4

Proteins identified from ~100kDa protein band

Proteins identified from U937 on G ₃ RGDG-adsorbed TCPS with 0 or 80μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	Staphylococcal nuclease domain containing protein 1	99,628	gi 799177	NLPGLVQEGEPFSEEATLFTK	52
	glycoprotein 96 (GP96)	65,912	gi 62088648	GVVDSDDDLPLNVSR	68
	ATP-dependent RNA helicase A (DDX)	141,984	gi 1082769	ELDALDANDELTPPLGR	60
80	N/D ^a	---	---	---	---

^aN/D = None detected

Table 5

Proteins identified from ~70/65kDa protein band^a

Proteins identified from U937 on G ₃ RGDG-adsorbed TCPS with 0, 20, 60 or 80 μ M AG18					
[AG18]	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	122
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	68
				APDFVFYAPR	58
				IGFPWSEIR	53
				VLGETLSVNDPPDVLDLR	65
20	moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	122
				TQEQLALEMAELTAR	117
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	84
				ALTSELANAR	65
				APDFVFYAPR	58
				EGILNDDIYCPPETAVLLASYAVQSK	55
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	51
				IAQDLEXYGVNYFSIK	60
				NFILDQTNVSAAAQR	94
				GVVDSIEDLPLNISR	55
60	moesin (Membrane organizing spike protein)	67,778	gi 188492	IINEPTAAAIAYGLDK	51
				TQEQLALEMAELTAR	122
				TQEQLALEMAELTAR	118
				KTQEQLALEMAELTAR	72
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	66
				KTQEQLALEMAELTAR	46
				VLGETLSVNDPPDVLDLR	80
				VLGETLSVNDPPDVLDLR	52
				FGGGINPELLTQMVSK	53
	interleukin enhancer-binding factor 3	48,742	gi 1770456	trifunctional enzyme alpha subunit, mitochondrial (78 kDa gastrin-binding protein)	

Proteins identified from U937 on G ₃ RGDG-adsorbed TCPS with 0, 20, 60 or 80 μM AG18					
[AG18]	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
	scaffold attachment factor A	90,423	gi 3202000	NFILDQTNVSAAQQR	98
	heat shock protein HSP 90-beta	83,242	gi 9082289	GVVDSDDLPLNISR	81
				GVVDSDDLPLNISR	55
	heat shock 70 kDa protein 1L	70,355	gi 188492	IINEPTAAAIAYGLDK	87
80	moesin (Membrane organizing spike protein)	67,778	gi 5419633	APDFVFYAPR	54

Proteins identified from U937 on G ₃ RGDG- or PBS-adsorbed TCPS with 20μM AG18						
Surface	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score	
G ₃ RGDG	moesin (Membrane organizing spike protein)	67,778	gi 5419633	TQEQLALEMAELTAR	122	
				TQEQLALEMAELTAR	117	
				TAMSTPHVAEPAENEQDEQDENGAEASADLR	84	
				ALTSELANAR	65	
				APDFVFYAPR	58	
					EGILNDDIYCPPETAVLLASYAVQSK	55
					TAMSTPHVAEPAENEQDEQDENGAEASADLR	51
					IAQDLEXYGVNYFSIK	60
					gi 8569618	
					gi 3202000	
	scaffold attachment factor A	90,423	gi 3202000	NFILDQTNVSAQAQR	94	
	heat shock protein HSP 90-beta	83,242	gi 306891	GVVDSEDLPLNISR	55	
	heat shock 70 kDa protein 1L	70,355	gi 188492	IINEPTAAAIAYGLDK	51	
PBS	titin ^b	2,991,589	gi 1212992	LEPPELILDANMAR	46	

^aCommon proteins are highlighted in bold text

^bTyrosine phosphorylated

Table 6

Proteins identified from ~52kDa protein band^a

Proteins identified from U937 on G ₃ PHSRNG-adsorbed TCPS with 0, 40 or 60 μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	serum albumin	29,229	gi 6650826	VPQVSTPTLVEVSR	55
				TIGGGDDSFNTFFSETGAGK	82
	alpha-tubulin ^b	50,120	gi 340021	AVFVDLEPTVIDEVR	53
				IHFPLATYAPVISAEK	51
40	nucleolin (Protein C23)	76,298	gi 128841	FGYVDFESAEDLEK	52
60	tubulin 5-beta	49,599	gi 35959	YLTVAAVFR	56
	growth regulated nuclear 68 protein	66,881	gi 226021	APILLATDVAAR	52

^aCommon proteins are highlighted in bold text

^bTyrosine phosphorylated

Table 7

Proteins identified from ~42kDa protein band^a

AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	beta actin variant	41,738	gi 62897625	SYELPDGQVITIGNER	78
				DSYVGDEAQSK	74
				DLXANTVLSGGTTMYPGIADR	68
				GYSFTTTAER	55
	interleukin enhancer binding factor 2	44,669	gi 532313	VAPEEHPVLLTEAPLNPK	48
				ILPTLEAVAAIGNK	69
				VLQSALAIR	68
				VLNNMEIGTSLFDEGAK	71
	phosphoglycerate kinase	44,586	gi 41350401	VLPGVDAISNI	47
				TAENATSGETLEENEAGD	80
				ITSGPPEPDLYK	73
				SEMEVQDAELK	66
	erbB3 binding protein EBP1	38,035	gi 4099506	THQNPTDQOK	65
				ALQSSASR	46
				IFVGGGLSPDTPEEK	82
				NNASTDYDLSDK	60
	heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0)	35,801	gi 181914	GIYAYGFEKPSAIQQR	54
				IIEDQQLSLNK	63
				GLVYETSVLDPDEGIR	52
				LSSPAVINSR	46
	ribosomal protein L3	45,440	gi 337580	FETTKYYITIIDAPGHR	48
	eukaryotic initiation factor 4A-II	46,365	gi 485388		
	La 4.1 protein	33,737	gi 34209		
	citrate synthase	51,673	gi 3288815		
	PRSS2 protein	25,671	gi 20988034		
	Eef1a2 ^b	50,438	gi 11137514		
20	beta actin variant	41,738	gi 62897625	DLXANTVLSGGTTMYPGIADR	82
				SYELPDGQVITIGNER	79
				SYELPDGQVITIGNER	75
				DLXANTVLSGGTTMYPGIADR	64
				VAPEEHPVLLTEAPLNPK	57

Proteins identified from U937 on G ₃ RGDG-adsorbed TCPS with 0 or 20μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
				VAPEHPVLLTEAPLNPK	51
	interleukin enhancer binding factor 2	44,669	gi 5322313	ILPTLEAAALGNK	77
				ILPTLEAAALGNK	58
	phosphoglycerate kinase	44,586	gi 41350401	LGDVYVNDAFGTAHR	46
	erbB3 binding protein EBP1	38,035	gi 4099506	ITSGPFEPDLYK	83
				ITSGPFEPDLYK	52
	heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0)	35,801	gi 181914	IFVGGLSPTDTPPEEK	74
				IFVGGLSPTDTPPEEK	74
	Ribosomal protein L3	46,091	gi 4250148	AHLMEIQVNGGTVAEK	47
	vimentin	53,653	gi 37852	ILLAELEQLK	68
				ILLAELEQLK	49
	plasminogen activator inhibitor type 2	46,597	gi 189563	GSYPDAILQAQAADK	64

Proteins identified from U937 on G ₆ - or PBS- adsorbed TCPS with 40μM AG18					
Surface	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
	48 kda histamine receptor subunit peptide 4 {internal fragment}	3,021	gi 998467	SYELPDGQVITIGNER	53
G6	Eukaryotic translation elongation factor 1 alpha 1	50,091	gi 48734966	YYVTIDAPGHR	73
	plasminogen activator inhibitor 2	46,615	gi 15214956	SLSSAINASTGNYLLESVNIK	70
PBS	N/D ^c	---	---	---	---

^aCommon proteins are highlighted in bold text

^bTyrosine phosphorylated

^cN/D = None Detected

Table 8

Comparisons of ~23kDa protein band^a

Proteins identified from U937 on G ₆ -adsorbed TCPS with 0 or 60μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	myeloid cell nuclear differentiation antigen	45,807	gi 55665063	EASSVSDFNQNFEVFN	57
	high mobility group box 1 (HMG-1)	24,968	gi 478813	MSSYAFFVQTCR	49
	alkyl-N-purine-DNA glycosylase	25,549	gi 178654	IVETEAYLGPEDAAHSR	48
	caspase recruitment domain protein 5 (CARD5)	21,613	gi 14488059	GALLSMDALDLTDK	74
60	N/D ^b	---	---	ESQSYLVEDLER	66
Proteins identified from U937 on G ₃ RGDG-adsorbed TCPS with 0 or 80μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	hnRNP protein A2 (heterogeneous ribonuclear protein A2)	35,984	gi 500638	LFIGGLSFETTESLR	89
				LFIGGLSFETTESLR	76
				IDTIEITDR	55
				IDTIEITDR	55
	heterogeneous nuclear ribonucleoprotein H2 (H')	49,232	gi 6065880	STGEAFVQFASQEIAEK	97
				STGEAFVQFASQEIAEK	89
				FNADFEFDMVAKK	88
				LATQLTGPVMPVR	47
	60S ribosomal protein L10-like	24,503	gi 42542826	LATQLTGPVMPVR	45
	60S ribosomal protein L13	24,247	gi 13436332	LATQLTGPVMPVR	45
80	hnRNP protein A2 (heterogeneous ribonuclear protein A2)	35,984	gi 500638	IDTIEITDR	48
				ILTFDQLALDSPK	74
				ILTFDQLALDSPK	62
				TAVVVGTTDDVR	48
				VLNSYWVGEDSTYK	64

Proteins identified from U937 on G ₃ PHSRNG-adsorbed TCPS with 0, 20 or 60μM AG18					
AG18 (μM)	Protein	Molecular Wt. (Da)	Accession Number	Peptide Identified	Mascot Score
0	hnRNP protein A2 (heterogeneous ribonuclear protein A2)	35,984	gi 500638	LFIGGLSFETTESLR	55
				IDTIEHTDR	48
				IDTIEHTDR	48
	ribosomal protein L7 (a)	29,164	gi 35903	IALTDNALIAR	69
				STGEAFVQFASQEIAEK	96
				STGEAFVQFASQEIAEK	85
20	60S ribosomal protein L13	24,247	gi 13436332	STESLQANVQR	75
				ISSLLEEQFQGGK	47
				ISSLLEEQFQGGK	47
60	hnRNP protein A2 (heterogeneous ribonuclear protein A2)	35,984	gi 500638	LFIGGLSFETTESLR	102
				IDTIEHTDR	61
				IDTIEHTDR	57
	ribosomal protein L7 (a)	29,164	gi 35903	IALTDNALIAR	72
				IVEPYIAWGYPNLK	62
				IALTDNALIAR	54
	metabotropic glutamate receptor 5 B ribosomal protein L18	132,495	gi 625527	SSSSQGSLMEQISSVVTR	46
				ILTFDQLALDSPK	66
				LVAIVDVIDQNR	68
	60S ribosomal protein L14 (CAG-ISL 7)	24,145	gi 12006350	VLNSYWVGEDSTYK	89

^aCommon proteins are highlighted in bold text

^bN/D = None detected