Published in final edited form as:

Exp Neurol. 2017 January ; 287(Pt 1): 14–20. doi:10.1016/j.expneurol.2016.10.011.

Identification of KRT16 as a Target of an Autoantibody Response in Complex Regional Pain Syndrome

Maral Tajerian, PhD^{1,2,3,*}, Victor Hung, BSc^{1,3}, Hamda Khan, BSc^{1,3}, Lauren J Lahey, BSc^{1,3,4}, Yuan Sun, PhD^{1,2,3}, Frank Birklein, MD⁵, Heidrun H. Krämer, PhD⁶, William H Robinson, PhD^{1,3,4}, Wade S Kingery, MD^{1,3,7}, and J David Clark, MD, PhD^{1,2,3}

¹Veterans Affairs Palo Alto Health Care System Palo Alto, CA, USA

²Department of Anesthesiology, Stanford University School of Medicine, Stanford, CA, USA

³Palo Alto Veterans Institute for Research, Palo Alto, CA, USA

⁴Division of Immunology and Rheumatology, Stanford University, Stanford, CA, USA

⁵Department of Neurology, University Medical Center, Mainz, Germany

⁶Department of Neurology, Justus Liebig University, Giessen, Germany

⁷Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA

Abstract

Objective—Using a mouse model of complex regional pain syndrome (CRPS), our goal was to identify autoantigens in the skin of the affected limb.

Methods—A CRPS-like state was induced using the tibia fracture/cast immobilization model. Three weeks after fracture, hindpaw skin was homogenized, run on 2-d gels, and probed by sera from fracture and control mice. Spots of interest were analyzed by liquid chromatography-mass spectroscopy (LC-MS) and the list of targets validated by examining their abundance and subcellular localization. In order to measure the autoantigenicity of selected protein targets, we quantified the binding of IgM in control and fracture mice sera, as well as in control and CRPS human sera, to the recombinant protein.

*Correspondence should be addressed to Maral Tajerian, PhD, Anesthesia Service Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304, Tel: 650-493-5000 ext. 68825, Fax: 650-852-3423, maral@stanford.edu.

COMPETING INTERESTS

This study was supported by the National Institutes of Health (Bethesda, Maryland; grant no. NS072168 to Drs. Kingery and Clark). Dr. Tajerian was supported by NIH Grant 5T32DA035165-02. Dr. Birklein was supported by the EU, FP7 ncRNAPain, grant 602133, the Deutsche Forschungsgemeinschaft Bi579/8-1 and the Dietmar-Hopp Foundation. The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization: M.T., W.S.K., & J.D.C.; Methodology: M.T., W.H.R., F.B., H.H.K., & J.D.C.; Formal analysis: M.T.; Investigation: M.T., V.H., L.X., & H.K.; Writing-original draft: M.T.; Writing- Review and Editing: M.T., V.H., L.X., H.K., Y.S., F.B., H.H.K., W.H.R., W.S.K., & J.D.C.; Supervision: W.S.K., & J.D.C.; Project administration: W.S.K., & J.D.C.; Funding acquisition: F.B., W.S.K. & J.D.C.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Results—We show unique binding between fracture skin extracts and fracture sera, suggesting the presence of auto-antigens. LC-MS analysis provided us a list of potential targets, some of which were upregulated after fracture (KRT16, eEF1a1, and PRPH), while others showed subcellular-redistribution and increased membrane localization (ANXA2 and ENO3). No changes in protein citrullination or carbamylation were observed. In addition to increased abundance, KRT16 demonstrated autoantigenicity, since sera from both fracture mice and CRPS patients showed increased autoantibody binding to recombinant kRT16 protein.

Conclusions—Pursuing autoimmune contributions to CRPS provides a novel approach to understanding the condition and may allow the development of mechanism-based therapies. The identification of autoantibodies against KRT16 as a biomarker in mice and in humans is a critical step towards these goals, and towards redefining CRPS as having an autoimmune etiology.

Keywords

Complex Regional Pain Syndrome; Autoimmunity; Antigen Identification; Keratin 16; Skin; Chronic Pain; Animal models of Pain

INTRODUCTION

Complex Regional Pain Syndrome (CRPS) is a chronic and debilitating condition comprised of a host of seemingly unrelated signs and symptoms including bone demineralization, skin growth changes, vascular dysfunction and pain. There are an estimated 50,000 new cases in the US each year, and in most cases, the symptoms are limited to a single extremity (de Mos et al., 2007). Despite the fact that CRPS was described over a century ago, we still lack a comprehensive mechanistic understanding of the syndrome, thus often preventing effective treatment or cure.

Historically, CRPS research has focused on areas such as sympathetic nervous system dysfunction, neurogenic inflammation, and peripheral and spinal nociceptive sensitization and brain reorganization as potential mechanisms (Tajerian and Clark, 2016). More recently, however, a collection of clinical and preclinical observations has suggested an autoimmune etiology. Agonistic anti-sympathetic autoantibodies have been shown in a subgroup of CRPS patients (Kohr et al., 2011) and, clinically, low dose intravenous immunoglobulin controlled CRPS symptoms in some patients (Goebel et al., 2010). In an animal model of mild tissue trauma, IgG from CRPS patients worsened the existing nociceptive sensitization (Tekus et al., 2014). Moreover, in a well-characterized mouse tibia fracture/cast model of CRPS, we reported attenuated CRPS-like symptoms in subjects treated with anti-CD20 and in mu-MT mice lacking mature B cells (Li et al., 2014). Finally, we observed increased IgM deposition in the skin of the affected hindpaw not explained by vascular leak of immune complexes, thereby suggesting the presence of auto-antigens in skin tissue, which could be responsible for regionalized trophic changes, allodynia, and other signs of the syndrome (Li et al., 2014).

In the current study we employed the mouse tibia fracture/cast immobilization model to address the critical issue of identifying the target antigen(s) of potentially CRPS-related autoantibodies in skin. The identification of such proteins would both support the

autoimmune hypothesis and provide biomarkers useful in following disease activity and responses to treatments.

MATERIALS AND METHODS

Animals

Male C57/B6J mice aged 12–14 weeks (Jackson Labs) were housed in groups of 4 (12-hr light/dark cycle, ambient temperature of $22 \pm 3^\circ\text{C}$, with food and water available *ad libitum*). All animal procedures and experimental designs were approved by the *Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee* and followed the “animal subjects” guidelines of the *International Association for the Study of Pain*.

Limb fracture and cast immobilization

Mice were anesthetized with 1.5% isoflurane and underwent a distal tibial fracture in the right hind limb. Immediately following fracture and while still under anesthesia, a cast was placed around the injured hindlimb as previously described (Li et al., 2014). Three weeks after surgery, the mice were anesthetized (isoflurane) and the casts were removed.

Sample preparation

All analysis was done blind to the identity and experimental condition of the subject or tissue.

Mice—Mice were deeply anesthetized using isoflurane, and blood was collected via cardiac puncture. The ventral hindpaw skin was then dissected and stored -20°C . Following clotting at room temperature, blood was centrifuged at $10,000\times g$ and the supernatant serum was collected.

Total protein from skin and serum samples—Skin was homogenized using T-PER Protein Extraction Reagent (Thermo Scientific) with proteinase and phosphatase inhibitors (Roche Applied Science), centrifuged at $12,000\times g$, and supernatant fractions were collected.

Protein extracts from subcellular fractions—Cytoplasmic, membrane, nuclear soluble, chromatin-bound, and cytoskeletal protein extracts from skin samples were separated using a subcellular fractionation kit (Thermo Scientific).

Human sera—The study protocol was approved by the institutional review board at the Justus Liebig University (Gießen, Germany). Sera were collected from CRPS patients, and 5 serum samples were chosen at random to represent both acute and chronic stages of the syndrome (see Table 1 for details; all patients fulfilled the CRPS research criteria). Commercially available normal human serum was used as control (Sigma).

Gel electrophoresis

2-Dimensional—200 μg of protein was precipitated in acetone, centrifuged at 7800g, and the pellet was rehydrated and incubated with the IPG strips (PH 5–8, 11cm; BioRad). One-dimensional isoelectric focusing was carried out using an IEF protean cell (Biorad). The IPG

strips were then equilibrated and subjected to two-dimensional separation according to standard protocols.

1-Dimensional (vertical)—Vertical gel electrophoresis was performed (in preparation for Western blotting) according to standard sodium dodecyl sulfate polyacrylamide gel electrophoresis procedures.

Mass spectrometry

In-Gel Digestion—Per standard procedures, gel bands were washed with MilliQ water, destained, dehydrated, and dried in a speed-vac (Thermo Savant). The gel pieces were rehydrated and incubated at 56°C for 20min. After discarding the supernatant, the gel pieces were incubated in 15mM iodoacetamide, washed with water and dehydrated and dried as before. The dried gel pieces were rehydrated and the reaction mixture was then acidified and desalted. Peptides were eluted and lyophilized in a SpeedVac (Thermo Savant).

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS)—Each digestion mixture was analyzed by UHPLC-MS/MS. LC was performed on an Easy-nLC 1000 UHPLC system (Thermo). The LC was interfaced to a quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher) via nano-electrospray ionization using a source with an integrated column heater (Thermo Easy Spray source). The column was heated to 50°C. An electrospray voltage of 2.2kV was applied. Tandem mass spectra from the top 20 ions in the full scan from 400 – 1200m/z were acquired. Dynamic exclusion was set to 15s, singly-charged ions were excluded, isolation width was set to 1.6Da, full MS resolution to 70,000 and MS/MS resolution to 17,500. Normalized collision energy was set to 25, automatic gain control to 2e5, max fill MS to 20ms, max fill MS/MS to 60ms and the underfill ratio to 0.1%.

Data Processing and Library Searching—MGF files were searched using X!Tandem using both the native and k-score scoring algorithms and by OMSSA. XML output files were parsed and non-redundant protein sets determined using Proteome Cluster. MS1-based features were detected and peptide peak areas were calculated using OpenMS. Proteins considered in further analyses were required to have 1 or more unique peptides across the analyzed samples with Evalue <0.01. Ingenuity Pathway analysis was used to interrogate the list of targets generated by LC-MS for involvement in known autoimmune pathways.

Protein visualization and quantification

Antibodies used in this report are listed in Supplemental Table 2.

2-Dimensional blots—To visualize serum binding to target antigens in skin, 2d gels of total skin protein were transferred into a membrane and incubated overnight with fracture/cast or control mouse serum or control or CRPS human serum (1:500 dilution) followed by incubation with the appropriate anti-IgM secondary antibody. To visualize total protein content on the blot, Coomassie staining was used.

Western blots—Membranes were stained by overnight incubation with the primary antibody followed by 1-hr incubation with the appropriate secondary antibody. GAPDH was used as an internal control.

Dot blots—2 μ l of human KRT16 recombinant protein (Novus) was applied to a nitrocellulose membrane and incubated for 1hr with fracture or control mouse serum or CRPS or control human serum (1:500 dilution) followed by 1-hr incubation with the appropriate anti-IgM secondary antibody.

The signals were detected using Odyssey (LI-COR Biosciences) and quantified using Image Studio Lite© (LI-COR Biosciences).

Anti-citrullinated protein antibody arrays

In-house arrays were carried out using mouse sera as previously described (Sohn et al., 2015).

ELISA

Mouse anti-cyclic citrullinated peptide IgM in mouse sera (Alpha Diagnostic International) and mouse anti-carbamylated proteins in hindpaw skin (Cell Biolabs Inc.) were measured using standard ELISA procedures.

RNA isolation and qPCR

Total RNA was isolated from hindpaw skin using the RNeasy Mini Kit (Qiagen). Real-time PCR was performed in an ABI prism 7900HT system (Applied Biosystems). Data were normalized to 18S mRNA expression. The following *krt16* primers were used (SABiosciences): LEFT: AGGCCTGGTTCCTGAGAAAG; RIGHT: TTTCATGCTGAGCTGGGACT.

Statistical analysis

All data are expressed as mean $\pm$ SEM. To evaluate the overall differences between the fracture and control groups, 2-tailed student's t-test was used. Significance was set at $p < 0.05$ (Prism 7.0; GraphPad Software, La Jolla, CA). Sample sizes are indicated in the figure legends.

RESULTS

Unique antigen-antibody binding of fracture serum to fracture skin proteins

In order to identify the presence of specific antigens involved in CRPS-related autoimmunity in the hindpaw skin, proteins from fracture and control mice were separated using 2-d gel electrophoresis (Fig. 1A) and incubated with sera from fracture and control mice. Unique binding was observed only when fracture serum was added to fracture skin (Fig. 1B, 3rd row, right). We observed a similar pattern when probing fracture mouse skin with human CRPS patient sera (Fig. 1B, 5th row, right). To determine the identity of the observed spots, proteins were harvested from additional Coomassie stained gels and analyzed by LC-MS (Fig. 1B, bottom row).

The list of proteins identified using this approach that demonstrated increased expression in fracture skin is shown in Table 3. The LC-MS protein data were subsequently processed using Ingenuity Pathway Analysis (IPA) employing a biased search for proteins with known autoimmune involvement such as psoriasis and rheumatoid arthritis (Fig. 1C).

Characterization of the identified candidate autoantigens

In order to validate selected proteins as autoantigens and investigate the mechanisms of autoantigenicity in our list of targets (chosen on the basis of autoimmune involvement [Fig. 1C] and LCMS data [Table 3]), we carried out a series of experiments to measure antigen abundance and changes in subcellular localization as well as post-translational modifications that could incite an autoimmune response.

Of the proteins the levels of which were measured in the fracture and control groups (KRT16, eEF1a1, PRPH, ANXA2, ENO3, ALDOA, AKT2, Serpinb1), only eEF1a1, PRPH (Fig. 2A) and KRT16 (Fig. 3B) showed increased protein abundance after fracture, while ANXA2 and ENO3 showed changes in their subcellular location where they were shown to localize in the membrane fraction of the cell in the fracture group only (Fig. 2B, insets). None of the other targets analyzed showed cellular re-distribution.

Another potential mechanism of autoimmunity is the post-translational modification of native proteins, including protein citrullination (arginine to citrulline) and carbamylation (cyanate binding to primary amino or thiol groups). We therefore measured total levels of carbamylated proteins in the hindpaw skin and found no differences between the two groups (Fig. 2C, left). Additionally, we found no differences in the levels of anti-citrullinated protein antibodies in the sera of mice belonging to the two groups (Fig. 2C, right). Similarly, an in-house protocol that measures serum binding to an antigen panel for rheumatoid arthritis showed negligible immunoglobulin binding for the majority of antigens, with non-specific binding to native and citrullinated forms of select antigens observed similarly between groups (Fig. 2D). Finally, our LC-MS analysis showed no significant differences in post-translational modifications in proteins from fracture skin (data not shown).

Anti KRT16: a serum biomarker in fracture mice and CRPS patients

In addition to the potential targets shown in Fig. 2, we measured levels of KRT16, our top target from the LCMS analysis. This choice was further bolstered by the overall abundance of keratin species in the LCMS analysis as well as their known involvement in other autoimmune conditions such as rheumatoid arthritis and psoriasis (Fig. 1C).

We show increased KRT16 both at the mRNA (Fig 3A) and protein (Fig. 3B) levels in the mouse skin 3w following fracture. In order to confirm the auto-immunoreactivity of KRT16, dot blot analysis was performed using recombinant KRT16 and probed with mouse and human sera. Our data show increased binding of fracture (mouse, Fig. 3C) and CRPS (human, Fig. 3D, E) sera to KRT16. Similar dot blots were also conducted for PRPH recombinant protein with no differences between the injured and control groups in either species (data not shown).

No correlation was observed between KRT16 immunoreactivity and CRPS duration in patients (Fig. 3F).

DISCUSSION

In the present study, we provide further evidence for an autoimmune origin for CRPS, a mechanism that could account for the multitude of seemingly disparate signs and symptoms associated with it. Additionally, we propose anti-KRT16 as a potential biomarker both in our murine model and in CRPS patients, based both on serum binding experiments and the identification of KRT16 through exploratory immunoblotting experiments. The data presented regarding CRPS-related autoantigen identification complements prior findings of surface-binding autoantibodies against autonomic neurons (Kohr et al., 2009) as well as the presence of autoantibodies binding to and activating the M-2 muscarinic and the β 2-adrenergic receptors (Kohr et al., 2011) in some CRPS patients.

The mechanistic link between limb trauma and autoimmunity remains unclear, but dysregulation of the immune system is probably crucial for the pathogenesis of post-traumatic autoimmunity. For instance, lowered thresholds of T and B lymphocyte tolerance and activation could account for increased ability to bind “self” antigens (Kil and Hendriks, 2013). Similarly, dead or dying cells – presumably abundant after tissue trauma- could be a source of autoantigens (Mahajan et al., 2016), particularly in cases where apoptotic clearance of these cells is decreased. Furthermore, autoimmunity could be the result of changes in the localization of the antigen within the cell. In our model, we found ANXA2 and ENO3 in the membrane fraction of the cell in the fracture group only, which could make it more accessible to various immunoglobulins. In Sjogrens syndrome, for example, major epithelial autoantigens display cellular re-distribution into the cell membrane, therefore making them more available to auto-antibodies (Katsiogiannis et al., 2015). Re-localization of autoantigens can even occur extracellularly; The nuclear DNA sensor IFI16, elevated in various autoimmune conditions, has been shown to re-localize from the nucleus to the extracellular milieu (Sporn and Vilcek, 1996). Finally, post-translational modifications of native proteins have been known to be implicated in pain-related autoimmunity in diseases such as rheumatoid arthritis, where an increase in anti-citrullinated protein antibodies in patient sera has been observed and thought to participate in the initiation and propagation of synovial inflammation (Sohn et al., 2015). However, we found no changes in anti-citrullinated protein antibody levels in fracture mouse sera and no differences in the binding of sera to select citrullinated and native proteins; nor did we observe changes in the levels of carbamylated proteins in fracture mouse skin samples.

KRT16 was both elevated in abundance in fracture mouse skin and appeared to be reactive with IgM in sera from fracture mice as well as sera from CRPS patients. These data are in agreement with findings from another regional autoimmune condition that affects the hair follicle, alopecia areata, where KRT16 co-localizes with serum from alopecia patients in the outer root sheath of the hair follicle (Leung et al., 2010). This suggests that, despite the ubiquitous distribution of KRT16, it may be a marker for regional autoimmunity. Notably, increased KRT16 might not be maladaptive in itself, but simply a response to skin damage that is associated with fracture/casting (mice) or extremity trauma (humans); KRT16 is

instrumental in regulating the production of innate danger signals and the over-activation of cytokines and various regulators of skin barrier function after epidermal barrier breach (Lessard et al., 2013) and could parallel the trophic changes that often accompany CRPS. For example, cytokine overproduction has been shown in our fracture model (Li et al., 2010), and could potentially drive innate and adaptive immune system activation, both of which are demonstrated in CRPS.

While the identification of KRT16 and possibly other skin-related autoantigens may reflect a contributing autoimmune component in CRPS, our search was not exhaustive. Neural tissue, for example, may contain additional autoantigens, and previous reports suggested that autoantibodies targeting cell surface receptors may functionally contribute to the clinical manifestations of the syndrome. Furthermore, we have screened 5 CRPS patients corresponding to a wide range of disease duration as well as differing CRPS causes (bone fracture, contusion, and surgery). It is possible that a larger sample size in addition to increased variation in disease etiology/duration would provide us with a more representative image of the heterogeneous CRPS population. Finally, since all of our patients list some form of trauma as the CRPS cause, it is possible that KRT16 immunoreactivity is due to the trauma itself rather than the experience of CRPS. Additional control samples from pain free individuals who have experienced prior injuries would be useful.

CONCLUSIONS

Our demonstration of the auto-antigenicity of KRT16 in a murine CRPS model and CRPS patients further bolsters the idea of autoimmune involvement in CRPS, and suggests that novel diagnostic tests and therapeutic strategies might be developed by pursuing these findings.

Acknowledgments

The authors thank Orr Sharpe, BSc. (Palo Alto Veterans Institute for Research, Palo Alto, California), David Leu, BSc. (Palo Alto Veterans Institute for Research, Palo Alto, California), Jeremy Sokolove MD (Palo Alto Veterans Institute for Research, Palo Alto, California), Xiao You Shi, Ph.D. (Palo Alto Veterans Institute for Research, Palo Alto, California), and Krupal Patel, BSc. for technical assistance.

References

- de Mos M, de Bruijn AG, Huygen FJ, Dieleman JP, Stricker BH, Sturkenboom MC. The incidence of complex regional pain syndrome: a population-based study. *Pain*. 2007; 129:12–20. [PubMed: 17084977]
- Goebel A, Baranowski A, Maurer K, Ghiai A, McCabe C, Ambler G. Intravenous immunoglobulin treatment of the complex regional pain syndrome: a randomized trial. *Ann Intern Med*. 2010; 152:152–158. [PubMed: 20124231]
- Katsiogiannis S, Tenta R, Skopouli FN. Endoplasmic reticulum stress causes autophagy and apoptosis leading to cellular redistribution of the autoantigens Ro/Sjogren's syndrome-related antigen A (SSA) and La/SSB in salivary gland epithelial cells. *Clin Exp Immunol*. 2015; 181:244–252. [PubMed: 25845745]
- Kil LP, Hendriks RW. Aberrant B cell selection and activation in systemic lupus erythematosus. *Int Rev Immunol*. 2013; 32:445–470. [PubMed: 23768157]
- Kohr D, Singh P, Tschernatsch M, Kaps M, Pouokam E, Diener M, Kummer W, Birklein F, Vincent A, Goebel A, Wallukat G, Blaes F. Autoimmunity against the beta2 adrenergic receptor and

- muscarinic-2 receptor in complex regional pain syndrome. *Pain*. 2011; 152:2690–2700. [PubMed: 21816540]
- Kohr D, Tschernatsch M, Schmitz K, Singh P, Kaps M, Schafer KH, Diener M, Mathies J, Matz O, Kummer W, Maihofner C, Fritz T, Birklein F, Blaes F. Autoantibodies in complex regional pain syndrome bind to a differentiation-dependent neuronal surface autoantigen. *Pain*. 2009; 143:246–251. [PubMed: 19375222]
- Lessard JC, Pina-Paz S, Rotty JD, Hickerson RP, Kaspar RL, Balmain A, Coulombe PA. Keratin 16 regulates innate immunity in response to epidermal barrier breach. *Proc Natl Acad Sci U S A*. 2013; 110:19537–19542. [PubMed: 24218583]
- Leung MC, Sutton CW, Fenton DA, Tobin DJ. Trichohyalin is a potential major autoantigen in human alopecia areata. *J Proteome Res*. 2010; 9:5153–5163. [PubMed: 20722389]
- Li WW, Guo TZ, Li XQ, Kingery WS, Clark JD. Fracture induces keratinocyte activation, proliferation, and expression of pro-nociceptive inflammatory mediators. *Pain*. 2010; 151:843–852. [PubMed: 20934254]
- Li WW, Guo TZ, Shi X, Czirr E, Stan T, Sahbaie P, Wyss-Coray T, Kingery WS, Clark JD. Autoimmunity contributes to nociceptive sensitization in a mouse model of complex regional pain syndrome. *Pain*. 2014; 155:2377–2389. [PubMed: 25218828]
- Mahajan A, Herrmann M, Munoz LE. Clearance Deficiency and Cell Death Pathways: A Model for the Pathogenesis of SLE. *Front Immunol*. 2016; 7:35. [PubMed: 26904025]
- Sohn DH, Rhodes C, Onuma K, Zhao XY, Sharpe O, Gazitt T, Shiao R, Fert-Bober J, Cheng DY, Lahey LJ, Wong HDH, Van Eyk J, Robinson WH, Sokolove J. Local Joint Inflammation and Histone Citrullination in a Murine Model of the Transition From Preclinical Autoimmunity to Inflammatory Arthritis. *Arthritis Rheumatol*. 2015; 67:2877–2887. [PubMed: 26227989]
- Sporn MB, Vilcek JT. The inauguration of Cytokine & Growth Factor Reviews. *Cytokine Growth Factor Rev*. 1996; 7:1. [PubMed: 8864348]
- Tajerian M, Clark JD. New Concepts in Complex Regional Pain Syndrome. *Hand Clin*. 2016; 32:41–49. [PubMed: 26611388]
- Tekus V, Hajna Z, Borbely E, Markovics A, Bagoly T, Szolcsanyi J, Thompson V, Kemeny A, Helyes Z, Goebel A. A CRPS-IgG-transfer-trauma model reproducing inflammatory and positive sensory signs associated with complex regional pain syndrome. *Pain*. 2014; 155:299–308. [PubMed: 24145209]

Highlights

An autoimmune mechanism of pain in a murine model of CRPS is proposed

Multiple potential target antigens are identified by LC-MS

Keratin16 is a validated target both in the mouse model and in CRPS patients

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

(A) Mice were randomly divided to control (C) and tibia fracture/cast (F) groups. 3 weeks after fracture, ipsilateral hindpaw skin protein was extracted and separated by 2-dimensional gel electrophoresis. (B) When probed with fracture and control mouse sera and CRPS and control human sera, total protein extracts harvested from 3w fracture mice were shown to bind differentially to mouse fracture serum and sera collected from CRPS patients (yellow ellipses). These same spots were excised from 2-d gels stained with Coomassie brilliant blue (red ellipses) and processed for LC-MS. (C) Proteins that were shown (by LC-MS) to be increased in the fracture group were analyzed by Ingenuity Pathway Analysis and plotted based on known involvement in autoimmune conditions. Darker colors indicate increased evidence for involvement. Asterisks indicate some of the targets that were chosen for further analysis.

Figure 2.

Multiple mechanisms can be associated with auto-antigenicity, including antigen abundance, localization, and post-translational modification. **(A)** Both eEF1a1 and PRPH are upregulated in hindpaw skin 3w after fracture. **(B)** ANXA2 and ENO3 did not show significant increase in protein levels, however, they did show unique localization within the cell following fracture (inset, fracture skin is on the bottom blots, yellow ellipses. I=cytoplasm, II=Membrane, III= Soluble nuclear, IV= chromatin-bound nuclear, and V= cytoskeletal). **(C)** No differences in total levels of carbamylated proteins in hindpaw skin were observed (left panel) and no differences in serum anti-citrullinated protein antibody (right panel) were observed. **(D)** Finally, sera from 3w fracture and control mice were run on an anti-citrullinated protein antibody array (Sohn et al., 2015). Negligible immunoglobulin binding was detected for the majority of antigens, with non-specific binding to native and citrullinated forms of select antigens observed similarly between groups. N=6–8/group. * $p < 0.05$, 2-tailed student's t-test. C= control; F= fracture; PTM= post translational modification.

Figure 3.

(**A, B**) 3 weeks after fracture, fracture mice show increased levels of KRT16 both in the mRNA (**A**) and protein (**B**) levels in the hindpaw skin. (**C**) Mouse fracture serum showed increased binding to KRT16 recombinant protein using dot blots. N=6–8/group (pooled sera, 5 technical replicates). (**D, E**) We confirmed immunoreactivity to KRT16 in CRPS patients using both pooled sera (**D**), as well as individual serum samples (**E**) (n=5 patients; 4–8 technical replicates/condition). (**F**) No correlation was observed between KRT16 immunoreactivity and CRPS duration. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. 2-tailed student's t-test. C= control; F= fracture; FI= fluorescence, Av=average.

Table 1

CRPS Serum Donor Information

	Subject ID				
	1	2	3	4	5
Age	24	43	44	45	50
Sex	Female	Female	Male	Female	Male
CRPS duration	4 months	11 months	2 weeks	7 years	4 weeks
CRPS location	Right foot	Left hand	Left hand	Right foot	Left hand
CRPS cause	Surgery	Fracture	Fracture	Fracture	Contusion
Edema	Yes	Yes	Yes	No	Yes
Allodynia	Yes	No	No	Yes	No
Pain NRS	6	9	7	8	8.5

NRS= Numeric Rating Scale

Table 2

List of Antibodies Used

Antibody	Supplier; Cat. #	Dilution	Application
Primary Antibodies			
Rabbit monoclonal anti eEF1A1	Abcam; 157455	1:20,000	WB
Mouse monoclonal anti PRPH	Abcam; 129007	1:5,000	WB
Rabbit Polyclonal anti ANXA2	Abcam; 41803	1:1,000	WB
Rabbit polyclonal anti ENO3	Abcam; 96334	1:500	WB
Rabbit monoclonal anti GAPDH	Abcam; 181602	1:5,000	WB
Rabbit polyclonal anti KRT16	Abcam; 182791	1:500	WB
Secondary Antibodies			
IrDye 800CW Goat anti mouse IgM	LI-COR Biosciences; 926-32280	1:20,000	WB/DB
DyLight 800 Goat anti human IgM	Thermo Fisher; SA5-10108	1:10,000	WB/DB
IrDye 800CW Goat anti rabbit IgG	LI-COR Biosciences; 925-32211	1:20,000	WB
IrDye 800CW Goat anti mouse IgG	LI-COR Biosciences; 926-32210	1:20,000	WB

WB= Western blot, DB= Dot blot

Table 3

Top LC-MS targets that are uniquely expressed in fracture skin

Protein	Gene name	Description	Peak intensity
Q9CV13	Krt16	Keratin 16	1.925669386
Q3ZAW8	Krt16	Keratin 16	1.924395393
Q9CRS6	Krt16	Keratin 16	1.910517686
Q678L1	Krt16	Keratin 16	1.910517686
Q9Z2K1	Krt16	Keratin 16	1.910517686
E9PXX7	Tuba1c	Tubulin alpha-1C	1.764922985
Q3TEE8	Tuba1c	Tubulin, alpha 1C	1.764922985
Q9JJZ2	Tuba1a	Tubulin alpha-1A	1.747042617
Q9NSB2	Krt4	Keratin 4	1.658202253
Q3UX10	Tuba1b	Tubulin alpha-1B	1.595342648
Q3TLD5	Tuba4a	Tubulin alpha-4A	1.563006187
Q6PHC1	Eno1	Alpha-enolase	1.52511714
G3UZR1	Tuba1b	Tubulin alpha-1B	1.496652939
Q3U9Q0	Krt78	Keratin 78	1.422753941
B0QZL1	Eno2	Gamma-enolase	1.408239965
P17182	Eno2	Gamma-enolase	1.408239965
Q922A0	Eno2	Gamma-enolase	1.408239965
D3YVD3	Eno2	Gamma-enolase	1.408239965
Q3UP82	KRT84	Keratin 84	1.390005676
Q9DC69	Krt72	Keratin 72	1.386185483
Q3UNH6	Eef1a1	Elongation factor 1-alpha 1	1.373908815
P10126	Eef1a1	Elongation factor 1-alpha 1	1.373246796
Q3UJ20	Eno3	Beta-enolase	1.370225809
F7B9M9	Eno3	Beta-enolase	1.370188404
K3W4Q6	Eno1	Alpha-enolase	1.370095804
Q0VJ69	Tuba8	Tubulin alpha-8	1.33725954
Q3TC83	Krt84	Keratin 84	1.335257256
Q9CZ13	Tuba3a	Tubulin alpha-3A	1.332034277
Q4L0E6	Krt20	Keratin 20	1.323664536
O35493	Eef1a1	Elongation factor 1-alpha 1	1.3232521
P06797	Eef1a1	Elongation factor 1-alpha 1	1.3232521
Q3UA81	Eef1a1	Elongation factor 1-alpha 1	1.3232521
Q58E64	Eef1a2	Elongation factor 1-alpha 2	1.3232521
B2FDE4	Eno1	Alpha-enolase	1.315130317
B1ARR6	Eno1	Alpha-enolase	1.315130317
B1ARR7	Eno2	Gamma-enolase	1.315130317
P17183	Eno2	Gamma-enolase	1.315130317

Protein	Gene name	Description	Peak intensity
Q543U3	Prph	Peripherin	1.303358941
D3YU63	Eno3	Beta-enolase	1.182414652
Q9EQ83	Prph	Peripherin	1.16790781
Q3UHD6	Prph	Peripherin	1.16790781
Q8K088	Tubb5	Tubulin beta-5	1.152288344
E0CYF3	Krt8	Keratin 8	1.037426498
D3Z2S4	Eno3	Beta-enolase	1.026941628
D3Z6E4	Eno3	Beta-enolase	1.026941628
Q6GQV1	Eno3	Beta-enolase	1.026941628
D3Z710	Ilf2	Interleukin enhancer-binding factor 2	1.024299267
P62631	Eno1	Alpha-enolase	1.007747778
Q5XJF8	Serpinb1a	Leukocyte elastase inhibitor A	1.006893708
Q99KA2	Serpinb1a	Leukocyte elastase inhibitor A	1.006893708
F2Z3W1	Anxa2	Annexin A2	0.887249795

Protein names in bold letters indicate some of the targets that were chosen for further analysis.